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International Rules for Seed Testing 2018

Introduction to the ISTA Rules
Chapters 1–19

Including changes and editorial corrections adopted at the Ordinary General Meeting 2017, Denver, USA

Effective from 1 January 2018

Note on the use of the translations

The electronic version of the International Rules for Seed Testing includes the English, French and German versions. If there are any questions on interpretation of the ISTA Rules, the English version is the definitive version.

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Preface to the 2018 Edition of the ISTA Rules

Since 2014, the *International Rules for Seed Testing* (ISTA Rules) are primarily available in electronic form only. The ISTA Rules can be downloaded as a complete PDF file or as individual chapters from:

http://www.ingentaconnect.com/content/ista/rules

If required, users of the ISTA Rules can print their own copies. For further information on the ISTA Rules, see:

http://www.seedtest.org/rules

The electronic version includes the English, French and German versions of the ISTA Rules. If there are any questions on interpretation of the ISTA Rules, the English version is the definitive version.

Seed health testing methods

Previously, the seed health testing methods were published as a separate Annexe to Chapter 7 of the ISTA Rules. They are now available as separate method sheets from the ISTA web site at:

http://www.seedtest.org/seedhealthmethods

Details of changes

The 2018 changes are editorial corrections or Rules changes adopted at the Ordinary General Meeting held at Denver, USA, in June 2017. Edits were made in Adobe InDesign by Vanessa Sutcliffe of HeartWood Editorial (www.heartwoodeditorial.co.uk).

The changes in the text content from the previous edition of the ISTA Rules are listed below. They can be displayed as yellow highlighted text as a 'layer' within the electronic copy with comments on what has changed.

For the previous history of amendments to the ISTA Rules, see the Prefaces for 2003 to 2017 on the ISTA web site.

Dr. Steve Jones, ISTA Rules Committee Chair

Ernest Allen, ISTA Rules Committee Vice-Chair

ISTA Secretariat

Changes to the ISTA Rules for 2018

General editorial: Change to British English as a house style, e.g., 'authorization' to 'authorisation'

Introduction

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2.5.4.1 b): Deletion of last sentence in 'Seed lot size' for consistency on Orange International Certificate requirements

2.9.1.1 and 2.9.2.1: Number of seeds tested from each independent container-sample reduced to 2 500

Table 2A Part 1: *Brassica carinata* A. Braun added; *Beta vulgaris* L. divided to make provision for different sample sizes for multi- and mono-germ seed

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Table 5A Part 1: *Brassica carinata* A. Braun added

Table 5F: Errors corrected in Germination Tolerance table to make consistent with Tolerance and Confidence Interval Calculator for Germination Tests

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7.2.3: Seed treatment link to 2.2.11 renumbered to 2.2.12

Editorial modifications to ensure consistency in method structures

Method 7-004: New version of method

Method 7-015: Pathogen names updated according to information provided by *Index Fungorum*

Method 7-031: Addition of new method

Method 7-032: Addition of new method

Chapter 8

8.3.2: Wording of 'Testing principles' updated

8.10.3: Addition of DNA-based method for variety verification in *Zea mays*

Chapter 11

11.2.5.4.1: Removal of 'encrusted seed' in description of maximum seed lot size; deletion of last sentence for consistency on Orange International Certificate requirements

Chapter 13

13.6: Update to wording for checking the reliability of a test result, for consistency with Table 13C

Chapter 15

15.8.2.5.2: Revision of concentration of sodium hypochlorite solution used to clean equipment

Chapter 18

Revision of entire Chapter with a clearer and more logical structure, as proposed by the Bulking and Sampling Committee

Introduction to the ISTA Rules

I-1 General information

The International Seed Testing Association (ISTA) was established in 1924 to work towards a vision of uniformity in seed testing internationally. ISTA's current mission is to develop, adapt and publish standard procedures for sampling and testing seeds, and to promote uniform application of these procedures for evaluation of seeds moving in international trade. The need for seed testing methods that are reliable and reproducible among its accredited member laboratories is therefore a basic need for ISTA. This is achieved through the publication of the International Rules for Seed Testing (hereafter 'ISTA Rules').The primary aim of the ISTA Rules is to provide testing methods for seeds designated for growing of crops or production of plants. In addition, most of the testing methods can also be applied for evaluation of the quality of seeds used as food or for technical purposes.

ISTA's seed sampling and testing methods have been developed by its members since its formation in 1924. Methods have gone through appropriate validation studies to ensure that test procedures give reliable and reproducible results. Following agreement between ISTA's member countries, the validated methods have been included in the ISTA Rules.

Seed quality testing therefore requires test methods and equipment that have been tested to ensure they are fit for purpose, i.e. validated. The ISTA Method Validation Programme (see Section I-2) provides the mechanism for the inclusion of test methods in the ISTA Rules.

Seed is a living biological product, and its behaviour cannot be predicted with the certainty that characterises the testing of inert or non-biological material. The test methods used must be based on scientific knowledge and the accumulated experience of those working in seed testing and quality control. This expertise is provided largely by the members of ISTA's Technical Committees.

The ISTA Rules contain 19 chapters, 17 of which provide internationally accepted test methods for various attributes of seed quality. Chapter 2 (Sampling) provides the required methods for sampling of seed lots, because for ISTA, a direct connection between the seed lot from which the sample was drawn and the results of quality tests conducted on that seed lot must always be evident. The 'end product' for an accredited ISTA laboratory following quality tests on a seed lot is an ISTA Certificate. Information on how to use ISTA Certificates is presented in Chapter 1.

Each of the 17 chapters on test methods includes sections on the Object (of the test), Definitions (of terms used in the chapter), General Principles (for the test), Apparatus (required for the test), Procedure (how to conduct the test), Calculation and Expression of Results (specific to each test), Reporting Results (how to report results correctly on an ISTA Certificate), and Tolerances (statistical tables for use in determining whether test results are acceptable or not acceptable). Note that where, to provide adequate guidance, it has been necessary in the Apparatus section to refer to a particular manufacturer's piece of equipment, this should not be construed that ISTA endorses that piece of equipment in preference to, or to the exclusion of, equivalent products from other manufacturers.

The ISTA Rules are designed for the principal crop species of the world. Species are broadly classified as agricultural and vegetable, tree and shrub, and flower, spice, herb and medicinal. ISTA encourages proposals for the addition of new species to the ISTA Rules.

ISTA Certificates can only be issued by ISTA accredited laboratories. For seed quality test results to be reported on an ISTA Certificate, it is mandatory that all the requirements of the ISTA Rules are strictly followed.

ISTA also recommends that the ISTA Rules be used by all seed testing laboratories (including non-ISTA member laboratories) when testing seed for trade transactions which do not require the use of an ISTA Certificate (e.g. within a country), and for the enforcement of national laws for the control of seed quality.

For further information on the ISTA Rules and their use, please contact:

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CH-8303 Bassersdorf
Switzerland
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or visit the ISTA website: www.seedtest.org

I-2 Guidelines for ISTA Rules proposals

Proposals to amend the ISTA Rules or to introduce new species are welcomed from any source. ISTA operates an open system, and proposals are not restricted to ISTA members only. Any external proposal needs to have been submitted to the ISTA Secretariat by 1 November.

Following receipt, the ISTA Secretariat may send the proposal to the relevant ISTA Technical Committee or directly to the ISTA Rules Committee, which will review all the proposals received. The ISTA Executive Committee will then either approve a proposal for consideration by the ISTA membership or request further work on the proposal. All approved Rules proposals are then sent to the ISTA membership two months before the Ordinary Meeting. At the Ordinary Meeting, the ISTA voting delegates may vote to accept a proposal (which will then be implemented in the ISTA Rules, effective 1 January of the following year), to withdraw a proposal (for further consideration), or to reject a proposal.

I-2.1 Proposals concerning test methods

All seed quality test methods proposed for inclusion in the ISTA Rules must have gone through the ISTA Method Validation Programme. This is required for both new test methods (i.e. not currently in the ISTA Rules) and modifications to existing methods already included in the ISTA Rules. A four-step process is involved:

1) method selection and development;
2) validation through comparative testing;
3) review of comparative test results and preparation of a Method Validation Report;
4) approval of validation status by the relevant ISTA Technical Committee and preparation and of an ISTA Rules proposal for the method.

Final acceptance of the proposal by vote of the ISTA membership at an Ordinary Meeting will allow publication of the validated method in the ISTA Rules.

Further information on the ISTA Method Validation Programme can be obtained from the ISTA Secretariat.

I-2.2 Proposals for new species

For a proposal to introduce a new species, Form 1 on pages 5–9 may be used.
The following information must be supplied by the applicant:

1. **Names of species.** The scientific name (including author) plus common names and synonyms must be given. The common names will be used by the ISTA Nomenclature Committee to update the Multilingual Glossary of Common Plant Names. The ISTA Nomenclature Committee will stabilise the scientific name for at least six years so that laws and trade agreements do not have to be altered frequently. For assistance in determining the correct scientific name and its author, the ISTA Nomenclature Committee may be contacted.

2. **Maximum lot size and sample sizes.** Proposals for maximum lot size should take into account the general principles that have been applied to species already in the ISTA Rules and to the feasibility of achieving reasonably homogenous seed lots. Seed size is generally the significant factor in determining maximum lot size, but this is also influenced by whether the species is for agriculture or horticulture use, a tree or shrub species, or a flower, spice, herb or medicinal species. This, in turn, will determine whether the species should be placed in Part 1, 2 or 3, respectively, of Table 2A. Proposals for maximum lot size and submitted sample size should then be based on those already to be found in the corresponding part of Table 2A. For agricultural and horticultural species, the submitted sample is larger in relation to the purity working sample, based on the weight of 2500 seeds, than for the other species, to allow for determination of other species by number based on 10 times the purity weight.

3. **Pure Seed Definition.** The ISTA Rules and the *Handbook of Pure Seed Definitions* already list many pure seed definitions. The appropriate one should be given. If none of them apply, a proposal for a new definition should be submitted.

4. **Validated germination test methods.** The methods proposed must have been validated, either by multi-laboratory collaborative testing or peer validation (see ISTA Method Validation Programme). Advice as to requirements can be obtained from the ISTA Germination Committee. Please specify the data as required for insertion in Table 5A.

5. **Validated tetrazolium test procedures.** Procedures for tetrazolium testing should be given if known. A proposal to amend Chapter 6 may be submitted following the appropriate method validation.

6. **Validated moisture content determination methods.** A validated method for moisture determination must be provided if the method is different to the reference (i.e. low-constant-temperature) method.

7. **Thousand-seed weight**

8. **Varietal identification.** Using current techniques, it is possible to verify a descriptor to check varietal purity in some species. Please indicate validated techniques.

9. **Seed health tests.** The methods proposed must have been validated, either by multi-laboratory collaborative testing or peer validation (see ISTA Method Validation Programme). Advice as to requirements can be obtained from the ISTA Seed Health Committee.

I-2.3 Other proposals

Within a chapter of the ISTA Rules, a change to the existing text (e.g. amendment of a definition) or introduction of new text (e.g. introduction of a new definition) may be proposed. Providing the proposal does not directly involve a test method or new species, it should be sent directly to the ISTA Secretariat.

Thousand-seed weight of small-seeded varieties of *Poa pratensis*

Before a small-seeded variety can be included in Table 3A, a determination of the thousand-seed weight must be performed on at least 20 samples from different seed lots, representing seeds grown either in two different harvest years or in two different countries.

The determination of the thousand-seed weight must be carried out on pure seeds, obtained by blowing a 1 g sample of *Poa pratensis* using the standard blower setting (factor 1.00). Only seed remaining in the heavy fraction may be used for the thousand-seed weight. See Chapter 10 of the ISTA Rules for the weight determination procedure.

Results should be submitted to the ISTA Purity Committee with a request to change the ISTA Rules.

Form 1: Proposal for inclusion of new species in the ISTA Rules

Note: this form is also available on the ISTA web site (www.seedtest.org/mv-prog)

1. Scientific name of proposed species

(Family)	Genus	Species	(Nominated Authority)

Genus and species names appear in List of Stabilised Plant Names: Yes/No
Known synonyms: _____
Common plant name: _____ in _____ (Member country)
(required for Multilingual Glossary)

2. Lot and sample weights

(Information as it should appear in Table 2A)

Species	Maximum weight of lot (kg)	Minimum submitted sample (g)	Minimum working samples (g)	
			Purity analysis (3.5.1)	Count of other species (4.5.1)

3. Pure Seed Definition

(Table 3B Part 1)
The following Pure Seed Definition (PSD) covers the proposed species:

Genus	Family	PSD number	Chaffiness

No existing definition covers this species:	

Distinguishing characteristics of this species:

(List distinguishing characteristics. Attach drawings, if available, and be prepared to send to the Secretariat five seed samples from well-processed, as well as from incompletely cleaned, seed.)

4. Validated germination test method(s)

(Information as it should appear in Table 5A)

Species	Prescriptions for:				Additional directions incl. recommendations for breaking dormancy
	Substrate	Temperature (°C)	First count (d)	Final count (d)	

5. Validated tetrazolium test procedure

(Information as it should appear in Table 6A)

Species	Pretreatment: type/minimum time (h)	Preparation before staining	Staining solution (%)	Optimum staining time (h)	Preparation for evaluation	Permitted non-viable tissue	Remarks

(If no existing drawings apply, attach if available)

6. Validated moisture test methods

Specify appropriate methods or details for inclusion in Table 9A Part 1 or 2:

Species	Grinding/cutting (9.1.5.4, 9.1.5.5)	High temperature	Drying at high temperature (h)	Predrying requirement (9.1.5.6)	Remarks
(Part 1)					(Not applicable)
(Part 2)		(Not applicable)	(Not applicable)	(Not applicable)	

7. Thousand-seed weight = _____ g

8. Validated varietal identification method (attach separate sheet, if necessary)

_____

Supporting evidence for proposal

9. Number of national seed analysis certificates issued per year:

_____

10. Other countries or laboratories testing the proposed species:

_____ _____

_____ _____

Submitted by:

Signature:

Date:

Chapter 1: ISTA Certificates

1.1 Object

The object is to prescribe rules for the issue of ISTA Certificates for seed analysis. The completed certificates are available only from accredited member laboratories of ISTA and must only be issued in accordance with the ISTA Rules currently in force.

1.2 Definitions

Blank ISTA Certificates for seed analysis are produced by ISTA, and only provided to accredited laboratories (see 1.2.6) for reporting the results of tests. These completed certificates are the property of ISTA and may only be issued under the authority of ISTA.

1.2.1 Orange International Seed Lot Certificate

An Orange International Seed Lot Certificate is issued when both sampling from the lot and testing of the sample are carried out under the responsibility of an accredited laboratory, or when sampling from the lot and testing of the sample are carried out under the authority of different accredited laboratories. Where the accredited laboratory carrying out the sampling is different to the one carrying out the testing, this must be stated (see 1.4.2). The procedure followed links the Orange International Seed Lot Certificate with the seed lot. The certificate is coloured orange.

An Orange International Seed Lot Certificate may also be issued, without further sampling or testing, for a portion (sublot) of a seed lot which has been sampled and tested and for which an Orange International Seed Lot Certificate has been issued. The certificate for the sublot must carry the same test results as reported for the original seed lot.

The results reported on an Orange International Seed Lot Certificate refer strictly to the lot as a whole at the time of sampling.

1.2.2 Blue International Seed Sample Certificate

A Blue International Seed Sample Certificate is issued when sampling from the lot is not under the responsibility of an accredited laboratory. The accredited laboratory is responsible only for testing the sample as submitted. It is not responsible for the relationship between the sample and any seed lot from which it may have been derived. The certificate is coloured blue.

The results reported on a Blue International Seed Sample Certificate refer strictly to the sample at the time of receipt.

1.2.3 Original certificate

An original certificate is an ISTA Certificate issued after the completion of a test or tests. It is marked ORIGINAL.

1.2.4 Duplicate certificate

A duplicate certificate is an exact printed copy, not a photocopy, of a completed original ISTA Certificate, marked DUPLICATE.

1.2.5 Provisional certificate

A provisional certificate is an ISTA Certificate issued before the completion of a test or tests. It is marked PROVISIONAL, and must include a statement under ‘Other determinations’ that a final original certificate will be issued upon completion.

1.2.6 Accredited laboratory

An accredited laboratory is an ISTA-accredited member laboratory authorised by the ISTA Executive Committee under Article 4(i) of the Articles of ISTA to sample and test seeds and to issue ISTA Certificates.

1.3 Conditions for issuance of ISTA Certificates

ISTA Certificates must be issued only on forms obtained from the ISTA Secretariat and approved by the ISTA Executive Committee. There are two kinds of certificates: Orange International Seed Lot Certificates and Blue International Seed Sample Certificates, as defined in 1.2.

On request of the applicant, duplicate and provisional certificates as defined in 1.2 may be issued.

A duplicate certificate may be issued for an original certificate. More than one duplicate certificate may be issued.

A provisional certificate may be issued for any ISTA test result(s) that are later combined onto an original certificate. More than one provisional certificate may be issued.

If an applicant cancels testing, an ISTA Certificate does not need to be issued.

An ISTA Certificate may be issued only by the seed testing laboratory which either carried out all the tests to be reported, or subcontracted sampling and/or some of the tests to be reported (see 1.4.2 and 1.4.3), and under the conditions listed below:

a) The issuing laboratory must be currently authorised to do so by the Executive Committee.
b) The seed tested must be of a species listed in Table 2A (Lot and sample weights) of the ISTA Rules. Where in other tables, such as Table 5A and Table 6A, methods are prescribed for a group of species, only those species specifically listed in Table 2A may be considered to be covered.
   Consequently, no certificates may be issued for species not listed in Table 2A of the current ISTA Rules, except in the case of seed mixtures, where for the species tested it is shown as 'Seed mixture'.
c) The tests must be carried out in accordance with the ISTA Rules. However, additionally and on request, results of tests not covered by these Rules may be reported on an ISTA Certificate (see 1.5.2.22).
   Results of analyses not covered by the current ISTA Rules may be included on a certificate only if results of at least one test covered by the ISTA Rules are also being reported.
d) For the result of a determination of moisture content to be reported on an ISTA Certificate, the sample must be submitted in an intact, moisture-proof container from which as much air as possible has been excluded (see 9.1.5.1).
e) To report results of tests which are in the ISTA Rules, the laboratory must be accredited for these tests, either directly or through subcontracting to another laboratory accredited for these tests.
f) The assessment of any attribute reported on a certificate must be calculated from tests carried out on one submitted sample.
g) In the case of Orange International Seed Lot Certificates:
   – the seed lot must comply with the requirements prescribed in 2.5.4;
   – the submitted sample must be drawn and dealt with in accordance with 2.5.4.
h) For an Orange International Seed Lot Certificate, each container in the lot must be marked, labelled and sealed in accordance with 2.5.4.3.
i) For an Orange International Seed Lot Certificate to be issued for a sublot, the sublot must represent a minimum size of 20 % of the weight of the original seed lot. A maximum of five Orange International Seed Lot Certificates may be issued for sublots of any one original seed lot.
j) For an Orange International Seed Lot Certificate, the submitted sample must be tested by an accredited laboratory. The issuing laboratory must ensure that sampling, sealing, identification, testing and issuance of the certificate is in accordance with the ISTA Rules, although subcontracting of sampling and/or testing to another accredited laboratory is permissible. The laboratory which carries out sampling must provide all the information that is necessary to complete the Orange International Seed Lot Certificate.
   The seed lot identification ('Marks of the lot'; see 2.2.10) may take the form of a sequential series of characters or a single reference character. Each container within the lot or sublot must be identified in such a way that the containers can be readily recognised by the information provided on the certificate issued. Each container of a sublot must be marked with the identification of the original seed lot. A sublot-specific identification is not necessary.
   When the seed lot is located in a different country to the sampling laboratory, the country where the seed lot has been sampled must be reported either under 'Sampling by' or under 'Additional observations'.

1.4 Completing ISTA Certificates

1.4.1 General

ISTA Certificates must be completed using a typewriter or machine-printer and can be completed in any language. No certificate may be issued that shows signs of amendment, alteration or erasure.

A completed certificate must show the following information:

a) The name and address of the issuing laboratory; the laboratory must be on the ISTA list of accredited member laboratories.
b) Dates, written in the ISO 8601 format: year in full – month – day, with two figures for both month and day (e.g. 2007-07-25).
c) The scientific name of the species tested, as listed in the current ISTA Rules and (in most cases) also the *ISTA List of Stabilised Plant Names*. Where it is impossible to determine the species with certainty on the basis of seed characters, only the genus name must be stated (example: *Malus* sp.). In the case of seed mixtures, for the species tested 'Seed mixture' must be entered.
d) The name and address of the applicant. Other information stated by the applicant, such as country of origin, species, cultivar, weight of lot or sublot, certification category and applicant's lot reference must be entered as stated by the applicant.
   Note: at the request of the applicant the name and address of the applicant may be omitted.
e) The signature of the Head of the issuing laboratory or their assignee. It may be either a physical or an electronic signature, the use of which is authorised by the Head of the issuing laboratory.
f) Under 'Status of certificate', the word 'ORIGINAL', 'PROVISIONAL' or 'DUPLICATE', as appropriate.

1.4.2 Orange International Seed Lot Certificate

It is stated on the back of the Orange International Seed Lot Certificate:

'I certify that sampling, sealing and testing have been carried out in accordance with the International Rules for Seed Testing of the ISTA and that the tests have been made at a laboratory accredited by the International Seed Testing Association to issue International Seed Analysis Certificates.'

The completed Orange International Seed Lot Certificate must show the following information:

a) name, address, ISTA member code and stamp (seal) of issuing laboratory;
b) name and ISTA member code of laboratory responsible for sampling;
c) seed lot identification (i.e. marks of lot);
d) Under 'Seal of lot': the method of sealing (e.g. stitching, metal seal) and/or the authority (e.g. ISTA laboratory, Ministry).
e) either the number of containers for which the certificate is issued; or 'N/A' for 'not applicable';
f) date of sampling;
g) date that the sample was received by the testing laboratory;
h) date test was concluded;
i) place, country and date of issue of the certificate;
j) test or sample number of the testing laboratory;
k) analysis results;
l) In the case of certificates for sublots, under 'Other determinations': 'The results reported represent the sample drawn from the original seed lot of … kg.'
m) country where the seed lot was sampled, when the seed lot is located in a different country to the sampling laboratory, reported under either 'Sampling by' or 'Additional observations';
n) the signature of the Head of the issuing laboratory or their assignee which confirms the statement on the back of the certificate as true;
o) under the signature it must state at least, the job position of the person signing or "Authorised signatory".

1.4.3 Blue International Seed Sample Certificate

The Blue International Seed Sample Certificate refers only to the sample submitted for testing.

It is stated on the back of the Blue International Seed Sample Certificate:

'I certify that testing has been carried out in accordance with the International Rules for Seed Testing of the ISTA and that the tests have been made at a laboratory accredited by the International Seed Testing Association to issue International Seed Analysis Certificates.'

The completed certificate must show the following information:

a) name, address, ISTA member code and stamp (seal) of issuing laboratory;
b) date that the sample was received by the testing laboratory;
c) date test was concluded;
d) place, country and date of issue of the certificate;
e) test or sample number of the testing laboratory;
f) results of tests;
g) the signature of the Head of the issuing laboratory, or their assignee which confirms the statement on the back of the certificate as true;

h) under the signature it must state at least, the job position of the person signing or "Authorised signatory".

1.4.4 Duplicate certificate

A duplicate ISTA Certificate may be issued on request of the applicant.

1.4.5 Provisional certificate

A provisional ISTA Certificate may be issued on request of the applicant.

1.5 Reporting results

1.5.1 Sampling and testing

From one sampling operation, only one sample may be submitted for testing. The sample may be subjected to one or more of the tests described in the ISTA Rules as requested by the applicant. However, in certain situations (see 2.5.1.6) the submission of separate moisture-proof-packed subsample(s) from the same sampling operation attached to the submitted sample is required.

1.5.2 Certificates

The results of tests may be reported on one or more ISTA Certificates, separately or combined.

Test results must be reported in accordance with the rules for calculating, expressing and reporting results in the appropriate chapter of the ISTA Rules. If there is a space on the certificate for certain determinations which are not made or applicable, 'N' for 'not tested' must be placed in the space.

1.5.2.1 Sampling: heterogeneity testing for seed lots in multiple containers

1.5.2.1.1 The H value heterogeneity test

The result of the H value heterogeneity test for seed lots in multiple containers must be reported under 'Other determinations', as follows:

- $\overline{X}$: mean of all X values determined for the lot in respect of the adopted attribute;
- N: number of independent container samples;
- No: number of containers in the lot;
- the calculated H value;
- the statement: 'This H value does/does not indicate significant heterogeneity.'

Note: the H value must not be calculated or reported if $\overline{X}$ is outside the following limits:

- purity components: above 99.8 % or below 0.2 %;
- germination: above 99.0 % or below 1.0 %;
- number of specified seeds: below two per sample.

1.5.2.1.2 The R value heterogeneity test

The result of the R value heterogeneity test for seed lots in multiple containers must be reported under 'Other determinations', as follows:

- $\overline{X}$: mean of all X values determined for the lot in respect of the adopted attribute;
- N: number of independent container samples;
- No: number of containers in the lot;
- the calculated R value;
- the statement: 'This R value does/does not indicate significant heterogeneity.'

1.5.2.2 Purity

The results of a purity test must be reported in the spaces provided as follows:

- The scientific name of the species of pure seed, in accordance with Table 2A (e.g. *Triticum aestivum*). Where it is impossible to determine the species with certainty on the basis of seed characteristics, reporting must be done to the most precise taxon possible.
- The percentage by weight of pure seed, inert matter and other seeds, given to one decimal place. The percentage of all components must total 100 %. Components amounting to less than 0.05 % must be reported as 'Trace' or 'TR' (for 'Trace'). If no inert matter or other seeds are found, this must be reported as '0.0'.
- The kind of inert matter.
- The scientific name of every species of other seeds found, in accordance, where applicable, with the current *ISTA List of Stabilised Plant Names*, available at www.seedtest.org/stablist (e.g. *Elytrigia repens*).
- When the weight of the working sample tested for purity equals or is no more than 10 % higher than the weight specified in Table 2A, column 4 (Purity analysis), no statement regarding the weight of the working sample is required on the ISTA Certificate.

– When the weight of the working sample tested for purity deviates from that specified in Table 2A, column 4, the actual weight of the working sample weighed according to 3.5.1 must be reported on the ISTA Certificate using one of the following, as applicable:
  a) When testing a weight that exceeds by 10 % the weight specified in Table 2A, column 4, report under other determinations as:
  'Purity: ... g'
  b) When testing a weight estimated to contain 2500 seed units, report under other determinations as:
  'Purity: …g (approx. 2500 seeds)'
  c) When the submitted sample received for purity testing weighs less than the weight in Table 2A, column 4, report under other determinations and use the current statement, according to 2.5.4.5:
  'The submitted sample weighed only … g and is not in accordance with the *International Rules for Seed Testing*.'
– The percentage of winged seed (as defined in Pure Seed Definitions 47 and 51), if winged seeds are found.

Upon request, the following information must be reported under 'Other determinations' as follows:
– The percentage by weight of a specified species, entered immediately after the name of the species to the nearest 0.1 %. Species for which the percentage by weight has been requested are listed first.
– Other seeds may be divided into 'other crop seeds' and 'weed seeds'. In this case, the words 'Other crop seeds' must be entered, followed by the percentage by weight of other crop seeds and the name(s) of the species found. This procedure must also be used for 'Weed seeds'.
– Multiple seed units must be reported as '% MSU'.
– Seeds with appendages attached must be reported as '% seeds with appendages attached'.
– The kinds of inert matter, together with the percentage by weight of any particular kind (to one decimal place).
– The percentage by weight of broken pure seed.

The percentages may be reported to more than one decimal place if requested.

1.5.2.3 Purity tests on coated seeds

The result of a purity test on coated seeds must be reported as follows:
– Following the species name, the words 'seed pellets', 'encrusted seeds', 'seed granules', 'seed tapes' or 'seed mats', as applicable, must be clearly entered.
– The results must be reported to one decimal place, and the percentage of all components must total 100 %. Components amounting to less than 0.05 % must be reported as 'Trace' or 'TR' (for 'Trace'). If no inert matter or other seeds are found, this must be reported as '0.0'.
– In the case of pelleted seeds only, the percentages of pure pelleted seeds, inert matter and unpelleted seeds must be reported in the spaces provided for 'Pure seeds', 'Inert matter', and 'Other seeds', respectively.
– The name and number of the seeds of each species found in the examination of the 100 seeds removed from the pellets or tapes must be reported under 'Other determinations'.

Upon request, the following information may be reported under 'Other determinations' as follows:
– Purity test on depelleted seeds. The component parts (pure seed, other seeds and inert matter) may be reported as percentages of their total weight, ignoring the pelleting material. The percentage of pelleting material must be reported separately only on request. The result of this test is to be reported: 'weight of … material excluded'.
– Purity of seeds removed from tapes. The component parts (pure seed, other seeds, and inert matter) may be reported as percentages of their total weight, ignoring the tape material. The result of this test is to be reported: 'weight of … material excluded'.

1.5.2.4 Determination of other seeds by number

The result of a determination of other seeds by number must be reported under 'Other determinations' as follows:
– The actual weight of seed examined to the minimum number of decimal places indicated in Table 4.1.
– The scientific name and number of seeds of each species sought and found in this weight. If no other seeds are found, this must be indicated on the certificate.
– Where it is impossible to determine with certainty on the basis of seed characteristics, reporting must be done to the most precise taxon possible.

– If the full weight prescribed in Table 2A was examined for all other species present, then the words 'Complete test' must be entered, alongside the weight of seed examined.
– If the examination was for only a limited range of other species, then the words 'Limited test' must be entered.
– If the weight examined for all other species was less than the prescribed weight, then the words 'Reduced test' must be entered.
– If the weight examined was less than the weight prescribed in Table 2A, and only a limited range of other species was examined, then the words 'Reduced-limited test' must be entered.
– If a sample of at least 25 000 seeds was examined, and this sample was below the weight prescribed in Table 2A, then the weight of seed examined and the statement 'Test based on at least 25 000 seeds' must be entered.

Upon request, the results may in addition be expressed in some other way, such as 'weight of seeds found' or 'number of seeds per kilogram'.

Upon request, the presence of *Orobanche* species can only be reported on a Blue International Seed Sample Certificate (see 1.2.2) and must be reported as: Test for presence of *Orobanche* species: '… seeds of *Orobanche* spp. were found in … g of seed examined.'

If no seeds were found it can be reported as: 'No seeds of *Orobanche* spp. were found in … g of seed examined.'

The sample weight examined must be reported according to the minimum number of decimals indicated in Table 4.1.

1.5.2.5 Determination of other seeds by number on coated seeds

The result of a determination of other seeds by number on coated seeds must be reported as follows:
– Following the species name, the words 'seed pellets', 'encrusted seeds', 'seed granules', 'seed tapes' or 'seed mats', as applicable, must be clearly entered.
– Under 'Other determinations', the actual weight (or length of tape, or area of mat) and approximate number of pelleted seeds examined must be entered, together with the scientific name and number of seeds of each species sought and found in this weight, length or area.

Upon request, the result may in addition be expressed in some other way, such as number of seeds per kilogram, per metre or per square metre.

1.5.2.6 Germination

The result of a germination test must be reported in the spaces provided as follows:
– the actual duration of the test (in days, excluding the period of special treatment or method used for promoting germination);
– the percentages, calculated to the nearest whole number (5.8.2), of normal seedlings, hard seeds, fresh seeds, abnormal seedlings and dead seeds. If the result for any of these categories is found to be zero, it must be reported as '0'.
– If an applicant requests that the test be terminated when the sample reaches a predetermined germination percentage, before the final count, then only the percentage of normal seedlings is reported. The results of the other categories (abnormal seedlings, hard seeds, fresh seeds and dead seeds) must be reported as 'N', because they have not been determined.

The following additional information must be reported under 'Other determinations':
– the number of seeds tested, if less than 400 seeds;
– the germination method using the abbreviations used in Table 5A, including at least substrate and temperature;
– any special treatment or method used for promoting germination (5.6.3);
– the duration in days of any special treatment or method used for promoting germination, except in the case of prestorage;
– the germination percentage obtained within the prescribed time, if the germination period was extended beyond the period indicated in Table 5A. The statement must be entered as follows: 'After the prescribed period of … days, there were … % normal seedlings.'
– the method for evaluating fresh seeds (dissection, tetrazolium or excised embryo – see paragraph 5.6.5.3.) when 5 % or more of fresh seeds are believed to be present.
– If an applicant requests that the germination test be terminated when the sample reaches a predetermined germination percentage, the following statement: 'Upon request of the applicant, the germination test was terminated after … days. The prescribed test period is … days.'

When double tests are prescribed in Table 5A Part 2, the result of the first test, with treatment for breaking dormancy, is reported in the appropriate space on the ISTA Certificate, and the result of the second test, without treatment for breaking dormancy, is reported under 'Other determinations'.

Upon request, the following information may be reported as follows:
- the result of parallel tests or any additional test;
- the viability of ungerminated seeds and the method used to determine it;
- the categories of ungerminated seeds (as listed in 5.6.5.3) and the method used to determine them;
- in the case of multigerm seed units: the number of normal seedlings produced by 100 units, the number of units which have produced one, two or more than two normal seedlings, or the proportion of units producing one, two or more than two normal seedlings. The proportion is expressed as a percentage of the total number of units which have produced at least one normal seedling.

1.5.2.7 Germination of coated seeds

The result of a germination test on coated seeds must be reported as follows:
- Following the species name, the words 'seed pellets', 'encrusted seeds', 'seed granules', 'seed tapes' or 'seed mats', as applicable, must be clearly entered in the space provided.
- The percentage of pellets or seed in tapes with normal seedlings, with abnormal seedlings and without seedlings.
- The duration of the test.

The following additional information must also be reported under 'Other determinations':
- The method used for the germination test.
- For seed tapes or mats: the number of normal seedlings per metre of tape or square metre of mat.

Seedlings that are obviously not of the species stated by the applicant, even if otherwise normal, must not be included in the germination result, but their number must be reported separately.

1.5.2.8 Tetrazolium test

The result of a tetrazolium test must be reported under 'Other determinations' as follows:
- The statement 'Tetrazolium test: …% of seeds were viable' must be entered.
- In cases where the testing procedure deviates from that prescribed in Table 6A, any deviating procedure must also be reported.
  The only variations permitted from procedures given in Table 6A are for premoistening time, tetrazolium concentration, staining temperature or staining time. Precise prescriptions about the limitation of the variations are given in 6.5.
- If individual seeds are tested at the end of the germination test, the result must be reported in accordance with 1.5.2.6 and 5.9.

In addition, in the case of species of *Fabaceae*, one of the following, and only one, must be reported:
either (in cases where the viability percentage of the hard seed is not determined) 'Tetrazolium test: ...% of seeds were viable, ...% of hard seeds found in the test.'
or (in cases where the viability percentage of the hard seed is determined) 'Tetrazolium test: ...% of seeds were viable, ...% of hard seeds included in the percentage of viable seed'

At the discretion of the seed testing laboratory, further information may be reported, e.g. percentage of seeds that were empty, with larvae, broken or decayed.

1.5.2.9 Tetrazolium test on coated seeds

The result of a tetrazolium test on coated seeds must be reported as follows:
- Following the species name, the words 'seed pellets', 'encrusted seeds', 'seed granules', 'seed tapes' or 'seed mats', as applicable, must be clearly entered.

The following additional information must be reported under 'Other determinations':
- The statement 'Number of seeds (of the species stated by the applicant) included in 100 seed pellets' (or 'encrusted seeds', or 'seed granules');
- or the statement 'Number of seeds (of the species stated by the applicant) included in one metre of seed tape';
- or the statement 'Number of seeds (of the species stated by the applicant) included in one seed mat or in one square metre of seed mat'.
- The statement 'Tetrazolium test: …% were viable' must be entered.
- In cases where the testing procedure deviates from that prescribed in Table 6A, any deviating procedure must also be reported. The only areas where variations from procedures given in Table 6A are permitted are for premoistening time, tetrazolium concentration, staining temperature and staining time. For precise guidance about the limitation of the variations permitted, see 6.5.

– If individual seeds are tested at the end of the germination test, the result must be reported in accordance with 5.9.

In addition, in the case of species of *Fabaceae*, one of the following, and only one, must be reported:

either (in cases where the percentage of the viability of hard seed is not determined) 'Tetrazolium test: ...% of seeds were viable, ...% of hard seeds found in the test'

or (in cases where the percentage of the viability of hard seed is determined) 'Tetrazolium test: ...% of seeds were viable, ...% of hard seeds included in the percentage of viable seed'

1.5.2.10 Seed health test

The results of a test for seed health must be reported under 'Other determinations' as follows:

– either qualitative or quantitative results, as specified in the individual methods;
– negative and positive results, as specified in the individual methods;
– the scientific name of the pathogen detected;
– the percentage of infected seeds;
– the method used, including any pretreatment (7.2.2);
– the size of the sample or fraction examined;
– any additional permitted procedure used.

The absence of a statement concerning the health condition of the seed does not necessarily imply that the health condition is satisfactory.

1.5.2.11 Species and variety testing

The results of species and variety testing must be reported under 'Other determinations', and in addition the following information must be given:

a) the request of the applicant;

b) the trait(s) and the method(s) used;

c) the kind of preparation of the working sample (e.g. the whole working sample excluding the inert matter or only the pure seed fraction, washing);

d) whether an authentic standard sample or a standard reference was used; if a standard reference was used, its origin must be indicated;

e) the number of seeds, seedlings or plants examined. When it is difficult to determine the total number of plants examined in field plots, the mass of seed sown must be reported.

1.5.2.11.1 Results of examination of individual seeds or seedlings

Suggested phrases for reporting divergent seeds or seedlings depending upon the result are as follows:

a) if none was found: 'The test performed revealed nothing to indicate that the species (and/or variety) stated by the applicant is incorrect.'

b) if non-conforming seeds were found: 'Out of … seeds examined, … seeds do not conform to the authentic standard sample of the species (and/or variety) stated by the applicant.'

c) if non-conforming seedlings were found: 'Out of .... seeds producing normal seedlings, …% do not conform to the authentic standard sample of the species (and/or variety) stated by the applicant.'

d) if the total working sample was found to be of a species and/or variety other than that stated by the applicant: 'The sample does not conform to the authentic standard sample of the species (and/or variety) stated by the applicant.'

1.5.2.11.2 Results of a field plot examination

The result of a field plot examination must, whenever possible, be reported as a percentage of each other species, other variety or aberrant found. When the expression of the result as a percentage is not possible, appropriate comments regarding the conformity of the sample may be reported.

If nothing worthy of special comment was found the following statement is suggested: 'The results of a field plot examination of this sample revealed nothing to indicate that the species (and/or variety) stated by the sender is (are) incorrect.'

1.5.2.12 Moisture content

This Rule is applicable to both the oven method (9.1.7) and the moisture meter method (9.2.2.7).

The result of a moisture content test must be reported in the space provided to the nearest 0.1 %.

The following additional information must also be reported under 'Other Determinations':

– For the oven method (9.1.7), the method (i.e. duration and temperature) must be reported.
– For the moisture meter method (9.2.2.7), the statement: 'A moisture meter was used' must be entered.
– If germinating seeds were present in the sample, the following statement must be entered: 'Germinating seeds were found in the submitted moisture sample.'
– If mouldy seeds were present in the sample, the following statement must be entered: 'Mouldy seeds were found in the submitted moisture sample.'
– In the case of pelleted seeds (see Chapter 11), the following statement must be entered: 'The seeds of the submitted moisture sample were pelleted, and the moisture content reported is the average of seed and pelleting materials.'
– For *Arachis hypogaea*, one of the following statements must be entered: 'The submitted sample for moisture determination consisted of seeds in their pod' or 'The submitted sample for moisture determination consisted of seeds with the pod removed (shelled seeds)'.

1.5.2.13 Weight determination

The result of a weight determination test must be reported under 'Other determinations' to the number of decimal places used in the determination (10.5.3).

The method used ('Counting the entire working sample' or 'Counting replicates') and the result as calculated according to 10.6 must be reported under 'Other Determinations'.

1.5.2.14 Excised embryo

The result of an excised embryo test must be reported under 'Other determinations' as follows: 'Excised embryo test: ……% of seeds had viable embryos'

Further details may be given at the discretion of the seed testing laboratory, e.g. percentages of seeds that were empty, insect-damaged or physically damaged.

1.5.2.15 Weighed replicates

The result of a weighed replicates test must be reported in the space provided as follows:

– The result of the purity test (if requested), in the spaces provided for purity tests.
– 'N' must be entered in all the spaces provided for reporting the percentages of the components of the germination tests.

The following additional information must also be reported under 'Other determinations':

– average weight of four replicates;
– average number of normal seedlings in four replicates;
– number of normal seedlings per kilogram;
– other information as specified in 1.5.2.6 and 5.9.

Upon request, other seeds found to be present in the weighed replicates may be reported, giving the scientific name(s) and number(s) of seeds found.

1.5.2.16 X-ray test

The results of an X-ray test must be reported under 'Other determinations' as percentages of filled, empty, insect-damaged or physically damaged seeds, as follows:

'X-ray test results:
……% filled
……% empty
……% insect-damaged
……% physically damaged'.

1.5.2.17 Seed vigour test

1.5.2.17.1 Conductivity test

The result of a seed vigour test using the conductivity test method must be reported under 'Other determinations' as follows:

– The result must be expressed in $\mu S\ cm^{-1}\ g^{-1}$ to the nearest 0.1 $\mu S\ cm^{-1}\ g^{-1}$.
– The seed moisture content before the test must be reported. Where the moisture content has been adjusted before the test, both the initial moisture content and the calculated moisture content after adjustment must be reported.
– The results must be accompanied by a statement of the specific variables used in the test (soaking time and temperature)

1.5.2.17.2 Accelerated ageing test

The result of a seed vigour test using the accelerated aging (AA) method must be reported under 'Other determinations' as follows:

– Results are expressed as a percentage, calculated to the nearest whole number (5.8.1) of normal seedlings, abnormal seedlings, hard seeds, fresh seeds and dead seeds. If the result for any of these categories is found to be zero, it must be reported as '0'.
– The seed moisture content before the test must be reported. Where the moisture content has been adjusted before the test, both the initial moisture content and the calculated moisture content after adjustment must be reported.
– The results must be accompanied by a statement of the specific variables used in the test (seed weight per AA box both before and after ageing, ageing time and temperature)

1.5.2.17.3 Controlled deterioration test

The result of a seed vigour test using the controlled deterioration test method must be reported under 'Other determinations' as follows for the two alternative methods of assessing deterioration in 15.8.3.4.3:

a) CD germination test

– Results are expressed as a percentage, calculated to the nearest whole number (5.8.1), and stated as 'Total germinated seeds (normal plus abnormal seedlings) … %' and 'Normal seedlings … %'. If the result for either of these is found to be zero, it must be reported as '0'.
– The results must be accompanied by a statement of the specific variables used in the test (method used to raise seed moisture content, raised seed moisture content, deterioration period and temperature).

b) Conductivity test after deterioration

– The result must be expressed in $\mu S\ cm^{-1}\ g^{-1}$ to the nearest 0.1 $\mu S\ cm^{-1}\ g^{-1}$.
– The results must be accompanied by a statement of the specific variables used during deterioration (method used to raise seed moisture content, raised seed moisture content, deterioration period and temperature) and in the conductivity test (soaking time and temperature).

1.5.2.17.4 Radicle emergence test

The result of a seed vigour test using the radicle emergence test must be reported under 'Other Determinations' as follows:

– Results are reported as a percentage of seeds with emerged radicles calculated to the nearest whole number (5.8.1). If the result is found to be nil, it must be entered as '0'.
– The results must be accompanied by a statement of the temperature used for the test and the time of the radicle emergence counts in hours, e.g. 'Radicle emergence test 90 % with emerged radicles after 66 h at 20 °C.'

1.5.2.17.5 Tetrazolium vigour test

The result of a seed vigour test using the TZ method must be reported under 'Other determinations'. Results are expressed as a percentage, calculated to the nearest whole number of vigorous seeds, e.g.: "Tetrazolium vigour tests using 0.1 % TZ solution for 3 h at 35 °C: 90 % vigorous seeds."

1.5.2.18 Size and grading of seeds

The result of a screening analysis test for size and grading of seeds must be reported under 'Other determinations' as the average of two screening analyses falling within the permitted tolerance limits.

1.5.2.19 Weighted average test for seed lots transported loose in bulk containers

The result of a weighted average test performed on seed lots, as described in Chapter 17, must be reported in the normal way, except that:

a) across the date of sampling, date sample received, date test concluded and test number boxes insert the statement: 'Seed loose in bulk container(s) – see under Other determinations.'

b) Under 'Other determinations', list the test number, date of sampling and date test concluded of all constituent lots together with the statement: 'The test results reported represent the weighted average of the results reported on these certificates which were not significantly different from each other.'

1.5.2.20 Seed mixtures

The results of tests on seed mixtures can only be reported on a Blue International Seed Sample Certificate (see 1.2.2).

For the species tested, 'Seed mixture' together with the mixture composition according to the declaration of the applicant, must be entered.

1.5.2.20.1 Purity and component analysis

The results of the purity analysis are reported according to Chapter 3.

The actual weight of sample examined to the minimum number of decimal places indicated in Table 4.1 must be reported under 'Other determinations', i.e. 'Purity and composition analysis: … g of seed examined.'

The mixture composition is reported under 'Other determinations' in one of the following formats, as requested by the applicant:

1. The percentage by weight of the pure seeds of the mixture components using the total weight of the pure seed fraction. In addition, if applicable, the percentage by weight of the 'inert material according to declaration' referred to the sum of the weights of all mixture components (pure seeds and inert material according to declaration) must be given to one decimal place under 'Other determinations'.
2. The percentage by weight of mixture components, pure seeds or inert material according to declaration using the sum of the weights of the pure seed fraction and the declared inert material.
3. The percentage by number of the pure seeds of the mixture components using the total number of seeds of the pure seed fraction.

In addition, if applicable, the percentage by weight of the 'inert material according to declaration' using the sum of the weights of all mixture components must be given to one decimal place under 'Other determinations'.

1.5.2.20.2 Determination of other seeds by number

The results of a determination of other seeds by number on a seed mixture must be reported according to 4.7.

1.5.2.20.3 Germination, seed viability, seed vigour and other tests using replicates of 100 seeds

Test results are reported only for those species for which methods are given in the appropriate Chapter of the ISTA Rules. The results of these tests must be reported under 'Other determinations'.

Germination test results are not reported in the 'Germination' section of the certificate (an 'N' must be entered there), but under 'Other determinations'. When 100 or more seeds are tested, the percentage results of the test for each mixture component tested are reported to the nearest whole number. The number of seeds tested is also reported. Tolerances as described in the appropriate Chapters are applied to tests of 400, 300, 200 and 100 seeds.

When fewer than 100 seeds are tested, the actual number of seeds in each category (e.g. normal seedlings or viable seeds) is reported, together with the total number of seeds tested.

The method used in the test must be reported on the certificate according to the appropriate Chapter for each component species tested.

1.5.2.20.4 Weight determination

The results as calculated according to 18.7 must be reported under 'Other determinations'.

1.5.2.21 Genetically modified organisms

The result of a genetically modified organism test must be reported under 'Other determinations' as follows:

- the request of the applicant;
- the name and scope (with reference to the target) of the method(s) used;
- a description of the working sample (e.g. pure seed fraction, inert matter present, other seeds present, washed seed);
- the number of seeds in the working sample;
- a description and the source of the reference material used (e.g. certified reference material, provider);
- the limit of detection of the method (when testing seed groups or seed bulk);
- the limit of quantification of the method (when testing seed bulk with a quantitative method).

1.5.2.21.1 Qualitative test results

Suggested phrases for reporting the detection of test targets depending upon the result are as follows:

a) If the test target(s) was(were) not detected: 'The test target was not detected.'

b) If the test target(s) was (were) detected: 'The test target was detected.'

1.5.2.21.2 Quantitative results obtained by multiple qualitative tests of individuals or groups of seeds or seedlings

Results should be reported relative to the percentage of seeds or seedlings showing the test target specified by the applicant. The total number of seeds tested, the number of groups, and the number of seeds per group must be reported. Suggested phrases for reporting such results depending upon the result are as follows:

a) If the test target(s) was (were) not detected: 'The test target(s) was (were) not detected.'

b) If the test target(s) was (were) detected: 'The % of seeds in the lot with the test target(s) was determined to be …%, with a 95 % confidence interval of […%, …%].'
or
'For the test target(s) specified by the applicant, the seed lot meets the specification of ...% (maximum or minimum) with …% confidence.'

If the results do not show evidence that the seed lot meets a given specification with some confidence, then the applicant will report the point estimate with the 95 % confidence interval.

1.5.2.21.3 Quantitative measurements of GMO in bulk samples

Results should be reported relative to the percentage of the test target specified by the applicant by mass or number of DNA copies. The testing plan (e.g. number of replicate seed samples, number of replicate flour samples per seed sample, number of extracts per flour sample, number of replicate measurements per extract) must be indicated.

Required phrases for reporting depending upon the results are as follows:

a) If the test target was not detected (no signal or below the limit of detection): 'The test target was not detected at a level above the limit of detection.'

b) If the test target was detected at a level above the limit of detection and below the limit of quantification: 'The test target was detected at a level below the limit of quantification of the method used.'

c) If seeds showing the test target were found at a level above the limit of quantification: 'The test target(s) percentage in the seed lot was determined to be …% by mass or number of copies, with a 95 % confidence interval of […%, …%]'
or
'For the test target(s) specified by the applicant, the seed lot meets the specification of ...% (maximum or minimum) by mass or number of copies with …% confidence.'

If the results do not show evidence that the seed lot meets a given specification with some confidence, then the applicant will report the point estimate with the 95 % confidence interval.

1.5.2.22 Reporting of results of tests not covered by the Rules

Results must be reported under 'Other determinations'. The test method must be reported and followed by:
'(This method is not covered by the *International Rules for Seed Testing*).'

1.5.3 Reporting of uncertainty of measurement on ISTA Certificates

Uncertainties of measurements associated with test results are accessible through the tolerance tables in the ISTA Rules and are not reported on the ISTA Certificates.

1.5.4 Statement referring to compliance with legislative requirements

In addition to results of tests carried out, it is permissible, at the issuing laboratory's own risk, to make a statement that the seed lot tested meets particular legislative standards. ISTA takes no responsibility for such statements.

1.6 Validity of ISTA Certificates

The results on an original ISTA Certificate are valid until superseded, or partly superseded, by new results on another valid original ISTA Certificate, issued, for the same particular test(s).

If an original certificate is re-issued because of new or amended test results, it must carry a statement indicating that the new results replace previous results, and referring to the Reg. No. of the superseded certificate. In this case, the date entered on the certificate is the new date of issuing.

If an original, duplicate or provisional certificate is lost, a replacement certificate can be issued. In this case, the date entered on the certificate is the same as on the lost certificate.

A new original Orange International Seed Lot Certificate may be issued to supersede a previous certificate for the same seed lot or sublot under the same reference (i.e. seed lot seal and identification) and the same particular test(s), provided that a new submitted sample from that lot or sublot is taken and tested. The new certificate is only valid for the particular lot or sublot that was re-sampled. If a sublot is re-sampled it becomes a new seed lot and must be given a new seed lot identification mark.

A new original Blue International Seed Sample Certificate may be issued to supersede a previous certificate for the same sample and the same particular test(s), provided that the same sample is re-tested. If a new sample is submitted and tested, it must be regarded as independent from the previous sample, and the results on the previous certificate are not superseded.

Previously issued certificates do not need to be returned to the issuing laboratory.

The reference dates are (in order of priority) the date of sampling, the date the test was concluded, and the date of issuing the certificate.

1.7 Disputed results

If the results reported on an ISTA Certificate are contradicted by subsequent test results at another accredited laboratory and the problem cannot readily be resolved, the laboratory issuing the certificate should contact the ISTA Secretariat to determine the correct course of action.

Chapter 2: Sampling

2.1 Object

The object of sampling is to obtain a sample of a size suitable for tests, in which the probability of a constituent being present is determined only by its level of occurrence in the seed lot.

2.2 Definitions

2.2.1 Seed lot

A seed lot is a specified quantity of seed that is physically and uniquely identifiable.

2.2.2 Sublot

A sublot is a portion of not less than 20 % of the seed lot. Each container of a sublot must be marked with the identification of the seed lot.

2.2.3 Primary sample

A primary sample is a portion taken from the seed lot during one single sampling action.

2.2.4 Composite sample

The composite sample is formed by combining and mixing all the primary samples taken from the seed lot.

2.2.5 Subsample

A subsample is a portion of a sample obtained by reducing a sample.

2.2.6 Submitted sample

A submitted sample is a sample that is to be submitted to the testing laboratory and may comprise either the whole of the composite sample or a subsample thereof. The submitted sample may be divided into subsamples packed in different material meeting conditions for specific tests (e.g. moisture or health).

2.2.7 Duplicate sample

A duplicate sample is another sample obtained for submission from the same composite sample and marked 'Duplicate sample'.

2.2.8 Working sample

The working sample is the whole of the submitted sample or a subsample thereof, on which one of the quality tests described in these ISTA Rules is made and must be at least the weight prescribed by the ISTA Rules for the particular test.

2.2.9 Sealed

Sealed means that a container in which seed is held is closed in such a way, that it cannot be opened to gain access to the seed and closed again, without either destroying the seal or leaving evidence of tampering. This definition refers to the sealing of seed lots, as well as of seed samples.

2.2.10 Self-sealing containers

The 'valve-pack' bag is a specific type of self sealing container. It is filled through a sleeve-shaped valve which is automatically closed by the completion of filling the bag.

2.2.11 Marked/labelled

A container of a seed lot can be considered as marked or labelled when there is a unique identification mark on the container, which defines the seed lot to which the container belongs. All containers of a seed lot must be marked with the same unique seed lot designation (numbers, characters or combination of both). Should the unique identification mark be indicated on a label attached to the container, it must not be possible to remove the label and replace it with another label without showing signs of tampering. Marking of samples and subsamples must ensure that there is always an unambiguous link between the seed lot and the samples and subsamples.

2.2.12 Treated seed

'Seed treatment' is a generic term which indicates that a seed lot has been subjected to:

a) the application of a compound including chemicals, nutrients or hormones
b) the application of a biological product including micro-organisms
c) a process including wetting and drying
d) an energy form including heat, radiation, electricity or magnetism

but does not specify the application method.

Seed treatment does not significantly change the size, shape or add to the weight of the seeds in the lot.

2.2.13 Coated seeds

Coated seeds are seeds covered with material that may contain pesticides, fungicides, dyes or other additives. The following types of coated seeds are defined:

Seed pellets. More or less spherical units, usually incorporating a single seed with the size and shape of the seed no longer readily evident.

Encrusted seed. Units more or less retaining the shape of the seed with the size and weight changed to a measurable extent.

Seed granules. Units more or less cylindrical, including types with more than one seed per granule.

Seed tapes. Narrow bands of material, such as paper or other degradable material, with seeds spaced randomly, in groups or in a single row.

Seed mats. Broad sheets of material, such as paper or other degradable material, with seeds placed in rows, groups or at random throughout the sheets.

2.2.14 Small seed lots

Small seed lots are seed lots of high-value seed, where obtaining a submitted sample of standard size could have a substantial effect on the quantity of the remaining seed lot. High-value seed includes, but is not limited to, hybrid vegetable seeds that are sold per seed, or seed that is not commercially available and is used for research or for higher generation multiplication.

2.3 General principles

A composite sample is obtained from the seed lot by taking primary samples from different positions in the whole seed lot and combining them. From this composite sample, subsamples are obtained by sample reduction procedures at one or more stages forming the submitted sample and finally the working samples for testing. For issuing ISTA Certificates, specific requirements have to be fulfilled as given under 2.5.4. Further information on seed sampling can be found in the current *ISTA Handbook on Seed Sampling*.

2.4 Apparatus

Sampling and sample reduction must be performed using appropriate techniques and equipment that is clean and in good condition as described in 2.5.1 and 2.5.2.2.

Containers used to collect primary samples, composite samples and during mixing and dividing must be static-free to avoid chaff or small seeds adhering to the inside of the containers.

2.5 Procedures

2.5.1 Procedures for sampling a seed lot

2.5.1.1 Preparation of a seed lot and conditions for sampling

At the time of sampling, the seed lot must be as uniform as practicable. If the seed lot is found to be obviously heterogeneous, sampling must be refused or stopped. In cases of doubt heterogeneity can be determined as described under 2.9.

Seed may be sampled in containers or when it enters containers. The containers must be fit for purpose, i.e. must not damage the seed, and must be clean to avoid cross contamination. The containers must be labelled or marked before or just after sampling is completed.

The seed lot must be so arranged that each part of the seed lot is conveniently accessible.

2.5.1.2 Minimum sampling intensity

For seed lots in containers holding up to and including 100 kg, the minimum sampling intensity is the following:

a) For containers holding between 15 kg and 100 kg (inclusive) of seed, the number of primary samples according to Table 2.1.
b) For containers holding less than 15 kg of seed, containers must be combined into sampling units not exceeding 100 kg, e.g. 20 containers of 5 kg, 33 containers of 3 kg or 100 containers of 1 kg. The sampling units must be regarded as containers as described in Table 2.1.
c) For seed pellets, seed granules, seed tapes and seed mats, containers of less than 300 000 seed units must be combined to sampling units not exceeding 2 000 000 seeds. The sampling units must be regarded as containers as described in Table 2.1.

Table 2.1. Minimum sampling intensity for seed lots in containers holding up to and including 100 kg seed

Number of containers	Minimum number of primary samples to be taken
1–4	3 primary samples from each container
5–8	2 primary samples from each container
9–15	1 primary sample from each container
16–30	15 primary samples, one each from 15 different containers
31–59	20 primary samples, one each from 20 different containers
60 or more	30 primary samples, one each from 30 different containers

When sampling seed in containers holding more than 100 kg of seed, or from streams of seed entering containers, the sampling intensity according to Table 2.2 must be regarded as the minimum requirement.

Table 2.2. Minimum number of primary samples to be taken from seed lots in containers holding more than 100 kg of seed, or from seed streams

Seed lot size	Number of primary samples to be taken
Up to 500 kg	At least five primary samples
501–3 000 kg	One primary sample for each 300 kg, but not less than five
3 001–20 000 kg	One primary sample for each 500 kg, but not less than 10
20 001 kg and above	One primary sample for each 700 kg, but not less than 40

When sampling a seed lot of up to 15 containers, regardless of their size, the same number of primary samples must be taken from each container.

Sampling intensity for coated seeds is as described in Tables 2.1 and 2.2.

2.5.1.3 Taking primary samples

When defining the number and/or the size of primary samples, the seed sampler needs to ensure (besides meeting the minimum sampling intensity) that the minimum amount of seed required for the requested test(s) is sent to the testing laboratory and enough seed remains available for obtaining duplicate samples if requested.

Primary samples of approximately equal size must be taken from a seed lot, irrespective of where in the lot or container the primary sample is taken.

When the seed lot is in containers, the containers to be sampled must be selected at random or according to a systematic plan throughout the seed lot. Primary samples must be drawn from the top, middle and bottom of containers, but not necessarily from more than one position in any container, unless so specified in Tables 2.1 and 2.2.

When the seed is in bulk or in large containers, the primary samples must be drawn from random positions.

Containers must be opened or pierced for abstraction of primary samples. The sampled containers must then be closed or the contents transferred to new containers.

When seed is to be packed in special types of containers (e.g. small, not penetrable, or moisture-proof containers), it should be sampled, if possible, either before or during the filling of the containers.

Sampling seed lots of seed tapes and seed mats should be done by taking packets or pieces of tape or mat.

The instruments being used must neither damage the seed nor select according to seed size, shape, density, chaffiness or any other quality trait. All sampling apparatus must be clean before use to prevent cross contaminations. Triers must be long enough so that the opening at the tip reaches at least half of the diameter of the container. When the container is not accessible from opposite sides, the trier must be long enough to reach the opposite side. Sampling seed lots may be done by one of the methods listed below.

a) Automatic sampling from a seed stream. Seed may be sampled by automatic sampling devices, provided that the instrument uniformly samples the cross section of the seed stream and the material entering the instrument does not bounce out again. It may be operated either under manual or automatic control. The intervals between taking primary samples should be constant.

b) Manual sampling from a seed stream. Seed streams may also be sampled by using manual instruments when fulfilling the requirements listed under a).

c) Sampling stick. The sampling stick (e.g. stick trier, sleeve type trier, spiral trier) consists of two parts, one of which fits loosely inside the other, but tightly enough so that seed or impurities do not slip between them. The outer part has a solid pointed end. Both parts have slots in their walls so that the cavity of the inner part can be opened and closed by moving the two parts against each other by either a twisting or a push-pull motion.

The sampling stick may be used horizontally, diagonally or vertically. The spiral trier has slots in a spiral arrangement for their subsequent opening from the tip to the handle and may only be used for seeds of a size smaller than *Triticum aestivum*.

However, when used vertically or diagonally downwards, the sampling stick must either have partitions dividing the instrument into a number of compartments or have slots in a spiral arrangement. The minimum inside diameter should be wide enough to allow the smooth and free flow of seed and contaminants into the sampling stick.

When using the sampling stick, insert it in the closed position into the container, gently push it so that the point reaches the required position, open the sampling stick, agitate it slightly to allow it to fill completely, gently close and withdraw it and empty the primary sample into a container. Care should be exercised in closing the sampling stick so that seeds are not damaged.

d) Nobbe trier. The Nobbe trier (dynamic spear) is a pointed tube with an opening near the pointed end. Seed passes through the tube and is collected in a container. The minimum internal diameter of the Nobbe trier should be wide enough to allow the smooth and free flow of seed and contaminants through the trier.

When using the Nobbe trier, insert it at an angle of about 30° to the horizontal plane with the opening facing down, push the trier until it reaches the required position and revolve it through 180°. Withdraw it with decreasing speed from the container, gently agitating the trier to help maintain an even flow of seed, and collect the seed sample coming from the trier in a suitable container.

e) Cargo sampler. The cargo sampler (bulk sampler) consists of a special type of chamber that is fixed to a shaft. The lower part of the chamber is cone-shaped with a pointed end. To reach a greater depth, the shaft may be lengthened by screwing on successive extensions. There is a closing system in the chamber that may be a collar on the outside of the instrument, a wing connected to a door or a valve with a spring. Some cargo samplers can be closed before they are drawn back from the sampling position; others cannot be closed, so that the filled chamber is open during withdrawal. For all species, the minimum inside diameter can be about 35 mm and the depth 75 mm. When using the cargo sampler, insert it in the closed position into the container, gently push it vertically into the seed so that the point reaches the required position, pull the cargo sampler back about 10 cm or turn it (depending on the closing system), agitate it slightly to allow it to fill completely, gently close if possible and withdraw it and empty the primary sample into a container. Care should be exercised in closing the cargo sampler, so that the seeds are not damaged.

f) Sampling by hand. This method can be used for all species and may be the most suitable method for seed that may be damaged by the use of triers, seeds with wings, seeds with low moisture content, seed tapes and seed mats.

For hand sampling seed in containers, all positions inside the containers must be accessible. Containers with layers which are not accessible from the regular opening may have to be cut open, sampled and repackaged. Containers may also be partially or completely emptied during the sampling process to gain access to all positions in the containers. For sampling by hand, clean the hand and roll the sleeve up if necessary, insert the open hand into the container to the required position, close and withdraw the hand, taking great care that the fingers remain tightly closed about the seeds so none may escape, and empty the hand into a receiving pan.

2.5.1.4 Obtaining the composite sample

Where possible, the primary samples are compared with each other during sampling. The primary samples can only be combined to form the composite sample if they appear to be uniform. If not, the sampling procedure must be stopped. When primary samples are collected directly into one container, the content of this container may be regarded as the composite sample only if it appears uniform. If not, it must not be used for obtaining a submitted sample.

2.5.1.5 Obtaining the submitted sample

The submitted sample must be obtained by reducing the composite sample to an appropriate size by one of the methods referred to in 2.5.2.2. In the case of very large composite samples, a method according to 2.5.1.3 may also be used. Obtaining subsamples such as for moisture testing must be carried out in such a way that changes in moisture content are minimal.

The composite sample can be submitted to the seed testing laboratory if it is of appropriate size or if it is difficult to mix and reduce the composite sample properly under warehouse conditions.

Duplicate samples, which were requested not later than at the time of sampling, must be prepared in the same way as the submitted sample.

2.5.1.6 Dispatch of the submitted sample

The submitted sample must be marked with the same identification as the seed lot. For an Orange International Seed Lot Certificate, the sample must be sealed. The additional information required according to 1.4.2 as well as the name of any chemical treatment applied must be provided.

Submitted samples must be packed so as to prevent damage during transit. Submitted samples should be packed in breathable containers.

Subsamples for moisture testing, and samples from seed lots which have been dried to low moisture content, must be packed in moisture-proof containers which contain as little air as possible. Submitted samples for germination tests, viability tests and health tests may only be packed in moisture-proof containers if suitable storage conditions can be assured.

Submitted samples must be dispatched to the seed testing laboratory without delay.

2.5.1.7 Storage of submitted samples before testing

Every effort must be made to start testing a submitted sample on the day of receipt. Storage of orthodox seeds, when necessary, should be in a cool, well-ventilated room.

Non-orthodox (i.e. recalcitrant or intermediate) seeds should be tested as soon as possible after obtaining the submitted sample from the composite sample without any storage. Handling of the submitted sample and, if necessary, storage should be done under species specific optimum conditions.

2.5.2 Procedures for obtaining the submitted and working sample

2.5.2.1 Minimum size of working sample

Minimum sizes of working samples are prescribed in the appropriate chapter for each test. The working sample weights for purity analyses given in Table 2A are calculated to contain at least 2 500 seeds. These weights are recommended for normal use in purity tests, see 3.5.1.

The sample weights in column 5 of Table 2A, Part 1, for counts of other species are 10 times the weights in column 4, subject to a maximum of 1000 g.

Working samples of all coated seeds except those defined as treated seed in 2.2.11 must contain at least the number of pellets, seeds or granules indicated in column 3 of Table 2B, Part 1 and Part 2. If a smaller sample is used, the actual number of pellets, seeds or granules in the sample must be reported.

2.5.2.2 Sample reduction methods

If the seed sample needs to be reduced to a size equal to or greater than the size prescribed, the seed sample must first be thoroughly mixed. The submitted/working sample must then be obtained either by repeated halving or by abstracting and subsequently combining small random portions. The apparatus and methods for sample reduction are described in 2.5.2.2.1 to 2.5.2.2.4. One, two or more of these methods may be used in one sample reduction procedure. When using one of the dividers described for seed pellets the distance of fall must not exceed 250 mm.

After obtaining a working sample the remainder must be re-mixed before a second working sample is obtained.

Except in the case of seed health, the method of hand halving must be restricted to certain genera listed in 2.5.2.2.4. Only the spoon method and the hand halving method may be used in the laboratory to obtain working samples for seed health testing where other samples or equipment may be contaminated by spores or other propagating material.

For seed tapes and mats take pieces of tape or mat at random, to provide sufficient seeds for the test.

To obtain the submitted sample for moisture content determination (2.5.4.5 c), subsamples must be taken in the following way: first, mix the composite sample. Then, take a minimum of three samples from different positions and combine them to create the subsample for moisture of the required size. The subsample for moisture must be taken as soon as possible to avoid changes in moisture content.

To obtain the working sample for moisture content determination (9.1.5.2) subsamples must be taken in the following way: before taking the subsample, mix the sample by either stirring the sample in its container with a spoon or by placing the opening of the original container against the opening of a similar container and pour the seed back and forth between the two containers. Take a minimum of three subsamples with a spoon from different positions and combine them to create the subsample of the required size. The seed must not be exposed to the air during sample reduction for more than 30 s.

2.5.2.2.1 Mechanical divider method

This method is suitable for all kinds of seeds except some very chaffy seeds. The apparatus divides a sample passed through it into two or more approximately equal parts. The submitted sample can be mixed by passing it through the divider, recombining the parts and passing the whole sample through a second time, and similarly, a third time if necessary. The sample is reduced by passing the seed through repeatedly and removing parts on each occasion. This process of reduction is continued until a working sample of approximately, but not less than, the required size is obtained.

a) Conical divider. The conical divider (Boerner type) consists of a hopper, cone, and series of baffles directing the seed into two spouts. The baffles form alternate channels and spaces of equal width. They are arranged in a circle and are directed inward and downward, the channels leading to one spout and the spaces to an opposite spout. A valve or gate at the base of the hopper retains the seed. When the valve is opened the seed falls by gravity over the cone where it is evenly distributed to the channels and spaces, then passes through the spouts into the seed pans.
Dividers with more than 18 channels have been found to be suitable. Channels must be wide enough to allow the smooth free flow of seed and contaminants.

b) Soil divider. The soil divider (riffle divider) consists of a hopper with about 18 attached channels or ducts alternately leading to opposite sides. Channels must be wide enough to allow the smooth free flow of seed and contaminants.
In using the divider the seed is placed evenly into a pouring pan and then poured in the hopper at approximately equal rates along the entire length. The seed passes through the channels and is collected in two receiving pans.

c) Centrifugal divider. In the centrifugal divider (Gamet type) the seed flows downward through a hopper onto a shallow cup or spinner. Upon rotation of the spinner by an electric motor the seeds are thrown out by centrifugal force and fall downward. The circle or area where the seeds fall is equally divided into two parts by a stationary baffle so that approximately half the seeds fall in one spout and half in the other spout.
The centrifugal divider tends to give variable results unless the spinner is operated after having poured the seed centrally into the hopper.

d) Rotary divider. The rotary divider comprises a rotating crown unit with 6 to 10 attached subsample containers, a vibration chute and a hopper. In using the divider the seed is poured into the hopper and the rotary divider is switched on so that the crown unit with the containers rotates with approx. 100 rpm and the vibration chute starts to feed the seed into the inlet cylinder of the rotating crown. The feeding rate and therefore the duration of the dividing operation can be adjusted by the distance between the funnel of the hopper and the chute and the vibration intensity of the chute.

There are two principles: (i) The inlet cylinder feeds the seed centrally onto a distributor within the rotating crown distributing the seed to all containers simultaneously. (ii) The inlet cylinder feeds the seed de-centrally into the inlets of the containers rotating underneath the inlet cylinder so that the seed stream is subdivided into a lot of subsamples.

e) **Variable sample divider.** The variable sample divider consists of a pouring hopper and a tube underneath that rotates with about 40 rpm. The tube distributes the seed stream from the pouring hopper onto the inner surface of a further hopper, which is well fitted into a third hopper all being concentric. In the second and the third hopper there are slots that comprise 50 % of the perimeter of the hoppers. 50 % of the seed will pass through the two hoppers into a collecting pan. The other 50 % will stay within the hoppers and will then go into a second collecting pan. The two hoppers can be twisted against each other resulting in more narrow slots. The effect is that a smaller percentage will pass through the slots. Either the smaller sample outside the hoppers or the bigger sample inside the hoppers can be used as the required sample. The position of the two hoppers in relation to each other can be adjusted accurately, resulting in pre-determined subsample sizes.

2.5.2.2.2 Modified halving method

The apparatus comprises a tray into which fits a grid of equal-sized cubical cells, open at the top and every alternate one having no bottom. After preliminary mixing, the seed is poured evenly over the grid. When the grid is lifted, approximately half the sample remains on the tray. The submitted sample is successively halved in this way until a working sample, of approximately but not less than the required size, is obtained.

2.5.2.2.3 Spoon method

The spoon method is recommended for sample reduction for seed health testing (7.4.1). For other tests it is restricted to species with seeds smaller than *Triticum* spp., to the genera *Arachis*, *Glycine* and *Phaseolus*, and to tree genera *Abies*, *Cedrus* and *Pseudotsuga*. A tray, a spatula and a spoon with a straight edge are required. After preliminary mixing, pour the seed evenly over the tray; do not shake the tray thereafter. With the spoon in one hand, the spatula in the other, and using both, remove small portions of seed from not less than five random places. Sufficient portions of seed are taken to constitute a subsample of the required size.

2.5.2.2.4 The hand halving method

This method is restricted to the following genera of chaffy seeds:

Agrimonia, Andropogon, Anthoxanthum, Arrhenatherum, Astrebla, Beckmannia, Bouteloua, Brachiaria, Briza, Cenchrus, Chloris, Dichanthium, Digitaria, Echinochloa, Ehrharta, Elymus, Eragrostis, Gomphrena, Gossypium (linted seed only), *Melinis, Oryza, Pennisetum* (non *glaucum*)*, Psathyrostachys, Scabiosa, Sorghastrum, Stylosanthes* (non *guianensis*), *Trisetum;*

to the following genera of easily damaged fragile seeds:

Arachis, Glycine and *Phaseolus*;

and to the following genera and species of tree and shrub seeds:

Acer, Aesculus, Ailanthus, Castanea, Cedrela, Corylus, Fagus, Fraxinus, Juglans, Liriodendron, Pinus cembra, Pinus pinea, Platanus, Populus, Quercus, Salix, Tectona, Ulmus.

The hand halving method can also be used with the species where all other dividing methods are extremely difficult or impossible to use.

For all other species it can be used only to obtain working samples in the laboratory for seed health tests (7.4.1).

For applying the hand halving method, pour the sample evenly onto a smooth clean surface, thoroughly mix the seed into a mound with a flat-edged spatula, divide the mound into half and halve each half again – giving four portions – and halve each portion again – giving eight portions, arrange the portions in two rows of four, combine and retain alternate portions: e.g. combine the first and third portions in the first row with the second and fourth in the second row, remove the remaining four portions. Repeat the procedure using the retained portions until obtaining the required sample size.

2.5.3 Storage of samples after testing

The primary aim of storage of samples after testing is to be able to repeat the original tests carried out on the submitted sample. Therefore, storage conditions should be such that changes in the seed quality traits tested are minimal. For example, in the case of the purity test or other seed count, the sample should be stored in such a way that the physical identity is kept. In the case of germination, viability or health test of orthodox seeds the sample should be stored under cool and dry conditions. For such tests in recalcitrant and intermediate seeds of tropical and subtropical species, long term storage is not possible. For such seed of temperate species storability depends on the fungal status and to some extent whether the seed is dormant or not. All factors pertaining to storage need to be determined on a species basis. Protection against insects and rodents may be necessary.

To provide for re-testing by the original or by another seed testing laboratory, samples on which ISTA Certificates have been issued must be stored at least for one year from the receipt of the sample. Submitted samples in moisture proof containers, and samples of recalcitrant or intermediate species, must be stored under appropriate conditions for as long as it can be expected that the results of a re-test are not affected by the storage.

When a re-test in a different testing laboratory is required, a portion must be drawn from the stored sample in accordance with 2.5.2.2, and submitted to the designated testing laboratory. The remainder must be retained in store.

2.5.4 Conditions for issuing Orange International Seed Lot Certificates

The sampling methods laid down in the ISTA Rules must be followed when seed samples are drawn for the issue of Orange International Seed Lot Certificates. Further conditions have to be fulfilled as listed below.

2.5.4.1 Seed lot size

The seed lot must not exceed the quantity indicated in column 2 of Table 2A, subject to a tolerance of 5 % with the exception of:

a) seed being transported loose in bulk containers. The conditions under which this exception may be permitted are laid down in Chapter 17.

b) seed pellets, seed granules, seed tapes or seed mats. The maximum number of seeds that a seed lot of seed pellets, seed granules, seed tapes or seed mats may contain is 1 000 000 000 (10 000 units of 100 000) except that the weight of the seed lot, including the coating material may not exceed 40 000 kg subject to a tolerance of 5 % (42 000 kg).

c) seed lots of species of *Poaceae* produced in a seed company that has been approved to make larger seed lots. The conditions under which this may be permitted are laid down in 2.5.4.2.

d) seed lots of species of *Poaceae* produced in a seed company that has applied for approval to make larger seed lots according to 2.5.4.2. The heterogeneity of the seed lot must be tested according to 2.9 and the seed lot must not show significant heterogeneity.

Maximum lot size for treated and encrusted seeds is defined by applying the quantities indicated in Table 2A to the seeds without coating material.

A seed lot in excess of the prescribed quantity must be subdivided into seed lots not larger than the prescribed quantity, each of which must be labelled or marked with a separate seed lot identification.

2.5.4.2 Large seed lots of *Poaceae*

2.5.4.2.1 Definitions

Large seed lots of *Poaceae* species may have a maximum size of 25 000 kg (with a 5 % tolerance), if produced by an approved production plant.

For the purposes of large seed lots of *Poaceae* species, the following species with similar characteristics are regarded as two species groups:

Species group 1:

Lolium perenne, Lolium multiflorum, Lolium ×hybridum (previously *Lolium ×boucheanum*), *×Festulolium, Festuca pratensis, Festuca arundinacea* and *Phleum pratense*.

Species group 2:

Festuca rubra, Festuca ovina, Festuca filiformis, Festuca heterophylla, Dactylis glomerata, Poa pratensis and *Poa trivialis*.

Approval which was granted following heterogeneity testing of any species of a group is also valid for all other species of the same group.

For all other species of *Poaceae*, approval must be requested and granted separately for each individual species.

2.5.4.2.2 Approval

Approval is granted after heterogeneity testing of six large seed lots of the species group or individual species for which the approval is requested. Heterogeneity testing must be carried out according to 2.9, and must as a minimum be based on purity and other seed count. At least five of the six tested seed lots must have a non-significant level of heterogeneity.

2.5.4.2.3 Check sampling and testing

After approval, the large seed lots of a production plant must be monitored by check sampling and further heterogeneity testing, according to 2.9, and as a minimum based on purity and other seed count.

Of the first 100 large seed lots per species group, 4 are randomly selected (4 % check sampling) and tested for heterogeneity. If none of these are heterogeneous, the check-sampling rate is reduced to 3 % for the following 100 lots, and to 2 % for subsequent lots.

However, if a check sample is found to show significant heterogeneity, the check-sampling rate must remain at 4 %, or again be increased from 3 to 4 % or from 2 to 3 %, as applicable (Fig. 2.1).

In six consecutive check samples tested, a maximum of one sample may show significant heterogeneity.

Hence, a heterogeneous sample must be followed by at least five non-heterogeneous samples in order for approval to be retained (Fig. 2.1).

2.5.4.2.4 Withdrawal of approval

If more than one of the last six consecutive check samples tested shows significant heterogeneity, approval must be withdrawn for the species or species group and production plant concerned, and the company must re-apply for approval (Fig. 2.1).

2.5.4.2.5 Responsibility

The Certifying or Designated Authority in a country is responsible for:

- the decision of approval of the seed company (production plant);
- ensuring that each production plant is approved separately, if a seed company has more than one production plant;
- ensuring that the testing is done by an ISTA-accredited laboratory;
- the check-sampling programme.

2.5.4.3 Marking/labelling and sealing of containers

The seed lot must be in marked/labelled containers which are self-sealing, sealed (or capable of being sealed) or under the control of the seed sampler.

Where the seed lot is already marked/labelled and sealed before sampling, the seed sampler must verify the marking/labelling and sealing on the containers. Otherwise the sampler has to mark/label the containers and must seal every container before the seed lot leaves their control.

The samplers are personally responsible for the seals, labels and bags supplied to them and it is their duty to ensure that primary, composite or submitted samples must never be left in the hands of persons not authorised by the seed testing laboratory unless they are sealed in such a way that they cannot be tampered with.

2.5.4.4 Sampling from the seed lot

For sampling from the seed lot methods listed under 2.5.1 must be used. Automatic seed samplers must be approved by the ISTA seed testing laboratory according to the "Protocol for the approval of automatic seed samplers" as approved by the ISTA membership and published on the ISTA website.

An Orange International Seed Lot Certificate issued on a seed lot (see 2.2.1) is still valid after re-packaging the seed lot in new containers provided that:

a) The identity of the seed in the initial seed lot is preserved.

b) The seed lot designation (see 2.2.10) is not changed.

c) The moving of the seed into the new containers is done under the control of an ISTA seed sampler.

d) There is no processing of the seed during filling of the new containers.

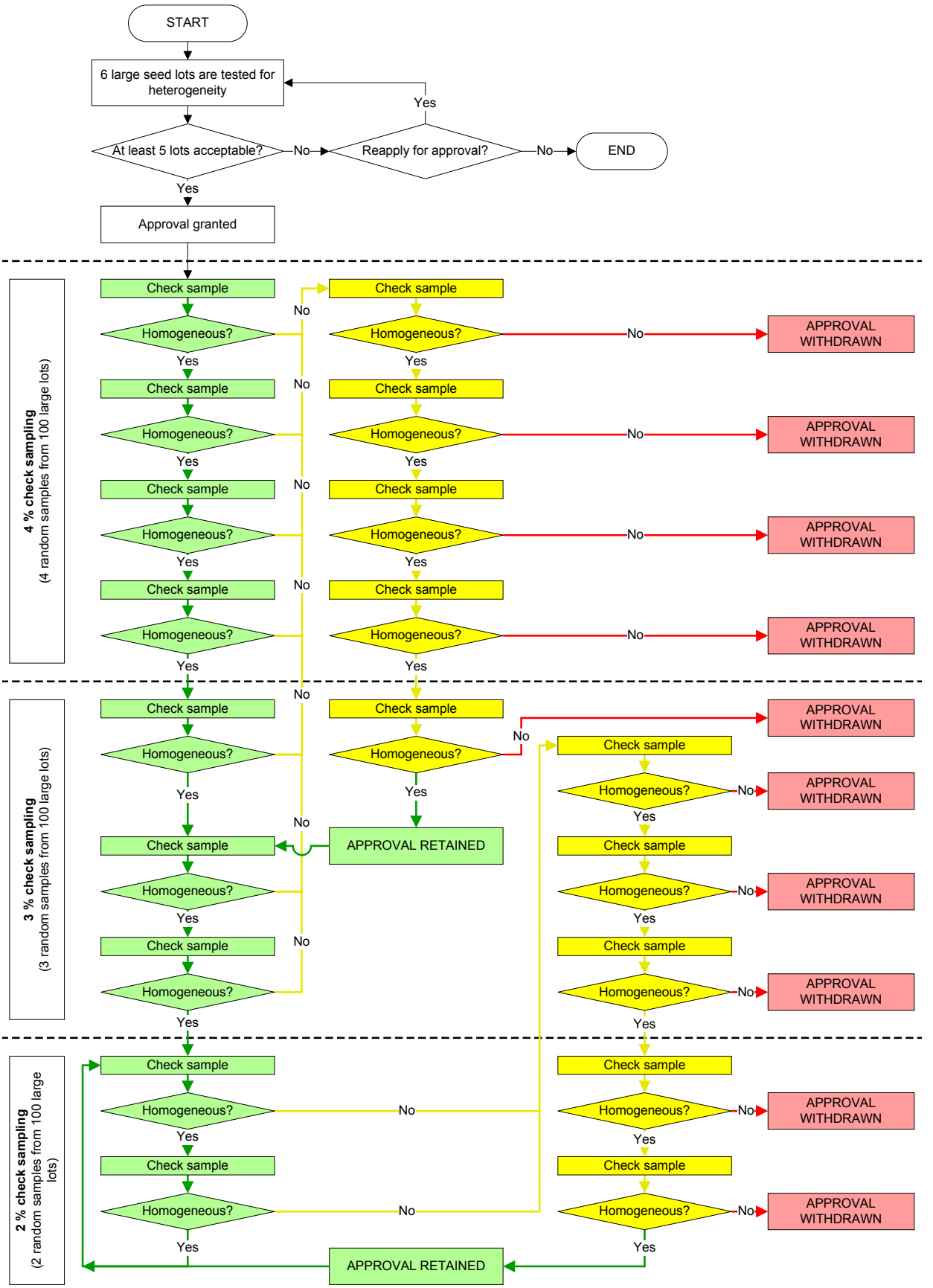

Figure 2.1. Flow chart describing the approval procedure and check-sampling programme with regard to large seed lots of *Poaceae* species (2.5.4.2.2–4).

2.5.4.5 Submitted sample

The minimum sizes of submitted samples are as follows:
– If a determination of other seeds by number is required: the weight prescribed in Table 2A, column 3;

or

– if a determination of other seeds by number is not required: the weight prescribed for the working sample for purity analysis in Table 2A, column 4, or in 3.5.1.

For certain tests or under certain conditions, the following exceptions apply:

a) For coated seeds, if a determination of other seeds by number or size grading is required: the number of seeds indicated in Table 2B, Parts 1 and 2, column 2.

b) For coated seeds, if a determination of other seeds by number or size grading is not required: the number of seeds indicated for the working sample for purity analysis in Table 2B, Parts 1 and 2, column 3.

c) For moisture determination of species that must be ground (see Table 9A): 100 g. For all other species: 50 g.

   When moisture meters are to be used for testing, a larger sample size may be necessary. Contact the accredited ISTA laboratory for specific instructions.

d) For verification of species and variety: as prescribed in Chapter 8.

e) For germination or viability tests of small seed lots (2.2.14): the number of seeds required to complete one of these tests plus 25 seeds for identity assurance.

f) For determination of other seeds of small seed lots (2.2.14): the amount necessary to complete this test according to Chapter 4.

If the submitted sample is smaller than prescribed above, the sampler must be notified accordingly and analysis withheld until sufficient seed is received in a single submitted sample.

The submitted sample must be sealed and labelled or marked.

2.5.4.6 Sample reduction

For sample reduction, methods listed under 2.5.2.2 must be used.

2.5.4.7 Storage of submitted samples after testing

Submitted samples on which ISTA Certificates have been issued must be stored. In the case of small seed lots (see 2.2.14), the remainder of the submitted sample, minus 25 seeds for assurance of identity, may be sent back to the applicant. The seed testing laboratory cannot be held responsible for any deterioration of the sample during storage.

2.6 Calculation and expression of results

No specific calculation or expression of results required except under 2.9 for heterogeneity tests.

2.7 Reporting of results

No specific calculation or expression of results required except under 2.9 for heterogeneity tests.

2.8 Tables for lot size and sample sizes

Table 2A is referred to in various chapters of the ISTA Rules and indicates weights of lots and samples for different species, and the specific names to be used in reporting test results. Each sample size is derived from a nominal thousand-seed weight (TSW) for each species which, on the available evidence, is expected to be adequate for the majority of samples tested.

Where a weight is not given in the table and a count of other species is requested, the submitted sample must contain a minimum of 25 000 seeds.

Note 1: Names with an asterisk are not included in the ISTA List of Stabilised Plant Names. Names without an asterisk are included in the ISTA List of Stabilised Plant Names (but not the synonym which follows some of these names), or, in the case of generic names (e.g. *Pyrus* spp.) conserved by the International Botanical Congress and listed in the International Code of Nomenclature. Changes in the stabilised list agreed at the 2013 ISTA Congress are included in this version of Table 2A. Where plant names have been changed, the old name is included with a cross reference to the new name. This applies only to 2013 Congress changes; previous cross references have been removed.

Note 2: For all species the maximum seed lot size stated can be exceeded by no more than 5 %, except for:

a) seed being transported loose in bulk containers. The conditions under which this exception may be permitted are stated in Chapter 17;

b) seed pellets, seed granules, seed tapes or seed mats (see 2.5.4.1);

c) species of *Poaceae* listed in Table 2A Part 1 (see 2.5.4.2).

For production plants approved under 2.5.4.2, the maximum seed lot weight for *Poaceae* species listed in Table 2A Part 1 is 25 000 kg (with a 5 % tolerance).

Table 2A Part 1. Lot sizes and sample sizes: agricultural and vegetable seeds

Species	Maximum weight of lot (kg) (except see 2.8 Note 2)	Minimum submitted sample (g)	Minimum working samples (g)	
			Purity analysis (3.5.1)	Other seeds by number (4.5.1)
1	2	3	4	5
Abelmoschus esculentus (L.) Moench	20 000	1 000	140	1 000
Achillea millefolium L.	10 000	5	0.5	5
Aeschynomene americana L.	10 000	120	12	120
Agropyron cristatum (L.) Gaertn.	10 000	40	4	40
Agropyron desertorum (Fisch. ex Link) Schult.	10 000	60	6	60
Agrostis canina L.	10 000	5	0.25	2.5
Agrostis capillaris L.	10 000	5	0.25	2.5
Agrostis gigantea Roth	10 000	5	0.25	2.5
Agrostis stolonifera L. (includes *A. palustris* Hudson)	10 000	5	0.25	2.5
Allium cepa L.	10 000	80	8	80
Allium fistulosum L.	10 000	50	5	50
Allium porrum L.	10 000	70	7	70
Allium schoenoprasum L.	10 000	30	3	30
Allium tuberosum Rottler ex Spreng.	10 000	100	10	100
Alopecurus pratensis L.	10 000	30	3	30
Alysicarpus vaginalis (L.) DC.	10 000	40	4	40
Andropogon gayanus Kunth	10 000	80	8	80
Andropogon gerardi Vitman	10 000	70	7	70
Andropogon hallii Hack.	10 000	100	10	100
Anethum graveolens L.	10 000	40	4	40
Anthoxanthum odoratum L.	10 000	20	2	20
Anthriscus cerefolium (L.) Hoffm.	10 000	60	6	60
Anthyllis vulneraria L.	10 000	60	6	60
Apium graveolens L.	10 000	10	1	10
Arachis hypogaea L.	30 000	1 000	1 000	1 000
Arctium lappa L.	10 000	50	5	50
Arrhenatherum elatius (L.) P.Beauv. ex J.Presl & C.Presl	10 000	80	8	80
Asparagus officinalis L.	20 000	1 000	100	1 000
Astragalus cicer L.	10 000	90	9	90
Astrebla lappacea (Lindl.) Domin	10 000	200	20	200
Atriplex hortensis L.	5 000	10	2.5	–
Atropa belladonna L.	10 000	30	3	30
Avena nuda L.	30 000	1 000	120	1 000
Avena sativa L.	30 000	1 000	120	1 000
Avena strigosa Schreb.	30 000	500	50	500
Axonopus compressus (Sw.) P.Beauv.	10 000	10	1	10
Axonopus fissifolius (Raddi) Kuhlm.	10 000	10	1	10
Beckmannia eruciformis (L.) Host	10 000	20	2	20
Beta vulgaris L. (multi-germ varieties)	20 000	500	50	500
Beta vulgaris L. (mono-germ varieties)	20 000	500	30	300
Borago officinalis L.	10 000	450	45	450
Bothriochloa insculpta (Hochst. ex A.Rich.) A.Camus	10 000	20	2	20
Bothriochloa pertusa (L.) A.Camus	10 000	10	1	10
Bouteloua gracilis (Kunth) Lag. ex Griffiths	10 000	60	6	60
Brachiaria brizantha (Hochst. ex A.Rich) Stapf	10 000	100	10	100
Brachiaria decumbens Stapf	10 000	100	10	100
Brachiaria humidicola (Rendle) Schweick.	10 000	100	10	100
Brachiaria mutica (Forssk.) Stapf	10 000	30	3	30
Brachiaria ramosa (L.) Stapf	10 000	90	9	90
Brachiaria ruziziensis R.Germ. & C.M.Evrard	20 000	150	15	150
Brassica carinata A. Braun	10 000	100	10	100
Brassica juncea (L.) Czern.	10 000	40	4	40

Table 2A Part 1. Lot sizes and sample sizes: agricultural and vegetable seeds (continued)

Species	Maximum weight of lot (kg) (except see 2.8 Note 2)	Minimum submitted sample (g)	Minimum working samples (g)	
			Purity analysis (3.5.1)	Other seeds by number (4.5.1)
1	2	3	4	5
Brassica napus L.	10 000	100	10	100
Brassica napus L. var. *napobrassica* (L.) Rchb.*	10 000	100	10	100
Brassica nigra (L.) W.D.J.Koch	10 000	40	4	40
Brassica oleracea L. (all varieties)	10 000	100	10	100
Brassica rapa L. (includes *B. campestris* L. and species previously known as *B. chinensis, B. pekinensis* and *B. perviridis*)	10 000	70	7	70
Bromus arvensis L.	10 000	60	6	60
Bromus carinatus Hook. & Arn.	10 000	200	20	200
Bromus catharticus Vahl	10 000	200	20	200
Bromus erectus Huds.	10 000	100	10	100
Bromus hordeaceus L.	10 000	50	5	50
Bromus inermis Leyss.	10 000	90	9	90
Bromus marginatus Steud.	10 000	200	20	200
Bromus riparius Rehmann	10 000	90	9	90
Bromus sitchensis Trin.	10 000	200	20	200
Cajanus cajan (L.) Huth	20 000	1 000	300	1 000
Calopogonium mucunoides Desv.	20 000	400	40	400
Camelina sativa (L.) Crantz	10 000	40	4	40
Cannabis sativa L.	10 000	600	60	600
Capsicum spp.	10 000	150	15	150
Carthamus tinctorius L.	25 000	900	90	900
Carum carvi L.	10 000	80	8	80
Cenchrus ciliaris L. (fascicles)	10 000	60	6	60
Cenchrus setiger Vahl	20 000	150	15	150
Centrosema molle Mart. ex Benth. (previously *Centrosema pubescens* Benth.)	20 000	600	60	600
Centrosema pascuorum Mart. ex Benth.	20 000	550	55	550
(*Centrosema pubescens* Benth. see *Centrosema molle* Mart. ex Benth.)				
Chamaecrista rotundifolia (Pers.) Greene	10 000	100	10	100
Chloris gayana Kunth	10 000	10	1	10
Cicer arietinum L.	30 000	1 000	1 000	1 000
Cichorium endivia L.	10 000	40	4	40
Cichorium intybus L.	10 000	50	5	50
Citrullus lanatus (Thunb.) Matsum. & Nakai	20 000	1 000	250	1 000
Claytonia perfoliata Donn ex Willd.	10 000	20	2	20
Corchorus capsularis L.	10 000	150	15	150
Corchorus olitorius L.	10 000	150	15	150
Coriandrum sativum L.	10 000	400	40	400
Crambe abyssinica Hochst. ex R.E.Fr.	10 000	200	20	200
Crotalaria brevidens Benth. (includes *Crotalaria intermedia* Kotschy)	10 000	150	15	150
Crotalaria juncea L.	10 000	700	70	700
Crotalaria lanceolata E.Mey.	10 000	70	7	70
Crotalaria pallida Aiton	10 000	150	15	150
Crotalaria spectabilis Roth	10 000	350	35	350
Cucumis melo L.	10 000	150	70	–
Cucumis sativus L.	10 000	150	70	–
Cucumis spp.	10 000	150	70	–
Cucurbita maxima Duchesne	20 000	1 000	700	1 000

Table 2A Part 1. Lot sizes and sample sizes: agricultural and vegetable seeds (continued)

Species	Maximum weight of lot (kg) (except see 2.8 Note 2)	Minimum submitted sample (g)	Minimum working samples (g)	
			Purity analysis (3.5.1)	Other seeds by number (4.5.1)
1	2	3	4	5
Cucurbita moschata Duchesne	10000	350	180	–
Cucurbita pepo L.	20000	1000	700	1000
Cucurbita spp.	10000	350	180	–
Cucurbita hybrids	10000	350	180	–
Cuminum cyminum L.	10000	60	6	60
Cyamopsis tetragonoloba (L.) Taub.	20000	1000	100	1000
Cynara cardunculus L.	10000	900	90	900
Cynodon dactylon (L.) Pers.	10000	10	1	10
Cynosurus cristatus L.	10000	20	2	20
Dactylis glomerata L.	10000	30	3	30
Daucus carota L.	10000	30	3	30
Deschampsia cespitosa (L.) P.Beauv.	10000	10	1	10
Deschampsia flexuosa (L.) Trin.	10000	10	1	10
Desmodium intortum (Mill.) Urb.	10000	40	4	40
Desmodium uncinatum (Jacq.) DC.	20000	120	12	120
Dichanthium aristatum (Poir.) C.E.Hubb.	10000	30	3	30
Dichondra micrantha Urb. (previously *Dichondra repens* J.R.Forst. & G.Forst.)	10000	50	5	50
Digitaria eriantha Steud. (includes *Digitaria decumbens* Stent)	10000	12	1.2	12
Echinochloa crus-galli (L.) P.Beauv.	10000	80	8	80
Ehrharta calycina Sm.	10000	40	4	40
Eleusine coracana (L.) Gaertn.	10000	60	6	60
Elymus lanceolatus (Scribn. & J.G.Sm.) Gould	10000	80	8	80
Elymus trachycaulus (Link) Gould ex Shinners	10000	80	8	80
Elytrigia elongata (Host) Nevski	10000	200	20	200
Elytrigia intermedia (Host) Nevski	10000	150	15	150
Elytrigia repens (L.) Desv. ex Nevski	10000	100	10	100
Eragrostis curvula (Schrad.) Nees	10000	10	1	10
Eragrostis tef (Zuccagni) Trotter	10000	10	1	10
Eruca sativa Mill.	10000	40	4	40
Fagopyrum esculentum Moench	10000	600	60	600
Festuca arundinacea Schreb.	10000	50	5	50
Festuca filiformis Pourr.	10000	25	2.5	25
Festuca heterophylla Lam.	10000	60	6	60
Festuca ovina L. (all varieties)	10000	25	2.5	25
Festuca pratensis Huds.	10000	50	5	50
Festuca rubra L. s.l. (all varieties)	10000	30	3	30
Festuca trachyphylla (Hack.) Krajina (synonym *Festuca brevipila* R.Tracey)	10000	25	2.5	25
×*Festulolium* Asch. & Graebn.	10000	60	6	60
Foeniculum vulgare Mill.	10000	180	18	180
Fragaria spp.	10000	10	1	10
Galega orientalis Lam.	10000	200	20	200
Glycine max (L.) Merr.	30000	1000	500	1000
Gossypium spp.	25000	1000	350	1000
Hedysarum coronarium L. (fruit)	10000	300	30	300
Hedysarum coronarium L. (seed)	10000	120	12	120
Helianthus annuus L.	25000	1000	200	1000
Hibiscus cannabinus L.	10000	700	70	700
Holcus lanatus L.	10000	10	1	10

Table 2A Part 1. Lot sizes and sample sizes: agricultural and vegetable seeds (continued)

Species	Maximum weight of lot (kg) (except see 2.8 Note 2)	Minimum submitted sample (g)	Minimum working samples (g)	
			Purity analysis (3.5.1)	Other seeds by number (4.5.1)
1	2	3	4	5
Hordeum vulgare L.	30 000	1 000	120	1 000
Ipomoea aquatica Forssk.	20 000	1 000	100	1 000
Koeleria macrantha (Ledeb.) Schult.	10 000	10	1	10
Kummerowia stipulacea (Maxim.) Makino	10 000	50	5	50
Kummerowia striata (Thunb.) Schindl.	10 000	40	4	40
Lablab purpureus (L.) Sweet	20 000	1 000	600	1 000
Lactuca sativa L.	10 000	30	3	30
Lagenaria siceraria (Molina) Standl.	20 000	1 000	500	1 000
Lathyrus cicera L.	20 000	1 000	140	1 000
Lathyrus hirsutus L.	10 000	700	70	700
Lathyrus sativus L.	20 000	1 000	450	1 000
Lens culinaris Medik.	30 000	600	60	600
Lepidium sativum L.	10 000	60	6	60
Lespedeza juncea (L. f.) Pers.	10 000	30	3	30
Leucaena leucocephala (Lam.) de Wit	20 000	1 000	100	1 000
Linum usitatissimum L.	10 000	150	15	150
Listia bainesii (Baker) B.-E. van Wyk & Boatwr. (previously *Lotononis bainesii* Baker)	10 000	10	1	10
Lolium ×*hybridum* Hausskn. (previously *Lolium* ×*boucheanum* Kunth)	10 000	60	6	60
Lolium multiflorum Lam.	10 000	60	6	60
Lolium perenne L.	10 000	60	6	60
Lolium rigidum Gaudin	10 000	60	6	60
(*Lotononis bainesii* Baker see *Listia bainesii* (Baker) B.-E. van Wyk & Boatwr.)				
Lotus corniculatus L.	10 000	30	3	30
Lotus tenuis Waldst. & Kit. ex Willd.	10 000	30	3	30
Lotus uliginosus Schkuhr	10 000	20	2	20
Luffa acutangula (L.) Roxb.	20 000	1 000	400	1 000
Luffa aegyptiaca Mill.	20 000	1 000	250	1 000
Lupinus albus L.	30 000	1 000	450	1 000
Lupinus angustifolius L.	30 000	1 000	450	1 000
Lupinus luteus L.	30 000	1 000	450	1 000
(*Lycopersicon esculentum* Mill. see *Solanum lycopersicum* L.)				
(*Lycopersicon* spp. see *Solanum* (sect. *Lycopersicon*) spp.)				
(*Lycopersicon* hybrids see *Solanum* (sect. *Lycopersicon*) hybrids)				
Macroptilium atropurpureum (DC.) Urb.	20 000	350	35	350
Macroptilium lathyroides (L.) Urb.	20 000	200	20	200
Macrotyloma axillare (E.Mey.) Verdc.	20 000	250	25	250
Macrotyloma uniflorum (Lam.) Verdc.	20 000	800	80	800
Medicago arabica (L.) Huds. (in burr)	10 000	600	60	600
Medicago arabica (L.) Huds. (out of burr)	10 000	50	5	50
Medicago italica (Mill.) Fiori (includes *Medicago tornata* (L.) Mill.)	10 000	100	10	100
Medicago littoralis Rohde ex Loisel.	10 000	70	7	70
Medicago lupulina L.	10 000	50	5	50
Medicago orbicularis (L.) Bartal.	10 000	80	8	80
Medicago polymorpha L.	10 000	70	7	70
Medicago rugosa Desr.	10 000	180	18	180
Medicago sativa L.	10 000	50	5	50

Table 2A Part 1. Lot sizes and sample sizes: agricultural and vegetable seeds (continued)

Species	Maximum weight of lot (kg) (except see 2.8 Note 2)	Minimum submitted sample (g)	Minimum working samples (g)	
			Purity analysis (3.5.1)	Other seeds by number (4.5.1)
1	2	3	4	5
Medicago scutellata (L.) Mill.	10000	400	40	400
Medicago truncatula Gaertn.	10000	100	10	100
Melilotus albus Medik.	10000	50	5	50
Melilotus indicus (L.) All.	10000	50	5	50
Melilotus officinalis (L.) Lam.	10000	50	5	50
Melinis minutiflora P.Beauv.	10000	5	0.5	5
Momordica charantia L.	20000	1000	450	1000
Mucuna pruriens (L.) DC. (includes species previously known as *M. aterrima* (Piper & Tracy) Holland, *M. cochinchinensis* (Lour.) A.Chev. and *Stizolobium deeringianum* Bort.)	20000	1000	1000	1000
Nasturtium officinale R.Br.	10000	5	0.5	5
Neonotonia wightii (Wight & Arn.) J.A.Lackey	10000	150	15	150
Nicotiana tabacum L.	10000	5	0.5	5
Ocimum basilicum L.	10000	40	4	40
Oenothera biennis L.	10000	10	1	10
Onobrychis viciifolia Scop. (fruit)	10000	600	60	600
Onobrychis viciifolia Scop. (seed)	10000	400	40	400
Origanum majorana L.	10000	5	0.5	5
Origanum vulgare L.	10000	5	0.5	5
Ornithopus compressus L.	10000	120	12	120
Ornithopus sativus Brot.	10000	90	9	90
Oryza sativa L.	30000	700	70	700
Panicum antidotale Retz.	10000	20	2	20
Panicum coloratum L.	10000	20	2	20
Panicum maximum Jacq.	10000	20	2	20
Panicum miliaceum L.	10000	150	15	150
Panicum virgatum L.	10000	30	3	30
Papaver somniferum L.	10000	10	1	10
Pascopyrum smithii (Rydb.) Barkworth & D.R.Dewey	10000	150	15	150
Paspalum dilatatum Poir.	10000	50	5	50
Paspalum notatum Flüggé	10000	70	7	70
Paspalum plicatulum Michx.	10000	40	4	40
Paspalum scrobiculatum L.	10000	80	8	80
Paspalum urvillei Steud.	10000	30	3	30
Paspalum virgatum L. (previously *Paspalum wettsteinii* Hack.)	10000	30	3	30
Pastinaca sativa L.	10000	100	10	100
Pennisetum clandestinum Hochst. ex Chiov.	10000	70	7	70
Pennisetum glaucum (L.) R.Br.	10000	150	15	150
Petroselinum crispum (Mill.) Fuss	10000	40	4	40
Phacelia tanacetifolia Benth.	10000	50	5	50
Phalaris aquatica L.	10000	40	4	40
Phalaris arundinacea L.	10000	30	3	30
Phalaris canariensis L.	10000	200	20	200
Phaseolus coccineus L.	30000	1000	1000	1000
Phaseolus lunatus L.	30000	1000	1000	1000
Phaseolus vulgaris L.	30000	1000	700	1000
Phleum nodosum L.	10000	10	1	10
Phleum pratense L.	10000	10	1	10
Physalis pubescens L.	10000	20	2	20
Pimpinella anisum L.	10000	70	7	70

Table 2A Part 1. Lot sizes and sample sizes: agricultural and vegetable seeds (continued)

Species	Maximum weight of lot (kg) (except see 2.8 Note 2)	Minimum submitted sample (g)	Minimum working samples (g)	
			Purity analysis (3.5.1)	Other seeds by number (4.5.1)
1	2	3	4	5
Piptatherum miliaceum (L.) Coss.	10 000	20	2	20
Pisum sativum L. s.l.	30 000	1 000	900	1 000
Plantago lanceolata L.	10 000	60	6	60
Poa annua L.	10 000	10	1	10
Poa bulbosa L.	10 000	30	3	30
Poa compressa L.	10 000	5	0.5	5
Poa nemoralis L.	10 000	5	0.5	5
Poa palustris L.	10 000	5	0.5	5
Poa pratensis L.	10 000	5	1	5
Poa secunda J.Presl (includes *Poa ampla* Merr.)	10 000	15	1.5	15
Poa trivialis L.	10 000	5	1	5
Portulaca oleracea L.	10 000	5	0.5	5
Psathyrostachys juncea (Fisch.) Nevski	10 000	60	6	60
Pseudoroegneria spicata (Pursh) Á.Löve	10 000	80	8	80
Psophocarpus tetragonolobus (L.DC.	20 000	1 000	1 000	1 000
Pueraria lobata (Willd.) Ohwi	10 000	350	35	350
Pueraria phaseoloides (Roxb.) Benth.	20 000	300	30	300
Raphanus sativus L.	10 000	300	30	300
Rheum rhaponticum L.	10 000	450	45	450
Ricinus communis L.	20 000	1 000	500	1 000
Rosmarinus officinalis L.	10 000	30	3	30
Rumex acetosa L.	10 000	30	3	30
Sanguisorba minor Scop.	10 000	250	25	250
Satureja hortensis L.	10 000	20	2	20
Schizachyrium scoparium (Michx.) Nash	10 000	50	5	50
Scorzonera hispanica L.	10 000	300	30	300
Secale cereale L.	30 000	1 000	120	1 000
Securigera varia (L.) Lassen	10 000	100	10	100
Sesamum indicum L.	10 000	70	7	70
Setaria italica (L.) P.Beauv.	10 000	90	9	90
Setaria sphacelata (Schumach.) Stapf & C.E.Hubb.	10 000	30	3	30
Sinapis alba L.	10 000	200	20	200
Solanum (sect. *Lycopersicon*) spp. (previously *Lycopersicon* spp.)	10 000	15	7	–
Solanum (sect. *Lycopersicon*) hybrids (previously *Lycopersicon* hybrids)	10 000	15	7	–
Solanum lycopersicum L. (previously *Lycopersicon esculentum* Mill.)	10 000	15	7	–
Solanum melongena L.	10 000	150	15	150
Solanum nigrum L.	10 000	25	2.5	25
Solanum tuberosum L.	10 000	25	10	–
Sorghastrum nutans (L.) Nash	10 000	70	7	70
Sorghum ×*almum* Parodi	30 000	200	20	200
Sorghum bicolor (L.) Moench	30 000	900	90	900
Sorghum bicolor (L.) Moench × *S. sudanense* (Piper) Stapf	30 000	300	30	300
Sorghum halepense (L.) Pers.	10 000	90	9	90
Sorghum sudanense (Piper) Stapf	10 000	250	25	250
Spergula arvensis L.	10 000	40	4	40
Spinacia oleracea L.	10 000	250	25	250
Stylosanthes guianensis (Aubl.) Sw.	10 000	70	7	70
Stylosanthes hamata (L.) Taub.	10 000	70	7	70

Table 2A Part 1. Lot sizes and sample sizes: agricultural and vegetable seeds (continued)

Species	Maximum weight of lot (kg) (except see 2.8 Note 2)	Minimum submitted sample (g)	Minimum working samples (g)	
			Purity analysis (3.5.1)	Other seeds by number (4.5.1)
1	2	3	4	5
Stylosanthes humilis Kunth	10 000	70	7	70
Stylosanthes scabra Vogel	10 000	80	8	80
Taraxacum officinale F.H.Wigg., s.l.	10 000	30	3	30
Tetragonia tetragonoides (Pall.) Kuntze	20 000	1 000	200	1 000
Thymus vulgaris L.	10 000	5	0.5	5
Tragopogon porrifolius L.	10 000	400	40	400
Trifolium alexandrinum L.	10 000	60	6	60
Trifolium campestre Schreb.	10 000	5	0.5	5
Trifolium dubium Sibth.	10 000	20	2	20
Trifolium fragiferum L.	10 000	40	4	40
Trifolium glomeratum L.	10 000	10	1	10
Trifolium hirtum All.	10 000	70	7	70
Trifolium hybridum L.	10 000	20	2	20
Trifolium incarnatum L.	10 000	80	8	80
Trifolium lappaceum L.	10 000	20	2	20
Trifolium michelianum Savi (includes *Trifolium balansae* Boiss.)	10 000	20	2	20
Trifolium pratense L.	10 000	50	5	50
Trifolium repens L.	10 000	20	2	20
Trifolium resupinatum L.	10 000	20	2	20
Trifolium semipilosum Fresen.	10 000	20	2	20
Trifolium squarrosum L.	10 000	150	15	150
Trifolium subterraneum L.	10 000	250	25	250
Trifolium vesiculosum Savi	10 000	30	3	30
Trigonella foenum-graecum L.	10 000	450	45	450
Trisetum flavescens (L.) P.Beauv.	10 000	5	0.5	5
×*Triticosecale Wittm.* ex A.Camus	30 000	1 000	120	1 000
Triticum aestivum L.	30 000	1 000	120	1 000
Triticum dicoccon Schrank	30 000	1 000	270	1 000
Triticum durum Desf.	30 000	1 000	120	1 000
Triticum spelta L.	30 000	1 000	270	1 000
Urochloa mosambicensis (Hack.) Dandy	10 000	30	3	30
Valerianella locusta (L.) Laterr.	10 000	70	7	70
Vicia benghalensis L.	30 000	1 000	120	1 000
Vicia ervilia (L.) Willd.	30 000	1 000	120	1 000
Vicia faba L.	30 000	1 000	1 000	1 000
Vicia narbonensis L.	30 000	1 000	600	1 000
Vicia pannonica Crantz	30 000	1 000	120	1 000
Vicia sativa L. (includes *V. angustifolia* L.)	30 000	1 000	140	1 000
Vicia villosa Roth (includes *V. dasycarpa* Ten.)	30 000	1 000	100	1 000
Vigna angularis (Willd.) Ohwi & H.Ohashi	30 000	1 000	250	1 000
Vigna marina (Burm.) Merr.	30 000	800	80	800
Vigna mungo (L.) Hepper	30 000	1 000	700	1 000
Vigna radiata (L.) R.Wilczek	30 000	1 000	120	1 000
Vigna subterranea (L.) Verdc.	30 000	1 000	500	1 000
Vigna unguiculata (L.) Walp.	30 000	1 000	400	1 000
Zea mays L.	40 000	1 000	900	1 000
Zoysia japonica Steud.	10 000	10	1	10

Table 2A Part 2. Lot sizes and sample sizes: tree and shrub seeds

Species	Maximum weight of lot (kg) (except see 2.8 Note 2)	Minimum submitted sample (g)	Minimum working sample for purity analysis (3.5.1) (g)
1	2	3	4
Abies alba Mill.	1 000	240	120
Abies amabilis Douglas ex J.Forbes	1 000	200	100
Abies balsamea (L.) Mill.	1 000	40	20
Abies cephalonica Loudon	1 000	360	180
Abies cilicica (Antoine & Kotschy) Carrière	1 000	1 000	500
Abies concolor (Gordon & Glend.) Lindl. ex Hildebr.	1 000	160	80
Abies firma Siebold & Zucc.	1 000	200	100
Abies fraseri (Pursh) Poir.	1 000	40	20
Abies grandis (Douglas ex D.Don) Lindl.	1 000	100	50
Abies homolepis Siebold & Zucc.	1 000	80	40
Abies lasiocarpa (Hook.) Nutt.	1 000	50	25
Abies magnifica A.Murray	1 000	400	200
Abies nordmanniana (Steven) Spach	1 000	360	180
Abies numidica de Lannoy ex Carrière	1 000	500	250
Abies pinsapo Boiss.	1 000	320	160
Abies procera Rehder	1 000	160	80
Abies sachalinensis (F.Schmidt) Mast.	1 000	60	30
Abies veitchii Lindl.	1 000	40	20
Acacia spp.	1 000	70	35
Acer campestre L.	1 000	400	200
Acer negundo L.	500	200	100
Acer palmatum Thunb.	500	100	50
Acer platanoides L.	500	700	350
Acer pseudoplatanus L.	500	600	300
Acer rubrum L.	500	100	50
Acer saccharinum L.	500	1 000	500
Acer saccharum Marshall	500	360	180
Aesculus hippocastanum L.	5 000	500 seeds	500 seeds
Ailanthus altissima (Mill.) Swingle	1 000	160	80
Alnus cordata (Loisel.) Duby	1 000	12	6
Alnus glutinosa (L.) Gaertn.	1 000	8	4
Alnus incana (L.) Moench	1 000	4	2
Alnus rubra Bong.	1 000	4	2
Amorpha fruticosa L.	1 000	1 000	150
Berberis aquifolium Pursh (previously *Mahonia aquifolium* (Pursh) Nutt.)	1 000	60	30
Betula papyrifera Marshall	300	10	3
Betula pendula Roth	300	10	1
Betula pubescens Ehrh.	300	10	1
Calocedrus decurrens (Torr.) Florin	300	160	80
Caragana arborescens Lam.	1 000	160	80
Carica papaya L.	1 000	100	50
Carpinus betulus L.	1 000	500	250
Castanea sativa Mill.	5 000	500 seeds	500 seeds
Catalpa spp.*	1 000	120	60
Cedrela spp.	1 000	80	40
Cedrus atlantica (Endl.) G.Manetti ex Carrière	1 000	400	200
Cedrus deodara (Roxb. ex D.Don) G.Don	1 000	600	300
Cedrus libani A.Rich.	1 000	400	200
Chamaecyparis lawsoniana (A.Murray) Parl.	1 000	20	6
Chamaecyparis nootkatensis (D.Don) Spach	1 000	20	10
Chamaecyparis obtusa (Siebold & Zucc.) Endl.	1 000	12	6
Chamaecyparis pisifera (Siebold & Zucc.) Endl.	1 000	10	3
Chamaecyparis thyoides (L.) Britton et al.	1 000	10	3

Table 2A Part 2. Lot sizes and sample sizes: tree and shrub seeds (continued)

Species	Maximum weight of lot (kg) (except see 2.8 Note 2)	Minimum submitted sample (g)	Minimum working sample for purity analysis (3.5.1) (g)
1	2	3	4
Cornus mas L.	1 000	1 000	600
Cornus sanguinea L.	1 000	300	150
Corylus avellana L.	5 000	500 fruits	500 fruits
Corymbia citriodora (Hook.) K.D.Hill & L.A.S.Johnson (previously *Eucalyptus citriodora* Hook.)	1 000	40	15
Corymbia ficifolia (F.Muell.) K.D.Hill & L.A.S.Johnson (previously *Eucalyptus ficifolia* F.Muell.)	1 000	40	15
Corymbia maculata (Hook.) K.D.Hill & L.A.S.Johnson (previously *Eucalyptus maculata* Hook.)	1 000	40	15
Cotoneaster spp.*	1 000	40	20
Crataegus monogyna Jacq.	1 000	400	200
Cryptomeria japonica (L. f.) D.Don	1 000	20	10
Cupressus arizonica Greene	1 000	60	30
Cupressus macrocarpa Hartw.	1 000	40	20
Cupressus sempervirens L.	1 000	40	20
Cydonia oblonga Mill.	1 000	50	25
Cytisus scoparius (L.) Link	1 000	40	20
Elaeagnus angustifolia L.	1 000	800	400
Eucalyptus astringens (Maiden) Maiden	1 000	40	15
Eucalyptus botryoides Sm.	1 000	15	5
Eucalyptus bridgesiana R.T.Baker	1 000	30	10
Eucalyptus camaldulensis Dehnh.	1 000	15	5
Eucalyptus cinerea F.Muell. ex Benth.	1 000	30	10
(*Eucalyptus citriodora* Hook. see *Corymbia citriodora* (Hook.) K.D.Hill & L.A.S.Johnson)			
Eucalyptus cladocalyx F.Muell.	1 000	40	15
Eucalyptus cloeziana F.Muell.	1 000	40	15
Eucalyptus cypellocarpa L.A.S.Johnson	1 000	30	10
Eucalyptus dalrympleana Maiden	1 000	30	10
Eucalyptus deanei Maiden	1 000	15	5
Eucalyptus deglupta Blume	1 000	10	2
Eucalyptus delegatensis R.T.Baker	1 000	40	15
Eucalyptus elata Dehnh.	1 000	40	15
Eucalyptus fastigata H.Deane & Maiden	1 000	40	15
(*Eucalyptus ficifolia* F.Muell. see *Corymbia ficifolia* (F.Muell.) K.D.Hill & L.A.S.Johnson)			
Eucalyptus glaucescens Maiden & Blakely	1 000	40	15
Eucalyptus globulus Labill. (includes *E. maidenii* F.Muell. and *E. saint-johnii* (R.T.Baker) R.T.Baker)	1 000	60	20
Eucalyptus grandis W.Hill ex Maiden	1 000	15	5
Eucalyptus gunnii Hook. f.	1 000	15	5
Eucalyptus largiflorens F.Muell.	1 000	15	5
Eucalyptus leucoxylon F.Muell.	1 000	30	10
Eucalyptus macrorhyncha F.Muell. ex Benth.	1 000	40	15
(*Eucalyptus maculata* Hook. see *Corymbia maculata* (Hook.) K.D.Hill & L.A.S.Johnson)			
Eucalyptus mannifera Mudie	1 000	15	5
Eucalyptus melliodora A.Cunn. ex Schauer	1 000	30	10
Eucalyptus microtheca F.Muell.	1 000	15	5
Eucalyptus moluccana Roxb.	1 000	30	10
Eucalyptus muelleriana A.W.Howitt	1 000	60	20
Eucalyptus nitens (H.Deane & Maiden) Maiden	1 000	30	10
Eucalyptus pauciflora Sieber ex Spreng. (includes *E. niphophila* Maiden & Blakely)	1 000	60	20

Table 2A Part 2. Lot sizes and sample sizes: tree and shrub seeds (continued)

Species	Maximum weight of lot (kg) (except see 2.8 Note 2)	Minimum submitted sample (g)	Minimum working sample for purity analysis (3.5.1) (g)
1	2	3	4
Eucalyptus pilularis Sm.	1 000	60	20
Eucalyptus polybractea R.T.Baker	1 000	60	20
Eucalyptus radiata Sieber ex DC.	1 000	40	15
Eucalyptus regnans F.Muell.	1 000	30	10
Eucalyptus resinifera Sm.	1 000	30	10
Eucalyptus robusta Sm.	1 000	15	5
Eucalyptus rudis Endl.	1 000	15	5
Eucalyptus saligna Sm.	1 000	15	5
Eucalyptus sideroxylon A.Cunn. ex Woolls	1 000	30	10
Eucalyptus sieberi L.A.S.Johnson	1 000	40	15
Eucalyptus smithii R.T.Baker	1 000	30	10
Eucalyptus tereticornis Sm.	1 000	15	5
Eucalyptus viminalis Labill.	1 000	30	10
Euonymus europaeus L.	1 000	200	100
Fagus sylvatica L.	5 000	1 000	600
Fraxinus spp.	1 000	400	200
Ginkgo biloba L.	5 000	500 seeds	500 seeds
Gleditsia triacanthos L.	1 000	800	400
Ilex aquifolium L.	1 000	200	90
Juniperus communis L. (berries)	1 000	300	150
Juniperus communis L. (seeds)	1 000	40	20
Juniperus scopulorum Sarg.	1 000	70	35
Juniperus virginiana L.	1 000	100	50
Koelreuteria paniculata Laxm.	1 000	800	400
Laburnum alpinum (Mill.) J.Presl	1 000	140	70
Laburnum anagyroides Medik.	1 000	140	70
Larix decidua Mill.	1 000	35	17
Larix ×*eurolepis* A.Henry	1 000	35	16
Larix gmelinii (Rupr.) Rupr.	1 000	25	10
Larix kaempferi (Lamb.) Carrière	1 000	24	10
Larix laricina (D.Roi) K.Koch	1 000	25	10
Larix occidentalis Nutt.	1 000	25	10
Larix sibirica Ledeb.	1 000	25	10
Ligustrum vulgare L.	1 000	100	50
Liquidambar styraciflua L.	300	30	15
Liriodendron tulipifera L.	1 000	180	90
(*Mahonia aquifolium* (Pursh) Nutt. see *Berberis aquifolium* Pursh)			
Malus spp. (except *M. sargentii, M. sylvestris*)	1 000	50	25
Malus sargentii Rehder	1 000	24	12
Malus sylvestris (L.) Mill.	1 000	160	80
Malva sylvestris L.	5 000	30	15
Morus spp.	1 000	20	5
Nothofagus alpina (Poepp. & Endl.) Oerst.	1 000	50	25
Nothofagus obliqua (Mirb.) Blume	1 000	60	30
Picea abies (L.) H.Karst.	1 000	40	20
Picea engelmannii Parry ex Engelm.	1 000	16	8
Picea glauca (Moench) Voss	1 000	10	5
Picea glehnii (F.Schmidt) Mast.	1 000	25	9
Picea jezoensis (Siebold & Zucc.) Carrière	1 000	25	7
Picea koyamae Shiras.	1 000	25	9
Picea mariana (Mill.) Britton et al.	1 000	6	3
Picea omorika (Pančić) Purk.	1 000	25	8
Picea orientalis (L.) Link	1 000	30	15

Table 2A Part 2. Lot sizes and sample sizes: tree and shrub seeds (continued)

Species	Maximum weight of lot (kg) (except see 2.8 Note 2)	Minimum submitted sample (g)	Minimum working sample for purity analysis (3.5.1) (g)
1	2	3	4
Picea polita (Siebold & Zucc.) Carrière	1 000	80	40
Picea pungens Engelm.	1 000	30	15
Picea rubens Sarg.	1 000	25	9
Picea sitchensis (Bong.) Carrière	1 000	12	6
Pinus albicaulis Engelm.	1 000	700	350
Pinus aristata Engelm.	1 000	100	50
Pinus banksiana Lamb.	1 000	25	9
Pinus brutia Ten.	1 000	100	50
Pinus canariensis C.Sm.	1 000	60	30
Pinus caribaea Morelet	1 000	100	50
Pinus cembra L.	1 000	1 000	700
Pinus cembroides Zucc.	1 000	1 000	700
Pinus clausa (Chapm. ex Engelm.) Vasey ex Sarg.	1 000	40	20
Pinus contorta Douglas ex Loudon	1 000	25	9
Pinus coulteri D.Don	1 000	1 000	900
Pinus densiflora Siebold & Zucc.	1 000	60	30
Pinus echinata Mill.	1 000	50	25
Pinus edulis Engelm.	1 000	1 000	700
Pinus elliottii Engelm.	1 000	160	80
Pinus flexilis E.James	1 000	500	250
Pinus glabra Walter	1 000	80	40
Pinus halepensis Mill.	1 000	100	50
Pinus heldreichii Christ	1 000	120	60
Pinus jeffreyi Balf.	1 000	600	300
Pinus kesiya Royle ex Gordon ('*khasya*')	1 000	80	40
Pinus koraiensis Siebold & Zucc.	1 000	2 000	1 000
Pinus lambertiana Douglas	1 000	1 000	500
Pinus merkusii Jungh. & de Vriese	1 000	120	60
Pinus monticola Douglas ex D.Don	1 000	90	45
Pinus mugo Turra	1 000	40	20
Pinus muricata D.Don	1 000	50	25
Pinus nigra J.F.Arnold	1 000	100	50
Pinus oocarpa Schiede ex Schltdl.	1 000	70	35
Pinus palustris Mill.	1 000	500	250
Pinus parviflora Siebold & Zucc.	1 000	500	250
Pinus patula Schltdl. & Cham.	1 000	40	20
Pinus peuce Griseb.	1 000	240	120
Pinus pinaster Aiton	1 000	240	120
Pinus pinea L.	1 000	1 000	1 000
Pinus ponderosa P.Lawson & C.Lawson	1 000	200	100
Pinus pumila (Pall.) Regel	1 000	40	20
Pinus radiata D.Don	1 000	160	80
Pinus resinosa Aiton	1 000	50	25
Pinus rigida Mill.	1 000	40	20
Pinus strobus L.	1 000	90	45
Pinus sylvestris L.	1 000	40	20
Pinus tabuliformis Carrière	1 000	100	50
Pinus taeda L.	1 000	140	70
Pinus taiwanensis Hayata	1 000	100	50
Pinus thunbergii Parl.	1 000	70	35
Pinus virginiana Mill.	1 000	50	25
Pinus wallichiana A.B.Jacks.	1 000	250	125
Platanus spp.	1 000	25	6
Platycladus orientalis (L.) Franco	1 000	120	60
Populus spp.	50	5	2

Table 2A Part 2. Lot sizes and sample sizes: tree and shrub seeds (continued)

Species	Maximum weight of lot (kg) (except see 2.8 Note 2)	Minimum submitted sample (g)	Minimum working sample for purity analysis (3.5.1) (g)
1	2	3	4
Prunus avium (L.) L.	1 000	900	450
Prunus padus L.	1 000	360	180
Prunus persica (L.) Batsch	5 000	500 seeds	500 seeds
Prunus serotina Ehrh.	1 000	500	250
Prunus spp. (TSW ≤ 200 g	1 000	1 000	500
Prunus spp. (TSW > 200 g	1 000	500 seeds	500 seeds
Pseudotsuga menziesii (Mirb.) Franco	1 000	60	30
Pyrus spp.	1 000	180	90
Quercus spp.	5 000	500 seeds	500 seeds
Robinia pseudoacacia L.	1 000	100	50
Rosa spp.	1 000	50	25
Salix spp.	50	5	2
Sequoia sempervirens (D.Don) Endl.	1 000	25	12
Sequoiadendron giganteum (Lindl.) J.Buchholz	1 000	25	12
Sorbus spp.	1 000	25	10
Spartium junceum L.	1 000	40	20
Styphnolobium japonicum (L.) Schott	1 000	100	50
Syringa spp.	1 000	30	15
Taxodium distichum (L.) Rich.	300	500	250
Taxus spp.	1 000	320	160
Tectona grandis L. f.	1 000	2 000	1 000
Thuja occidentalis L.	1 000	25	4
Thuja plicata Donn ex D.Don	1 000	10	3
Tilia cordata Mill.	1 000	180	90
Tilia platyphyllos Scop.	1 000	500	250
Tsuga canadensis (L.) Carrière	1 000	25	7
Tsuga heterophylla (Raf.) Sarg.	1 000	10	4
Ulmus americana L.	1 000	30	15
Ulmus parvifolia Jacq.	1 000	20	8
Ulmus pumila L.	1 000	30	15
Viburnum opulus L.	1 000	160	80
Zelkova serrata (Thunb.) Makino	1 000	60	30

Table 2A Part 3. Lot sizes and sample sizes: flower, spice, herb and medicinal species

Species	Maximum weight of lot (kg) (except see 2.8 Note 2)	Minimum submitted sample (g)	Minimum working sample for purity analysis (3.5.1) (g)
1	2	3	4
Abutilon ×*hybridum* hort. ex Voss	5000	40	10
Achillea clavennae L.	5000	5	0.5
Achillea filipendulina Lam.	5000	5	0.5
Achillea ptarmica L.	5000	5	0.5
Achillea umbellata Sm.	5000	5	0.5
Adonis vernalis L.	5000	20	5
Ageratum houstonianum Mill.	5000	5	0.5
Agrimonia eupatoria L.	5000	200	50
Alcea rosea L.	5000	80	20
Althaea hybrids	5000	80	20
Althaea officinalis L.	5000	80	20
Alyssum argenteum All.	5000	10	3
Alyssum montanum L.	5000	10	3
Amaranthus caudatus L.	5000	10	2
Amaranthus cruentus L.	5000	10	2
Amaranthus hybridus L.	5000	10	2
Amaranthus tricolor L.	5000	10	2
Amberboa moschata (L.) DC.	5000	40	10
Ammobium alatum R.Br.	5000	5	1
Anagallis arvensis L.	5000	10	2
Anchusa azurea Mill.	5000	100	25
Anchusa capensis Thunb.	5000	40	10
Anemone coronaria L.	5000	10	3
Anemone pulsatilla L.	5000	10	3
Anemone sylvestris L.	5000	10	3
Angelica archangelica L.	5000	40	10
Antirrhinum majus L.	5000	5	0.5
Aquilegia alpina L.	5000	20	4
Aquilegia canadensis L.	5000	20	4
Aquilegia chrysantha A.Gray	5000	20	4
Aquilegia ×*cultorum* Bergmans	5000	20	4
Aquilegia vulgaris L.	5000	20	4
Arabis alpina L.	5000	10	2
Arabis ×*arendsii* H.R.Wehrh.	5000	10	2
Arabis blepharophylla Hook. & Arn.	5000	10	2
Arabis caucasica Willd.	5000	10	2
Arabis procurrens Waldst. & Kit.	5000	10	2
Arabis scopoliana Boiss.	5000	10	2
Arctotis stoechadifolia P.J.Bergius	5000	20	4
Armeria maritima (Mill.) Willd.	5000	20	5
Artemisia absinthium L.	5000	5	0.5
Artemisia dracunculus L.	5000	5	0.5
Artemisia maritima L.	5000	5	0.5
Artemisia vulgaris L.	5000	5	0.5
Asclepias tuberosa L.	5000	130	13
Asparagus aethiopicus L. (previously *Asparagus densiflorus* (Kunth) Jessop)	10000	200	60
Asparagus plumosus L. (previously *Asparagus setaceus* (Kunth) Jessop)	10000	200	50
Aster alpinus L.	5000	20	5
Aster amellus L.	5000	20	5
Aster dumosus L.	5000	20	5

Table 2A Part 3. Lot sizes and sample sizes: flower, spice, herb and medicinal species (continued)

Species	Maximum weight of lot (kg) (except see 2.8 Note 2)	Minimum submitted sample (g)	Minimum working sample for purity analysis (3.5.1) (g)
1	2	3	4
Aubrieta deltoidea (L.) DC. (includes *A. graeca* Griseb.)	5000	5	1
Aurinia saxatilis (L.) Desv.	5000	10	3
Bassia scoparia (L.) A.J.Scott (previously *Kochia scoparia* (L.) Schrad.)	5000	10	3
Begonia Semperflorens-Cultorum Group	5000	5	0.1
Begonia ×*tuberhybrida* Voss	5000	5	0.1
Bellis perennis L.	5000	5	0.5
Brachyscome iberidifolia Benth.	5000	5	0.3
Briza maxima L.	5000	40	10
Browallia viscosa Kunth	5000	5	0.5
Brunnera macrophylla (Adams) I.M.Johnst.	5000	40	10
Calceolaria ×*herbeohybrida* Voss	5000	5	0.1
Calceolaria polyrrhiza Cav.	5000	5	0.1
Calendula officinalis L.	5000	80	20
Callistephus chinensis (L.) Nees	5000	20	6
Campanula carpatica Jacq.	5000	5	0.2
Campanula fragilis Cirillo	5000	5	1
Campanula garganica Ten.	5000	5	0.5
Campanula glomerata L.	5000	5	0.2
Campanula lactiflora M.Bieb.	5000	5	1
Campanula medium L.	5000	5	0.6
Campanula persicifolia L.	5000	5	0.2
Campanula portenschlagiana Schult.	5000	5	0.5
Campanula pyramidalis L.	5000	5	1
Campanula rapunculus L.	5000	5	1
Celosia argentea L.	5000	10	2
(*Centaurea americana* Nutt. see *Plectocephalus americana* (Nutt.) D.Don)			
Centaurea benedicta (L.) L. (previously *Cnicus benedictus* L.)	5000	300	75
Centaurea cyanus L.	5000	40	10
(*Centaurea dealbata* Willd. see *Psephellus dealbatus* (Willd.) K.Koch)			
Centaurea gymnocarpa Moris & D.Not.	5000	40	10
Centaurea imperialis Hausskn. ex Bornm.	5000	40	10
Centaurea macrocephala Muss. Puschk. ex Willd.	5000	40	10
Centaurea montana L.	5000	40	10
Centaurea ragusina L.	5000	40	10
Cerastium tomentosum L.	5000	10	2
Chelidonium majus L.	5000	5	1
Chrysanthemum indicum L.	5000	30	8
Clarkia amoena (Lehm.) A.Nelson & J.F.Macbr.	5000	5	1
Clarkia pulchella Pursh	5000	5	1
Clarkia unguiculata Lindl.	5000	5	1
Cleome hassleriana Chodat	5000	20	5
(*Cnicus benedictus* L. see *Centaurea benedicta* (L.) L.)			
Cobaea scandens Cav.	5000	200	50
Coix lacryma-jobi L.	5000	600	150
Coleostephus multicaulis (Desf.) Durieu	5000	30	8
(*Coleus blumei* Benth. see *Plectranthus scutellarioides* (L.) R.Br.)			
Consolida ajacis (L.) Schur	5000	30	8

Table 2A Part 3. Lot sizes and sample sizes: flower, spice, herb and medicinal species (continued)

Species	Maximum weight of lot (kg) (except see 2.8 Note 2)	Minimum submitted sample (g)	Minimum working sample for purity analysis (3.5.1) (g)
1	2	3	4
Consolida regalis Gray	5000	30	8
Convolvulus tricolor L.	5000	100	25
Coreopsis basalis (A.Dietr.) S.F.Blake (includes *C. drummondii* (D.Don) Torr. & A.Gray)	5000	20	5
Coreopsis lanceolata L.	5000	20	5
Coreopsis maritima (Nutt.) Hook. f.	5000	5	1
Coreopsis tinctoria Nutt.	5000	5	1
Cosmos bipinnatus Cav.	5000	80	20
Cosmos sulphureus Cav.	5000	80	20
Cyclamen persicum Mill.	5000	100	30
Cymbalaria muralis G.Gaertn. et al.	5000	5	0.2
Cynoglossum amabile Stapf & J.R.Drumm.	5000	40	10
Dahlia pinnata Cav.	5000	80	20
Datura metel L.	5000	100	25
Datura stramonium L.	5000	100	25
Delphinium ×belladonna hort. ex Bergmans	5000	20	4
Delphinium cardinale Hook.	5000	20	4
Delphinium ×cultorum Voss	5000	20	4
Delphinium formosum Boiss. & A.Huet	5000	20	4
Delphinium grandiflorum L.	5000	20	4
Dianthus barbatus L.	5000	10	3
Dianthus caryophyllus L.	5000	20	5
Dianthus chinensis L.	5000	10	3
Dianthus deltoides L.	5000	20	0.5
Dianthus plumarius L.	5000	20	5
Digitalis lanata Ehrh.	5000	5	1
Digitalis purpurea L.	5000	5	0.2
Dimorphotheca pluvialis (L.) Moench	5000	40	10
Dimorphotheca tragus (Aiton) B.Nord.	5000	40	10
Doronicum orientale Hoffm.	5000	10	2
Dorotheanthus bellidiformis (Burm. f.) N.E.Br.	5000	5	0.5
Echinacea purpurea (L.) Moench	5000	20	5
Echinops ritro L.	5000	80	20
Echium candicans L. f.	5000	40	10
Echium plantagineum L.	5000	40	10
Erigeron speciosus (Lindl.) DC.	5000	5	0.5
Erysimum cheiri (L.) Crantz	5000	10	3
Erysimum ×marshallii (Henfr.) Bois	5000	10	3
Eschscholzia californica Cham.	5000	20	5
Fatsia japonica (Thunb.) Decne. & Planch.	5000	60	15
Freesia refracta (Jacq.) Klatt	5000	100	25
Gaillardia aristata Pursh	5000	30	8
Gaillardia pulchella Foug.	5000	20	6
Galega officinalis L.	5000	80	20
Galeopsis segetum Neck.	5000	20	4
Gazania rigens (L.) Gaertn.	5000	20	5
Gentiana acaulis L.	5000	5	0.7
Geranium hybrids	5000	40	10
Gerbera jamesonii Adlam	5000	40	10
Geum coccineum Sm.	5000	20	5
Geum quellyon Sweet	5000	20	5
Gilia tricolor Benth.	5000	5	1

Table 2A Part 3. Lot sizes and sample sizes: flower, spice, herb and medicinal species (continued)

Species	Maximum weight of lot (kg) (except see 2.8 Note 2)	Minimum submitted sample (g)	Minimum working sample for purity analysis (3.5.1) (g)
1	2	3	4
Glandularia canadensis (L.) Nutt.	5000	20	6
Glebionis carinata (Schousb.) Tzvelev	5000	30	8
Glebionis coronaria (L.) Cass. ex Spach	5000	30	8
Glebionis segetum (L.) Fourr.	5000	30	8
Gomphrena globosa L.	5000	40	10
Goniolimon tataricum (L.) Boiss.	5000	20	5
Grevillea robusta A.Cunn. ex R.Br.	5000	80	20
Gypsophila elegans M.Bieb.	5000	10	2
Gypsophila paniculata L.	5000	10	2
Gypsophila repens L.	5000	10	2
Helenium autumnale L.	5000	5	0.9
Helianthemum nummularium (L.) Mill.	5000	20	5
Helianthus debilis Nutt.	10000	150	40
(*Helichrysum bracteatum* (Vent.) Andrews see *Xerochrysum bracteatum* (Vent.) Tzvelev)			
Heliopsis helianthoides (L.) Sweet	5000	40	10
Heliotropium arborescens L.	5000	5	1
(*Helipterum humboldtianum* (Gaudich.) DC. see *Rhodanthe humboldtiana* (Gaudich.) Paul G.Wilson)			
(*Helipterum manglesii* (Lindl.) F.Muell. ex Benth. see *Rhodanthe manglesii* Lindl.)			
(*Helipterum roseum* (Hook.) Benth. see *Rhodanthe chlorocephala* (Turcz.) Paul G.Wilson)			
Hesperis matronalis L.	5000	20	5
Heteranthemis viscidehirta Schott	5000	30	8
Heuchera sanguinea Engelm.	5000	5	0.1
Hibiscus trionum L.	5000	40	10
Hippeastrum hybrids	5000	80	20
Hypericum perforatum L.	5000	5	0.3
Hyssopus officinalis L.	5000	10	3
Iberis amara L.	5000	20	6
Iberis gibraltarica L.	5000	10	3
Iberis sempervirens L.	5000	10	3
Iberis umbellata L.	5000	10	3
Impatiens balsamina L.	5000	100	25
Impatiens walleriana Hook. f.	5000	10	2
Inula helenium L.	5000	20	4
Ipomoea alba L.	10000	400	100
Ipomoea purpurea (L.) Roth	10000	400	100
Ipomoea quamoclit L.	10000	200	50
Ipomoea tricolor Cav.	10000	400	100
Jacobaea maritima (L.) Pelser & Meijden (previously *Senecio cineraria* DC.)	5000	5	0.5
Kalanchoe blossfeldiana Poelln.	5000	5	0.1
Kalanchoe crenata (Andrews) Haw.	5000	5	0.1
Kalanchoe globulifera H.Perrier	5000	5	0.1
Kniphofia uvaria (L.) Oken	5000	10	3
(*Kochia scoparia* (L.) Schrad. see *Bassia scoparia* (L.) A.J.Scott)			
Lathyrus latifolius L.	10000	400	100
Lathyrus odoratus L.	10000	600	150
Lavandula angustifolia Mill.	5000	10	2
Lavatera trimestris L.	5000	40	10

Table 2A Part 3. Lot sizes and sample sizes: flower, spice, herb and medicinal species (continued)

Species	Maximum weight of lot (kg) (except see 2.8 Note 2)	Minimum submitted sample (g)	Minimum working sample for purity analysis (3.5.1) (g)
1	2	3	4
Legousia speculum-veneris (L.) Chaix	5000	5	1
Leontopodium nivale (Ten.) Hand.-Mazz. (previously *Leontopodium alpinum* Cass.)	5000	5	0.1
Leonurus cardiaca L.	5000	10	2
Leucanthemum maximum (Ramond) DC.	5000	20	5
Leucanthemum vulgare Lam.	5000	20	5
Levisticum officinale W.D.J.Koch	5000	30	8
Liatris pycnostachya Michx.	5000	30	8
Liatris spicata (L.) Willd.	5000	30	8
Lilium regale E.H.Wilson	5000	40	10
Limonium bellidifolium (Gouan) Dumort.	5000	20	5
Limonium bonduellei (T.Lestib.) Kuntze	5000	200	50
Limonium gerberi Soldano	5000	20	5
Limonium sinuatum (L.) Mill. (heads)	5000	200	50
Limonium sinuatum (L.) Mill. (seeds)	5000	20	6
Linaria bipartita (Vent.) Willd.	5000	5	0.2
Linaria maroccana Hook. f.	5000	5	0.4
Linaria vulgaris Mill.	5000	5	0.2
Linum flavum L.	5000	20	5
Linum grandiflorum Desf.	5000	40	10
Linum narbonense L.	5000	20	5
Linum perenne L.	5000	20	5
Lobelia cardinalis L. (includes *L. fulgens* Humb. & Bonpl. ex Willd.)	5000	5	0.1
Lobelia erinus L.	5000	5	0.2
Lobularia maritima (L.) Desv.	5000	5	1
Lomelosia caucasica (M.Bieb.) Greuter & Burdet (previously *Scabiosa caucasica* M.Bieb.)	5000	80	20
Lonas annua (L.) Vines & Druce	5000	5	0.6
Lunaria annua L.	5000	80	20
Lupinus hartwegii Lindl.	10000	200	60
Lupinus hybrids	10000	200	60
Lupinus nanus Douglas ex Benth.	10000	200	60
Lupinus polyphyllus Lindl.	10000	200	60
Malcolmia maritima (L.) R.Br.	5000	10	3
Malope trifida Cav.	5000	20	5
Marrubium vulgare L.	5000	10	2
Matricaria chamomilla L. (previously *Matricaria recutita* L.)	5000	5	0.5
Matthiola incana (L.) R.Br.	5000	20	4
Matthiola longipetala (Vent.) DC.	5000	10	2
Melissa officinalis L.	5000	10	2
Mentha ×piperita L.	5000	5	0.5
Mimosa pudica L.	5000	40	10
Mimulus cardinalis Douglas ex Benth.	5000	5	0.2
Mimulus cupreus hort. ex Dombrain	5000	5	0.2
Mimulus ×hybridus hort. ex Voss	5000	5	0.2
Mimulus luteus L.	5000	5	0.2
Mirabilis jalapa L.	10000	800	200
Moluccella laevis L.	5000	100	25
Myosotis hybrids	5000	10	2
Myosotis scorpioides L.	5000	10	2
Myosotis sylvatica Hoffm.	5000	10	2

Table 2A Part 3. Lot sizes and sample sizes: flower, spice, herb and medicinal species (continued)

Species	Maximum weight of lot (kg) (except see 2.8 Note 2)	Minimum submitted sample (g)	Minimum working sample for purity analysis (3.5.1) (g)
1	2	3	4
Nemesia strumosa Benth.	5000	5	1
Nemesia versicolor E.Mey. ex Benth.	5000	5	1
Nemophila maculata Benth. ex Lindl.	5000	20	5
Nemophila menziesii Hook. & Arn.	5000	20	5
Nepeta cataria L.	5000	10	2
Nicotiana alata Link & Otto	5000	5	0.2
Nicotiana ×sanderae W.Watson	5000	5	0.2
Nicotiana suaveolens Lehm.	5000	5	0.5
Nierembergia hippomanica Miers	5000	5	0.5
Nigella damascena L.	5000	20	6
Nigella hispanica L.	5000	20	6
Nigella sativa L.	5000	40	10
Oenothera macrocarpa Nutt.	5000	40	10
Osteospermum ecklonis (DC.) Norl.	5000	40	10
Papaver alpinum L.	5000	5	0.5
Papaver glaucum Boiss. & Hausskn.	5000	5	0.5
Papaver nudicaule L.	5000	5	0.5
Papaver orientale L.	5000	5	1
Papaver rhoeas L.	5000	5	0.5
Pelargonium Zonale Group	5000	80	20
Penstemon barbatus (Cav.) Roth	5000	10	2
Penstemon hartwegii Benth.	5000	10	2
Penstemon hybrids	5000	10	2
Pericallis cruenta (Masson ex L'Hér.) Bolle (previously *Senecio cruentus* (Masson ex L'Hér.) DC.)	5000	5	0.5
Perilla frutescens (L.) Britton	5000	10	3
Petunia ×atkinsiana (Sweet) D.Don ex W.H.Baxter (previously *Petunia ×hybrida* hort. ex E.Vilm.)	5000	5	0.2
Phacelia campanularia A.Gray	5000	10	2
Phlox drummondii Hook.	5000	20	5
Phlox paniculata L.	5000	20	5
Phlox subulata L.	5000	20	5
Pholistoma auritum (Lindl.) Lilja	5000	20	5
Physalis alkekengi L.	5000	20	4
Pimpinella major (L.) Huds.	5000	20	5
Pimpinella saxifraga L.	5000	20	5
Plectocephalus americana (Nutt.) D.Don (previously *Centaurea americana* Nutt.)	5000	100	35
Plectranthus scutellarioides (L.) R.Br. (previously *Coleus blumei* Benth.)	5000	10	2
Portulaca grandiflora Hook.	5000	5	0.3
Primula auricula L.	5000	5	1
Primula denticulata Sm.	5000	5	0.5
Primula elatior (L.) Hill	5000	10	2
Primula japonica A.Gray	5000	5	1
Primula ×kewensis W.Watson	5000	5	0.5
Primula malacoides Franch.	5000	5	0.5
Primula obconica Hance	5000	5	0.5
Primula praenitens Ker Gawl.	5000	5	1
Primula veris L.	5000	5	1
Primula vulgaris Huds.	5000	5	1

Table 2A Part 3. Lot sizes and sample sizes: flower, spice, herb and medicinal species (continued)

Species	Maximum weight of lot (kg) (except see 2.8 Note 2)	Minimum submitted sample (g)	Minimum working sample for purity analysis (3.5.1) (g)
1	2	3	4
Psephellus dealbatus (Willd.) K.Koch (previously *Centaurea dealbata* Willd.)	5000	40	10
Psylliostachys suworowii (Regel) Roshkova	5000	20	5
Ranunculus asiaticus L.	5000	5	1
Reseda odorata L.	5000	10	3
Rheum palmatum L.	5000	100	30
Rhodanthe humboldtiana (Gaudich.) Paul G.Wilson (previously *Helipterum humboldtianum* (Gaudich.) DC.)	5000	30	8
Rhodanthe manglesii Lindl. (previously *Helipterum manglesii* (Lindl.) F.Muell. ex Benth.)	5000	30	8
Rhodanthe chlorocephala (Turcz.) Paul G.Wilson (includes *Helipterum roseum* (Hook.) Benth.)	5000	30	8
Rudbeckia fulgida Aiton	5000	10	2
Rudbeckia hirta L.	5000	5	1
Ruta graveolens L.	5000	20	6
Saintpaulia ionantha H.Wendl.	5000	5	0.1
Salpiglossis sinuata Ruiz & Pav.	5000	5	1
Salvia coccinea Buc'hoz ex Etl.	5000	30	8
Salvia farinacea Benth.	5000	20	5
Salvia officinalis L.	5000	30	20
Salvia patens Cav.	5000	30	8
Salvia pratensis L.	5000	30	8
Salvia sclarea L.	5000	80	20
Salvia splendens Sellow ex Schult.	5000	30	8
Salvia viridis L.	5000	20	5
Sanvitalia procumbens Lam.	5000	10	2
Saponaria calabrica Guss.	5000	20	5
Saponaria ocymoides L.	5000	20	5
Saponaria officinalis L.	5000	20	5
Scabiosa atropurpurea L.	5000	60	15
(*Scabiosa caucasica* M.Bieb. see *Lomelosia caucasica* (M.Bieb.) Greuter & Burdet)			
Schefflera elegantissima (hort. Veitch ex Mast.) Lowry & Frodin	5000	20	6
Schizanthus pinnatus Ruiz & Pav.	5000	10	2
(*Senecio cineraria* DC. see *Jacobaea maritima* (L.) Pelser & Meijden)			
(*Senecio cruentus* (Masson ex L'Hér.) DC. see *Pericallis cruenta* (Masson ex L'Hér.) Bolle)			
Senecio elegans L.	5000	5	0.5
Silene chalcedonica (L.) E.H.L.Krause	5000	5	1
Silene coronaria (L.) Clairv.	5000	20	5
Silene pendula L.	5000	10	2
Silybum marianum (L.) Gaertn.	5000	200	50
Sinningia speciosa (Lodd. et al.) Hiern	5000	5	0.2
(*Solanum diflorum* Vell. see *Solanum pseudocapsicum* L.)			
Solanum giganteum Jacq.	5000	20	5
Solanum laciniatum Aiton	5000	20	5
Solanum marginatum L. f.	5000	20	5
Solanum pseudocapsicum L. (previously *Solanum diflorum* Vell.)	5000	20	5
Stachys macrantha (K.Koch) Stearn	5000	20	5
Tagetes erecta L.	5000	40	10

Table 2A Part 3. Lot sizes and sample sizes: flower, spice, herb and medicinal species (continued)

Species	Maximum weight of lot (kg) (except see 2.8 Note 2)	Minimum submitted sample (g)	Minimum working sample for purity analysis (3.5.1) (g)
1	2	3	4
Tagetes patula L.	5 000	40	10
Tagetes tenuifolia Cav.	5 000	20	5
Tanacetum achilleifolium (M.Bieb.) Sch. Bip.	5 000	30	8
Tanacetum cinerariifolium (Trevir.) Sch. Bip.	5 000	10	3
Tanacetum coccineum (Willd.) Grierson	5 000	30	8
Tanacetum parthenium (L.) Sch. Bip.	5 000	20	5
Thunbergia alata Bojer ex Sims	5 000	200	50
Thymus serpyllum L.	5 000	5	0.5
Torenia fournieri Linden ex E.Fourn.	5 000	5	0.2
Tripleurospermum inodorum (L.) Sch. Bip. (previously *Tripleurospermum perforatum* (Mérat) M.Laínz)	5 000	5	0.5
Tripleurospermum maritimum (L.) W.D.J.Koch	5 000	5	0.5
(*Tripleurospermum perforatum* (Mérat) M.Laínz see *Tripleurospermum inodorum* (L.) Sch. Bip.)			
Tropaeolum majus L.	10 000	1 000	350
Tropaeolum peltophorum Benth.	10 000	1 000	350
Tropaeolum peregrinum L.	10 000	1 000	350
Vaccaria hispanica (Mill.) Rauschert	5 000	20	5
Valeriana officinalis L.	5 000	10	2
Verbascum densiflorum Bertol.	5 000	5	0.3
Verbascum phlomoides L.	5 000	5	0.5
Verbascum thapsus L.	5 000	5	0.5
Verbena bonariensis L.	5 000	20	6
Verbena Hybrida Group	5 000	20	6
Verbena rigida Spreng.	5 000	10	2
Vinca minor L.	5 000	20	5
Viola cornuta L.	5 000	10	3
Viola odorata L.	5 000	10	3
Viola tricolor L.	5 000	10	3
Xeranthemum annuum L.	5 000	10	3
Xerochrysum bracteatum (Vent.) Tzvelev (previously *Helichrysum bracteatum* (Vent.) Andrews)	5 000	10	2
Zinnia elegans Jacq.	5 000	80	20
Zinnia haageana Regel	5 000	20	6

Table 2B Part 1. Sample sizes (numbers of seeds) for pelleted seeds, encrusted seed and seed granules

Determinations	Minimum submitted sample	Minimum working sample
Purity analysis (including verification of species)	2 500	2 500
Weight determination	2 500	Pure pellet fraction
Germination	2 500	400
Determination of other seeds	10 000	7 500
Determination of other seeds (encrusted seeds and seed granules)	25 000	25 000
Size grading	5 000	1 000

Table 2B Part 2. Sample sizes (number of seeds) for seed tapes and mats

Determinations	Minimum submitted sample	Minimum working sample
Verification of species	300	100
Germination	2 000	400
Purity analysis (if required)	2 500	2 500
Determination of other seeds	10 000	7 500

2.9 Heterogeneity testing for seed lots in multiple containers

The object of heterogeneity testing is to detect the presence of heterogeneity which makes the seed lot technically unacceptable for sampling according to the object as defined in 2.1.

2.9.1 The H value test

2.9.1.1 Definitions of terms and symbols

The testing of predominantly in-range heterogeneity of an attribute adopted as an indicator involves a comparison between the observed variance and the acceptable variance of that attribute. The container-samples of a seed lot are samples drawn independently of each other from different containers. The examinations of container-samples for the indicating attribute must also be mutually independent. Since there is only one source of information for each container, heterogeneity within containers is not directly involved. The acceptable variance is calculated by multiplying the theoretical variance caused by random variation with a factor *f* for additional variation, taking into account the level of heterogeneity which is achievable in good seed production practice. The theoretical variance can be calculated from the respective probability distributions, which is the binomial distribution in the case of purity and germination, and the Poisson distribution in the case of the other seed count.

***N*o number of containers in the lot**

N number of independent container-samples

n number of seeds tested from each container-sample (1 000 for purity, 100 for germination and 2 500 for other seed count, see 2.9.1.3)

X test result of the adopted attribute in a container-sample

$\sum$ symbol for sum of all values

f factor for multiplying the theoretical variance to obtain the acceptable variance (see Table 2C)

Mean of all *X* values determined for the lot in respect of the adopted attribute:

$$\overline{X} = \frac{\sum X}{N}$$

Acceptable variance of independent container-samples in respect of purity or germination percentages:

$$W = \frac{\overline{X} \cdot (100 - \overline{X})}{n} \cdot f$$

Acceptable variance of independent container-samples in respect of number of other seeds:

$$W = \overline{X} \cdot f$$

Observed variance of independent container-samples based on all *X* values in respect of the adopted attribute:

$$V = \frac{N \sum X^2 - (\sum X)^2}{N(N-1)}$$

H value:

$$H = \frac{V}{W} - f$$

Negative H values are reported as zero.

Table 2C. Factors for additional variation in seed lots to be used for calculating *W* and finally the H value

Attributes	Non-chaffy seeds	Chaffy seeds
Purity	1.1	1.2
Other seed count	1.4	2.2
Germination	1.1	1.2

Remarks:
- For purity and germination calculate to two decimal places if *N* is less than 10 and to three decimal places if *N* is 10 or more.
- For the number of other seeds, calculate to one decimal place if *N* is less than 10, and to two decimal places if *N* is 10 or more.
- For definition of non-chaffy and chaffy seeds see 3.6.6 of the ISTA Rules. The chaffiness of various genera is listed in Table 3B Part 1.

Table 2D. Sampling intensity and critical H values. Number of independent container samples to be drawn as depending on the number of containers in the lot and critical H values for seed lot heterogeneity at a significance level of 1 % probability

Number of containers in the lot	Number of independent container samples	Critical H value for purity and germination attributes		Critical H value for other seed count attributes	
		non-chaffy seeds	chaffy seeds	non-chaffy seeds	chaffy seeds
5	5	2.55	2.78	3.25	5.10
6	6	2.22	2.42	2.83	4.44
7	7	1.98	2.17	2.52	3.98
8	8	1.80	1.97	2.30	3.61
9	9	1.66	1.81	2.11	3.32
10	10	1.55	1.69	1.97	3.10
11–15	11	1.45	1.58	1.85	2.90
16–25	15	1.19	1.31	1.51	2.40
26–35	17	1.10	1.20	1.40	2.20
36–49	18	1.07	1.16	1.36	2.13
50 or more	20	0.99	1.09	1.26	2.00

2.9.1.2 Sampling the lot

The number of independent container samples must be not less than presented in Table 2D.

Sampling intensity has been chosen such that in a lot containing about 10 % deviating containers, at least one deviating container is selected with a probability of p = 90 %. Since the detection of a deviating container is conditional on selection, the power of both tests to detect heterogeneity is at best close to equal, but usually lower than the chosen selection probability. (Reference: Steiner, A. M. and Meyer, U. (1990), H value and R value heterogeneity testing of seed lots; properties, sampling intensity and precision. Agribiological Research 43, 103–114.)

The containers to be sampled are chosen strictly at random. The sample taken from the container must adequately represent the whole contents, e.g. the top, middle and bottom of a bag. The weight of each container-sample must be not less than half that specified in the Table 2A, column 3.

2.9.1.3 Testing procedure

The attribute adopted to indicate heterogeneity may be:

a) percentage by weight of any purity component,
b) percentage of any germination test component, or
c) the total number seeds or the number of any single species in the determination of other seeds by number.

In the laboratory, a working sample is drawn from each container-sample and tested independently of any other sample for the chosen attribute.

a) The percentage by weight of any component may be used, provided it can be separated as in the purity analysis, e.g. pure seed, other seeds, or empty seeds of grasses. The working sample should be of such weight as is estimated to contain 1000 seeds counted from each container-sample. Each working sample is separated into two fractions: the selected component and the remainder.

b) Any kind of seed or seedling determinable in a standard germination test may be used, e.g. normal seedlings, abnormal seedlings or hard seeds. From each container-sample a germination test of 100 seeds is set up simultaneously and completed in accordance with conditions specified in Table 5A.

c) The seed count may be of any component that can be counted, e.g. a specified seed species, or all other seeds together. Each working sample must be of a weight estimated to contain about 2500 seeds and a count is made in it of the number of seeds of the kind selected (i.e. other seed count).

2.9.1.4 Use of Table 2D

Table 2D shows the critical H values which would be exceeded in only 1 % of tests from seed lots with an acceptable distribution of the attribute adopted as indicator. If the calculated H value exceeds the critical H value belonging to the sample number *N*, the attribute and the chaffiness in Table 2D, then the lot is considered to show significant heterogeneity in the in-range, or possibly also the off-range sense. If, however, the calculated H value is less than or equal to the tabulated critical H value, then the lot is considered to show no heterogeneity in the in-range, or possibly off-range sense with respect to the attribute being tested.

2.9.1.5 Reporting results

The result of the H value heterogeneity test for seed lots in multiple containers must be reported under 'Other determinations', as follows:

- $\overline{X}$: mean of all *X* values determined for the lot in respect of the adopted attribute;
- *N*: number of independent container samples;
- *No*: number of containers in the lot;
- the calculated H value;
- the statement: 'This H value does/does not indicate significant heterogeneity.'

Note: the H value must not be calculated or reported if $\overline{X}$ is outside the following limits:

- purity components: above 99.8 % or below 0.2 %;
- germination: above 99.0 % or below 1.0 %;
- number of specified seeds: below two per sample.

2.9.2 The R value test

The object of this test is to detect off-range heterogeneity of the seed lot using the attribute adopted as an indicator. The test for off-range heterogeneity involves comparing the maximum difference found between samples of similar size drawn from the lot with a tolerated range. This tolerated range is based on the acceptable standard deviation, which is achievable in good seed production practice.

Each independent container-sample is taken from a different container, so that heterogeneity within containers is not directly involved. Information about heterogeneity within containers is contained, however, in the acceptable standard deviation which is in fact incorporated into the tabulation of tolerated ranges. The acceptable standard deviation was calculated by the standard deviation due to random variation according to the binomial distribution in the case of purity and germination, and to the Poisson distribution in the case of the other seed count, multiplied by the square root of the factor *f* given in Table 2C, respectively. The spread between containers is characterised by the calculated range to be compared with the corresponding tolerated range.

2.9.2.1 Definitions of terms and symbols

No number of containers in the lot

N number of independent container-samples

n number of seeds tested from each container-sample (1 000 for purity, 100 for germination and 2 500 for other seed count, see 2.9.1.3)

X test result of the adopted attribute in a container-sample

$\sum$ symbol for sum of all values

Mean of all *X* values determined for the lot in respect of the adopted attribute:

$$\overline{X} = \frac{\sum X}{N}$$

Range found as maximum difference between independent container samples of the lot in respect of the adopted attribute:

$$R = X_{max} - X_{min}$$

Note: for precision of *X* for the R value test, see 2.9.1.1 'Remarks' to the H value test.

2.9.2.2 Sampling the lot

Sampling for the R value test is the same as for the H value test (see 2.9.1.2); the same samples must be used.

2.9.2.3 Testing procedure

The same testing procedures of purity, germination and the other seed count are used for the R value test as are used for the H value test (see 2.9.1.3). For calculations, the same set of data must be used.

2.9.2.4 Use of tables

Seed lot off-range heterogeneity is tested by using the appropriate table for tolerated, i.e. critical range:
- Table 2E for components of pure seed analyses,
- Table 2F for germination determinations, and
- Table 2G for numbers of other seeds.

Find the value $\overline{X}$ in the 'Average' columns of the appropriate table. When entering the table, round averages following the usual procedure; read off the tolerated range which would be exceeded in only 1 % of tests from seed lots with an acceptable distribution of the attribute:
- in columns 5–9 for cases when $N = 5$ to 9,
- in columns 10–19 for cases when $N = 10$ to 19, or
- in column 20 when $N = 20$.

If the calculated R value exceeds this tolerated range, then the lot is considered to show significant heterogeneity in the off-range sense. If, however, the calculated R value is less than or equal to the tabulated tolerated range, then the lot is considered to show no heterogeneity in the off-range sense with respect to the attribute being tested.

When using the tables, round averages to the next tabulated value (if in the middle, then downwards).

2.9.2.5 Reporting results

The result of the R value heterogeneity test for seed lots in multiple containers must be reported under 'Other determinations', as follows:

- $\overline{X}$: mean of all X values determined for the lot in respect of the adopted attribute;
- N: number of independent container samples;
- No: number of containers in the lot;
- the calculated R value;
- the statement: 'This R value does/does not indicate significant heterogeneity.'

2.9.3 Interpretation of results

Whenever either of the two tests, the H value test or the R value test, indicates significant heterogeneity, then the lot must be declared heterogeneous. When, however, neither of the two tests indicates significant heterogeneity, then the lot must be adopted as non-heterogeneous, having a non-significant level of heterogeneity.

Table 2E Part 1. Maximum tolerated ranges for the R value test at a significance level of 1 % probability using components of purity analyses as the indicating attribute in non-chaffy seeds

Average % of the component and its complement		Tolerated range for number of independent samples (N)		
		5–9	10–19	20
99.9	0.1	0.5	0.5	0.6
99.8	0.2	0.7	0.8	0.8
99.7	0.3	0.8	0.9	1.0
99.6	0.4	1.0	1.1	1.2
99.5	0.5	1.1	1.2	1.3
99.4	0.6	1.2	1.3	1.4
99.3	0.7	1.3	1.4	1.6
99.2	0.8	1.4	1.5	1.7
99.1	0.9	1.4	1.6	1.8
99.0	1.0	1.5	1.7	1.9
98.5	1.5	1.9	2.1	2.3
98.0	2.0	2.1	2.4	2.6
97.5	2.5	2.4	2.7	2.9
97.0	3.0	2.6	2.9	3.2
96.5	3.5	2.8	3.1	3.4
96.0	4.0	3.0	3.4	3.7
95.5	4.5	3.2	3.5	3.9
95.0	5.0	3.3	3.7	4.1
94.0	6.0	3.6	4.1	4.5
93.0	7.0	3.9	4.4	4.8
92.0	8.0	4.1	4.6	5.1
91.0	9.0	4.4	4.9	5.4
90.0	10.0	4.6	5.1	5.6
89.0	11.0	4.8	5.4	5.9
88.0	12.0	5.0	5.6	6.1
87.0	13.0	5.1	5.8	6.3
86.0	14.0	5.3	5.9	6.5
85.0	15.0	5.4	6.1	6.7
84.0	16.0	5.6	6.3	6.9
83.0	17.0	5.7	6.4	7.0
82.0	18.0	5.9	6.6	7.2
81.0	19.0	6.0	6.7	7.4
80.0	20.0	6.1	6.8	7.5
78.0	22.0	6.3	7.1	7.8
76.0	24.0	6.5	7.3	8.0
74.0	26.0	6.7	7.5	8.2
72.0	28.0	6.9	7.7	8.4
70.0	30.0	7.0	7.8	8.6
68.0	32.0	7.1	8.0	8.7
66.0	34.0	7.2	8.1	8.9
64.0	36.0	7.3	8.2	9.0
62.0	38.0	7.4	8.3	9.1
60.0	40.0	7.5	8.4	9.2
58.0	42.0	7.5	8.4	9.2
56.0	44.0	7.6	8.5	9.3
54.0	46.0	7.6	8.5	9.3
52.0	48.0	7.6	8.6	9.4
50.0	50.0	7.6	8.6	9.4

Table 2E Part 2. Maximum tolerated ranges for the R value test at a significance level of 1 % probability using components of purity analyses as the indicating attribute in chaffy seeds

Average % of the component and its complement		Tolerated range for number of independent samples (N)		
		5–9	10–19	20
99.9	0.1	0.5	0.6	0.6
99.8	0.2	0.7	0.8	0.9
99.7	0.3	0.9	1.0	1.1
99.6	0.4	1.0	1.1	1.2
99.5	0.5	1.1	1.3	1.4
99.4	0.6	1.2	1.4	1.5
99.3	0.7	1.3	1.5	1.6
99.2	0.8	1.4	1.6	1.7
99.1	0.9	1.5	1.7	1.8
99.0	1.0	1.6	1.8	1.9
98.5	1.5	1.9	2.2	2.4
98.0	2.0	2.2	2.5	2.7
97.5	2.5	2.5	2.8	3.1
97.0	3.0	2.7	3.0	3.3
96.5	3.5	2.9	3.3	3.6
96.0	4.0	3.1	3.5	3.8
95.5	4.5	3.3	3.7	4.1
95.0	5.0	3.5	3.9	4.3
94.0	6.0	3.8	4.2	4.6
93.0	7.0	4.1	4.6	5.0
92.0	8.0	4.3	4.8	5.3
91.0	9.0	4.6	5.1	5.6
90.0	10.0	4.8	5.4	5.9
89.0	11.0	5.0	5.6	6.1
88.0	12.0	5.2	5.8	6.4
87.0	13.0	5.4	6.0	6.6
86.0	14.0	5.5	6.2	6.8
85.0	15.0	5.7	6.4	7.0
84.0	16.0	5.8	6.6	7.2
83.0	17.0	6.0	6.7	7.4
82.0	18.0	6.1	6.9	7.5
81.0	19.0	6.3	7.0	7.7
80.0	20.0	6.4	7.1	7.8
78.0	22.0	6.6	7.4	8.1
76.0	24.0	6.8	7.6	8.4
74.0	26.0	7.0	7.8	8.6
72.0	28.0	7.2	8.0	8.8
70.0	30.0	7.3	8.2	9.0
68.0	32.0	7.4	8.3	9.1
66.0	34.0	7.5	8.5	9.3
64.0	36.0	7.6	8.6	9.4
62.0	38.0	7.7	8.7	9.5
60.0	40.0	7.8	8.8	9.6
58.0	42.0	7.9	8.8	9.7
56.0	44.0	7.9	8.9	9.7
54.0	46.0	7.9	8.9	9.8
52.0	48.0	8.0	8.9	9.8
50.0	50.0	8.0	8.9	9.8

Table 2F Part 1. Maximum tolerated ranges for the R value test at a significance level of 1 % probability using components of germination tests as the indicating attribute in non-chaffy seeds

Average % of the component	its complement	Tolerated range for number of independent samples (N)		
		5–9	10–19	20
99	1	5	6	6
98	2	7	8	9
97	3	9	10	11
96	4	10	11	12
95	5	11	12	13
94	6	12	13	15
93	7	13	14	16
92	8	14	15	17
91	9	14	16	17
90	10	15	17	18
89	11	16	17	19
88	12	16	18	20
87	13	17	19	20
86	14	17	19	21
85	15	18	20	22
84	16	18	20	22
83	17	19	21	23
82	18	19	21	23
81	19	19	22	24
80	20	20	22	24
79	21	20	23	25
78	22	20	23	25
77	23	21	23	25
76	24	21	24	26
75	25	21	24	26
74	26	22	24	26
73	27	22	25	27
72	28	22	25	27
71	29	22	25	27
70	30	23	25	28
69	31	23	26	28
68	32	23	26	28
67	33	23	26	28
66	34	23	26	29
65	35	24	26	29
64	36	24	26	29
63	37	24	27	29
62	38	24	27	29
61	39	24	27	29
60	40	24	27	30
59	41	24	27	30
58	42	24	27	30
57	43	24	27	30
56	44	24	27	30
55	45	25	27	30
54	46	25	27	30
53	47	25	28	30
52	48	25	28	30
51	49	25	28	30
50	50	25	28	30

Table 2F Part 2. Maximum tolerated ranges for the R value test at a significance level of 1 % probability using components of germination tests as the indicating attribute in chaffy seeds

Average % of the component	its complement	Tolerated range for number of independent samples (N)		
		5–9	10–19	20
99	1	6	6	7
98	2	8	8	9
97	3	9	10	11
96	4	10	12	13
95	5	11	13	14
94	6	12	14	15
93	7	13	15	16
92	8	14	16	17
91	9	15	17	18
90	10	16	17	19
89	11	16	18	20
88	12	17	19	21
87	13	17	20	21
86	14	18	20	22
85	15	18	21	23
84	16	19	21	23
83	17	19	22	24
82	18	20	22	24
81	19	20	23	25
80	20	21	23	25
79	21	21	24	26
78	22	21	24	26
77	23	22	24	27
76	24	22	25	27
75	25	22	25	27
74	26	23	25	28
73	27	23	26	28
72	28	23	26	28
71	29	23	26	29
70	30	24	26	29
69	31	24	27	29
68	32	24	27	29
67	33	24	27	30
66	34	24	27	30
65	35	25	27	30
64	36	25	28	30
63	37	25	28	30
62	38	25	28	31
61	39	25	28	31
60	40	25	28	31
59	41	25	28	31
58	42	25	28	31
57	43	25	28	31
56	44	26	29	31
55	45	26	29	31
54	46	26	29	31
53	47	26	29	31
52	48	26	29	31
51	49	26	29	31
50	50	26	29	31

Table 2G Part 1. Maximum tolerated ranges for the R value test at a significance level of 1 % probability using components of other seed count analyses as the indicating attribute in non-chaffy seeds

Average count of other seeds	Tolerated range for number of independent samples (N)		
	5–9	10–19	20
1	6	7	7
2	8	9	10
3	10	11	12
4	11	13	14
5	13	14	15
6	14	15	17
7	15	17	18
8	16	18	19
9	17	19	21
10	18	20	22
11	19	21	23
12	19	22	24
13	20	23	25
14	21	23	26
15	22	24	26
16	22	25	27
17	23	26	28
18	24	26	29
19	24	27	30
20	25	28	30
21	25	28	31
22	26	29	32
23	27	30	33
24	27	30	33
25	28	31	34
26	28	32	35
27	29	32	35
28	29	33	36
29	30	33	37
30	30	34	37
31	31	34	38
32	31	35	38
33	32	36	39
34	32	36	39
35	33	37	40
36	33	37	41
37	34	38	41
38	34	38	42
39	34	39	42
40	35	39	43
41	35	40	43
42	36	40	44
43	36	41	44
44	37	41	45
45	37	41	45
46	37	42	46
47	38	42	46
48	38	43	47
49	39	43	47
50	39	44	48

Average count of other seeds	Tolerated range for number of independent samples (N)		
	5–9	10–19	20
51	39	44	48
52	40	45	49
53	40	45	49
54	40	45	50
55	41	46	50
56	41	46	51
57	42	47	51
58	42	47	51
59	42	47	52
60	43	48	52
61	43	48	53
62	43	49	53
63	44	49	54
64	44	49	54
65	44	50	54
66	45	50	55
67	45	50	55
68	45	51	56
69	46	51	56
70	46	52	56
71	46	52	57
72	47	52	57
73	47	53	58
74	47	53	58
75	48	53	58
76	48	54	59
77	48	54	59
78	49	54	60
79	49	55	60
80	49	55	60
81	49	55	61
82	50	56	61
83	50	56	61
84	50	56	62
85	51	57	62
86	51	57	62
87	51	57	63
88	52	58	63
89	52	58	64
90	52	58	64
91	52	59	64
92	53	59	65
93	53	59	65
94	53	60	65
95	54	60	66
96	54	60	66
97	54	61	66
98	54	61	67
99	55	61	67
100	55	62	67

Average count of other seeds	Tolerated range for number of independent samples (N)		
	5–9	10–19	20
101	55	62	68
102	55	62	68
103	56	62	68
104	56	63	69
105	56	63	69
106	57	63	69
107	57	64	70
108	57	64	70
109	57	64	70
110	58	65	71
111	58	65	71
112	58	65	71
113	58	65	72
114	59	66	72
115	59	66	72
116	59	66	73
117	59	67	73
118	60	67	73
119	60	67	73
120	60	67	74

Average count of other seeds	Tolerated range for number of independent samples (N)		
	5–9	10–19	20
121	60	68	74
122	61	68	74
123	61	68	75
124	61	68	75
125	61	69	75
126	62	69	76
127	62	69	76
128	62	70	76
129	62	70	76
130	63	70	77
131	63	70	77
132	63	71	77
133	63	71	78
134	64	71	78
135	64	71	78
136	64	72	78
137	64	72	79
138	64	72	79

For higher other seed counts, tolerances (R) are calculated by using the following formula and rounding up to the next whole number:

For N = 5–9: $R = \sqrt{(\text{average count of other seed})} \times 5.44$

For N = 10–19: $R = \sqrt{(\text{average count of other seed})} \times 6.11$

For N = 20: $R = \sqrt{(\text{average count of other seed})} \times 6.69$

Table 2G Part 2. Maximum tolerated ranges for the R value test at a significance level of 1 % probability using components of other seed count analyses as the indicating attribute in chaffy seeds

Average count of other seeds	Tolerated range for number of independent samples (N)		
	5–9	10–19	20
1	7	8	9
2	10	11	12
3	12	14	15
4	14	16	17
5	16	18	19
6	17	19	21
7	19	21	23
8	20	22	24
9	21	23	26
10	22	25	27
11	23	26	28
12	24	27	30
13	25	28	31
14	26	29	32
15	27	30	33
16	28	31	34
17	29	32	35
18	29	33	36
19	30	34	37
20	31	35	38
21	32	36	39
22	33	36	40
23	33	37	41
24	34	38	42
25	35	39	42
26	35	40	43
27	36	40	44
28	37	41	45
29	37	42	46
30	38	42	46
31	38	43	47
32	39	44	48
33	40	44	49
34	40	45	49
35	41	46	50
36	41	46	51
37	42	47	51
38	43	48	52
39	43	48	53
40	44	49	54
41	44	50	54
42	45	50	55
43	45	51	55
44	46	51	56
45	46	52	57
46	47	52	57
47	47	53	58
48	48	54	59
49	48	54	59
50	49	55	60

Average count of other seeds	Tolerated range for number of independent samples (N)		
	5–9	10–19	20
51	49	55	60
52	50	56	61
53	50	56	62
54	51	57	62
55	51	57	63
56	52	58	63
57	52	58	64
58	52	59	64
59	53	59	65
60	53	60	65
61	54	60	66
62	54	61	66
63	55	61	67
64	55	62	68
65	56	62	68
66	56	63	69
67	56	63	69
68	57	64	70
69	57	64	70
70	58	65	71
71	58	65	71
72	58	65	72
73	59	66	72
74	59	66	73
75	60	67	73
76	60	67	74
77	60	68	74
78	61	68	75
79	61	69	75
80	62	69	75
81	62	69	76
82	62	70	76
83	63	70	77
84	63	71	77
85	63	71	78
86	64	71	78
87	64	72	79
88	65	72	79
89	65	73	80
90	65	73	80
91	66	74	80
92	66	74	81
93	66	74	81
94	67	75	82
95	67	75	82
96	67	75	83
97	68	76	83
98	68	76	83
99	68	77	84
100	69	77	84

Average count of other seeds	Tolerated range for number of independent samples (N)		
	5–9	10–19	20
101	69	77	85
102	69	78	85
103	70	78	86
104	70	79	86
105	70	79	86
106	71	79	87
107	71	80	87
108	71	80	88
109	72	80	88
110	72	81	88
111	72	81	89
112	73	81	89
113	73	82	90
114	73	82	90
115	74	83	90
116	74	83	91
117	74	83	91
118	75	84	92
119	75	84	92
120	75	84	92

Average count of other seeds	Tolerated range for number of independent samples (N)		
	5–9	10–19	20
121	76	85	93
122	76	85	93
123	76	85	93
124	76	86	94
125	77	86	94
126	77	86	95
127	77	87	95
128	78	87	95
129	78	87	96
130	78	88	96
131	79	88	96
132	79	88	97
133	79	89	97
134	79	89	98
135	80	89	98
136	80	90	98
137	80	90	99
138	81	90	99

For higher other seed counts, tolerances (R) are calculated by using the following formula and rounding up to the next whole number:

For N = 5–9: $R = \sqrt{(\text{average count of other seed})} \times 6.82$

For N = 10–19: $R = \sqrt{(\text{average count of other seed})} \times 7.65$

For N = 20: $R = \sqrt{(\text{average count of other seed})} \times 8.38$

Chapter 3: The purity analysis

3.1 Object

The object of the purity analysis is to determine:

a) The percentage composition by weight of the sample being tested and by inference the composition of the seed lot
b) The identity of the various species of seeds and inert particles constituting the sample.

3.2 Definitions

3.2.1 Pure seed

The pure seed must refer to the species stated by the applicant, or found to predominate in the test, and must include all botanical varieties and cultivars of that species including:

1. The following structures (even if immature, undersized, shrivelled, diseased or germinated, providing they can be definitely identified as of that species) unless transformed into partially or fully ergotised visible fungal sclerotia, smut balls or nematode galls (see 3.5.2.5.1 for exceptions when the uniform blowing method is used):
   1. Intact seed units (= commonly found dispersal units i.e. achenes and similar fruits, schizocarps, florets etc.) as defined for each genus or species in the Pure Seed Definitions (PSDs) in Table 3B Part 2.
      In *Poaceae*:
      a) florets with an obvious caryopsis containing endosperm,
      b) free caryopses.
   2. Pieces of seed units larger than one-half their original size.

2. From the above main principles, exceptions are made for certain genera of *Poaceae* (Table 3B Part 2):
   a) a minimum size of caryopsis is required (3.5.2.2);
   b) the presence of caryopses in spikelets and florets is not always obligatory;
   c) the separation of pure seed and inert matter is done by a uniform blowing procedure (see 3.5.2.5);
   d) multiple seed units (MSU) are left intact in the pure seed fraction;
   e) attached sterile florets are not removed (3.5.2.2);
   f) for certain genera appendages are left on the seed but reported according to 3.5.2.8.

3.2.2 Other seeds

Other seeds must include seed units of any plant species other than that of pure seed. With respect to classification as other seeds or inert matter the distinguishing characteristics described in the pure seed definitions (Table 3B Part 2) must also be applicable except that:

1. Seed units of species for which a uniform blowing procedure applies are evaluated without blowing.

2. Multiple seed units (MSU) must be separated and the single units classified according to the general principles in 3.2.

3. *Cuscuta* spp. seed units which are fragile or ashen grey to creamy white in colour are classified as inert matter.

4. For schizocarps with two or more seeds, the individual seeds (mericarps) contained in a schizocarp are to be counted separately.

For species and genera without pure seed definitions in Table 3B Part 2 the definitions in 3.2.1 must apply. Multiple structures, capsules, pods are opened and the seeds are removed and the non-seed material placed in the inert matter, except for certain species or genera as indicated in the Pure Seed Definitions (Table 3B Part 2).

3.2.3 Inert matter

Inert matter must include seed units and all other matter and structures not defined as pure seed or other seed as follows:

1. Seed units in which it is readily apparent that no true seed is present.

2. Florets of those species listed in 3.5.2.2 with a caryopsis less than the minimum size prescribed. Sterile florets attached to a fertile floret are to be removed, except in certain genera listed in 3.5.2.2.

3. Pieces of broken or damaged seed units half or less than half the original size.

4. Those appendages not classed as being part of the pure seed in the pure seed definitions for the species (Table 3B Part 2). Appendages not mentioned in the pure seed definitions must be removed and included in the inert matter.

5. Seeds of *Berberidaceae, Brassicaceae, Cupressaceae, Fabaceae, Pinaceae, Taxaceae* and *Taxodiaceae* with the seed coat entirely removed. In *Fabaceae*, separated cotyledons are regarded as inert matter, irrespective of whether or not the radicle-plumule axis and/or more than half of the testa may be attached.

6. Seeds of *Cuscuta* spp. which are fragile or ashen grey to creamy white in colour.

7. Unattached sterile florets, empty glumes, lemmas, paleas, chaff, stems, leaves, cone scales, wings, bark, flowers, nematode galls, fungus bodies such as ergot, sclerotia and smut balls, soil, sand, stones and all other non seed matter.

8. All material left in the light fraction when the separation is made by the uniform blowing method (3.5.2.5) except other seeds (as defined in 3.2.2).
   In the heavy fraction, broken florets, and caryopses half or less than half the original size, and all other matter except pure seed (3.2.1) and other seed (3.2.2).

3.3 General principles

The working sample is separated into the following three component parts: pure seed, other seeds, inert matter, and the percentage of each part is determined by weight. All species of seed and each kind of inert matter present must be identified as far as possible and, if required for reporting, its percentage by weight must be determined.

3.4 Apparatus

Aids such as magnifiers, reflected light, sieves and blowers may be used in separating the working sample into its component parts.

3.4.1 Magnifiers, reflected light and sieves

Hand lenses and binocular microscopes are quite often necessary aids for an accurate identification and separation of small seed units and fragments.

Reflected light is very useful for separating sterile florets of grasses from fertile ones and may also be used for the detection of nematode galls and fungal bodies.

Sieves can be used as an aid for the purity analysis in separating trash, soil and other small particles from the working sample.

3.4.2 Seed blowers

Seed blowers can be used to separate light-weight material such as chaff and empty florets from the heavier seeds for all species as a tool for purity analysis.

Blowers that will give the most accurate separations normally handle only small samples (up to 5 g). A good blower should provide a uniform flow of air, be capable of standardisation and retain all the particles which it separates.

For certain species and varieties of *Poaceae*, seed blowers must be used by the uniform blowing method (3.5.2.5) to separate light-weight material such as chaff and empty florets from the heavier seeds.

In order to maintain a uniform flow of air the blower should have one or more air compression chambers and a fan driven by a uniform speed motor. The diameter of the blowing tube should be in proportion to the size of the working sample and the tube should be long enough to allow satisfactory separation of the sample. The valve or air gate that regulates the air flow should be capable of precise adjustment, should be calibrated and marked to permit easy reading, and its construction and location should prevent areas of strong and weak currents in the blowing tube.

A seed blower to be used for the uniform blowing method must be capable of:

a) blowing at different air velocities (determined by the use of the calibration samples) to suit different species;

b) maintaining a uniform flow of air at the velocity required by the crop species under test;

c) rapid adjustment to any velocity likely to be required. The setting to provide each velocity should be checked

annually by blowing a calibration sample issued under the authority of ISTA;

d) accurate time setting.

3.4.2.1 Calibration of the seed blower

The air gate openings and the equivalent air velocity (EAV) value (see 3.4.2.2) of the optimum blowing point for a General-type seed blower are determined by using the uniform calibration samples. Calibration samples are issued under the authority of ISTA and are available for *Dactylis glomerata* and *Poa pratensis*. Prior to calibration, the calibration samples must be exposed to room conditions overnight.

For those not having a General-type seed blower, please contact the ISTA Secretariat.

The air gate opening for the varieties of *Poa pratensis* listed in Table 3A, with an average thousand-seed weight less than 0.35 g, and for *Poa trivialis* is obtained by multiplying the value of the air gate setting for *Poa pratensis* by 0.82 (applies only for General-type seed blowers).

3.4.2.2 Determination of the equivalent air velocity

After a General-type seed blower has been calibrated according to 3.4.2.1, the EAV of the air gate opening must be measured using an anemometer. The following procedure must be used:

1. Set the blower at the optimum blowing point, i.e. the air gate opening, obtained with the ISTA uniform calibration sample for the relevant species, e.g. *Dactylis glomerata* or *Poa pratensis*. Do not change that air gate opening.

2. Remove the sample cup from the cup holder, insert the anemometer with digital display facing up, and align the fan of the anemometer over the blower opening where the air flows from the chamber into the sample cup holder.

3. Turn on the anemometer and select metres per second (m/s), hold the anemometer in a steady position and then turn on the blower.

4. Read the air velocity value after the digital display of the anemometer reaches a steady reading (typically about 30 s after the blower was turned on). Example: If the anemometer indicates 2.3 m/s most frequently and fluctuates between 2.2 and 2.4 m/s, the EAV value of that specific air-gate opening would be recorded as 2.3 ±0.1 m/s.

Once the optimum air velocity has been measured, the seed blower can be recalibrated using the anemometer, by adjusting the blower setting until the optimum air velocity for the blower and species or variety is reached. The EAV for one blower is not transferable to another blower.

The optimum blowing point must be verified using the ISTA uniform calibration sample after major servicing of the blower, such as changing parts of the motor or the glass column. In general, it is strongly recommended that the blowing point be verified annually using the ISTA uniform calibration sample.

Laboratories that can not, or do not, use the EAV to determine the blowing point must calibrate the blower with the ISTA uniform calibration sample.

Note: Frequent use of the ISTA uniform calibration sample can cause a shift in blowing point due to deterioration and monitoring the blowing point simply by air gate opening may be reliable in some blowers and not in others.

3.4.2.3 Anemometer type

Any suitable anemometers can be used as long as the anemometer fits in the sample cup holder compartment of the blower and has a scale calibrated in metres per second for reading the air velocity value.

3.4.2.4 Calibration of the anemometer

The anemometer should be calibrated at intervals set by the laboratory. In addition, the batteries should be replaced at least once a year.

3.5 Procedure

3.5.1 Working sample

The purity analysis must be made on a working sample taken from the submitted sample in accordance with 2.5.2, the submitted sample having been received in accordance with 2.5.1. Except for species of *Poaceae* for which the uniform blowing method is to be used, the size of the working sample must be:

either a weight estimated to contain at least 2500 seed units,
or not less than the weight indicated in column 4 of Table 2A.

The analysis may be made on one working sample of this weight or on two representative subsamples of at least half this weight.

The working sample (or each subsample) must be weighed in grams to the minimum number of decimal places necessary to calculate the percentage of its component parts to one decimal place, as indicated below.

Weight of working sample or subsample (g)	Minimum number of decimal places
Less than 1.000	4
1.000–9.999	3
10.00–99.99	2
100.0–999.9	1
1000 or more	0

3.5.2 Separation

1. The working sample (or subsample) after weighing, must be separated into its component parts as defined in 3.2. In general, the separation must be based on an examination of each particle in the sample, but in certain cases special procedures are obligatory, such as uniform blowing.

2. The separation of the pure seed must be on such a basis that it can be made by visible seed characteristics, mechanical aids or using pressure without impairing the capacity for germination.

3. When it is difficult or impossible to distinguish between species, one of the procedures described in 3.5.2.4 must be followed. Furthermore, for methods which may be used for clearly distinguishing species and cultivars, but are not permissible in the purity analysis, follow Chapter 8.

4. After separation, each component part (3.3) and any species of seed or kind of other matter for which a percentage is to be reported, must be weighed in grams to the minimum number of decimal places necessary to calculate the percentage to one decimal place (3.5.1). After weighing, the other seeds components must be retained and stored for reference until sample disposal (see 2.5.3 and 2.5.4.7).

3.5.2.1 All families except *Poaceae*

Achenes, schizocarps and mericarps, other fruits and seeds are to be examined superficially only, without the use of pressure, a diaphanascope or other special equipment. If it is obvious on such an examination that there is no seed in the structure, it is to be regarded as inert matter.

3.5.2.2 *Poaceae*

Caryopses

In *Lolium, Festuca, ×Festulolium* and *Elytrigia repens* a floret with a caryopsis one third or more of the length of the palea measured from the base of the rachilla is regarded as pure seed or other seed, but a floret with a caryopsis less than one third the length of palea is regarded as inert matter. In other genera or species a floret with any endosperm in the caryopsis is regarded as pure seed.

Sterile florets

In the following genera a sterile floret attached to a fertile floret is not removed, but left attached and included in the pure seed fraction: *Arrhenatherum, Avena, Bromus, Chloris, Dactylis, Festuca, ×Festulolium, Holcus, Koeleria, Lolium, Poa, Sorghum, Triticum dicoccon* and *Triticum spelta*.

3.5.2.3 Damaged seed

If the seed units mentioned in 3.2.1 show no evident damage to the testa or pericarp, they are regarded as pure seed or other seed, irrespective of whether they are empty or full, but difficulty may arise when there is an opening in the testa or pericarp. If possible, the analyst must decide whether the remaining solid portion of the seed unit is larger than one-half the original size and apply this rule accordingly. If such a determination cannot be readily made, the seed unit will be classed as pure seed or other seed. It is not necessary for individual seed units to be turned over to determine the presence or absence of holes or other damaged areas on the underside.

Broken florets and caryopses are classed as pure seed or other seed if the piece is larger than half the original size (3.2.1.1.2).

3.5.2.4 Indistinguishable species

When it is difficult or impossible to distinguish between species, one of the two following procedures may be followed:

a) Only the genus name is reported on the analysis Certificate, all seeds of that genus (e.g. both awned and awnless seeds of *Lolium*) being classed as pure seed; additional information may be reported under 'Other Determinations', or

b) The similar seeds are separated from the other components and weighed together. From this mixture at least 400 seeds, and preferably about 1000, are taken at random; a final separation is made on this portion and the proportion of each species determined by weight. From this proportion the percentage of each species in the entire sample can be calculated (3.6).

If this procedure is followed, the details must be reported including the number of seeds examined.

The procedures are applicable when the seed is described by the sender as a species of *Agrostis, Brassica, Lolium, Poa,* and *Festuca rubra* or *F. ovina*, and in other cases at the discretion of the analyst.

3.5.2.5 *Poa pratensis*, *Poa trivialis* and *Dactylis glomerata*

For *Poa pratensis, Poa trivialis* and *Dactylis glomerata*, the uniform blowing method (see 3.4.2) is obligatory.

The working sample size is 1 g for *Poa pratensis* and *Poa trivialis,* and 3 g for *Dactylis glomerata.*

The optimum blower settings for *Poa pratensis* and *Dactylis glomerata* are determined by means of a uniform calibration sample issued under the authority of ISTA (see 3.4.2.1). The optimum blower setting for the varieties of *Poa pratensis* listed in Table 3A, with an average thousand-seed weight less than 0.35 g, and for *Poa trivialis* is obtained by multiplying the value of the optimum blower setting for *Poa pratensis* by 0.82 (applies only for General-type seed blowers).

For those not having a General-type seed blower, please contact the ISTA Secretariat.

For blowing samples, set the seed blower to the optimum blower setting, obtained with the ISTA uniform calibration sample or the anemometer (see 3.4.2.1).

Place the working sample into the cup and blow for exactly 3 min.

Prior to blowing, the working sample must be exposed to room conditions to equilibrate with ambient conditions.

Table 3A. List of varieties of *Poa pratensis* with an average thousand-seed weight of less than 0.35 g.

Variety	Thousand-seed weight (g)
Balin	0.34
Compact	0.34
Julia	0.33
Limousine	0.33
Enprima	0.32
Oxford	0.32
Ikone	0.31
Sobra	0.31
Pegasus	0.29
Platini	0.29
Slezanka	0.28
Mardona	0.27
Tommy	0.26
Lato	0.24
Harmony	0.23

3.5.2.5.1 Separation of the heavy fraction

1. All seed units of the species under analysis remaining in the cup after blowing (i.e. the heavy fraction) are to be classified as pure seed including:
   1. Intact single florets. For *Dactylis glomerata* refer to Table 3B Part 2
   2. All intact multiple florets of *Poa pratensis* and *Poa trivialis* and multiple seed units of *Dactylis glomerata* (3.2.1.2.2)
   3. Florets with fungus bodies, such as ergot, entirely enclosed within lemma and palea
   4. Florets and free caryopses (lemma and palea missing) that are insect-damaged or diseased, including caryopses which are spongy, corky, white or crumbly
   5. Broken florets and caryopses larger than half the original size

2. Classify the following *Poa pratensis*, *Poa trivialis* or *Dactylis glomerata* florets and caryopses as inert matter:
   1. Florets with ergot exserted from the tip of the floret
   2. Broken florets and caryopses, half or less than half the original size

3. Other seeds (including other *Poa* spp.), sticks, stems, sand etc. must be classified in accordance with 3.2.2 and 3.2.3.

3.5.2.5.2 Separation of the light fraction

The light fraction comprises seed units and other material removed by blowing at the uniform blowing point.

1. All *Poa pratensis*, *Poa trivialis* or *Dactylis glomerata* florets and caryopses contained in the light fraction must be considered as inert matter.

2. Other seeds (including other *Poa* spp. in *P. pratensis* and *P. trivialis*), sticks, stems, sand etc. must be classified in accordance with 3.2.2 and 3.2.3. When fertile florets of some *Poa* spp. (e.g. *Poa compressa*) are present in a sample of *Poa pratensis* or *Poa trivialis* it is necessary to examine the entire light fraction under magnification. If seeds of these species are present in minor amounts (1–3 %) in a sample it is generally easier to remove all florets from the heavy and light fractions and determine the percentage of other seed on the basis of the total weight. When seeds of other *Poa* spp. are present in a sample of *Poa pratensis* or *Poa trivialis* in larger amounts (3–5 %) the analyst may use the alternative method described in 3.5.2.5.3.

3.5.2.5.3 Alternative procedure for other *Poa* spp. classified as other seed in *Poa pratensis* or *Poa trivialis*

Fertile florets of other cultivated *Poa* spp. are removed from the light fraction and thoroughly mixed with the florets of the heavy fraction. At least 400 florets, and preferably 1000, must be taken at random from this mixture (or from the florets in the heavy fraction if no other *Poa* spp. were present in the light fraction). These are separated under magnification into the different *Poa* spp. present. The percentage of each is then determined by weight (3.6).

3.5.2.5.4 Procedure for chemically treated seeds of species for which blowing is the prescribed method for the purity test

Where such chemical treatment affects the blowing characteristics of the seed, the purity of the sample must be determined using the hand method and the Certificate endorsed with the words: 'Because of the chemical treatment the purity test has been carried out by the hand method'. Where the seed lot has been tested before the treatment was applied and only a germination result after treatment is required, then the Certificate must be endorsed with the words: 'Because of the chemical treatment, the pure seed used for the germination was obtained by the hand method'.

3.5.2.6 Multiple seed units (MSU)

On the request of the applicant in the genera covered by PSD 33: multiple seed units are to be weighed separately and reported according to 3.7.

3.5.2.7 Procedure when individual impurities have an undue effect on results

Impurities which deviate considerably in size or weight from the seed size of the sample being tested may unduly affect test results. Such cases may arise with stones, large cereal kernels etc. in a small-seeded crop. If they are relatively easy to eliminate, for example by sieving, remove these impurities from the entire submitted sample (or a sample of at least 10 times the weight used for purity analysis) and perform a normal analysis on the cleaned material, in working samples of the usual weight. Such impurities must be reported and calculations must be made in accordance with 3.6.5.

3.5.2.8 Attached appendages

In certain genera (those covered by PSD 15, 38, 46 and 62) seeds/fruits may have various appendages (awns, stalk, etc.) attached. Such appendages must be left attached to the seeds, but on the request of the applicant the content of seeds with appendages longer than the greatest dimension must be reported according to 3.7.

3.5.2.9 Winged seed

For seeds with PSD 47, winged seeds are those which retain an integument, either with or without wing or a portion thereof. For seeds with PSD 51, winged seeds are those which retain the wing or a portion thereof. Whenever present, such appendages must be left attached to the seed and the content of 'winged' seed reported according to 3.7.

3.6 Calculation and expression of results

3.6.1 One whole working sample

3.6.1.1 Test for weight gain or loss during analysis

Add together the weights of all the component fractions from the working sample. This sum must be compared with the original weight as a check against gain or loss. If there is a discrepancy of more than 5 % of the initial weight, a retest must be made. The result of the retest is then reported.

3.6.1.2 Calculation of component percentages

The percentage by weight of each of the component parts to be reported on the analysis certificate (3.7) must be given to one decimal place. Percentages must be based on the sum of the weights of the components, not on the original weight of the working sample.

The percentage of seed of any particular species other than the pure seed, or of any particular kind of inert matter need not be calculated except as required by 3.7.

3.6.1.3 Rounding procedure

Fraction percentages must be rounded to one decimal place. After rounding, add together the percentages of all fractions. Fractions that are to be reported as a 'trace' (see 3.7) are excluded from this calculation; the other fractions must then together total 100.0 %. If the sum does not equal 100.0 % (either 99.9 or 100.1 %), add or subtract 0.1 % from the largest value (normally the pure seed fraction).

Note: If a correction of more than 0.1 % is necessary, check for a calculation error.

3.6.2 Two half working samples

3.6.2.1 Test for weight gain or loss during analysis

Add together the weights of all the component fractions independently for each half working sample. These sums must be compared with the original weight as a check against gain or loss. If there is a discrepancy of more than 5 % of the initial weight, a retest on two half working samples is required. The result of the retest is then reported.

3.6.2.2 Calculation of component percentages

For each half working sample, calculate the percentage by weight of each component (3.3) to at least two decimal places. Percentages must be based on the sum of the weights of the components in each half working sample, not on the original weights of the working samples. Add the appropriate percentages together from each half working sample and calculate the average percentage by weight for each component. (If desired, the percentages may now be rounded off to a minimum of two decimal places; but do not correct to 100.00 %). Check against tolerances and round off according to 3.6.2.3 and 3.6.2.4, respectively. The percentage of seed of any particular species other than the pure seed, or of any particular kind of inert matter need not be calculated except as required by 3.7. To determine the final reported percentages, add together the weights of pure seed, inert matter and other seeds in each replicate and recalculate the percentages based on the total weight of each fraction in the purity test.

3.6.2.3 Test for variation between the two half working samples

The difference for each component of the two half working samples must not be in excess of the tolerance given in Table 3C. Use the average of a component to find the relevant range of percentages in columns 1 or 2; columns 3 or 4 will give maximum permitted difference between the two values of the particular component. For the definition of chaffy, refer to 3.6.6.

Repeat this for all components. If all components are within tolerance, calculate the average for each component as prescribed in 3.6.2.2 and 3.6.2.4.

If any of the components are out of tolerance, employ the following procedure:

a) Analyse further pairs (but not more than four pairs in all) until a pair is obtained which has its members within tolerance.
b) Discard any pair in which the difference between its members exceeds twice the tolerance.
c) The percentage of a component finally recorded must be calculated from the weighted average of all remaining pairs.

It is advisable to try to find the cause of the variation encountered, especially if additional tests also show too much difference. In such cases use the procedure as outlined in 3.5.2.7.

3.6.2.4 Rounding procedure

If all replicates of all fractions are within tolerance, add the weights of the corresponding fractions together, calculate percentages and round the figures off to one decimal place. Refer to 3.6.1.3 for the correction procedure.

3.6.3 Two or more whole working samples

There are occasions when it is necessary to test a second whole working sample. When a second test is carried out the following procedure should be followed.

3.6.3.1 Procedure

Perform the analysis in accordance with 3.5 and the calculation as prescribed in 3.6.1.

3.6.3.2 Test for variation between samples

When two complete tests have been carried out, proceed as with duplicate analysis on half working samples (3.6.2), but use column 5 or 6 to determine the maximum permitted difference between the two values of the particular component for finding the appropriate tolerance. If an ISTA Certificate has been issued already on the basis of the first test, refer to 1.6.

If the difference between the results exceeds the tolerance, analyse one or two more working samples until a pair is obtained which has its components within tolerance (not more than four samples in all). Report the weighted average of the samples for which the highest and lowest results do not differ by more than twice the tolerance (according to 3.6.3.3), unless it is apparent that one or more of these results are due to an error and not to random sample variation. In that case, discard the test(s) with errors. If no pair of results is within tolerance, it is advisable to find the cause of the variation encountered (3.5.2.7).

3.6.3.3 Calculation and rounding procedure

For each of the samples to be included in the final result add the weights of each fraction together and perform the calculation according to 3.6.1.2 and round according to 3.6.1.3. Average the results and again round according to 3.6.1.3.

3.6.4 Calculation for species difficult to separate

When two or more species which are difficult to separate are in the sample being tested and a final separation is made on 400 to 1000 seeds as described in 3.5.2.4 and 3.5.2.5.3, the following calculation is made for calculating the percentage by weight of one of the contaminant species.

Calculate the percentage of the seeds of that species (A) on the basis of their weight in relation to the total weight of the 400 to 1000 seeds and the initial pure seed percentage (P_1):

$$A\,\% = \frac{\text{Weight of species A seeds}}{\text{Total weight of 400 to 1000 seeds}} \times P_1$$

This percentage is then added to the percentage of the other seeds component (as it was determined in the purity test disregarding these difficult to separate species); the pure seed percentage is decreased by the same amount to return the total for the purity test to 100.0 %.

3.6.5 Calculation for individual impurities having an undue effect on results

In the procedure as given in 3.5.2.7, if m (g) have been removed from a sample of M (g), and if the subsequent purity test on the cleaned material has given P_1 (%) pure seed, I_1 (%) inert matter, and OS_1 (%) other seeds, then the final purity result must be calculated as follows:

$$\text{Pure seed: } P_2 = P_1 \times \frac{M - m}{M}$$

Where
M = initial weight of seed from which impurities having an undue effect on results are taken and
m = the weight of the impurity having an undue effect on the results.

Inert matter: $I_2 = I_1 \times \frac{M - m}{M} + D_1$

Where
$D_1 = (m_1/M) \cdot 100$ and
m_1 = weight of impurities having an undue effect, removed and classified as inert matter.

Other seed: $OS_2 = OS_1 \times \frac{M - m}{M} + D_2$

Where
$D_2 = (m_2/M) \cdot 100$ and m_2 = weight of impurities having an undue effect, removed and classified as other seed.

(Check that $P_2 + I_2 + OS_2 = 100.0$ %)

3.6.6 Chaffy seed structures

Chaffy dispersal units are units which, due to their structure or texture:

1. are likely to adhere to each other or to other objects (woven bags, sampling equipment, dividers, etc.);

2. may cause other seeds to become trapped or otherwise caught on the crop seeds;

3. cannot easily be cleaned, mixed or sampled.

A sample is to be regarded as chaffy if the total of all chaffy structures (including chaffy inert matter) is one third or more. Chaffiness is indicated in Table 3B Part 1 by a 'C' in column 4.

3.7 Reporting results

The results of a purity test must be reported in the spaces provided as follows:

- The scientific name of the species of pure seed, in accordance with Table 2A (e.g. *Triticum aestivum*). Where it is impossible to determine the species with certainty on the basis of seed characteristics, reporting must be done to the most precise taxon possible.
- The percentage by weight of pure seed, inert matter and other seeds, given to one decimal place. The percentage of all components must total 100 %. Components amounting to less than 0.05 % must be reported as 'Trace' or 'TR' (for 'Trace'). If no inert matter or other seeds are found, this must be reported as '0.0'.
- The kind of inert matter.
- The scientific name of every species of other seeds found, in accordance, where applicable, with the current *ISTA List of Stabilised Plant Names*, available at www.seedtest.org/stablist (e.g. *Elytrigia repens*).
- When the weight of the working sample tested for purity equals or is no more than 10 % higher than the weight specified in Table 2A, column 4 (Purity analysis), no statement regarding the weight of the working sample is required on the ISTA Certificate.
- When the weight of the working sample tested for purity deviates from that specified in Table 2A, column 4, the actual weight of the working sample weighed according to 3.5.1 must be reported on the ISTA Certificate using one of the following, as applicable:
  a) When testing a weight that exceeds by 10 % the weight specified in Table 2A, column 4, report under other determinations as: 'Purity: ……g'
  b) When testing a weight estimated to contain 2500 seed units, report under other determinations as: 'Purity: ……g (approx. 2500 seeds)'
  c) When the submitted sample received for purity testing weighs less than the weight in Table 2A, column 4, report under other determinations and use the current statement, according to 2.5.4.5: 'The submitted sample weighed only … g and is not in accordance with the *International Rules for Seed Testing*.'
- The percentage of winged seed (as defined in Pure Seed Definitions 47 and 51), if winged seeds are found.

Upon request, the following information must be reported under 'Other determinations' as follows:

- The percentage by weight of a specified species, entered immediately after the name of the species to the nearest 0.1 %. Species for which the percentage by weight has been requested are listed first.
- Other seeds may be divided into 'other crop seeds' and 'weed seeds'. In this case, the words 'Other crop seeds' must be entered, followed by the percentage by weight of other crop seeds and the name(s) of the species found. This procedure must also be used for 'Weed seeds'.
- Multiple seed units must be reported as ' % MSU'.
- Seeds with appendages attached must be reported as ' % seeds with appendages attached'.

– The kinds of inert matter, together with the percentage by weight of any particular kind (to one decimal place).
– The percentage by weight of broken pure seed.

The percentages may be reported to more than one decimal place if requested.

3.8 Pure seed definitions

The pure seed definition (PSD) number for each genus is listed in Table 3B Part 1. Genera regarded as chaffy are indicated with a 'C', for the purpose of applying the correct column of the tables of tolerances (Tables 3C–E, column 4; see also 3.6.2.3 and 3.6.6).

The detailed pure seed definitions are listed in Table 3B Part 2. The structures described in the definitions in Part 2 are to be classed as pure seed. Appendages are not to be classed as pure seed, unless specifically referred to in Table 3B Part 2.

A glossary of terms listed in Table 3B Part 2 can be found in Table 3B Part 3.

Table 3B Part 1. Pure seed definition numbers and chaffiness of seeds, listed by genus

Genus	Family	PSD no.	Chaffiness
Abelmoschus	*Malvaceae*	10	
Abies	*Pinaceae*	51	C
Abutilon	*Malvaceae*	16	
Acacia	*Fabaceae*	50	
Acer	*Aceraceae*	52	C
Achillea	*Asteraceae*	1	
Adonis	*Ranunculaceae*	4	
Aeschynomene	*Fabaceae*	23	C
Aesculus	*Hippocastanaceae*	10	
Ageratum	*Asteraceae*	4	C
Agrimonia	*Rosaceae*	3	C
Agropyron	*Poaceae*	28	C
Agrostis	*Poaceae*	34	C
Ailanthus	*Simaroubaceae*	52	C
Alcea	*Malvaceae*	16	C
Allium	*Alliaceae*	10	
Alnus	*Betulaceae*	53	C
Alopecurus	*Poaceae*	34	C
Althaea	*Malvaceae*	16	C
Alysicarpus	*Fabaceae*	20	
Alyssum	*Brassicaceae*	11	C
Amaranthus	*Amaranthaceae*	10	
Amberboa	*Asteraceae*	4	C
Ammobium	*Asteraceae*	1	
Amorpha	*Fabaceae*	22	
Anagallis	*Primulaceae*	10	
Anchusa	*Boraginaceae*	18	C
Andropogon	*Poaceae*	42	C
Anemone	*Ranunculaceae*	4	C
Anethum	*Apiaceae*	15	C
Angelica	*Apiaceae*	15	C
Anthoxanthum	*Poaceae*	29	C
Anthriscus	*Apiaceae*	15	C
Anthyllis	*Fabaceae*	11	
Antirrhinum	*Scrophulariaceae*	10	C
Apium	*Apiaceae*	15	C
Aquilegia	*Ranunculaceae*	10	
Arabis	*Brassicaceae*	11	
Arachis	*Fabaceae*	21	C
Arctium	*Asteraceae*	4	
Arctotis	*Asteraceae*	4	C
Armeria	*Plumbaginaceae*	2	C
Arrhenatherum	*Poaceae*	35	C
Artemisia	*Asteraceae*	1	
Asclepias	*Asclepiadaceae*	10	C
Asparagus	*Asparagaceae*	10	
Aster	*Asteraceae*	4	
Astragalus	*Fabaceae*	11	
Astrebla	*Poaceae*	41	C
Atriplex	*Chenopodiaceae*	2	
Atropa	*Solanaceae*	10	
Aubrieta	*Brassicaceae*	11	
Aurinia	*Brassicaceae*	11	C
Avena	*Poaceae*	33	C
Axonopus	*Poaceae*	36	C
Bassia	*Chenopodiaceae*	2	C
Beckmannia	*Poaceae*	34	C
Begonia	*Begoniaceae*	10	
Bellis	*Asteraceae*	1	
Berberis	*Berberidaceae*	50	
Beta	*Chenopodiaceae*	46	C
Betula (see also Chapter 13)	*Betulaceae*	53	C
Borago	*Boraginaceae*	18	C
Bothriochloa	*Poaceae*	42	C
Bouteloua	*Poaceae*	42	C
Brachiaria	*Poaceae*	36	C
Brachyscome	*Asteraceae*	5	

Table 3B Part 1. Pure seed definition numbers and chaffiness of seeds, listed by genus (cont.)

Genus	Family	PSD no.	Chaffiness
Brassica	*Brassicaceae*	11	
Briza	*Poaceae*	34	C
Bromus	*Poaceae*	33	C
Browallia	*Solanaceae*	10	
Brunnera	*Boraginaceae*	18	C
Cajanus	*Fabaceae*	11	
Calceolaria	*Scrophulariaceae*	10	
Calendula	*Asteraceae*	1	C
Callistephus	*Asteraceae*	1	
Calocedrus	*Cupressaceae*	49	C
Calopogonium	*Fabaceae*	11	
Camelina	*Brassicaceae*	11	
Campanula	*Campanulaceae*	10	
Cannabis	*Cannabaceae*	4	
Capsicum	*Solanaceae*	10	
Caragana	*Fabaceae*	11	
Carica	*Caricaceae*	10	C
Carpinus	*Betulaceae*	57	C
Carthamus	*Asteraceae*	4	
Carum	*Apiaceae*	15	
Castanea	*Fagaceae*	57	
Catalpa	*Bignoniaceae*	48	C
Cedrela	*Meliaceae*	48	C
Cedrus	*Pinaceae*	51	C
Celosia	*Amaranthaceae*	10	
Cenchrus	*Poaceae*	43	C
Centaurea	*Asteraceae*	4	C
Centrosema	*Fabaceae*	11	
Cerastium	*Caryophyllaceae*	10	
Chamaecrista	*Fabaceae*	11	
Chamaecyparis	*Cupressaceae*	49	C
Chelidonium	*Papaveraceae*	13	C
Chloris (see also Chapter 13)	*Poaceae*	42	C
Chrysanthemum	*Asteraceae*	1	C
Cicer	*Fabaceae*	11	
Cichorium	*Asteraceae*	4	C
Citrullus	*Cucurbitaceae*	10	
Clarkia	*Onagraceae*	10	
Claytonia	*Portulacaceae*	10	
Cleome	*Capparidaceae*	10	
Cnicus (see *Centaurea*)			
Cobaea	*Polemoniaceae*	14	C
Coix	*Poaceae*	37	C
Coleostephus	*Asteraceae*	1	C
Coleus (see *Plectranthus*)			
Consolida	*Ranunculaceae*	10	C
Convolvulus	*Convolvulaceae*	10	
Corchorus	*Tiliaceae*	10	
Coreopsis	*Asteraceae*	8	C
Coriandrum	*Apiaceae*	15	
Cornus	*Cornaceae*	55	
Corylus	*Betulaceae*	57	
Corymbia (see also Chapter 13)	*Myrtaceae*	60	C
Cosmos	*Asteraceae*	4	C
Cotoneaster	*Rosaceae*	56	
Crambe	*Brassicaceae*	23	
Crataegus	*Rosaceae*	56	
Crotalaria	*Fabaceae*	11	
Cryptomeria	*Taxodiaceae*	49	C
Cucumis	*Cucurbitaceae*	10	
Cucurbita	*Cucurbitaceae*	10	
Cuminum	*Apiaceae*	15	C
Cupressus	*Cupressaceae*	49	C
Cyamopsis	*Fabaceae*	11	
Cyclamen	*Primulaceae*	10	
Cydonia	*Rosaceae*	10	
Cymbalaria	*Scrophulariaceae*	10	C
Cynara	*Asteraceae*	4	
Cynodon	*Poaceae*	28	C
Cynoglossum	*Boraginaceae*	18	C
Cynosurus	*Poaceae*	28	C
Cytisus	*Fabaceae*	50	
Dactylis	*Poaceae*	33	C
Dahlia	*Asteraceae*	9	C
Datura	*Solanaceae*	10	
Daucus	*Apiaceae*	15	C
Delphinium	*Ranunculaceae*	10	C
Deschampsia	*Poaceae*	28	C
Desmodium	*Fabaceae*	11	C
Dianthus	*Caryophyllaceae*	10	C
Dichanthium	*Poaceae*	42	C
Dichondra	*Convolvulaceae*	10	
Digitalis	*Scrophulariaceae*	10	
Digitaria	*Poaceae*	36	C
Dimorphotheca	*Asteraceae*	8	C
Doronicum	*Asteraceae*	4	C
Dorotheanthus	*Aizoaceae*	10	
Echinacea	*Asteraceae*	1	C
Echinochloa	*Poaceae*	36	C
Echinops	*Asteraceae*	26	C
Echium	*Boraginaceae*	18	C
Ehrharta	*Poaceae*	29	C
Elaeagnus	*Elaeagnaceae*	57	
Eleusine	*Poaceae*	61	
Elymus	*Poaceae*	28	C
Elytrigia	*Poaceae*	28	C
Eragrostis	*Poaceae*	28	
Erigeron	*Asteraceae*	4	C
Eruca	*Brassicaceae*	11	
Erysimum	*Brassicaceae*	11	
Eschscholzia	*Papaveraceae*	10	
Eucalyptus (see also Chapter 13)	*Myrtaceae*	60	C
Euonymus	*Celastraceae*	10	
Fagopyrum	*Polygonaceae*	2	C
Fagus	*Fagaceae*	57	C
Fatsia	*Araliaceae*	10	C

Table 3B Part 1. Pure seed definition numbers and chaffiness of seeds, listed by genus (cont.)

Genus	Family	PSD no.	Chaffiness
Festuca	*Poaceae*	33	C
×*Festulolium*	*Poaceae*	33	C
Foeniculum	*Apiaceae*	15	C
Fragaria	*Rosaceae*	1	
Fraxinus	*Oleaceae*	52	C
Freesia	*Iridaceae*	10	
Gaillardia	*Asteraceae*	4	C
Galega	*Fabaceae*	11	
Galeopsis	*Lamiaceae*	18	
Gazania	*Asteraceae*	4	C
Gentiana	*Gentianaceae*	10	C
Geranium	*Geraniaceae*	17	
Gerbera	*Asteraceae*	4	C
Geum	*Rosaceae*	4	C
Gilia	*Polemoniaceae*	10	
Ginkgo	*Ginkgoaceae*	10	
Glandularia	*Verbenaceae*	18	
Glebionis	*Asteraceae*	1	C
Gleditsia	*Fabaceae*	11	
Glycine	*Fabaceae*	11	
Gomphrena	*Amaranthaceae*	2	C
Goniolimon	*Plumbaginaceae*	27	C
Gossypium	*Malvaceae*	12	C
Grevillea	*Proteaceae*	14	C
Gypsophila	*Caryophyllaceae*	10	
Hedysarum	*Fabaceae*	23	C
Helenium	*Asteraceae*	4	C
Helianthemum	*Cistaceae*	10	
Helianthus	*Asteraceae*	4	
Helichrysum (see *Xerochrysum*)			
Heliopsis	*Asteraceae*	1	
Heliotropium	*Boraginaceae*	18	C
Helipterum (see *Rhodanthe*)			
Hesperis	*Brassicaceae*	11	
Heteranthemis	*Asteraceae*	1	C
Heuchera	*Saxifragaceae*	10	C
Hibiscus	*Malvaceae*	10	
Hippeastrum	*Amaryllidaceae*	10	
Holcus	*Poaceae*	35	C
Hordeum	*Poaceae*	62	
Hypericum	*Hypericaceae*	10	
Hyssopus	*Lamiaceae*	18	
Iberis	*Brassicaceae*	11	
Ilex	*Aquifoliaceae*	56	
Impatiens	*Balsaminaceae*	10	
Inula	*Asteraceae*	4	C
Ipomoea	*Convolvulaceae*	10	
Jacobaea	*Asteraceae*	4	C
Juniperus	*Cupressaceae*	11	
Kalanchoe	*Crassulaceae*	10	C
Kniphofia	*Asphodelaceae*	10	C
Kochia (see *Bassia*)			
Koeleria	*Poaceae*	33	C
Koelreuteria	*Sapindaceae*	10	
Kummerowia	*Fabaceae*	22	
Lablab	*Fabaceae*	11	
Laburnum	*Fabaceae*	11	
Lactuca	*Asteraceae*	4	C
Lagenaria	*Cucurbitaceae*	10	
Larix	*Pinaceae*	51	C
Lathyrus	*Fabaceae*	11	C
Lavandula	*Lamiaceae*	18	
Lavatera	*Malvaceae*	16	
Legousia	*Campanulaceae*	10	
Lens	*Fabaceae*	11	
Leontopodium	*Asteraceae*	1	C
Leonurus	*Lamiaceae*	18	
Lepidium	*Brassicaceae*	11	
Lespedeza	*Fabaceae*	22	
Leucaena	*Fabaceae*	11	
Leucanthemum	*Asteraceae*	1	C
Levisticum	*Apiaceae*	15	C
Liatris	*Asteraceae*	4	C
Ligustrum	*Oleaceae*	10	C
Lilium	*Liliaceae*	10	C
Limonium	*Plumbaginaceae*	27	C
Linaria	*Scrophulariaceae*	10	C
Linum	*Linaceae*	10	
Liquidambar	*Hamamelidaceae*	48	C
Liriodendron	*Magnoliaceae*	52	C
Listia	*Fabaceae*	11	
Lobelia	*Campanulaceae*	10	C
Lobularia	*Brassicaceae*	11	C
Lolium	*Poaceae*	33	C
Lomelosia	*Dipsacaceae*	6	C
Lonas	*Asteraceae*	4	C
Lotononis (see *Listia*)			
Lotus	*Fabaceae*	11	
Luffa	*Cucurbitaceae*	10	
Lunaria	*Brassicaceae*	11	
Lupinus	*Fabaceae*	11	
Lycopersicon (see *Solanum*)			
Macroptilium	*Fabaceae*	11	
Macrotyloma	*Fabaceae*	11	
Mahonia (see *Berberis*)			
Malcolmia	*Brassicaceae*	11	
Malope	*Malvaceae*	16	
Malus	*Rosaceae*	10	
Malva	*Malvaceae*	16	
Marrubium	*Lamiaceae*	18	
Matricaria	*Asteraceae*	1	C
Matthiola	*Brassicaceae*	11	C
Medicago	*Fabaceae*	11	
Melilotus	*Fabaceae*	21	
Melinis	*Poaceae*	36	C
Melissa	*Lamiaceae*	18	
Mentha	*Lamiaceae*	18	
Mimosa	*Fabaceae*	11	

Table 3B Part 1. Pure seed definition numbers and chaffiness of seeds, listed by genus (cont.)

Genus	Family	PSD no.	Chaffiness
Mimulus	*Scrophulariaceae*	10	
Mirabilis	*Nyctaginaceae*	1	
Moluccella	*Lamiaceae*	18	
Momordica	*Cucurbitaceae*	10	
Morus	*Moraceae*	57	
Mucuna	*Fabaceae*	11	
Myosotis	*Boraginaceae*	18	
Nasturtium	*Brassicaceae*	11	
Nemesia	*Scrophulariaceae*	10	C
Nemophila	*Hydrophyllaceae*	10	C
Neonotonia	*Fabaceae*	11	
Nepeta	*Lamiaceae*	18	
Nicotiana	*Solanaceae*	10	
Nierembergia	*Solanaceae*	10	C
Nigella	*Ranunculaceae*	10	
Nothofagus	*Fagaceae*	57	C
Ocimum	*Lamiaceae*	18	
Oenothera	*Onagraceae*	10	
Onobrychis	*Fabaceae*	21	C
Origanum	*Lamiaceae*	18	
Ornithopus	*Fabaceae*	23	C
Oryza	*Poaceae*	38	C
Osteospermum	*Asteraceae*	8	C
Panicum	*Poaceae*	36	C
Papaver	*Papaveraceae*	10	
Pascopyrum	*Poaceae*	28	C
Paspalum	*Poaceae*	36	C
Pastinaca	*Apiaceae*	15	C
Pelargonium	*Geraniaceae*	17	
Pennisetum	*Poaceae*	43	C
Penstemon	*Scrophulariaceae*	10	C
Pericallis	*Asteraceae*	4	C
Perilla	*Lamiaceae*	18	
Petroselinum	*Apiaceae*	15	C
Petunia	*Solanaceae*	10	
Phacelia	*Hydrophyllaceae*	10	C
Phalaris	*Poaceae*	29	C
Phaseolus	*Fabaceae*	11	
Phleum	*Poaceae*	28	C
Phlox	*Polemoniaceae*	10	
Pholistoma	*Hydrophyllaeae*	10	C
Physalis	*Solanaceae*	10	
Picea	*Pinaceae*	47	C
Pimpinella	*Apiaceae*	15	C
Pinus I (*P. palustris, P. rigida*)	*Pinaceae*	51	C
Pinus II (all other species)	*Pinaceae*	47	
Piptatherum	*Poaceae*	31	C
Pisum	*Fabaceae*	11	
Plantago	*Plantaginaceae*	10	
Platanus	*Platanaceae*	58	C
Platycladus	*Cupressaceae*	49	C
Plectocephalus	*Asteraceae*	4	C
Plectranthus	*Lamiaceae*	18	
Poa (*non bulbosa*)	*Poaceae*	41	C
Poa bulbosa	*Poaceae*	63	C
Populus	*Salicaceae*	12	C
Portulaca	*Portulacaceae*	10	
Primula	*Primulaceae*	10	
Prunus	*Rosaceae*	56	
Psathyrostachys	*Poaceae*	28	C
Psephellus	*Asteraceae*	4	C
Pseudoroegneria	*Poaceae*	28	C
Pseudotsuga	*Pinaceae*	51	C
Psophocarpus	*Fabaceae*	11	
Psylliostachys	*Plumbaginaceae*	27	C
Pueraria	*Fabaceae*	11	
Pyrus	*Rosaceae*	10	
Quercus	*Fagaceae*	57	
Ranunculus	*Ranunculaceae*	4	C
Raphanus raphanistrum	*Brassicaceae*	23	
Raphanus (all other species)	*Brassicaceae*	11	
Reseda	*Resedaceae*	10	
Rheum	*Polygonaceae*	2	C
Rhodanthe	*Asteraceae*	4	C
Ricinus	*Euphorbiaceae*	13	
Robinia	*Fabaceae*	11	
Rosa	*Rosaceae*	57	
Rosmarinus	*Lamiaceae*	18	
Rudbeckia	*Asteraceae*	1	C
Rumex	*Polygonaceae*	2	C
Ruta	*Rutaceae*	10	
Saintpaulia	*Gesneriaceae*	10	
Salix	*Salicaceae*	12	C
Salpiglossis	*Solanaceae*	10	
Salvia	*Lamiaceae*	18	
Sanguisorba	*Rosaceae*	3	C
Sanvitalia	*Asteraceae*	5	C
Saponaria	*Caryophyllaceae*	10	
Satureja	*Lamiaceae*	18	
Scabiosa	*Dipsacaceae*	6	C
Schefflera	*Araliaceae*	10	
Schizachyrium	*Poaceae*	42	C
Schizanthus	*Solanaceae*	10	
Scorzonera	*Asteraceae*	4	C
Secale	*Poaceae*	40	
Securigera	*Fabaceae*	21	
Senecio	*Asteraceae*	4	C
Sequoia	*Taxodiaceae*	49	C
Sequoiadendron	*Taxodiaceae*	49	C
Sesamum	*Pedaliaceae*	10	
Setaria	*Poaceae*	36	C
Silene	*Caryophyllaceae*	10	
Silybum	*Asteraceae*	4	
Sinapis	*Brassicaceae*	11	
Sinningia	*Gesneriaceae*	10	
Solanum	*Solanaceae*	10	
Solanum (sect. *Lycopersicon*)	*Solanaceae*	10	C

Table 3B Part 1. Pure seed definition numbers and chaffiness of seeds, listed by genus (cont.)

Genus	Family	PSD no.	Chaffiness
Sorbus	*Rosaceae*	10	
Sorghastrum	*Poaceae*	42	C
Sorghum	*Poaceae*	42	C
Spartium	*Fabaceae*	11	
Spergula	*Caryophyllaceae*	10	
Spinacia	*Chenopodiaceae*	2	C
Stachys	*Lamiaceae*	18	
Stylosanthes	*Fabaceae*	24	C
Styphnolobium	*Fabaceae*	20	
Syringa	*Oleaceae*	48	C
Tagetes	*Asteraceae*	4	C
Tanacetum	*Asteraceae*	1	C
Taraxacum	*Asteraceae*	4	C
Taxodium	*Taxodiaceae*	11	C
Taxus	*Taxaceae*	50	
Tectona	*Verbenaceae*	54	
Tetragonia	*Aizoaceae*	19	
Thuja	*Cupressaceae*	49	C
Thunbergia	*Acanthaceae*	10	
Thymus	*Lamiaceae*	18	
Tilia	*Tiliaceae*	57	C
Torenia	*Scrophulariaceae*	10	
Tragopogon	*Asteraceae*	4	C
Trifolium	*Fabaceae*	11	
Trigonella	*Fabaceae*	11	
Tripleurospermum	*Asteraceae*	1	C
Trisetum	*Poaceae*	28	C
×*Triticosecale*	*Poaceae*	40	
Triticum (excluding *T. spelta* and *T. dicoccon*)	*Poaceae*	40	
Triticum (only *T. spelta* and *T. dicoccon*)	*Poaceae*	33	C
Tropaeolum	*Tropaeolaceae*	16	
Tsuga	*Pinaceae*	51	C
Ulmus	*Ulmaceae*	52	C
Urochloa	*Poaceae*	36	C
Vaccaria	*Caryophyllaceae*	10	
Valeriana	*Valerianaceae*	7	C
Valerianella	*Valerianaceae*	25	C
Verbascum	*Scrophulariaceae*	10	
Verbena	*Verbenaceae*	18	
Viburnum	*Adoxaceae*	55	
Vicia	*Fabaceae*	11	
Vigna	*Fabaceae*	11	
Vinca	*Apocynaceae*	10	
Viola	*Violaceae*	13	
Xeranthemum	*Asteraceae*	4	C
Xerochrysum	*Asteraceae*	4	C
Zea	*Poaceae*	40	
Zelkova	*Ulmaceae*	59	C
Zinnia	*Asteraceae*	9	C
Zoysia	*Poaceae*	39	C

Table 3B Part 2. Numbered pure seed definitions

For the sake of brevity, several genera which have similar pure seed definitions have been combined under the same number. Exceptions to the general definition have been given in brackets. For more detailed individual definitions for agricultural and vegetable, and flower, spice, herb and medicinal plant seeds, refer to the *ISTA Handbook of Pure Seed Definitions*.

1. Achene, unless it is obvious that no seed is present.
   Piece of achene larger than one-half the original size, unless it is obvious that no seed is present.
   Seed, with the pericarp/testa partially or entirely removed.
   Piece of seed larger than one-half the original size, with the pericarp/testa partially or entirely removed.

2. Achene or cluster, with or without perianth or pedicel, unless it is obvious that no seed is present.
   Piece of achene or cluster larger than one-half the original size, unless it is obvious that no seed is present.
   Seed, with the pericarp/testa partially or entirely removed.
   Piece of seed larger than one-half the original size, with the pericarp/testa partially or entirely removed.
   Gomphrena: Achene with or without hairy perianth, unless it is obvious that no seed is present.

3. Achene, with or without hypanthium, unless it is obvious that no seed is present.
   Piece of achene larger than one-half the original size, unless it is obvious that no seed is present.
   Seed, with the pericarp/testa partially or entirely removed.
   Piece of seed larger than one-half the original size, with the pericarp/testa partially or entirely removed.

4. Achene, with or without beak, pappus or bracts, including achenes where two or more seed units are joined together by fused pericarps, unless it is obvious that no seed is present.
   Piece of achene larger than one-half the original size, unless it is obvious that no seed is present.
   Seed, with the pericarp/testa partially or entirely removed.
   Piece of seed larger than one-half the original size, with the pericarp/testa partially or entirely removed.

5. Achene, with or without wing and/or pappus or bristle, unless it is obvious that no seed is present.
   Piece of achene larger than one-half the original size, unless it is obvious that no seed is present.
   Seed, with the pericarp/testa partially or entirely removed.
   Piece of seed larger than one-half the original size, with the pericarp/testa partially or entirely removed.

6. Achene, with or without involucel, calyx or beak, unless it is obvious that no seed is present.
   Piece of achene larger than one-half the original size, unless it is obvious that no seed is present.
   Seed, with the pericarp/testa partially or entirely removed.
   Piece of seed larger than one-half the original size, with the pericarp/testa partially or entirely removed.

7. Achene, with or without feathery calyx, unless it is obvious that no seed is present.
   Piece of achene larger than one-half the original size, unless it is obvious that no seed is present.
   Seed, with the pericarp/testa partially or entirely removed.
   Piece of seed larger than one-half the original size, with the pericarp/testa partially or entirely removed.

8. Achene, with or without wing, unless it is obvious that no seed is present.
   Piece of achene larger than one-half the original size, unless it is obvious that no seed is present.
   Seed, with the pericarp/testa partially or entirely removed.
   Piece of seed larger than one-half the original size, with the pericarp/testa partially or entirely removed.

Table 3B Part 2. Numbered pure seed definitions (cont.)

9. Achene, with or without bristles, unless it is obvious that no seed is present.
   Piece of achene larger than one-half the original size, unless it is obvious that no seed is present.
   Seed, with the pericarp/testa partially or entirely removed.
   Piece of seed larger than one-half the original size, with the pericarp/testa partially or entirely removed.

10. Seed, with or without testa.
    Piece of seed larger than one-half the original size, with or without testa.
    Allium: Pairs of *Allium* seeds adhering together do not need to be separated.

11. Seed, provided a portion of the testa is attached.
    Piece of seed larger than one-half the original size, provided a portion of the testa is attached.
    Fabaceae: cotyledons that are broken apart but held together within the testa.
    Seeds and pieces of seed entirely without testa are regarded as inert matter.
    Fabaceae: separated cotyledons are regarded as inert matter, irrespective of whether the radicle-plumule axis and/or more than half of the testa is attached.

12. Seed, with or without testa, testa with or without hairs.
    Piece of seed larger than one-half the original size, with or without testa.

13. Seed, with or without testa, with or without strophiole/caruncle.
    Piece of seed larger than one-half the original size, with or without testa.

14. Seed, with or without testa, with or without wing.
    Piece of seed larger than one-half the original size, with or without testa.

15. Schizocarp/mericarp, with or without pedicel (of any length), unless it is obvious that no seeds are present.
    Piece of mericarp larger than one-half the original size, unless it is obvious that no seed is present.
    Seed, with the pericarp partially or entirely removed.
    Piece of seed larger than one-half the original size, with the pericarp partially or entirely removed.
    Fruits with pieces of pedicel longer than the length of the schizocarp/mericarp are reported according to 3.7 (see also 3.5.2.8).

16. Mericarp, unless it is obvious that no seed is present.
    Piece of mericarp larger than one-half the original size, unless it is obvious that no seed is present.
    Seed, with the pericarp/testa partially or entirely removed.
    Piece of seed larger than one-half the original size, with the pericarp/testa partially or entirely removed.

17. Mericarp, with or without beak, unless it is obvious that no seed is present.
    Piece of mericarp larger than one-half the original size, unless it is obvious that no seed is present.
    Seed, with the pericarp/testa partially or entirely removed.
    Piece of seed larger than one-half the original size, with the pericarp/testa partially or entirely removed.

18. Nutlet, unless it is obvious that no seed is present.
    Piece of nutlet larger than one-half the original size, unless it is obvious that no seed is present.
    Seed, with the pericarp/testa partially or entirely removed.
    Piece of seed larger than one-half the original size, with the pericarp/testa partially or entirely removed.

19. Nut-like fruit, with enclosing perianth, unless it is obvious that no seed is present.
    Piece of fruit larger than one-half the original size, unless it is obvious that no seed is present.
    Seed, with the pericarp/testa partially or entirely removed.
    Piece of seed larger than one-half the original size, with the pericarp/testa partially or entirely removed.

Table 3B Part 2. Numbered pure seed definitions (cont.)

20. Pod, or portion of pod with one seed.
Seed, provided a portion of the testa is attached.
Piece of seed larger than one-half the original size, provided a portion of the testa is attached.
Cotyledons that are broken apart but held together within the testa.
Seeds and pieces of seed without testa are regarded as inert matter. Separated cotyledons are regarded as inert matter, irrespective of whether the radicle-plumule axis and/or more than half of the testa is attached.

21. Pod, with or without calyx, with seed(s).
Seed, provided a portion of the testa is attached.
Piece of seed larger than one-half the original size, provided a portion of the testa is attached.
Cotyledons that are broken apart but held together within the testa.
Seeds and pieces of seed without testa are regarded as inert matter. Separated cotyledons are regarded as inert matter, irrespective of whether the radicle-plumule axis and/or more than half of the testa is attached.

22. Pod, with or without calyx or bracts, with one seed.
Seed, provided a portion of the testa is attached.
Piece of seed larger than one-half the original size, provided a portion of the testa is attached.
Cotyledons that are broken apart but held together within the testa.
Seeds and pieces of seed without testa are regarded as inert matter. Separated cotyledons are regarded as inert matter, irrespective of whether the radicle-plumule axis and/or more than half of the testa is attached.

23. One-seeded segment of pod or siliqua, with or without stalk or terminal beak, unless it is obvious that no seed is present.
Seed, provided a portion of the testa is attached.
Piece of seed larger than one-half the original size, provided a portion of the testa is attached.
Ornithopus compressus: one-seeded pod segment, with or without attached empty pod segments or partial segments.
Fabaceae: cotyledons that are broken apart but held together within the testa.
Seeds and pieces of seed without testa are regarded as inert matter.
Fabaceae: separated cotyledons are regarded as inert matter irrespective of whether the radicle-plumule axis and/or more than half of the testa is attached.

24. Pod, with or without beak, unless it is obvious that no seed is present.
Seed, provided a portion of the testa is attached.
Piece of seed larger than one-half the original size, provided a portion of the testa is attached.
Cotyledons that are broken apart but held together within the testa.
Seeds and pieces of seed without testa are regarded as inert matter. Separated cotyledons are regarded as inert matter, irrespective of whether the radicle-plumule axis and/or more than half of the testa is attached.

25. Dry, indehiscent fruit with 1–3 loculi, with or without calyx or pedicel or stalk fragment, unless it is obvious that no seed is present.
Seed, with or without testa.
Piece of seed larger than one-half the original size, with or without testa.

26. One-flowered capitulum, unless it is obvious that no achene is present.
Achene, with or without pappus, unless it is obvious that no seed is present.
Piece of achene larger than one-half the original size, unless it is obvious that no seed is present.
Seed, with the pericarp/testa partially or entirely removed.
Piece of seed larger than one-half the original size, with the pericarp/testa partially or entirely removed.

27. Flower head, with or without pedicel, unless it is obvious that no achenes are present.
Achene, with or without perianth, unless it is obvious that no seed is present.
Piece of achene larger than one-half the original size, unless it is obvious that no seed is present.
Seed, with the pericarp/testa partially or entirely removed.
Piece of seed larger than one-half the original size, with the pericarp/testa partially or entirely removed.

Table 3B Part 2. Numbered pure seed definitions (cont.)

28. Floret, with lemma and palea enclosing a caryopsis, with or without awn.
Caryopsis.
Piece of caryopsis larger than one-half the original size.
Elytrigia repens: floret with lemma and palea enclosing a caryopsis at least one-third the length of the palea measured from the base of the rachilla, with or without awn.

29. Floret, with lemma and palea enclosing a caryopsis, plus attached sterile lemmas, with or without awn.
Floret, with lemma and palea enclosing a caryopsis.
Caryopsis.
Piece of caryopsis larger than one-half the original size.
Phalaris: including protruding anthers if present.

30. (Deleted 1 January 2012)

31. Floret, with lemma and palea enclosing a caryopsis, with or without awn.
Piece of floret containing a caryopsis larger than one-half the original size.
Caryopsis.
Piece of caryopsis larger than one-half the original size.

32. (Deleted 1 July 1993; see PSD 33)

33. Floret, with lemma and palea enclosing a caryopsis with or without awn.
Festuca, Lolium, ×Festulolium: size of caryopsis at least one-third the length of the palea, measured from the base of the rachilla.
Caryopsis.
Piece of caryopsis larger than one-half the original size.
The floret may be with or without attached single fertile or sterile floret, provided that the attached floret does not extend to the tip of the fertile floret, excluding the awn (Fig. 3.1, 1–4).
Where a uniform blowing method is prescribed, see 3.5.2.5.

Multiple seed units

Seed units may consist of spikelets or parts of spikelets with more than one floret. Such structures with or without glumes are called multiple seed units (MSUs) when formed by the following structures:

– one fertile floret with one attached fertile or sterile floret that extends to or beyond the tip of the fertile floret, excluding the awns (Fig. 3.1, 8–12).
– one fertile floret with two or more attached fertile or sterile florets of any length (Fig. 3.1, 5–7).
– one fertile floret with basally attached sterile floret or glumes of any length (Fig. 3.1, 13–15).

MSUs are left intact and included in the pure seed fraction. However, the applicant may request that they be weighed and the percentage reported (see 3.5.2.6).
For *Triticum dicoccon* and *Triticum spelta* only: with or without rachis segment attached.
In *Triticum dicoccon* and *Triticum spelta*, combinations of MSUs may be found. These are not to be separated in the purity analysis.
MSUs of *Avena* of the type of structure 13 (Fig. 3.1), where the lemma of the basal floret envelops the inner fertile floret, need not be reported as MSUs. All other structures (Fig. 3.1, 5–12, 14–15) are to be considered MSU.

34. Spikelet, with glumes, lemma and palea enclosing a caryopsis, with or without awn.
Floret, with lemma and palea enclosing a caryopsis, with or without awn.
Caryopsis.
Piece of caryopsis larger than one-half the original size.
Alopecurus: palea absent.

35. Spikelet, with lemma and palea enclosing a caryopsis, plus attached staminate floret, with or without awn.
Floret, with lemma and palea enclosing a caryopsis, with or without awn.
Caryopsis.
Piece of caryopsis larger than one-half the original size.
Holcus: spikelet with glumes, lemma and palea enclosing a caryopsis, plus attached staminate floret, with or without awn.

36. Spikelet, with or without pedicel, with glumes, lemma and palea enclosing a caryopsis, plus attached sterile lemma.
Floret, with lemma and palea enclosing a caryopsis.
Caryopsis.
Piece of caryopsis larger than one-half the original size.
Axonopus: spikelet, with single glume, lemma and palea enclosing a caryopsis, plus attached sterile lemma.

Table 3B Part 2. Numbered pure seed definitions (cont.)

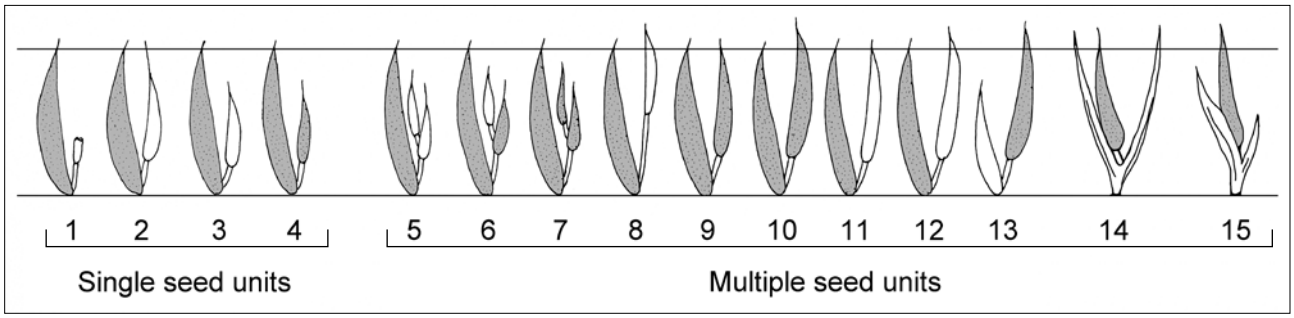

Figure 3.1. Classification of single and multiple seed units. The stippled portion represents fertile florets and the clear portion sterile florets.

Echinochloa and *Melinis:* attached sterile lemma with or without awn.
Panicum and *Digitaria*: no need to check for the presence of a caryopsis.

37. Spikelets (one fertile*, two sterile) enclosed in a bead-like involucre.
Caryopsis.
Piece of caryopsis larger than one-half the original size.
*The fertile spikelet consists of glumes, lemma and palea enclosing a caryopsis, plus attached sterile lemma.

38. Spikelet, with glumes, lemma and palea enclosing a caryopsis, including the awn irrespective of its size.
Floret, with or without sterile lemmas, with lemma and palea enclosing a caryopsis, including the awn irrespective of its size.
Floret with lemma and palea enclosing a caryopsis, including the awn irrespective of its size.
Caryopsis.
Piece of caryopsis larger than one-half the original size.
Seeds with awns longer than the length of the floret are reported according to 3.7 (see also 3.5.2.8).

39. Spikelet, with one glume*, lemma and palea enclosing a caryopsis.
Caryopsis.
Piece of caryopsis larger than one-half the original size.
*First glume absent, second glume completely infolding the thin lemma and palea, the palea sometimes obsolete.

40. Caryopsis.
Piece of caryopsis larger than one-half the original size.

41. Spikelet, with lemma and palea enclosing a caryopsis, with or without awn, plus attached sterile floret.
Floret, with lemma and palea enclosing a caryopsis, with or without awn.
Caryopsis.
Piece of caryopsis larger than one-half the original size.
Astrebla: spikelet and floret with or without caryopsis. Where a uniform blowing method is prescribed (*Poa pratensis, P. trivialis*) see 3.5.2.5.

42. Spikelet, with glumes enclosing a caryopsis with or without hyaline palea or lemmas, rachis segment(s), pedicel(s), awn(s), attached sterile or fertile floret(s).
Floret, with lemma and palea, with or without awn.
Caryopsis.
Piece of caryopsis larger than one-half the original size.
Bouteloua, Chloris: no need to check for the presence of a caryopsis.

43. Fascicle or burr with involucre of bristles and 1–5 spikelets, each comprising glumes, lemma and palea enclosing a caryopsis, plus attached sterile lemma.
Floret, with lemma and palea enclosing a caryopsis.
Caryopsis.
Piece of caryopsis larger than one-half the original size.
Cenchrus: burr or fascicle, with or without caryopsis.

Table 3B Part 2. Numbered pure seed definitions (cont.)

44.(Deleted 1 July 1993; see PSD 42)

45. (Deleted 1 July 1993; see PSD 42)

46. Cluster, or piece of cluster, with or without stalk, with or without pieces of leaf, unless it is obvious that no seed is present.
Seed, with the pericarp/testa partially or entirely removed.
Piece of seed larger than one-half the original size with the pericarp/testa partially or entirely removed.
Clusters with pieces of stalk or leaf protruding more than the largest dimension of the cluster are reported according to 3.7 (see also 3.5.2.8).

47. Seed, without wing or integument, provided a portion of the testa is attached.
Piece of seed larger than one-half the original size, without wing or integument, provided a portion of the testa is attached.
'Integument' refers to the tissue attaching the wing to the seed. In *Pinaceae* with PSD 47, the integument is not intimately associated with the seed and is usually removed in processing, thus removing the wing. However, if an integument (with or without wing) is still attached to any seed during the purity analysis, such seed will be regarded as 'winged seed' and must be left intact; neither the integument nor wing should be deliberately removed. Winged seed (i.e. seed with an attached integument with or without a wing of any size) must be weighed and reported as a separate percentage from 'pure seed' according to paragraphs 3.5.2.9 and 3.7. After weighing, the winged seed and pure seed fractions should be recombined and used in representative proportions for counting out the germination replicates.

48. Seed, with or without wing(s), unless it is obvious that no embryo is present, with or without testa.
Piece of seed larger than one-half the original size, unless it is obvious that no embryo is present, with or without testa.
Seeds are normally winged, are not weighed separately and are therefore all pure seed.

49. Seed, with or without wing(s), provided a portion of the testa is attached.
Piece of seed larger than one-half the original size, provided a portion of the testa is attached.
Seeds are normally winged, are not weighed separately and are therefore all pure seed.

50. Seed, provided a portion of the testa is attached, with or without aril.
Piece of seed larger than one-half the original size, provided a portion of the testa is attached.

51. Seed, without wing, with (but occasionally without) integument, provided a part of the testa is attached.
Piece of seed larger than one-half the original size, without wing, with (but occasionally without) integument, provided a portion of the testa is attached.
'Integument' refers to the tissue attaching the wing to the seed. In *Pinaceae* with PSD 51, the integument is fused to or intimately associated with the seed, is rarely removed in processing, and is impossible to consistently remove without causing damage. Hence, seed with fused or intimately associated integument attached is considered to be 'pure seed'. Winged seed (i.e. seed with an integument plus wing still attached) must be weighed and reported as a separate percentage from 'pure seed' according to paragraphs 3.5.2.9 and 3.7. After weighing, the winged seed and pure seed fractions should be recombined and used in representative proportions for counting out the germination replicates.

52. Samara (winged fruit), with or without wing(s).
Piece of samara larger than one-half the original size.
Seed with the pericarp/testa partially or entirely removed.
Piece of seed larger than one-half the original size, with the pericarp/testa partially or entirely removed.
Samaras are normally winged, are not weighed separately and are therefore all pure seed.

53. Samara (winged fruit), with or without wing(s), with or without attached styles.
Piece of samara larger than one-half the original size.
Seed, with the pericarp/testa partially or entirely removed.
Piece of seed larger than one-half the original size, with the pericarp/testa partially or entirely removed.
Samaras are normally winged, are not weighed separately and are therefore all pure seed.

Table 3B Part 2. Numbered pure seed definitions (cont.)

54. Fruit with or without calyx.
Piece of fruit, unless it is obvious that no seed is present.
Seed, with or without testa.
Piece of seed larger than one-half the original size, with or without testa.

55. Drupe, containing a pyrene (kernel, stone).
Pyrene, unless it is obvious that no seed is present.
Piece of pyrene larger than one-half the original size, unless it is obvious that no seed is present.
Seed, with the pericarp/testa partially or entirely removed.
Piece of seed larger than one-half the original size, with the pericarp/testa partially or entirely removed.

56. Pyrene (kernel, stone), unless it is obvious that no seed is present.
Piece of pyrene larger than one-half the original size, unless it is obvious that no seed is present.
Seed, with the pericarp/testa partially or entirely removed.
Piece of seed larger than one-half the original size, with the pericarp/testa partially or entirely removed.

57. Nut, unless it is obvious that no seed is present.
Piece of nut larger than one-half the original size, unless it is obvious that no seed is present.
Seed with the pericarp/testa partially or entirely removed.
Piece of seed larger than one-half the original size, with the pericarp/testa partially or entirely removed.

58. Nut, with or without hairs, unless it is obvious that no seed is present.
Piece of nut larger than one-half the original size, unless it is obvious that no seed is present.
Seed, with the pericarp/testa partially or entirely removed.
Piece of seed larger than one-half the original size, with the pericarp/testa partially or entirely removed.

59. Nut, with or without perianth, unless it is obvious that no seed is present.
Piece of nut larger than one-half the original size, unless it is obvious that no seed is present.
Seed, with the pericarp/testa partially or entirely removed.
Piece of seed larger than one-half the original size, with the pericarp/testa partially or entirely removed.

60. Seed, with or without testa.
Piece of seed more than one-half the original size, with or without testa.
In many species of *Eucalyptus* it is impossible to differentiate with certainty between seed and ovulodes (= unfertilised or inhibited ovules that did not develop into mature seed). In these cases, and upon request also for the species where one can make the distinction, a simplified procedure as described in Chapter 13 can be followed. Appropriate information on the method followed should be given on the Certificate.

61. Floret, with lemma and palea enclosing a caryopsis.
Caryopsis, with or without pericarp.
Piece of caryopsis larger than one-half the original size, with or without pericarp.

62. Floret, with lemma and palea enclosing a caryopsis, with or without awn or with or without rachis segment, irrespective of their length.
Piece of floret containing a caryopsis larger than one-half the original size.
Caryopsis.
Piece of caryopsis larger than one-half the original size.
Florets with awn or rachis segment longer than the length of the floret are reported according to 3.7 (see also 3.5.2.8).

63. Bulbil.
Piece of bulbil larger than one-half the original size.

Table 3B Part 3. Glossary

achene, achenium a dry, indehiscent, one-seeded fruit, formed from one free carpel (e.g. *Ranunculaceae, Geum*) with the seed coat distinct from the fruit coat; occasionally consisting of more than one carpel (*Asteraceae*)

anther the pollen-producing part of the stamen, borne at the top of the filament or stalk

aril, arillus (pl. arilli) a fleshy, often coloured covering or appendage of a seed growing out from the funicle or base of the ovule (see also caruncle, strophiole)

awn, arista slender, straight or bent bristle. In grasses: usually a continuation of the mid-nerve of lemmas or glumes

beak (-ed) a long, pointed prolongation of a fruit

bract a reduced leaf or scale-like structure subtending a flower or a grass spikelet in its axil

bristle a stiff hair; sometimes applied to the upper part of an awn, when the latter is bent

bulbil a small bulb, usually axillary or appearing instead of flowers as in *Poa bulbosa,* also a bulblet

calyx (pl. calyces) the outer floral envelope composed of the sepals

capitulum a dense inflorescence of usually sessile flowers

caruncle small outgrowth of the micropylar region (see also aril, strophiole)

caryopsis naked grass-fruit in which the testa is united with the pericarp

cluster a densely crowded inflorescence or, in *Beta,* part of an inflorescence

drupe indehiscent, one-seeded fruit with stony endocarp and fleshy outer layers

embryo the young plant enclosed in a seed

fascicle a tuft of branches arising from about the same place

fertile with functional sex organs; (for grass florets: having a caryopsis)

floret the lemma and palea with enclosed pistil and stamens or the mature caryopsis in *Poaceae;* for the purpose of the *Rules*, the term floret refers to the fertile floret with or without additional sterile lemmas

glume one of the two usually sterile bracts at the base of a grass spikelet

hair a uni- or multicellular outgrowth of the epidermis

hypanthium a ring-like, cup-like or tubular structure which surrounds the ovary and on which sepals, petals and stamens are borne

indehiscent not opening; fruits which do not open at maturity

integument the envelope of an ovule which becomes the seed coat or testa (generally two integuments present). In coniferous seeds integument also refers to the tissue attaching the wing to the seed

involucel a secondary involucre; often around a cluster of flowers

involucre ring of bracts or bristles surrounding the base of an inflorescence

lemma the outer (lower) bract of a grass floret, sometimes referred to as the flowering glume or the lower or outer palea. Bract enclosing the caryopsis on the outer (dorsal) side

locule, loculus (pl. loculi) compartment of the ovary containing the seeds

mericarp part of the schizocarp

nutlet a small nut

Table 3B Part 3. Glossary (cont.)

palea the upper (inner) bract of a grass floret, sometimes called the inner or upper palea. Bract enclosing the caryopsis on the inner ventral side

pappus a ring of fine, sometimes feathery hairs or scales, crowning an achene

pedicel the stalk of each single flower in an inflorescence

perianth the two floral envelopes (calyx and corolla) or any one of them

pericarp (fruit coat) the wall of the mature ovary or fruit

pod dehiscent dry fruit, especially of *Fabaceae*

pyrene seed enclosed by the hard endocarp from a drupe (or similar structures from multi-seeded fruits)

rachilla, rhachilla a secondary rachis. In particular in grasses the axis that bears the floret

rachis, rhachis (pl. rachides) the main axis of an inflorescence

seed unit commonly found dispersal unit, i.e. achenes and similar fruits, schizocarps, florets etc., as defined for each genus or species in the Pure Seed Definitions in Table 3B Parts 1 & 2

schizocarp a dry fruit which separates into two or more units (mericarps) at maturity

sessile without a stalk or pedicel

siliqua dehiscent, dry, two-valved fruit derived from two carpels, e.g. *Brassicaceae.*

spikelet the unit of a grass inflorescence comprising one or more florets subtended by one or two sterile glumes. For the purposes of the *Rules*, the term spikelet includes, as well as a fertile floret, either one or more additional fertile or completely infertile florets, or glumes

stalk the stem of any plant organ

staminate flower with stamens only

sterile without functional sex organs (for grass florets: without caryopsis)

strophiole small aril, wartlike outgrowth (see also aril, caruncle)

testa seed coat

wing a flat membranous outgrowth from a fruit or seed

3.9 Tolerance tables

Table 3C gives tolerances for comparing purity results on duplicate samples from the same submitted sample analysed in the same laboratory. It can be used for any component of a purity test. The table is used by entering it at the average of the two test results (columns 1 or 2). The appropriate tolerance is found in one of columns 3 to 6, determined as to whether the seeds are chaffy or non-chaffy and half or whole working samples have been analysed.

The tolerances in columns 5 and 6 are extracted from Miles (1963), Table P11, columns C and F respectively, and rounded to one decimal place. Those for half working samples, columns 3 and 4, are calculated from Table P11, columns C and F in Miles (1963) by multiplication with the square root of two.

Table 3D gives the tolerances for purity results made on two different submitted samples each drawn from the same lot and analysed in the same or a different laboratory. It can be used for any component of a purity test when the result of the second test is poorer than that of the first test. The table is used by entering it at the average of the two test results (columns 1 or 2). The appropriate tolerance is found in columns 3 or 4, determined as to whether the seeds are chaffy or non-chaffy.

The tolerances in columns 3 and 4 are extracted from columns D and G respectively of Table P1 in Miles (1963).

Table 3E gives the tolerances for purity results made on two different submitted samples each drawn from the same lot and analysed in the same or a different laboratory. It can be used for any component of a purity test to decide whether two estimates are compatible. The table is used by entering it at the average of the two test results (columns 1 or 2). The appropriate tolerance is found in columns 3 or 4, determined by whether the seeds are chaffy or non-chaffy.

The tolerances in columns 3 and 4 are extracted from columns D and G, respectively, of Table P7 in Miles (1963).

Table 3C. Tolerances for purity tests on the same submitted sample in the same laboratory (two-way test at 5 % significance level)

Average of the two test results		Tolerances for differences between			
		Half working samples		Whole working samples	
		Non-chaffy seeds	Chaffy seeds	Non-chaffy seeds	Chaffy seeds
1	2	3	4	5	6
99.95–100.00	0.00–0.04	0.20	0.23	0.1	0.2
99.90–99.94	0.05–0.09	0.33	0.34	0.2	0.2
99.85–99.89	0.10–0.14	0.40	0.42	0.3	0.3
99.80–99.84	0.15–0.19	0.47	0.49	0.3	0.4
99.75–99.79	0.20–0.24	0.51	0.55	0.4	0.4
99.70–99.74	0.25–0.29	0.55	0.59	0.4	0.4
99.65–99.69	0.30–0.34	0.61	0.65	0.4	0.5
99.60–99.64	0.35–0.39	0.65	0.69	0.5	0.5
99.55–99.59	0.40–0.44	0.68	0.74	0.5	0.5
99.50–99.54	0.45–0.49	0.72	0.76	0.5	0.5
99.40–99.49	0.50–0.59	0.76	0.82	0.5	0.6
99.30–99.39	0.60–0.69	0.83	0.89	0.6	0.6
99.20–99.29	0.70–0.79	0.89	0.95	0.6	0.7
99.10–99.19	0.80–0.89	0.95	1.00	0.7	0.7
99.00–99.09	0.90–0.99	1.00	1.06	0.7	0.8
98.75–98.99	1.00–1.24	1.07	1.15	0.8	0.8
98.50–98.74	1.25–1.49	1.19	1.26	0.8	0.9
98.25–98.49	1.50–1.74	1.29	1.37	0.9	1.0
98.00–98.24	1.75–1.99	1.37	1.47	1.0	1.0
97.75–97.99	2.00–2.24	1.44	1.54	1.0	1.1
97.50–97.74	2.25–2.49	1.53	1.63	1.1	1.2
97.25–97.49	2.50–2.74	1.60	1.70	1.1	1.2
97.00–97.24	2.75–2.99	1.67	1.78	1.2	1.3
96.50–96.99	3.00–3.49	1.77	1.88	1.3	1.3
96.00–96.49	3.50–3.99	1.88	1.99	1.3	1.4
95.50–95.99	4.00–4.49	1.99	2.12	1.4	1.5
95.00–95.49	4.50–4.99	2.09	2.22	1.5	1.6
94.00–94.99	5.00–5.99	2.25	2.38	1.6	1.7
93.00–93.99	6.00–6.99	2.43	2.56	1.7	1.8
92.00–92.99	7.00–7.99	2.59	2.73	1.8	1.9
91.00–91.99	8.00–8.99	2.74	2.90	1.9	2.1
90.00–90.99	9.00–9.99	2.88	3.04	2.0	2.2
88.00–89.99	10.00–11.99	3.08	3.25	2.2	2.3
86.00–87.99	12.00–13.99	3.31	3.49	2.3	2.5
84.00–85.99	14.00–15.99	3.52	3.71	2.5	2.6
82.00–83.99	16.00–17.99	3.69	3.90	2.6	2.8
80.00–81.99	18.00–19.99	3.86	4.07	2.7	2.9
78.00–79.99	20.00–21.99	4.00	4.23	2.8	3.0
76.00–77.99	22.00–23.99	4.14	4.37	2.9	3.1
74.00–75.99	24.00–25.99	4.26	4.50	3.0	3.2
72.00–73.99	26.00–27.99	4.37	4.61	3.1	3.3
70.00–71.99	28.00–29.99	4.47	4.71	3.2	3.3
65.00–69.99	30.00–34.99	4.61	4.86	3.3	3.4
60.00–64.99	35.00–39.99	4.77	5.02	3.4	3.6
50.00–59.99	40.00–49.99	4.89	5.16	3.5	3.7

Table 3D. Tolerances for purity tests on two different submitted samples from the same lot when a second test is made in the same or a different laboratory (one-way test at 1 % significance level)

Average of the two test results		Tolerance	
50–100 %	Less than 50 %	Non-chaffy seeds	Chaffy seeds
1	2	3	4
99.95–100.00	0.00–0.04	0.2	0.2
99.90–99.94	0.05–0.09	0.3	0.3
99.85–99.89	0.10–0.14	0.3	0.4
99.80–99.84	0.15–0.19	0.4	0.5
99.75–99.79	0.20–0.24	0.4	0.5
99.70–99.74	0.25–0.29	0.5	0.6
99.65–99.69	0.30–0.34	0.5	0.6
99.60–99.64	0.35–0.39	0.6	0.7
99.55–99.59	0.40–0.44	0.6	0.7
99.50–99.54	0.45–0.49	0.6	0.7
99.40–99.49	0.50–0.59	0.7	0,8
99.30–99.39	0.60–0.69	0.7	0.9
99.20–99.29	0.70–0.79	0.8	0.9
99.10–99.19	0.80–0.89	0.8	1.0
99.00–99.09	0.90–0.99	0.9	1.0
98.75–98.99	1.00–1.24	0.9	1.1
98.50–98.74	1.25–1.49	1.0	1.2
98.25–98.49	1.50–1.74	1.1	1.3
98.00–98.24	1.75–1.99	1.2	1.4
97.75–97.99	2.00–2.24	1.3	1.5
97.50–97.74	2.25–2.49	1.3	1.6
97.25–97.49	2.50–2.74	1.4	1.6
97.00–97.24	2.75–2.99	1.5	1.7
96.50–96.99	3.00–3.49	1.5	1.8
96.00–96.49	3.50–3.99	1.6	1.9
95.50–95.99	4.00–4.49	1.7	2.0
95.00–95.49	4.50–4.99	1.8	2.2
94.00–94.99	5.00–5.99	2.0	2.3
93.00–93.99	6.00–6.99	2.1	2.5
92.00–92.99	7.00–7.99	2.2	2.6
91.00–91.99	8.00–8.99	2.4	2.8
90.00–90.99	9.00–9.99	2.5	2.9
88.00–89.99	10.00–11.99	2.7	3.1
86.00–87.99	12.00–13.99	2.9	3.4
84.00–85.99	14.00–15.99	3.0	3.6
82.00–83.99	16.00–17.99	3.2	3.7
80.00–81.99	18.00–19.99	3.3	3.9
78.00–79.99	20.00–21.99	3.5	4.1
76.00–77.99	22.00–23.99	3.6	4.2
74.00–75.99	24.00–25.99	3.7	4.3
72.00–73.99	26.00–27.99	3.8	4.4
70.00–71.99	28.00–29.99	3.8	4.5
65.00–69.99	30.00–34.99	4.0	4.7
60.00–64.99	35.00–39.99	4.1	4.8
50.00–59.99	40.00–49.99	4.2	5.0

Table 3E. Tolerances for purity tests on two different submitted samples from the same seed lot when a second test is made in the same or a different laboratory (two-way test at 1 % significance level).

Average of the two test results		Tolerance	
50–100 %	Less than 50 %	Non-chaffy seeds	Chaffy seeds
1	2	3	4
99.95–100.00	0.00–0.04	0.2	0.2
99.90–99.94	0.05–0.09	0.3	0.4
99.85–99.89	0.10–0.14	0.4	0.5
99.80–99.84	0.15–0.19	0.4	0.5
99.75–99.79	0.20–0.24	0.5	0.6
99.70–99.74	0.25–0.29	0.5	0.6
99.65–99.69	0.30–0.34	0.6	0.7
99.60–99.64	0.35–0.39	0.6	0.7
99.55–99.59	0.40–0.44	0.6	0.8
99.50–99.54	0.45–0.49	0.7	0.8
99.40–99.49	0.50–0.59	0.7	0.9
99.30–99.39	0.60–0.69	0.8	1.0
99.20–99.29	0.70–0.79	0.8	1.0
99.10–99.19	0.80–0.89	0.9	1.1
99.00–99.09	0.90–0.99	0.9	1.1
98.75–98.99	1.00–1.24	1.0	1.2
98.50–98.74	1.25–1.49	1.1	1.3
98.25–98.49	1.50–1.74	1.2	1.5
98.00–98.24	1.75–1.99	1.3	1.6
97.75–97.99	2.00–2.24	1.4	1.7
97.50–97.74	2.25–2.49	1.5	1.7
97.25–97.49	2.50–2.74	1.5	1.8
97.00–97.24	2.75–2.99	1.6	1.9
96.50–96.99	3.00–3.49	1.7	2.0
96.00–96.49	3.50–3.99	1.8	2.1
95.50–95.99	4.00–4.49	1.9	2.3
95.00–95.49	4.50–4.99	2.0	2.4
94.00–94.99	5.00–5.99	2.1	2.5
93.00–93.99	6.00–6.99	2.3	2.7
92.00–92.99	7.00–7.99	2.5	2.9
91.00–91.99	8.00–8.99	2.6	3.1
90.00–90.99	9.00–9.99	2.8	3.2
88.00–89.99	10.00–11.99	2.9	3.5
86.00–87.99	12.00–13.99	3.2	3.7
84.00–85.99	14.00–15.99	3.4	3.9
82.00–83.99	16.00–17.99	3.5	4.1
80.00–81.99	18.00–19.99	3.7	4.3
78.00–79.99	20.00–21.99	3.8	4.5
76.00–77.99	22.00–23.99	3.9	4.6
74.00–75.99	24.00–25.99	4.1	4.8
72.00–73.99	26.00–27.99	4.2	4.9
70.00–71.99	28.00–29.99	4.3	5.0
65.00–69.99	30.00–34.99	4.4	5.2
60.00–64.99	35.00–39.99	4.5	5.3
50.00–59.99	40.00–49.99	4.7	5.5

Chapter 4: Determination of other seeds by number

4.1 Object

The object of the determination is to estimate the number of seeds of other species stated by the applicant either generally (e.g. all other species) or by reference to one category of seeds (e.g. species scheduled as noxious in a certain country), or specifically (e.g. *Elytrigia repens*).

In international trade this analysis is used mainly to determine the presence of seeds of noxious or undesirable species.

4.2 Definitions

4.2.1 Other seeds

Other seeds refer to species other than those under test as defined in Rule 3.2.2.

In determining the numbers of other seeds, the definitions prescribed in 3.2 must be observed. The extent of the determination of other seeds by number is for either all species or a selection of species in a working sample (see 4.5.1).

Determinations of numbers of dust-like seeds of *Orobanchaceae* species, such as *Orobanche* or *Striga*, is only completed upon request of the applicant (see 4.5.3).

4.2.2 Complete test

In a **complete test**, the whole working sample weight is examined for all other seeds present except for *Orobanchaceae* species. Testing for *Orobancheaceae* species is only completed upon request of the applicant.

4.2.3 Limited test

In a **limited test**, the whole working sample weight is examined, but for stated species only, as requested by the applicant.

4.2.4 Reduced test

In a **reduced test**, less than the whole working sample seed weight is examined for all other seeds present except for *Orobanchaceae* species.

In the case of very expensive seed (see 2.5.4.5), a reduced test can be performed.

4.2.5 Reduced-limited test

In a **reduced-limited test**, less than the whole working sample weight is examined for stated species only.

If a species under test is difficult to identify, a minimum of one fifth of the prescribed working sample weight only need be examined, i.e. a reduced-limited test can be performed.

4.3 General principles

The determination is made by count and expressed as number of seeds found in the quantity examined. When seeds found cannot be identified with certainty to the species level it is permitted to report the genus name only.

4.4 Apparatus

Sieves, blowers and other mechanical devices can be used to aid the analyst in examining the sample and reducing the work involved.

4.5 Procedure

4.5.1 Working sample

a) The size of the working sample must be either a weight estimated to contain at least 25 000 seed units or not less than the weight prescribed in Table 2A Part 1, column 5.
b) If a species under test is difficult to identify, a minimum of one fifth of the prescribed working sample weight only need be examined, i.e. a reduced-limited test can be performed.

4.5.2 Determination

The working sample is searched either for seeds of all other species or of certain stated species, as required by the applicant. The number of seeds found of each species sought is counted.

If the search is limited to certain stated species, the examination may be stopped when one or more seeds of one or all of the stated species (as appropriate to the applicant's requirements) has been found.

Seeds of the other species found must be retained and stored for reference until sample disposal (see 2.5.3 and 2.5.4.7).

4.5.3 Determination of *Orobanche* species

On request of the applicant, a determination for the presence of *Orobanche* spp. will be completed, allowing the number of *Orobanche* spp. found in a specified weight of a submitted subsample to be reported.

4.5.3.1 Background

Orobanche spp. are root parasites and can cause very significant reduction in crop yield of the host plants. The flowering shoots produce large numbers of very fine, dust-like seeds. Seed size, shape, colour and surface markings vary somewhat with each *Orobanche* species but all are basically similar. The seeds of all species of *Orobanche* are usually pear-shaped, under 0.5 mm long, often 0.2 to 0.3 mm long with a smaller width, seed width varies by species and seeds tend to adhere to the crop seed and other surfaces.

The determination requires microscopic analysis of the working sample and visual recognition of *Orobanche* species by the analyst. The working sample is obtained from the submitted or composite sample either by: a) washing and filtration, or b) dry sieving. The laboratory must decide on the most appropriate method to use to obtain the working sample, both have been proved to be satisfactory but effectiveness can vary with the size of the crop species seed under test. For crop species which have very small seeds either method is difficult but when a seed lot is very expensive, or needs to be returned to the customer, then the dry method is more appropriate.

4.5.3.2 Submitted subsample

The *Orobanche* determination requires a separate sealed submitted subsample or the whole of the composite sample to be submitted. The submitted subsample can be obtained from the composite sample by stirring the composite sample with a spoon, then take at a minimum three subsamples with a spoon from different positions and combine them to create the subsample of the required size. If the whole of the composite sample is submitted then the submitted subsample must be obtained by the laboratory from the composite sample.

The size of the submitted subsample must either be a weight estimated to contain at least 25 000 seed units or not less than the weight prescribed in Table 2A Part 1, column 5 (Other seeds by number) for the crop species under test.

The submitted subsample tested must be weighed in grams to the minimum number of decimal places indicated in Table 4.1.

4.5.3.3 Working sample

The working sample for visual analysis for the presence of *Orobanche* species is obtained by either: a) washing and filtration or b) dry sieving the whole weight of the submitted subsample.

a) Washing and filtration
The whole submitted subsample is washed in water containing a detergent and filtered, and the residue collected on the surface of the filter paper analysed. The seed weight to water volume ratio should be 1:2, e.g. 250 g of seed added to 500 mL of water containing one or two drops of surfactant. Large submitted samples may require washing of small batches but the whole submitted subsample is tested.

b) Dry sieving
The whole submitted subsample is sieved 'dry' using a sieve and a bottom collecting tray which are shaken by a mechanical shaker (e.g. Syntron shaker) or manually. The diameter of the hole in the screen-sieve should be adequate to retain the crop seed on top and allow the finer dust-like material to go through to the collection pan, e.g. for *Trifolium pratense* a suitable diameter of the sieve mesh (round holes) is 0.5 mm. Other combinations of sieves can be used depending on the size of the crop seed being tested.

Large submitted samples may require sieving of small batches to avoid overloading/plugging the holes of the sieve. The size of loading in every batch depends on the size of the crop seed, the diameter of the sieves and the number of holes in every square inch of sieve. For each sieving operation the sample should be shaken for at least 1 minute if a mechanical shaker is used. If the shaking is manual, the sample should be shaken vigorously for a longer period until the finer material is fully separated. The sievings collected in the bottom collecting tray from the whole submitted sample are then examined visually.

4.5.3.4 Visual analysis

Analysts must search the surface of the filter paper or dry sievings for *Orobanche* seeds using a microscope with at least ×10 magnification. The number of *Orobanche* seeds present is determined and reported according to 4.7.

4.6 Calculation and expression of results

The result is expressed as the number of seeds belonging to each stated species or category found in the actual quantity examined. In addition the number per unit weight (e.g. per kilogram) may be calculated.

If a second or more tests are carried out on the same sample, then the result must be expressed as the total number of seeds found in the total weight examined.

To decide whether two determinations, made in the same laboratory or in different laboratories, are significantly different, use Table 4A. The two samples compared must be of approximately the same weight.

4.7 Reporting results

The result of a determination of other seeds by number must be reported under 'Other determinations' as follows:

- The actual weight of seed examined to the minimum number of decimal places indicated in Table 4.1.
- The scientific name and number of seeds of each species sought and found in this weight. If no other seeds are found, this must be indicated on the certificate.
- Where it is impossible to determine with certainty on the basis of seed characteristics, reporting must be done to the most precise taxon possible.
- If the full weight prescribed in Table 2A was examined for all other species present, then the words 'Complete test' must be entered, alongside the weight of seed examined.
- If the examination was for only a limited range of other species, then the words 'Limited test' must be entered.
- If the weight examined for all other species was less than the prescribed weight, then the words 'Reduced test' must be entered.
- If the weight examined was less than the weight prescribed in Table 2A, and only a limited range of other species was examined, then the words 'Reduced-limited test' must be entered.
- If a sample of at least 25 000 seeds was examined, and this sample was below the weight prescribed in Table 2A, then the weight of seed examined and the statement 'Test based on at least 25 000 seeds' must be entered.

Upon request, the results may in addition be expressed in some other way, such as 'weight of seeds found' or 'number of seeds per kilogram'.

Upon request, the presence of *Orobanche* species can only be reported on a Blue International Seed Sample Certificate (see 1.2.2) and must be reported as: Test for presence of *Orobanche* species: '… seeds of *Orobanche* spp. were found in … g of seed examined.'

If no seeds were found it can be reported as: 'No seeds of *Orobanche* spp. were found in … g of seed examined.'

The sample weight examined must be reported according to the minimum number of decimals indicated in Table 4.1.

Table 4.1. Minimum number of decimal places for reporting weights of samples examined

Weight of sample (g)	Minimum number of decimal places for reporting
Lower than 1.000	4
1.000–9.999	3
10.00–99.99	2
100.0–999.9	1
1000 or greater	0

4.8 Tolerance tables

Table 4A gives the maximum difference in the numbers of other seeds, used to decide if two test results are compatible. The tests are to be made on the same or a different submitted sample in the same or a different laboratory. Both samples have to be of approximately the same weight. The table is used by entering it at the average of the two test results (column 1), and the maximum tolerated difference is found in column 2.

The tolerances are extracted from Table F1b (foreign seeds) in Miles (1963):

Miles, S. R. (1963). Handbook of Tolerances and Measures of Precision for Seed Testing. *Proceedings of the International Seed Testing Association,* **28 (3)**, 644.

Table 4B gives the tolerances for counts of number of other seeds, made on two different submitted samples each drawn from the same lot and analysed in the same or a different laboratory. Both samples are to be of approximately the same weight. The table can be used when the result of the second test is poorer than that of the first test. The table is used by entering it at the average of the two test results (column 1), and the maximum tolerated difference is found in column 2.

The tolerances appeared in the Report of the Rules Committee, International Seed Testing Association:

ISTA (1962). Revision of *International Rules for Seed Testing. Proceedings of the International Seed Testing Association,* **27**, 291–304.

Table 4A. Tolerances for the determination of other seeds by number when tests are made on the same or a different submitted sample in the same or a different laboratory (two-way test at 5 % significance level)

Average of the two test results	Tolerance	Average of the two test results	Tolerance	Average of the two test results	Tolerance
1	2	1	2	1	2
3	5	76–81	25	253–264	45
4	6	82–88	26	265–276	46
5–6	7	89–95	27	277–288	47
7–8	8	96–102	28	289–300	48
9–10	9	103–110	29	301–313	49
11–13	10	111–117	30	314–326	50
14–15	11	118–125	31	327–339	51
16–18	12	126–133	32	340–353	52
19–22	13	134–142	33	354–366	53
23–25	14	143–151	34	367–380	54
26–29	15	152–160	35	381–394	55
30–33	16	161–169	36	395–409	56
34–37	17	170–178	37	410–424	57
38–42	18	179–188	38	425–439	58
43–47	19	189–198	39	440–454	59
48–52	20	199–209	40	455–469	60
53–57	21	210–219	41	470–485	61
58–63	22	220–230	42	486–501	62
64–69	23	231–241	43	502–518	63
70–75	24	242–252	44	519–534	64

Table 4B. Tolerances for the determination of other seeds by number when tests are made on different submitted samples, the second being made in the same or in a different laboratory (one-way test at 5 % significance level)

Average of the two test results	Tolerance
1	2
3–4	5
5–6	6
7–8	7
9–11	8
12–14	9
15–17	10
18–21	11
22–25	12
26–30	13
31–34	14
35–40	15
41–45	16
46–52	17
53–58	18
59–65	19
66–72	20
73–79	21

Average of the two test results	Tolerance
1	2
80–87	22
88–95	23
96–104	24
105–113	25
114–122	26
123–131	27
132–141	28
142–152	29
153–162	30
163–173	31
174–186	32
187–198	33
199–210	34
211–223	35
224–235	36
236–249	37
250–262	38

Average of the two test results	Tolerance
1	2
263–276	39
277–290	40
291–305	41
306–320	42
321–336	43
337–351	44
352–367	45
368–386	46
387–403	47
404–420	48
421–438	49
439–456	50
457–474	51
475–493	52
494–513	53
514–532	54
533–552	55

Chapter 5: The germination test

5.1 Object

The object of the germination test is to determine the germination potential of a seed lot, which can then in turn be used to compare the quality of different lots and also estimate the field planting value.

Testing under field conditions is normally unsatisfactory, as the results cannot be repeated with reliability. Laboratory methods have, therefore, been evolved in which the external conditions are controlled to give the most regular, rapid and complete germination for the majority of samples of a particular species. The conditions have been standardised to enable the test results to be reproduced within limits as near as possible to those determined by random sample variation.

Further information on germination can be found in the current *ISTA Handbook on Seedling Evaluation*.

5.2 Definitions

5.2.1 Germination

Germination of a seed in an ISTA test is the emergence and development of the seedling to a stage where the aspect of its essential structures indicates whether or not it is able to develop further into a satisfactory plant under favourable conditions in the field.

5.2.2 Double test

A double test is where two tests are prescribed for certain tree and shrub seed species, and the results of both tests are reported.

5.2.3 Parallel tests

Parallel tests are where more than one test method from those prescribed is applied to a sample at the same time and the best result reported.

5.2.4 Germination percentage

The germination percentage reported on the ISTA Certificate indicates the proportion by number of seeds which have produced seedlings classified as normal under the conditions and within the period specified in Table 5A, i.e. the percentage of normal seedlings.

5.2.5 Essential seedling structures

A seedling, depending on the species being tested, consists of a specific combination of some of the following structures which are essential for its further development into a satisfactory plant:

- root system (primary root; in certain cases seminal roots);
- shoot axis (hypocotyl; epicotyl; in certain *Poaceae* mesocotyl; terminal bud);
- cotyledons (one to several);
- coleoptile (in all *Poaceae*).

For further details see 5.2.11.

5.2.6 The 50 % rule

The 50 % rule is used in the evaluation of cotyledons and primary leaves.

Cotyledon tissue:

- Seedlings are considered normal as long as half or more of the total cotyledon tissue is functional.
- Seedlings are abnormal when more than half of the cotyledon tissue is missing, necrotic, decayed or discoloured.

Primary leaves:

- Primary leaves need to be evaluated in species such as *Phaseolus*.
- Seedlings are considered normal as long as half or more of the primary leaf tissue is functional.
- Seedlings are abnormal when more than half of the primary leaf tissue is missing, necrotic, decayed or discoloured.

The 50 % rule does not apply if the two points of attachment of the cotyledons to the seedling axis or the terminal bud itself is necrotic or decayed; such seedlings are abnormal irrespective of the condition of the cotyledons or primary leaves. It does not apply also if one point of attachment of one cotyledon is necrotic or decayed and if the other cotyledon is not intact; such seedlings are also considered as abnormal.

Further details of how the 50 % rule is applied can be found in the *ISTA Handbook on Seedling Evaluation*.

5.2.7 Normal seedlings

Normal seedlings show the potential for continued development into satisfactory plants when grown in good quality soil and under favourable conditions of moisture, temperature and light. To be classified as normal a seedling must conform with one of the following categories:

intact seedlings: seedlings with all their essential structures well developed, complete, in proportion and healthy;

seedlings with slight defects: seedlings showing certain slight defects of their essential structures, provided they show an otherwise satisfactory and balanced development comparable to that of intact seedlings of the same test;

seedlings with secondary infection: seedlings which it is evident would have conformed with one of the above, but which have been affected by fungi or bacteria from sources other than the parent seed.

5.2.7.1 Intact seedlings

An intact seedling, depending on the species being tested, shows a specific combination of some of the following essential structures:

a) a well-developed **root system,** consisting of:
   - a long and slender **primary root**, usually covered with numerous root hairs and ending in a fine tip,
   - **secondary roots** when produced within the prescribed test period,
   - several seminal roots instead of one primary root in certain genera, including *Avena, Hordeum, Secale, Triticum, ×Triticosecale, Cyclamen;*

b) a well-developed **shoot axis,** consisting of:
   - a straight and usually slender and elongated **hypocotyl** in seedlings showing epigeal germination,
   - a well-developed **epicotyl** in seedlings showing hypogeal germination,
   - both an elongated **hypocotyl** and **epicotyl** in some genera with epigeal germination,
   - an elongated **mesocotyl** in certain genera of the *Poaceae*;

c) a specific number of **cotyledons**, i.e.:
   - **one** cotyledon in monocotyledons or exceptionally in dicotyledons (it may be green and leaf-like or modified and remaining wholly or partly within the seed),
   - **two** cotyledons in dicotyledons (in species with epigeal germination: green and leaf-like, the size and form varying with the species being tested; in seedlings with hypogeal germination: hemispherical and fleshy and remaining within the seed coat),
   - a **varying number** of cotyledons (2–18) in conifers (usually green, long and narrow);

d) green, expanding **primary leaves**:
   - **one** primary leaf, sometimes preceded by a few scale leaves in seedlings with alternating leaves, or
   - **two** primary leaves in seedlings with opposite leaves;

e) a **terminal bud** or **shoot apex**, the development of which varies with the species being tested;

f) a well-developed, straight **coleoptile** in *Poaceae*, containing a green leaf extending to the tip and eventually emerging through it;

g) in seedlings of tree species with epigeal germination: when the primary root and hypocotyl together exceed four times the length of the seed, provided all structures which have developed are intact.

5.2.7.2 Slight defects

The following defects are considered slight and therefore seedlings are classified as normal:
- primary root with limited damage (e.g. not affecting the conductive tissue) or slight growth retardation;
- primary root defective but with sufficiently well-developed secondary roots (in specific genera of *Fabaceae,* especially large-seeded genera such as *Phaseolus, Pisum* and *Vicia*, and *Poaceae*, e.g. *Zea,* and in all genera of *Cucurbitaceae*, e.g. *Cucumis, Cucurbita* and *Citrullus*, and *Malvaceae*, e.g. *Gossypium*. For a complete list see the current *ISTA Handbook on Seedling Evaluation*);

- at least three secondary roots, each of which is greater than or equal to half the length of the hypocotyl, in *Glycine max*, when the primary root is defective;
- only one strong seminal root in *Avena, Hordeum, Secale, Triticum* and ×*Triticosecale*, and two in *Cyclamen*;
- hypocotyl, epicotyl or mesocotyl with limited damage (e.g. not affecting the conductive tissue);
- cotyledons with limited damage (if half or more of the total tissue area is left functioning normally [i.e. the 50 % rule; see 5.2.6] and if there is no evidence of damage or decay to the shoot apex or surrounding tissues);
- only one normal cotyledon in dicotyledons (if there is no evidence of damage or decay to the shoot apex or surrounding tissues);
- three or more cotyledons instead of two (provided that they comply with the 50 % rule; see 5.2.6);
- fused cotyledons (provided that they comply with the 50 % rule; see 5.2.6);
- primary leaves with limited damage (if half or more of the total tissue area is left functioning normally [the 50 % rule; see 5.2.6]);
- only one normal primary leaf, e.g. in *Phaseolus* (if there is no evidence of damage or decay to the terminal bud);
- primary leaves of *Phaseolus* which are properly formed but reduced in size, as long as they are larger than a quarter of the normal size;
- three or more primary leaves instead of two, e.g. in *Phaseolus* (provided that they comply with the 50 % rule; see 5.2.6);
- coleoptile with limited damage;
- coleoptile with a split from the tip extending downward not more than one third of the length (for *Zea mays*; seedling with coleoptile defects described in Figure 5.1 may be classed as normal if the first leaf is intact or only slightly damaged, as defined in Figure 5.2);
- coleoptile loosely twisted or forming a loop (because it is trapped under the lemma and palea or fruit coat);
- coleoptile with a green leaf not extending to the tip but reaching at least half-way up the coleoptile.

5.2.7.3 Secondary infection

Seedlings which are seriously decayed by fungi or bacteria are classified as normal, if it is evident that the parent seed is not the source of infection, and if it can be determined that all the essential structures were present.

5.2.8 Abnormal seedlings

Abnormal seedlings do not show the potential to develop into a normal plant when grown in good quality soil and under favourable conditions of moisture, temperature and light. The following seedlings are classified as abnormal:

damaged: seedlings with any of the essential structures missing or so badly and irreparably damaged that balanced development cannot be expected;

deformed or unbalanced: seedlings with weak development or physiological disturbances or in which essential structures are deformed or out of proportion;

decayed: seedlings with any of their essential structures so diseased or decayed as a result of primary infection (see 5.2.11) that normal development is prevented.

5.2.8.1 Seedling abnormalities

One or more of the following defects in the seedling renders it abnormal.

0 Overall abnormalities
00 The seedling:
- 00/01 is deformed
- 00/02 is fractured
- 00/03 releases the cotyledons before the primary root from the seed coat
- 00/04 consists of fused twin seedlings
- 00/05 bears an endosperm collar
- 00/06 is yellow or white
- 00/07 is spindly
- 00/08 is glassy
- 00/09 is decayed as a result of primary infection
- 00/10 shows phytotoxic symptoms
- 00/11 is unbalanced
- 00/12 in *Poaceae*, detached endosperm

1 Abnormalities of the root system
11 The primary root:
- 11/01 is stunted
- 11/02 is stubby
- 11/03 is retarded
- 11/04 is missing
- 11/05 is deeply cracked or broken
- 11/06 is split from the tip or split right through
- 11/07 is trapped in the seed coat
- 11/08 shows negative geotropism
- 11/09 is constricted
- 11/10 is spindly
- 11/11 is glassy
- 11/12 is decayed as a result of primary infection

Note: secondary roots showing one or more of the above defects are abnormal and cannot replace an abnormal primary root in cases where the presence of several secondary roots (e.g. *Cucumis*) determines the value of a seedling.

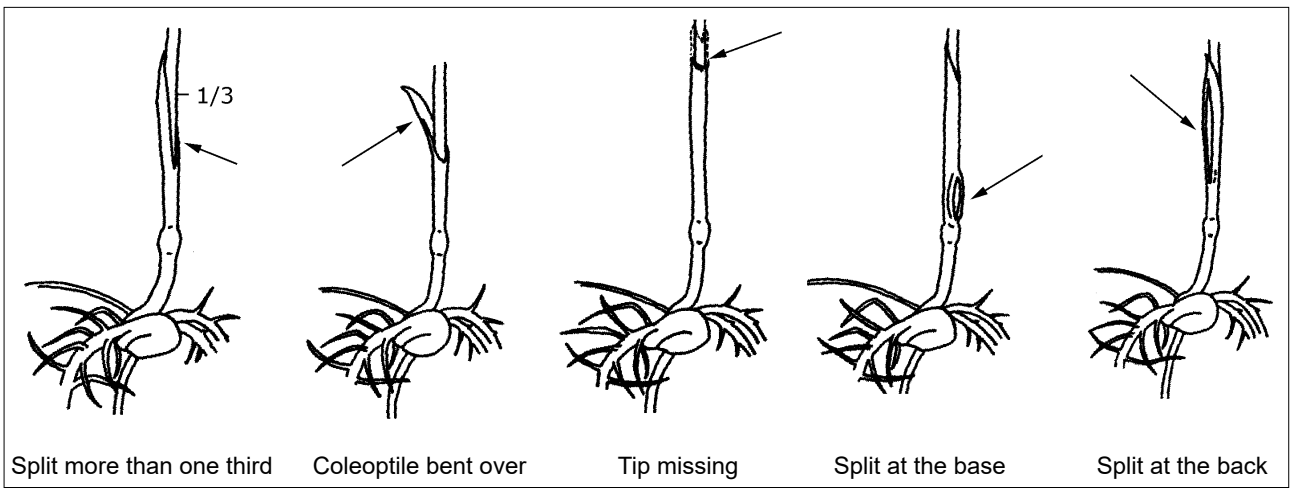

Figure 5.1. Evaluation of maize seedlings with coleoptile defects. Seedlings are normal if the primary leaf is intact or only slightly damaged, as defined in Figure 5.2. Seedlings are abnormal if primary leaf is damaged, as defined in Figure 5.2.

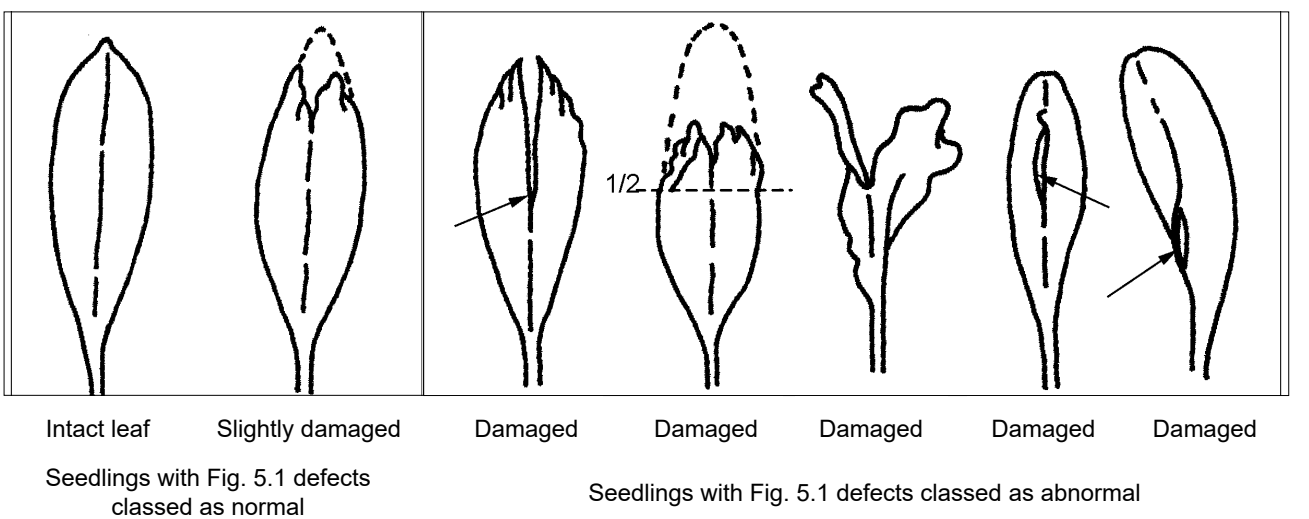

Figure 5.2. Evaluation of maize seedlings with coleoptile defects. Definition of intact, slightly damaged and damaged primary leaf.

12 The seminal roots:
- 12/01 are stunted
- 12/02 are stubby
- 12/03 are retarded
- 12/04 are missing
- 12/05 show negative geotropism
- 12/06 are glassy
- 12/07 are decayed as a result of primary infection

Note: at least one strong seminal root (e.g. *Triticum*), or two strong seminal roots (i.e. *Cyclamen*) are required for a normal seedling.

2 Abnormalities of the shoot system

21 The hypocotyl, epicotyl or mesocotyl:
- 21/01 is short and thick (except *Cyclamen*)
- 21/02 does not form a tuber (only in *Cyclamen*)
- 21/03 is deeply cracked or broken
- 21/04 is split right through
- 21/05 is missing
- 21/06 is bent over or forms a loop
- 21/07 forms a spiral
- 21/08 is tightly twisted
- 21/09 is constricted
- 21/10 is spindly
- 21/11 is glassy
- 21/12 is decayed as a result of primary infection
- 21/13 shows negative phototropism

22 The terminal bud and surrounding tissues:
- 22/01 are deformed
- 22/02 are damaged
- 22/03 are missing
- 22/04 are necrotic
- 22/05 are decayed as a result of primary infection

Note: irrespective of the presence of auxiliary buds (e.g. *Phaseolus*) or auxiliary shoots (e.g. *Pisum*) arising from the axils of the cotyledons or of the primary leaves, the seedling is considered abnormal if the main shoot fails to develop normally.

3 Abnormalities of the cotyledons and primary leaves

31 The cotyledons (apply the 50 % rule; see 5.2.6):
- 31/01 are swollen or curled
- 31/02 are deformed
- 31/03 are broken or otherwise damaged
- 31/04 are separate or missing
- 31/05 are discoloured or necrotic
- 31/06 are glassy
- 31/07 are decayed as a result of primary infection
- 31/08 are fused on both sides

Note: damage or decay of the cotyledons at the two points of attachment of the cotyledons to the seedling axis or near the terminal bud renders a seedling abnormal, irrespective of the 50 % rule. The 50 % rule also does not apply if one point of attachment of one cotyledon is necrotic or decayed and the other cotyledon is not intact; such seedlings are also considered as abnormal.

32 In *Allium* spp., the cotyledon:
- 32/01 is short and thick
- 32/02 is bent over or forms a loop
- 32/03 forms a spiral
- 32/04 does not show a definite 'knee'
- 32/05 is constricted
- 32/06 is spindly

33 The primary leaves (apply the 50 % rule; see 5.2.6):
- 33/01 are deformed
- 33/02 are damaged
- 33/03 are missing
- 33/04 are discoloured
- 33/05 are necrotic
- 33/06 are of normal shape, but less than one-quarter normal size (only in *Phaseolus*)
- 33/07 are decayed as a result of primary infection

4 Abnormalities of the coleoptile and primary leaf:

41 The coleoptile:
- 41/01 is stubby or otherwise deformed
- 41/02 is broken
- 41/03 is missing
- 41/04 is defective or has no tip
- 41/05 is strongly bent over or forms a loop
- 41/06 forms a spiral
- 41/07 is tightly twisted
- 41/08 is split for more than one-third of the length from the tip
- 41/09 is spindly
- 41/10 is decayed as a result of primary infection
- 41/11 is split other than from the tip
- 41/12 is trapped under the lemma or the testa.

Note: a seedling with its coleoptile trapped under the lemma or seed coat is considered normal, if development is otherwise normal. If the growth of such a seedling is stunted, it must be evaluated as abnormal.

Note: for *Zea mays* only: the seedling is abnormal if the coleoptile has any of the following defects together with damage to the primary leaf as defined in Figure 5.1:

1 if the primary leaf has emerged at time of evaluation:
- a. coleoptile split for more than one-third of the length from the tip
- b. coleoptile strongly bent over
- c. coleoptile tip damaged or missing
- d. coleoptile split at any location below the tip

2 if the primary leaf has not emerged at time of evaluation:
- a. tip of coleoptile damaged or missing
- b. coleoptile split for more than one-third of the length from the tip
- c. leaf protruding below the tip of the coleoptile

42 The primary leaf:
- 42/01 extends less than halfway up the coleoptile
- 42/02 is missing
- 42/03 is shredded or otherwise deformed
- 42/04 protrudes from the lower part of the coleoptile
- 42/05 is yellow or white (no chlorophyll)
- 42/06 is decayed as a result of primary infection

5.2.9 Multigerm seed units

Several types of seed units can produce more than one seedling:
- units containing more than one true seed (e.g. multiple seed units in *Dactylis, Festuca, ×Festulolium* and *Lolium*; unseparated schizocarps of *Apiaceae*; clusters of *Beta vulgaris*, and fruits of *Tectona grandis*);
- true seeds containing more than one embryo. This may occur normally in certain species (polyembryony) or exceptionally in other species (twins). In this case, frequently one of the seedlings is weak or spindly, but occasionally both are of nearly normal size;
- fused embryos. Occasionally two seedlings which are fused together are produced from one seed.

When a unit produces more than one seedling, these are evaluated separately. One normal seedling is considered sufficient to classify the unit as normal. If a unit produces more than one normal seedling, only one is counted for determining the germination percentage.

5.2.10 Ungerminated seeds

Seeds which have not germinated by the end of the test period, when tested under the conditions given in Table 5A, are classified as follows:

hard seeds: seeds which remain hard at the end of the test period, because they have not absorbed water;

fresh seeds: seeds, other than hard seeds, which because of dormancy have failed to germinate under the conditions of the germination test, but which remain clean and firm and have the potential to develop into a normal seedling;

dead seeds: seeds which at the end of the test period are neither hard nor fresh nor have produced any part of a seedling;

other categories: in some circumstances empty and ungerminated seeds may be further categorised according to classes described in 5.2.10.4.

5.2.10.1 Hard seeds

Hardseededness is a form of dormancy. It is common in many species of the *Fabaceae* but may also occur in other families. These seeds are not able to imbibe water under the conditions set out in Table 5A and remain hard.

5.2.10.2 Fresh seeds

Fresh seeds are able to imbibe water when provided with the conditions set out in Table 5A, but the germination process is blocked.

5.2.10.3 Dead seeds

Dead seeds absorb water, are usually soft or discoloured or frequently mouldy, and show no sign of seedling development.

5.2.10.4 Other categories

Ungerminated seeds may be further subdivided into:

empty seeds: seeds which are completely empty or contain only some residual tissue;

embryoless seeds: seeds which contain fresh endosperm or gametophytic tissue in which there is apparently no embryonic cavity nor embryo;

insect-damaged seeds: seeds which contain insect larvae, frass, or show other evidence of insect attack affecting the ability of the seed to germinate.

These categories may appear in all species of seeds, but are found more commonly in tree species.

5.2.11 Additional definitions

coleoptile the sheath enclosing and protecting the apex of the axis of the embryo and young seedling in certain monocotyledons (e.g. *Poaceae*)

cotyledon the first leaf or pair of leaves of an embryo and seedling (see **primary leaf**)

decay break-down of organic tissue, usually associated with the presence of micro-organisms

discoloration alteration or loss of colour

dicotyledons a group of plants so classified because the embryo usually has two cotyledons (see **monocotyledons**)

diseased showing the effect of the presence and activity of micro-organisms or of chemical deficiency

embryo rudimentary plant contained in a seed, usually consisting of a more or less differentiated axis and attached cotyledon(s)

endosperm nutritive tissue originating from fertilisation and retained at maturity in some seeds as a storage tissue for food reserves

epicotyl the part of the seedling axis immediately above the cotyledons and below the primary leaf or pair of leaves

epigeal germination a type of germination in which cotyledons and shoot are carried above soil level by the elongating hypocotyl (see **hypogeal germination**)

gametophytic tissue the nutritive tissue occurring within conifer seeds (it serves a function similar to endosperm)

geotropism plant growth response to gravity

positive geotropism downward growth (e.g. normal primary root)

negative geotropism upward growth (e.g. normal shoot)

hypocotyl the part of the seedling axis immediately above the primary root and below the cotyledons

hypogeal germination a type of germination in which the cotyledon(s) or comparable structure (e.g. scutellum) remain in the soil and within the seed. The shoot is carried above soil level by the elongating epicotyl in dicotyledons, or by the mesocotyl in some monocotyledons (see **epigeal germination**)

infection entrance and spread of disease organisms in living material (e.g. seedling structures), not necessarily but often causing disease symptoms and decay

primary infection disease organism present and active in the seed itself

secondary infection disease organism spreading from other seeds or seedlings

looped structure seedling structure (e.g. hypocotyl, coleoptile) which completes a loop or circle instead of being more or less straight

mesocotyl in some highly specialised monocotyledons (e.g. certain *Poaceae*) the part of the seedling axis between the point of attachment of the scutellum and the coleoptile

monocotyledons a group of plants so classified because the embryo usually has one cotyledon (see **dicotyledons**)

phototropism growth and response to a light stimulus

positive phototropism growth towards light

negative phototropism growth away from light

phytotoxic poisonous to plants

primary infection see 'infection'

primary leaf the first leaf or pair of leaves found after the cotyledons (see **cotyledon**)

primary root main root of the seedling, developing from the radicle of the embryo (see **radicle**)

radicle the rudimentary root of the embryo, developing into the primary root after emergence through the seed coat during germination (see **primary root**)

retarded root a root usually with an intact tip but much too short and weak to be in balance with the other structures of the seedling

root hair a fine tubular outgrowth of a surface cell of the root

scutellum a shield-shaped structure that is part of the cotyledon in some *Poaceae* and through which nutrients are absorbed from the endosperm into the embryo

secondary infection see **infection**

secondary root used in seed testing to mean any root other than the primary root

seedling a young plant developing from the embryo in a seed

seminal roots the primary root and a number of secondary roots arising from the embryo axis and forming the seedling root system in cereals

shoot apex terminal portion of the shoot, that contains the main growing point

stubby root the kind of root characteristic for seedlings with phytotoxic symptoms; usually short and club-shaped, though often with an intact root tip (see **stunted root**)

stunted root root with a missing or defective root tip, irrespective of the length of the root (see **stubby root**)

terminal bud the shoot apex enveloped by several more or less differentiated leaves

twisted structure seedling structure (e.g. hypocotyl, coleoptile) which twists around its main axis of elongation

loosely twisted turns completed over a long section of the structure

tightly twisted turns completed over a short section of the structure

5.3 General principles

Germination tests must be made with pure seeds, except where testing of seed by weighed replicates is allowed.

The pure seed definition for the species must be applied. The pure seed can be taken from either the pure seed fraction of a purity test carried out as prescribed in Chapter 3, or from a representative fraction of the submitted sample. When the seed lot has been coated, the pure pellet definition must be used, except in the case of tapes or mats where the seeds are tested without removing the seed.

Prescribed procedures for promoting germination are given in 5.6.3. Parallel testing is permitted. The rules for reporting parallel and double testing are defined in 5.9. If additional tests are undertaken after any procedure other than those given in 5.6.3, the test is not covered by the Rules, and the result and procedure must be reported under 'Other determinations' on the ISTA Certificate (see 1.5.2.22).

The seeds, arranged in replicates, are tested under favourable moisture conditions and in accordance with the methods prescribed in Table 5A. After the period indicated in Table 5A, the replicates are examined and counts made of the seedlings and seeds in the various categories required for reporting (5.9).

5.4 Growing media

5.4.1 Definition

Growing media used for germination tests are products which provide sufficient pore space for air and water, for the growth of the root system and for contact with solutions (water) needed for plant growth.

With paper as the base medium (see 5.6.2.1.1), any combination of growing media prescribed in Table 5A for that species is allowed, provided that each growing medium is verified and meets the specifications prescribed in 5.4.2.

5.4.2 Specifications

The following general specifications apply for all growing media and must be verified.

Composition: the growing medium can be paper, pure sand or mixtures of organic compounds with added mineral particles.

Water retention characteristics: when the appropriate amount of water is added, the particles of the growing medium should have the capacity to hold sufficient water to provide continuous movement of water to the seeds and seedlings, but also provide sufficient pore space for aeration required for optimal germination and root growth. The water content of the growing medium should be adjusted to correspond to the needs of the species being tested, based on the maximum water-holding capacity of the medium. The water retention is then expressed as a percentage of the maximum retention.

pH: the growing medium must have a pH value within the range 6.0–7.5 when checked in the substrate. Measurements of pH can be replaced by biological tests (see 5.4.5).

Conductivity: the salinity must be as low as possible and no more than 40 millisiemens per metre. Measurements of conductivity can be replaced by biological tests (see 5.4.5).

Cleanness and freedom from toxicity: the growing medium must be free from seeds, fungi, bacteria or toxic substances, which may interfere with the germination of seeds or the growth or evaluation of seedlings.

Re-use of substrates: it is strongly recommended that the growing medium is only used once.

Alternative measurements: it may be difficult to check all the specifications or to get growing media from suppliers with the requested specifications. It is permissible to replace the measure of conductivity with biological tests such as phytotoxicity. If not, all the characteristics described in 5.4.2 must be verified.

5.4.3 Growing media characteristics

5.4.3.1 Paper growing media

The paper must be wood, cotton or other purified vegetable cellulose. The paper may take the form of filter papers, blotters or towels. The paper should be such that:

– the roots of the seedlings will grow on and not into it;
– it possesses sufficient strength to enable it to resist tearing when handled during the test.

5.4.3.2 Sand growing media

At least 90 % of the particles must pass through a sieve with holes or meshes of 2.0 mm width. If the particle size characteristics given by the supplier are in accordance with these specifications then the laboratory does not need to perform a quality check of the sand particle size. In the absence of a supplier's specification sheet, the laboratory must check the particle size for each batch of sand received.

5.4.3.3 Organic growing media

Organic growing media are defined as containing the following elements in known proportions and fitting the requirements of 5.4.2:

Organic compounds: fibres such as peat, coconut fibres or wood fibres, with a recommended size less than 5 mm;

Mineral particles: for example sand, perlite, dolomite or vermiculite. The proportion should be between 15 and 30 % in volume. It is recommended that 90 % of the particles should pass through a sieve with holes or meshes of 3 mm width.

Any other mixture of organic compounds and mineral particles, fitting the specifications included in 5.4.2, can be used. The media composition must be clearly described.

5.4.4 Water

Demineralised water, deionised water, tap water and spring water are commonly used and permitted.

5.4.4.1 General specifications

Cleanness: the water used to moisten the substrate should be reasonably free from organic or inorganic impurities.

pH: the pH value must be within the range 6.0–7.5 when checked in the substrate, unless there is evidence that the pH outside this range does not have a negative influence on the germination test results.

5.4.5 Quality control

New deliveries of growing media must meet the requirements for the principal physical characteristics and be free of negative effects due to toxic substances or noxious micro-organisms.

The characteristics **composition, water retention, pH, cleanness and innocuity** (freedom from phytotoxic effects and negative effects due to micro-organisms) must be checked.

Alternative measurements: it may be difficult to check all the specifications or to get growing media from suppliers with the requested specifications. It is permissible to replace the measurements of pH and conductivity with biological tests, such as a test for phytotoxicity.

Examples of media quality control tests are given in the *ISTA Handbook on Seedling Evaluation*. Quality control tests can be performed by the seed testing laboratory or subcontracted to laboratories specialising in soil analyses or microbiology tests.

5.5 Material and apparatus

5.5.1 Containers

All kinds of plastic, glass, metal or pottery containers can be used, provided that they have no toxic effects, and are clean and free from micro-organisms.

5.5.2 Counting equipment

Planting using counting boards or vacuum counters is permissible, as long as using these tools does not influence the germination result or cause replicate results to be biased.

Examples of how to use the counting equipment are given in the *ISTA Handbook on Seedling Evaluation*.

5.5.2.1 Counting boards

Counting boards are often used for large seeds such as *Zea*, *Phaseolus* and *Pisum*.

5.5.2.2 Vacuum counters

Vacuum counters can in principle be used for all species, but are mostly used for species with regularly shaped and relatively smooth seeds such as cereals or species of *Brassica* or *Trifolium*.

5.5.3 Germination apparatus

5.5.3.1 The bell jar or Jacobsen apparatus (Copenhagen tank)

This apparatus usually consists of a germination plate upon which filter paper substrates with seeds are placed. The substrate is kept continuously moist by means of a wick, which extends down through slits or holes in the germination plate into the underlying waterbath.

To prevent drying out, the substrate is covered with a bell jar provided with a hole which allows for ventilation without undue evaporation. The temperature is conditioned either indirectly by heating/cooling the water in the waterbath, or directly by conditioning the germination plate, and is usually automatically regulated. The apparatus may be used for all prescribed constant or alternating temperatures.

5.5.3.2 The germination incubator and the room germinator

The incubator is used for germinating seeds in darkness or light, or providing seeds with pretreatments to break dormancy, such as prechilling. The room germinator is a modification of the incubator, but is large enough to permit workers to enter and place the tests within it. Germination incubators and room germinators are well insulated and are equipped with both heating and cooling systems to ensure the maintenance of required temperatures. The temperature must be evenly distributed to ensure that all samples placed in the incubator/room have a temperature within the prescribed temperature limits for the test (±2 °C) or pretreatment. If the incubator/room does not have a system capable of providing alternating temperatures, samples can be transferred from one incubator/room to another running at a different temperature to achieve the desired alternative temperature cycle. Tests must be supplied with sufficient water for germination and must not be allowed to dry out. This can be achieved through maintaining a high humidity by using 'wet' incubators or using humidifiers in germination rooms. Tests can also be enclosed in moisture-proof containers.

5.6 Procedure

5.6.1 Working sample

Four hundred seeds are taken at random from the well-mixed pure seed (5.3) and spaced uniformly and adequately apart on the moist substrate. Care must be taken to ensure that there is no selection of seeds thus causing biased results. Replicates of 100 seeds are normally used, spaced sufficiently far apart on the seed bed to minimise the effect of adjacent seeds on seedling development. To ensure adequate spacing, split replicates of 50 or even 25 seeds may be necessary, particularly where there are seed-borne pathogens or saprophytes present. When seeds grown on paper substrates are heavily infected, it may be necessary at an intermediate count to transfer remaining seeds and seedlings to fresh media.

Multigerm seed units, except for *Arachis*, are not broken up for the germination test but are tested as though they were single seeds.

For *Arachis*, although a pod is a pure seed unit, seed must be removed from the pod before use in a germination test.

The ISTA germination test is based on 400 seeds. In certain circumstances (see 2.5.4.5) it may be necessary to test fewer than 400 seeds. In such cases, at least 100 seeds must be tested in replicates of 25 or 50.

At the request of the applicant, a germination test can be carried out on 200 seeds, for issuance on a Blue International Seed Sample Certificate only. In this case, the number of seeds tested is less than 400 and must be reported under 'Other Determinations' (see 5.9).

When due to counting errors more than 5 seeds are lost or found during a germination test (i.e. ±1.25 % for a total of 400 seeds), then the test must be repeated.

If there are up to 5 seeds lost or found as extra in the test, then each replicate must be adjusted to 100 by calculation. For example, if one replicate had 80 normal seedlings, 10 abnormal seedlings and 9 dead seeds, with one seed missing, then the result must be adjusted to 100 with the following proportional calculation: 80 × 100 : 99 normal seedlings, 10 × 100 : 99 abnormal seedlings and 9 × 100 : 99 dead seeds. Rounding follows the principles described in 5.8.2.

Note: if the submitted sample is smaller than prescribed, the sampler must be notified accordingly and analysis withheld until sufficient seed is received in a single submitted sample, except that in the case of very expensive seed, the analysis may be completed to the extent possible, and the following statement inserted on the certificate:
'The sample submitted weighed only ... g and is not in accordance with the *International Rules for Seed Testing*.'
Or, in the case of pelleted seeds:
'The sample submitted contained only ... pellets (seeds) and is not in accordance with the *International Rules for Seed Testing*.'

5.6.2 Test conditions

Permitted substrates, temperatures, duration of tests and additional directions, including recommended procedures for breaking dormancy, are indicated in Table 5A. Substrates, temperatures and duration of test indicated are prescriptive and no others may be used.

5.6.2.1 Growing media

5.6.2.1.1 Methods using paper

Top of paper (TP): the seeds are germinated on top of one or more layers of paper which are placed:
- on the Jacobsen apparatus (5.5.3.1);
- into transparent boxes or Petri dishes. The appropriate quantity of water is added at the beginning of the test and evaporation may be minimised by a tightly fitting lid or by enclosing the dishes in plastic bags;
- directly on trays in germination incubators. The relative humidity in the incubators must then be maintained at a level that prevents tests drying out.

Moistened porous paper or absorbent cotton can be used as a base for the substrates.

Between paper (BP): the seeds are germinated between two layers of paper. This may be achieved:
- by loosely covering the seeds with an additional layer of filter paper;
- by placing the seeds into folded envelopes which may be placed in a flat or upright position;
- by placing the seeds in rolled paper towels (the rolls must be placed in an upright position).

The substrates are kept in closed boxes, wrapped in plastic bags or placed directly on trays in a cabinet germinator, provided the relative humidity in the germinator can be maintained very near saturation.

Pleated paper (PP): the seeds are placed in a pleated, accordion-like paper strip with 50 pleats, usually two to a pleat. The pleated strips are kept in boxes or directly in a 'wet' cabinet, with a flat strip often wrapped around the pleated paper to ensure uniform moisture conditions. This method may be used as an a alternative where TP or BP are prescribed.

5.6.2.1.2 Methods using sand or organic growing media

Sand and organic growing media are used as follows:

Top of sand (TS), top of organic growing medium (TO): the seeds are pressed into the surface of the sand or the organic growing medium.

Sand (S), organic growing medium (O): the seeds are planted on a level layer of moist sand or the organic growing medium and covered with 10–20 mm of uncompressed substrate, depending on the size of the seed. To ensure good aeration it is recommended that the bottom layer be loosened by raking before sowing.

Sand or organic growing media may be used instead of paper, even if not prescribed in Table 5A:
- when the evaluation of a diseased sample proves impracticable because of the spread of infection between seeds and seedlings on paper substrate;
- for investigative purposes and to confirm evaluation of seedlings in cases of doubt;
- when seedlings show phytotoxic symptoms.

5.6.2.1.3 Methods using a combination of paper and sand

Top of paper covered with sand (TPS): the seeds are germinated on top of a moistened sheet of crêpe cellulose paper which is covered with a 2 cm layer of dry sand. Crêpe cellulose paper is a multi-layered paper pad, e.g. Versa-Pak®.

5.6.2.1.4 Soil

Soil is generally not recommended as a primary growing medium. However, it may be used as an alternative to organic growing media when seedlings show phytotoxic symptoms or if evaluation of seedlings is in doubt on paper or sand. If soil is used it must meet the specifications given in 5.4.2.

5.6.2.2 Moisture and aeration

Precautions must be taken to ensure that the medium cannot dry out and contains sufficient water for the whole test period. Subsequent watering should be avoided wherever possible, as it is likely to increase variability between replicates and between tests. However, it may be necessary to add water at intermediate counts.

Special measures for aeration are not necessary for TP and PP tests enclosed in boxes or Petri dishes. For BP, however, care must be taken that envelopes and rolled paper towels are loose enough to allow for sufficient air around the seeds. For the same reason, sand and organic growing media must not be compressed.

5.6.2.3 Temperature

The temperatures prescribed in Table 5A for the germination of a species are those to which the seed is exposed on or inside the substrate. They should be as uniform as possible throughout the germination apparatus, incubator or room germinator. For any test, whether in darkness or under artificial light or in indirect daylight, variation from the prescribed temperature must not be more than ±2 °C.

Where alternating temperatures are indicated, the lower temperature should be maintained for 16 h and the higher for 8 h. A gradual changeover lasting no more than 3 h may be satisfactory, but a sharp changeover lasting one hour or less may be necessary for breaking dormancy.

Where a temperature range is given, no tolerances may be applied to the upper or lower temperatures. For example, when a prechilling temperature of 5 to 10 °C is prescribed, this means that the allowed temperature range is 5 to 10 °C, and not 5 ±2 °C to 10 ±2 °C.

5.6.2.4 Light

Seeds of most of the species in Table 5A will germinate either in light or in darkness. However, illumination of the substrate from an artificial source or by indirect daylight is generally recommended, as better developed seedlings, which are more easily evaluated are produced. Seedlings grown in complete darkness are etiolated and white and therefore more sensitive to attack by micro-organisms. Besides, certain defects, such as chlorophyll deficiency, cannot be detected.

In certain cases (e.g. some tropical and subtropical grasses), light may promote germination of dormant samples (5.6.3.1). In such cases, the light should be between 750 and 1250 lux from cool white lamps. There are also a few species (e.g. *Phacelia tanacetifolia*) which must be germinated in darkness, as light may be inhibitory. Specific recommendations for light or darkness are given in the last column of Table 5A.

5.6.2.5 Choice of method

When alternative methods are indicated in Table 5A, one of them (any combination of substrate and temperature) must be used. The choice of method will depend largely on the facilities and experience of the testing laboratory and to some extent on the provenance and condition of the sample.

5.6.3 Procedures for promoting germination of dormant seed

For various reasons (e.g. physiological dormancy, hard-seededness, inhibitory substances) a considerable number of hard or fresh seeds may remain at the end of the germination test. If dormancy is suspected, more complete germination may be obtained by retesting after one or a combination of dormancy-breaking procedures. For some species, recommended procedures are indicated in column 6 of Table 5A, but these and all other procedures listed in 5.6.3.1, 5.6.3.2 and 5.6.3.3 can be used for any species without restriction. The period of treatment is not included in the germination test period. Precise details and duration of the dormancy-breaking procedure must be reported on the ISTA Certificate.

For some tree and shrub seeds, where it is known from experience that a proportion of the seeds will not germinate because of dormancy, a second test incorporating a special dormancy-breaking procedure is prescribed which preferably should run concurrently with the normal test (double test).

Disinfection of the seed prior to the test is permitted and described in 5.6.3.4.

5.6.3.1 Procedures for breaking physiological dormancy

Prechilling: The replicates for germination are placed in contact with the moist substrate and kept at a low temperature for an initial period before they are moved to the temperature indicated in Table 5A column 3. Agricultural, vegetable, flower, spice, herb and medicinal seeds are usually kept at a temperature of 5 to 10 °C for an initial period of up to 7 days. In some cases it may be necessary to extend the prechilling period or to rechill.

Tree and shrub seeds are usually prechilled at a temperature of 1 to 5 °C for a period, ranging with the species, from 2 weeks to 12 months prior to the germination test, but care must be taken to avoid freezing. For seeds where a long period of prechilling is required and a germination test cannot be completed within two months, quick viability tests are recommended (e.g. tetrazolium test: see Chapter 6; excised embryo test: see Chapter 12). For some tree and shrub species with a varying degree of dormancy, duplicate tests with and without prechilling are prescribed ('double tests'), as indicated in Table 5A Part 2, which should if possible be set to germinate at the same time.

Preheating: The non-imbibed seeds of the replicates for germination are heated at a temperature of 30 to 35 °C with free air circulation for a period of up to 7 days before they are placed under the prescribed germination conditions. In some cases it may be necessary to extend the preheating period.

For certain tropical and subtropical species, preheating temperatures of 40 to 50 °C may be used (e.g. *Arachis hypogaea*: 40 °C; *Oryza sativa*: 50 °C).

Prestorage: For some temperate herbage grass species, the seed submitted for testing is stored at a temperature of 15 to 25 °C with free air circulation before they are tested. A prestorage period of up to one year can be used.

Light: The tests should be illuminated during at least 8 h in every 24 h cycle and during the high temperature period when the seeds are germinated at alternating temperatures. The quality and intensity of light may be important. The light intensity should be between 750 and 1250 lux from cool white lamps. Illumination is recommended especially for certain tropical and subtropical grasses (e.g. *Chloris gayana, Cynodon dactylon*).

Sealed polyethylene envelopes: Where a high proportion of fresh ungerminated seeds is found at the end of the standard test (e.g. in *Trifolium* spp.), retesting in a sealed polyethylene envelope, of just sufficient size to hold the test satisfactorily, will usually induce these seeds to germinate.

Gibberellic acid (GA_3): The GA_3 treatment is recommended mainly for *Avena sativa, Hordeum vulgare, Secale cereale, ×Triticosecale, Triticum aestivum* and *Valerianella locusta*. The germination substrate is moistened with 0.05 % solution of GA_3, prepared by dissolving 500 mg GA_3 in 1 litre of water. When dormancy is weaker, 0.02 % may be enough; when it is stronger, up to 0.1 % may be used routinely. If it is necessary to use concentrations higher than 0.1 %, care must be taken to ensure that the development of seedlings is not adversely affected. When a concentration higher than 0.08 % is required, dissolving the GA_3 in a phosphate buffer solution is recommended. The buffer solution is prepared by dissolving 1.7799 g of $Na_2HPO_4 \cdot 2H_2O$ and 1.3799 g of $NaH_2PO_4 \cdot H_2O$ in 1 L of distilled water.

Potassium nitrate (KNO_3): Instead of water, 0.2 % KNO_3 solution, prepared by dissolving 2 g KNO_3 in 1 L of water, is used to saturate the germination substrate at the beginning of the test. Water is used for moistening thereafter.

Acid scarification: The seeds are soaked in concentrated sulphuric acid (H_2SO_4) until the seed coat becomes pitted. Digestion may be rapid, or take more than one hour, but the seeds should be examined every few minutes. After digestion, seeds must be thoroughly washed in running water before the germination test is commenced (e.g. *Brachiaria* spp.). In the case of *Oryza sativa*, scarification may be performed by soaking the seed in 1 M nitric acid (HNO_3) for 24 h (after preheating at 50 ±2 °C).

Mechanical scarification: The seed is cut, pierced, filed or sandpapered to improve permeability to moisture and gasses. Care must be taken to scarify the seed coat at a suitable place in order to avoid damaging the embryo and the resulting seedling. The best places are either immediately above the tips of the cotyledons or to the sides of the cotyledons.

5.6.3.2 Procedures for removing hardseededness

For many species where hard seeds occur, no attempt is made to germinate them and the percentage found is reported. Where a fuller assessment is required on the request of the customer, some special procedure for removing hardseededness is essential. This procedure may be applied prior to the commencement of the germination test, or, if it is suspected that the procedure may adversely affect non-hard seeds, it should be carried out on the hard seeds remaining after the prescribed test period.

Soaking: Seeds with hard seed coats may germinate more readily after soaking for up to 24–48 h in water, or, for *Acacia* spp., after plunging seeds in about three times their volume of near boiling water until it cools. The germination test is commenced immediately after soaking.

Mechanical scarification: Careful piercing, chipping, filing or sandpapering of the seed coat may be sufficient (see 5.6.3.1).

Acid scarification: This procedure is effective with some species (e.g. *Desmodium* spp., *Macroptilium* spp., *Stylosanthes guianensis*) (see 5.6.3.1).

5.6.3.3 Procedures for removing inhibitory substances

Prewashing: Naturally occurring substances in the pericarp or seed coat which act as inhibitors of germination may be removed by washing the seeds in running water at a temperature of 25 ±2 °C before the germination test is made. After washing, the seeds must be dried at a temperature of 20 to 25 °C (e.g. *Beta vulgaris*). Pelleted seed must not be prewashed.

Removal of outer structures: Germination of certain species is promoted by removing outer structures such as involucre of bristles or lemma and palea of certain *Poaceae*.

5.6.3.4 Disinfection of the seed

For samples of *Arachis hypogaea* and *Beta vulgaris* only, a fungicide treatment may be applied by the laboratory before planting the seed for germination, when the seed lot is known not to have received such a treatment. Results are reported under 'Germination' in the spaces provided.

For samples of other species, laboratory-applied fungicide treatments are not covered by the ISTA Rules (see 1.5.2.22). Germination test results for other species treated with laboratory-applied fungicide must be reported under 'Other determinations' and followed by: 'This method is not covered by the *International Rules for Seed Testing*'. In addition, a test without applying a laboratory fungicide treatment must also be conducted and the results reported under 'Germination' in the spaces provided.

When a fungicide pretreatment is used, the name of the chemical, the percentage of active ingredients and the method of treatment must be reported on the ISTA Certificate under 'Other determinations'.

5.6.4 Duration of the test

The duration of the test for individual species is indicated in Table 5A. The duration of the treatment required to break dormancy (5.6.3) before or during the test is not taken as part of the germination test period.

If it seems advisable, when for example some seeds have just started to germinate, the prescribed test period may be extended:

a) by 7 days;
b) by up to half the prescribed period;
c) up to 21 days for *Lolium* spp.;
d) up to 32 days for *Festuca* spp. (except *F. arundinacea* and *F. pratensis*);
e) up to 42 days for *Poa* spp. (except *P. bulbosa*);
f) up to 54 days for *Poa bulbosa*.

If, on the other hand, the maximum germination of the sample has been obtained before the end of the prescribed test period, a test may be terminated. At the request of the applicant the germination test may be terminated when the sample reaches a predetermined germination percentage.

The time of the first count is approximate but must be sufficient to permit the seedlings to reach a stage of development which allows for accurate evaluation. The times indicated in Table 5A refer to the highest temperatures. If a lower temperature is chosen, the first count may have to be postponed. For tests in sand, organic growing media or soil lasting not more than 14 days, the first count may be omitted. Intermediate counts to remove seedlings which are sufficiently well developed are recommended in order to make counting easier and to prevent them from affecting the development of other seedlings. Number and date of intermediate counts may be left to the discretion of the analyst, but should be kept at a minimum to reduce the risk of damaging any seedlings which are not sufficiently developed. When samples are tested on paper, ungerminated seed and seedlings requiring additional time to

reach the stage of development that allows for accurate evaluation, can be transferred to fresh substrate at intermediate counts. In doing so, care must be taken to ensure the integrity of replicates and to avoid any damage to the transferred seeds and seedlings.

5.6.5 Evaluation

Every seedling must be evaluated in accordance with the general principles laid down in 5.2.5–5.2.8. For evaluation, the essential structures must be sufficiently developed to permit detection of any abnormality.

At the end of the germination test, the classification of ungerminated seeds must be determined as prescribed in 5.6.5.3.

5.6.5.1 Seedlings

Seedlings which have reached a stage when all essential structures can be accurately assessed must be removed from the test at the first and any other intermediate counts. Badly decayed seedlings should be removed in order to reduce the risk of secondary infection, but doubtful seedlings with other defects must be left on the substrate until the final count, unless it is obvious that they will never develop into normal seedlings, e.g. broken seedlings and white seedlings.

5.6.5.2 Multigerm seed units

When a unit produces more than one normal seedling, only one is counted for determining the germination percentage. On request, the number of normal seedlings produced by 100 units, or the number of units which have produced one, two or more than two normal seedlings, or the proportion of units producing one, two or more than two normal seedlings, may also be determined.

5.6.5.3 Ungerminated seeds

Hard seeds: At the end of a germination test, hard seeds are counted and reported as such on the ISTA Certificate.

Fresh seeds: When 5 % or more of fresh seeds are believed to be present, their potential to germinate must be determined by dissection, tetrazolium or excised embryo. Those determined to have the potential to germinate are reported as fresh. Those determined not to have the potential to germinate are reported as dead. After this determination, if there is any doubt as to whether the seed is fresh or dead, it must be classified as dead. If not already applied, measures described in 5.6.3 must be taken to break dormancy if 5 % or more of fresh ungerminated seeds are found.

Dead seeds: Obviously dead (soft, mouldy) seeds are counted and reported as such on the ISTA Certificate. If it can be seen that a seed has produced any part of a seedling (e.g. the tip of the primary root) even though decayed at the time of assessment, it is counted as an abnormal seedling and not as a dead seed.

Other categories: Upon request of the customer, the number of empty, embryoless or insect-damaged seeds may be determined and reported under 'Other Determinations' on the ISTA Certificate.

To detect these other categories of seeds, the following methods may be used:

a) Before the germination test:
   – X-ray test, which is conducted on the replicates used for the germination test;
   – cutting test, which is performed on four separate replicates of 100 seeds, soaked for up to 24 h at room temperature. Each seed is cut along its longitudinal axis and the content examined and classified as full, empty, embryoless or insect-damaged;

b) After the germination test:
   – cutting test or X-ray test of apparently fresh ungerminated seeds.

When a tetrazolium test is performed, the percentage of empty and insect-damaged seeds can also be determined during preparation and evaluation.

5.7 Retesting

The result of a test must be considered unsatisfactory and must not be reported, and a second test must be made by the same or an alternative method, under the following circumstances:

a) When dormancy is suspected (fresh ungerminated seeds), any procedure to break dormancy indicated in column 6 of Table 5A or in 5.6.3.1 may be applied in one or more additional tests. The best result achieved must be reported and the procedure must be indicated on the ISTA Certificate.

b) When the result may not be reliable because of phytotoxicity or spread of fungi or bacteria, retests must be made using one or more alternative methods as indicated in Table 5A, or in sand, organic growing media, or soil. If necessary, the distance between the seeds must be increased. The best result achieved must be reported, and the method used must be indicated on the ISTA Certificate.

c) When there is difficulty in deciding the correct evaluation of a number of seedlings, retests must be made using one or more alternative methods as prescribed in Table 5A, or in sand, organic growing media, or soil. The best result achieved must be reported and the method used must be indicated on the ISTA Certificate.

d) When there is evidence of errors in test conditions, seedling evaluation or counting, a retest must be made using the same method or an alternative method as described in Table 5A, and the result of the retest must be reported on the ISTA Certificate.

e) If a sample does not respond satisfactorily to the method selected, it will be necessary to retest it by one or more of the alternative methods. When seedlings occur which cannot be easily evaluated or show phytotoxic symptoms, a retest should be made in sand, organic growing media, or soil at the temperature prescribed in Table 5A. Planting another sample of the same cultivar, known to germinate satisfactorily, alongside, may provide a useful guide to evaluation of this retest. The best result achieved must be reported and the method used must be indicated on the ISTA Certificate.

f) When the range for the replicates exceeds the maximum tolerated range in Table 5B, a retest must be carried out using the same test method or an alternative method. If the results of the retest using the same method are compatible with the first (i.e. the difference does not exceed the tolerance indicated in either Table 5C, 5D or 5E), the average of the test results must be reported on the ISTA Certificate (see 5.8.1 Tolerances). If an alternative method is used and if the results are better and within accepted tolerances, then these results must be reported on the ISTA Certificate (see 5.8.1 Tolerances) and must not be averaged with the previous test results.
When retesting is carried out under the circumstances a), b), c) or e), the best results achieved must be indicated on the ISTA Certificate. The results of the other tests do not have to be reported on the ISTA Certificate, except on specific request by the applicant.

g) When due to counting errors more than 5 seeds are lost or found during a germination test (i.e. ±1.25 % for a total of 400 seeds), then the test must be repeated.

5.8 Calculation and expression of results

The result of the germination test is expressed as percentages by number of normal and abnormal seedlings and hard, fresh and dead seeds. The percentages are rounded to the nearest whole number. The sum of the percentages of normal and abnormal seedlings and hard, fresh and dead seeds must be 100 (see 5.8.2 Rounding results).

For multigerm seed units, only one normal seedling per unit is counted to calculate the result of the germination test. On request, the number of normal seedlings produced by 100 units; the number of units producing one, two or more than two normal seedlings; or the proportion of units producing one, two or more than two normal seedlings, may also be reported. The proportion is expressed as a percentage of the total number of units which have produced at least one normal seedling.

5.8.1 Tolerances

The result of a germination test can be relied upon only if the difference between the highest and the lowest replicates is within accepted tolerances. To check the reliability of a test result, the average percentage of the replicates is rounded to the nearest whole number and compared with Table 5B. The result is considered reliable, if the difference between the highest and the lowest replicate does not exceed the tolerance indicated. Tolerances are applied to at least the category of normal seedlings.

If the range of the replicates exceeds the maximum tolerated range in Table 5B, a retest must be made. If the second result, using the same method, is in tolerance with the first (i.e. the difference between the two test results does not exceed the tolerance indicated in Table 5C), the average of the two test results must be reported on the ISTA Certificate.

If the second result is not in tolerance with the first (i.e. the difference between the two test results exceeds the tolerance indicated in Table 5C), a third test must be made. If the three test results are in tolerance (i.e. the difference between the three test results does not exceed the tolerance indicated in Table 5D), the average of the three test results, using the same method, must be reported. If the three test results are not in tolerance (i.e. the difference between the three test results exceeds the tolerance indicated in Table 5D), the highest compatible result obtained

from comparison of the two test pairs of the three tests is reported (i.e. comparison of tests 1 and 3 and tests 2 and 3, tests 1 and 2 having already been found to be out of tolerance). If after carrying out the second retest no compatible result is obtained, a fourth test is carried out.

The average of the four test results, using the same method, must be reported if the four test results are in tolerance (i.e. the difference between the four test results does not exceed the tolerance indicated in Table 5E). If the four test results are not in tolerance (i.e. the difference between the four test results exceeds the tolerance indicated in Table 5E), the highest compatible result obtained from comparison of the three test trios of the four tests is reported (i.e. comparison of tests 1, 2 and 4; tests 1, 3 and 4; and tests 2, 3 and 4). If after carrying out the comparison of trios of tests no compatible result is obtained, the highest compatible result obtained from comparison of the three pairs of the four tests is reported (i.e. comparison of tests 1 and 4; tests 2 and 4; and tests 3 and 4). If after carrying out the comparison of these three pairs of tests no compatible result is obtained, no test result is reported, and the customer is informed that the sample appears to have unacceptable variation in germination.

Figure 5.3 illustrates, in the form of a flow chart, the retesting procedure to obtain compatible results within tolerance.

When the germination percentage is reported on the ISTA Certificate, the method used must be given. The ISTA germination test is based on 400 seeds. In cases where less than 400 seeds are tested, the number tested must be reported.

5.8.2 Rounding results

First, round the percentage of normal seedlings up or down to the nearest whole number (xx.0 and xx.25 are rounded down to xx; xx.50 and xx.75 are rounded up to xx + 1).

Add up the integer parts of the remaining percentages.

If the sum is 100, the procedure ends; otherwise, continue with the following steps:

1. Find the value with the greatest decimal part among the remaining percentages (abnormal seedlings, hard seeds, fresh seeds and dead seeds) and round this percentage to the upper whole number; keep this value as a final result.

2. Add up the integer parts of the remaining percentages.

3. If the sum is 100, the procedure ends; otherwise continue with further steps 1. and 2.

In the case of equal decimal parts, the priority order is abnormal seedlings — hard seeds — fresh seeds — dead seeds.

5.9 Reporting results

The result of a germination test must be reported in the spaces provided as follows:

- the actual duration of the test (in days, excluding the period of special treatment or method used for promoting germination);
- the percentages, calculated to the nearest whole number (5.8.2), of normal seedlings, hard seeds, fresh seeds, abnormal seedlings and dead seeds. If the result for any of these categories is found to be zero, it must be reported as '0'.
- If an applicant requests that the test be terminated when the sample reaches a predetermined germination percentage, before the final count, then only the percentage of normal seedlings is reported. The results of the other categories (abnormal seedlings, hard seeds, fresh seeds and dead seeds) must be reported as 'N', because they have not been determined.

The following additional information must be reported under 'Other determinations':

- the number of seeds tested, if less than 400 seeds;
- the germination method using the abbreviations used in Table 5A, including at least substrate and temperature;
- any special treatment or method used for promoting germination (5.6.3);
- the duration in days of any special treatment or method used for promoting germination, except in the case of prestorage;
- the germination percentage obtained within the prescribed time, if the germination period was extended beyond the period indicated in Table 5A. The statement must be entered as follows: 'After the prescribed period of … days, there were … % normal seedlings.'
- the method for evaluating fresh seeds (dissection, tetrazolium or excised embryo – see paragraph 5.6.5.3.) when 5 % or more of fresh seeds are believed to be present.
- If an applicant requests that the germination test be terminated when the sample reaches a predetermined germination percentage, the following statement: 'Upon request of the applicant, the germination test was terminated after … days. The prescribed test period is … days.'

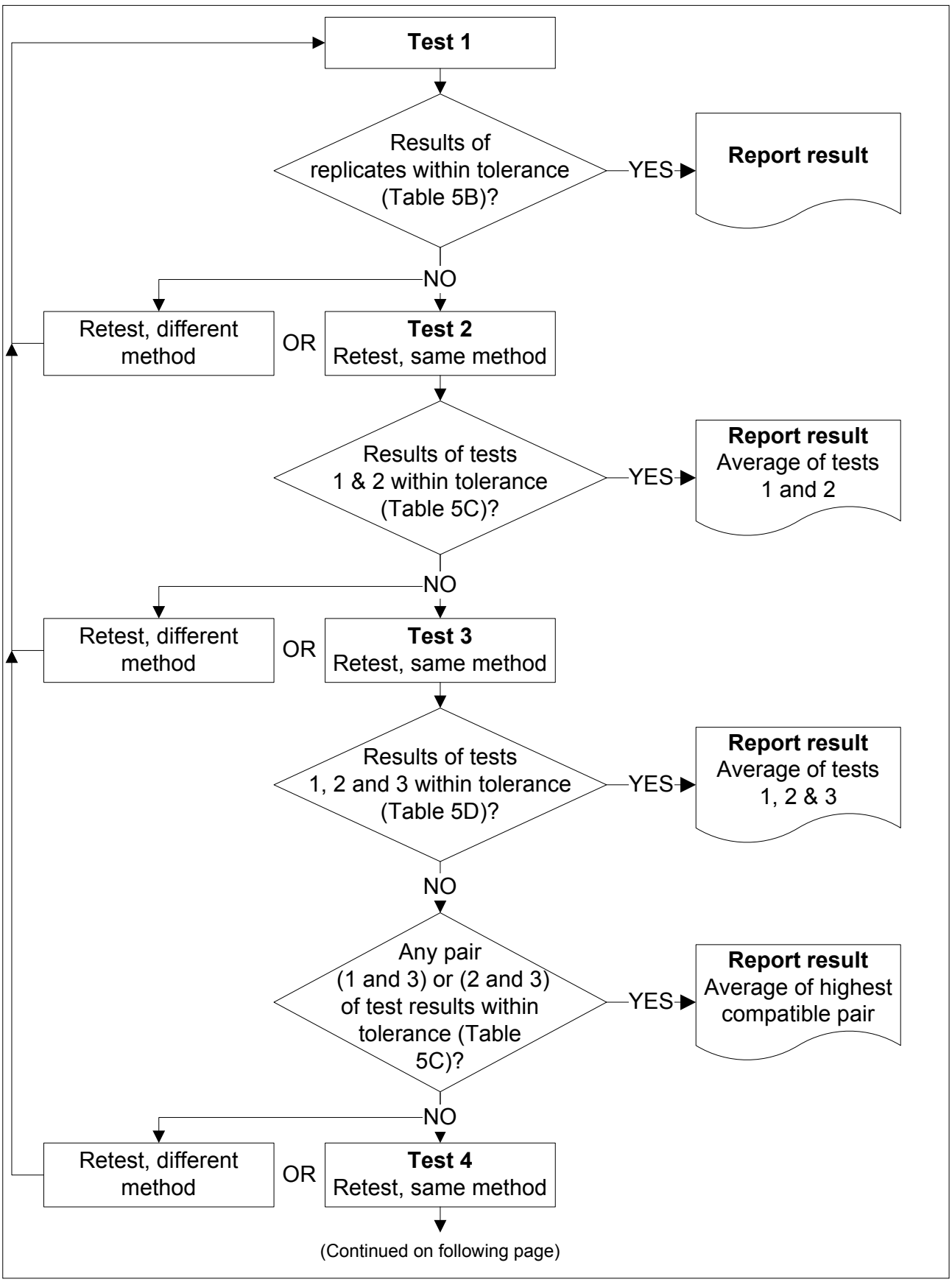

Figure 5.3. Flow chart to illustrate the retesting procedure when test replicates and repeat tests are out of tolerance.

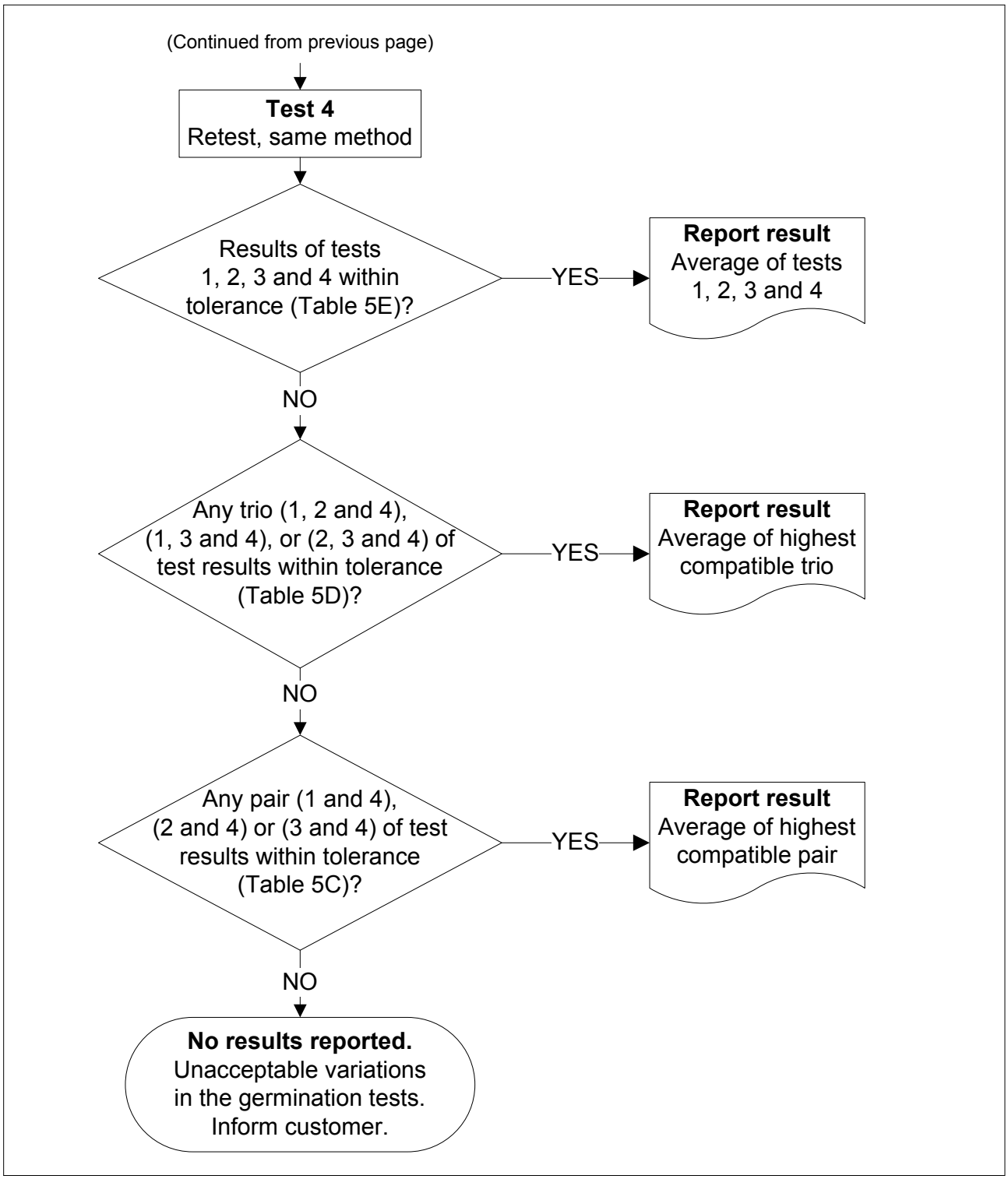

Figure 5.3. (Cont.) Flow chart to illustrate the retesting procedure when test replicates and repeat tests are out of tolerance.

When double tests are prescribed in Table 5A Part 2, the result of the first test, with treatment for breaking dormancy, is reported in the appropriate space on the ISTA Certificate, and the result of the second test, without treatment for breaking dormancy, is reported under 'Other determinations'.

Upon request, the following information may be reported as follows:

– the result of parallel tests or any additional test;
– the results of other tests made when retesting is necessary;
– the viability of ungerminated seeds and the method used to determine it;
– the categories of ungerminated seeds (as listed in 5.6.5.3) and the method used to determine them;
– in the case of multigerm seed units: the number of normal seedlings produced by 100 units, the number of units which have produced one, two or more than two normal seedlings, or the proportion of units producing one, two or more than two normal seedlings. The proportion is expressed as a percentage of the total number of units which have produced at least one normal seedling.

5.10 Germination methods

Table 5A indicates the prescribed substrates, temperatures and test durations, recommended procedures for breaking dormancy, additional directions and additional advice. Where methods are prescribed for a group of species, only those species specifically listed in Table 2A may be considered to be covered.

For certain species in Table 5A Part 2, 'double tests' (with and without prechilling) are mandatory, as indicated in column 6. Less desirable methods are placed in brackets, e.g. TTZ (or EET).

Substrates: The sequence of alternative substrates does not indicate any preference: TP; BP; TPS; S; O.
BP and TP may be replaced by PP (pleated paper).

Temperatures: The sequence of alternative temperatures is the same throughout and does not indicate any preference: alternating temperatures, highest first; constant temperatures, highest first. Alternating temperature regimes are indicated by the symbols '<=>' between temperatures; for example, 20<=>30 is an alternating temperature regime of 20 °C for 16 h and 30 °C for 8 h.

First count: The time for the first count is approximate and refers to the highest temperature alternative in paper substrates. If a lower temperature alternative is chosen or when the test is made in sand, the first count may have to be delayed. For tests in sand with a final count after 7–10 (14) days the first count may be omitted altogether.

Light: Illumination of the tests is generally recommended for better developed seedlings. If in certain cases light is required to promote germination of dormant samples, this is indicated in column 6. If light is inhibitory to germination and the substrates should be kept in darkness, this is indicated in column 7.
If tests are illuminated during an alternating temperature regime it is usually, at a minimum, for the duration of the higher of the two temperatures, i.e. for 8 h in a 20⇔30 alternating temperature regime.

Dormancy-breaking methods: Where more than one dormancy breaking method is indicated, the sequence of alternative methods does not indicate any preference, and any method or combination of methods can be used. However, if predrying or H_2SO_4 is used in combination with any other method, they must be used prior to the other methods.

Abbreviations

For further details see 5.6.2 and 5.6.3.

BP between paper
PP pleated paper
TP top of paper
TPS top of paper covered with sand

S sand
TS top of sand

O organic growing media
TO top of organic growing media

EET excised embryo test
GA_3 Use solution of gibberellic acid instead of water.
HNO_3 Soak seeds in 1 M nitric acid prior to the germination test.
H_2SO_4 Soak seeds in concentrated sulphuric acid prior to the germination test.
KNO_3 Use solution of 0.2 % potassium nitrate instead of water.
TTZ tetrazolium test

Table 5A Part 1. Detailed methods for germination tests: agricultural and vegetable seeds

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendations for breaking dormancy	Additional directions	Additional advice
1	2	3	4	5	6	7	8
Abelmoschus esculentus	TP; BP; S	20<=>30	4	21	–	–	–
Achillea millefolium	TP	20<=>30	5	14	–	–	–
Aeschynomene americana	TP	20<=>35; 20<=>30	4	14	–	–	–
Agropyron cristatum	TP	20<=>30; 15<=>25	5	14	KNO_3; prechill	–	–
Agropyron desertorum	TP	20<=>30; 15<=>25	5	14	KNO_3; prechill	–	–
Agrostis canina	TP	20<=>30; 15<=>25; 10<=>30	7	21	KNO_3; prechill	–	–
Agrostis capillaris	TP	20<=>30; 15<=>25; 10<=>30	7	28	KNO_3; prechill	–	–
Agrostis gigantea	TP	20<=>30; 15<=>25; 10<=>30	5	10	KNO_3; prechill	–	–
Agrostis stolonifera	TP	20<=>30; 15<=>25; 10<=>30	7	28	KNO_3; prechill	–	–
Allium cepa	TP; BP; S	20; 15	6	12	Prechill	–	–
Allium fistulosum	TP; BP; S	20; 15	6	12	Prechill	–	–
Allium porrum	TP; BP; S	20; 15	6	14	Prechill	–	–
Allium schoenoprasum	TP; BP; S	20; 15	6	14	Prechill	–	–
Allium tuberosum	TP	20<=>30; 20	6	14	Prechill	–	–
Alopecurus pratensis	TP	20<=>30; 15<=>25; 10<=>30	7	14	KNO_3; prechill	–	–
Alysicarpus vaginalis	BP	35	4	21	Pierce seed coat of swollen seeds at 21 d and continue test until 35 d. Swollen seeds may be placed at 20 °C for 2 d, then at 35 °C for 3 d	–	–
Andropogon gayanus	TP	20<=>35	7	14	KNO_3; light	–	–
Andropogon gerardi	TP	20<=>30	7	28	KNO_3; prechill	–	–
Andropogon hallii	TP	20<=>30	7	28	KNO_3; prechill	–	–
Anethum graveolens	TP; BP	20<=>30; 10<=>30	7	21	Prechill	–	–
Anthoxanthum odoratum	TP	20<=>30	6	14	–	–	–
Anthriscus cerefolium	TP; BP	20<=>30	7	21	Prechill	–	–
Anthyllis vulneraria	TP; BP	20	5	10	Prechill	–	–
Apium graveolens	TP	20<=>30	10	21	KNO_3; prechill, light	–	–
Arachis hypogaea	BP; S	20<=>30; 25	5	10	Remove shells; preheat at 40 ±2 °C	–	–

*The symbols '<=>' indicate alternating temperature regimes. 1st temperature: 16 h; 2nd temperature: 8 h

Chapter 5: The germination test

Chapter 5: The germination test

Table 5A Part 1. Detailed methods for germination tests: agricultural and vegetable seeds (continued)

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendations for breaking dormancy	Additional directions	Additional advice
1	2	3	4	5	6	7	8
Arctium lappa	BP; TP	20⇔30; 20	14	35	Prechill	–	For deeply dormant seed TTZ advisable
Arrhenatherum elatius	TP	20⇔30	6	14	Prechill	–	–
Asparagus officinalis	TP; BP; S	20⇔30	10	28	–	–	–
Astragalus cicer	BP; TP	15⇔25; 20	10	21	–	–	–
Astrebla lappacea	TP	32	7	14	KNO_3	–	–
Atriplex hortensis	TP; BP	20⇔30	7	28	–	–	–
Atropa belladonna	TP; BP	20⇔30	10	28	Prechill	–	–
Avena nuda	BP; S	20	5	10	Preheat at 30 to 35 °C; prechill	–	–
Avena sativa	BP; S	20	5	10	Preheat at 30 to 35 °C; prechill	–	–
Avena strigosa	BP; S	20	5	10	GA_3; prechill	–	–
Axonopus compressus	TP	20⇔35	10	21	KNO_3; light	–	–
Axonopus fissifolius	TP	20⇔35	10	21	KNO_3; light	–	–
Beckmannia eruciformis	TP	20⇔30	7	21	–	–	–
Beta vulgaris	TP; BP; S	20⇔30; 15⇔25; 20	4	14	Prewash (multigerm: 2 h; genetic monogerm: 4 h). Dry at max. 25 °C	–	–
Borago officinalis	TP; BP	20⇔30; 20	5	14	–	–	–
Bothriochloa insculpta	TP	20⇔35	3	21	KNO_3; light	–	–
Bothriochloa pertusa	TP	20⇔35	3	21	KNO_3; light	–	–
Bouteloua gracilis	TP	20⇔30; 15⇔30	7	28	KNO_3	–	–
Brachiaria brizantha	TP	20⇔35	7	21	Preheat; KNO_3	–	–
Brachiaria decumbens	TP	20⇔35	7	21	H_2SO_4; KNO_3; light	–	–
Brachiaria humidicola	TP	20⇔35	7	21	KNO_3	–	–
Brachiaria mutica	TP	20⇔35	7	21	H_2SO_4; KNO_3	–	–
Brachiaria ramosa	BP	20⇔30	4	14	Preheat; KNO_3	–	–
Brachiaria ruziziensis	TP	20⇔35	7	21	H_2SO_4; KNO_3	–	–
Brassica carinata	BP	20; 20⇔30	5	7	–	–	–
Brassica juncea	TP	20⇔30; 20	5	7	KNO_3; prechill	–	–
Brassica napus	BP; TP	20⇔30; 20	5	7	KNO_3; prechill	–	–
Brassica napus var. napobrassica	BP; TP	20⇔30; 20	5	14	Prechill	–	–
Brassica nigra	BP; TP	20⇔30; 20	5	10	KNO_3; prechill	–	–
Brassica oleracea	BP; TP	20⇔30; 20	5	10	KNO_3; prechill	–	–
Brassica perviridis	BP; TP	20⇔30; 20	5	7	Prechill	–	–

*The symbols '<=>' indicate alternating temperature regimes. 1st temperature: 16 h; 2nd temperature: 8 h

Table 5A Part 1. Detailed methods for germination tests: agricultural and vegetable seeds (continued)

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendations for breaking dormancy	Additional directions	Additional advice
1	2	3	4	5	6	7	8
Brassica rapa	BP; TP	20⇐⇒30; 20	5	7	KNO_3; prechill	–	–
Bromus arvensis	TP	20⇐⇒30; 15⇐⇒25	7	21	KNO_3; prechill	–	–
Bromus carinatus	TP	20⇐⇒30; 15⇐⇒25; 10⇐⇒30	7	14	KNO_3; prechill	–	–
Bromus catharticus	TP	20⇐⇒30	7	28	KNO_3; prechill	–	–
Bromus erectus	TP	20⇐⇒30; 15⇐⇒25	7	14	KNO_3; prechill	–	–
Bromus hordeaceus	TP	20⇐⇒30	7	14	Prechill	–	–
Bromus inermis	TP	20⇐⇒30; 15⇐⇒25	7	14	KNO_3; prechill	–	–
Bromus marginatus	TP	20⇐⇒30; 15⇐⇒25	7	14	KNO_3; prechill	–	–
Bromus riparius	TP	20⇐⇒30; 15⇐⇒25	7	14	KNO_3; prechill	–	–
Bromus sitchensis	TP	20⇐⇒30; 15⇐⇒25	7	21	Prechill	–	–
Cajanus cajan	BP; S	20⇐⇒30; 25	4	10	–	–	–
Calopogonium mucunoides	TP	25; 20	3	10	–	–	–
Camelina sativa	TP	20⇐⇒30	4	10	–	–	–
Cannabis sativa	TP; BP	20⇐⇒30; 20	3	7	–	–	–
Capsicum spp.	TP; BP; S	20⇐⇒30	7	14	KNO_3	–	–
Carthamus tinctorius	TP; BP; S	20⇐⇒30; 25	4	14	–	–	–
Carum carvi	TP	20⇐⇒30	7	21	–	–	–
Cenchrus ciliaris	TP; S	20⇐⇒35; 20⇐⇒30	7	28	Preheat; KNO_3; prechill	–	–
Cenchrus setiger	TP	20⇐⇒35	3	14	Preheat at 40 ±2 °C; KNO_3	–	–
Centrosema pascuorum	TP	35	3	7	–	–	–
Centrosema molle	TP	20⇐⇒35	4	10	–	–	–
Chamaecrista rotundifolia	TP	20⇐⇒30	4	14	–	–	–
Chloris gayana	TP	20⇐⇒35; 20⇐⇒30	7	14	KNO_3; prechill; light	–	Testing by weighed replicates also allowed (Chapter 13 Table 13B)
Cicer arietinum	BP; S	20⇐⇒30; 20	5	8	–	–	–
Cichorium endivia	TP	20⇐⇒30; 20	5	14	KNO_3	–	–
Cichorium intybus	TP	20⇐⇒30; 20	5	14	KNO_3	–	–
Citrullus lanatus	BP; S	20⇐⇒30; 25	5	14	–	–	PP advisable
Claytonia perfoliata	BP	10	7	21	–	–	–
Corchorus capsularis	TP; BP	30	3	5	–	–	–
Corchorus olitorius	TP; BP	30	3	5	–	–	–

*The symbols '<=>' indicate alternating temperature regimes. 1st temperature: 16 h; 2nd temperature: 8 h

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Table 5A Part 1. Detailed methods for germination tests: agricultural and vegetable seeds (continued)

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendations for breaking dormancy	Additional directions	Additional advice
1	2	3	4	5	6	7	8
Coriandrum sativum	TP; BP	20<=>30; 20	7	21	–	–	–
Crambe abyssinica	TP; BP	20<=>30; 20	4	7	KNO_3	–	–
Crotalaria brevidens	BP	20<=>30	4	10	–	–	–
Crotalaria juncea	BP; S	20<=>30	4	10	–	–	–
Crotalaria lanceolata	BP	20<=>30	4	10	–	–	–
Crotalaria pallida	BP	20<=>30	4	10	–	–	–
Crotalaria spectabilis	BP	20<=>30	4	10	–	–	–
Cucumis melo	BP; S	20<=>30; 25	4	8	–	–	PP advisable
Cucumis sativus	TP; BP; S	20<=>30; 25	4	8	–	–	PP advisable
Cucumis spp.	BP; S	20<=>30; 25	4	8	–	–	PP advisable
Cucurbita maxima	BP; S	20<=>30; 25	4	8	–	–	PP advisable
Cucurbita moschata	BP; S	20<=>30; 25	4	8	–	–	PP advisable
Cucurbita pepo	BP; S	20<=>30; 25	4	8	–	–	PP advisable
Cucurbita spp.	BP; S	20<=>30; 25	4	8	–	–	PP advisable
Cucurbita hybrids	BP; S	20<=>30; 25	4	8	–	–	PP advisable
Cuminum cyminum	TP	20<=>30	5	14	–	–	–
Cyamopsis tetragonoloba	BP	20<=>30	5	14	–	–	–
Cynara cardunculus	BP; S	15<=>20; 20	7	21	–	–	–
Cynodon dactylon	TP	20<=>35; 20<=>30	7	21	KNO_3; prechill; light	–	–
Cynosurus cristatus	TP	20<=>30	10	21	KNO_3; prechill	–	–
Dactylis glomerata	TP	20<=>30; 15<=>25	7	21	KNO_3; prechill	–	–
Daucus carota	TP; BP	20<=>30; 20	7	14	–	–	–
Deschampsia cespitosa	TP	20<=>30; 20	7	16	KNO_3; prechill	–	–
Deschampsia flexuosa	TP	20<=>30; 20	7	16	KNO_3; prechill	–	–
Desmodium intortum	TP	20<=>30	4	10	H_2SO_4	–	–
Desmodium uncinatum	TP	20<=>30	4	10	H_2SO_4	–	–
Dichanthium aristatum	TP	20<=>35	7	21	KNO_3	–	–
Dichondra micrantha	TP	20<=>30	7	21	–	–	–
Digitaria eriantha	TP	20<=>30	4	10	–	–	–
Echinochloa crus-galli	TP	20<=>30; 25	4	10	Preheat (40 ±2 °C)	–	–
Ehrharta calycina	TP	20	7	21	Prechill	–	–
Eleusine coracana	TP	20<=>30	4	8	KNO_3	–	–
Elymus trachycaulus	TP	20<=>30; 15<=>25	5	14	KNO_3; prechill	–	–
Elytrigia elongata	TP	20<=>30; 15<=>25	5	21	KNO_3; prechill	–	–

*The symbols '<=>' indicate alternating temperature regimes. 1st temperature: 16 h; 2nd temperature: 8 h

Table 5A Part 1. Detailed methods for germination tests: agricultural and vegetable seeds (continued)

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendations for breaking dormancy	Additional directions	Additional advice
1	2	3	4	5	6	7	8
Elytrigia intermedia	TP	20<=>30; 15<=>25	5	28	KNO_3; prechill	–	–
Elytrigia repens	TP	20<=>30; 15<=>25	7	21	KNO_3; prechill	–	–
Eragrostis curvula	TP	20<=>35; 15<=>30	6	10	KNO_3; prechill	–	–
Eragrostis tef	TP	20<=>30	4	10	KNO_3; prechill	–	–
Eruca sativa	TP; BP	20	4	7	–	–	–
Fagopyrum esculentum	TP; BP	20<=>30; 20	4	7	–	–	–
Festuca arundinacea	TP	20<=>30; 15<=>25	7	14	KNO_3; prechill	–	–
Festuca filiformis	TP	20<=>30; 15<=>25	7	14	KNO_3; prechill	–	–
Festuca heterophylla	TP	20<=>30; 15<=>25	7	14	KNO_3; prechill	–	–
Festuca ovina	TP	20<=>30; 15<=>25	7	14	KNO_3; prechill	–	–
Festuca pratensis	TP	20<=>30; 15<=>25	7	14	KNO_3; prechill	–	–
Festuca rubra	TP	20<=>30; 15<=>25	7	14	KNO_3; prechill	–	–
Festuca trachyphylla	TP	20<=>30; 15<=>25	7	14	KNO_3; prechill	–	–
×*Festulolium* spp.	TP	20<=>30; 15<=>25; 20	5	14	KNO_3; prechill	–	–
Foeniculum vulgare	TP; BP; TS	20<=>30	7	14	–	–	–
Fragaria spp.	TP	20<=>30; 20	7	28	–	–	–
Galega orientalis	TP; BP	20	5	14	–	–	–
Glycine max	BP; TPS; S	20<=>30; 25	5	8	–	–	–
Gossypium spp.	BP; S	20<=>30; 25	4	12	–	–	–
Hedysarum coronarium	TP; BP	20<=>30; 20	7	14	–	–	–
Helianthus annuus	BP; TPS; S; O	20<=>30; 25; 20	4	10	Preheat; prechill	–	–
Hibiscus cannabinus	BP; S	20<=>30	4	8	–	–	–
Holcus lanatus	TP	20<=>30	6	14	KNO_3; prechill	–	–
Hordeum vulgare	BP; S	20	4	7	Preheat at 30 to 35 °C; GA_3; KNO_3; prechill	–	–
Ipomoea aquatica	BP; S	30	4	10	–	–	–
Koeleria macrantha	TP	20<=>30	5	14	Prechill at 8 to 10 °C for 5 d; light	–	–
Kummerowia stipulacea	BP	20<=>35	5	14	–	–	–
Kummerowia striata	BP	20<=>35	7	14	–	–	–
Lablab purpureus	BP; S	20<=>30; 25	4	10	–	–	–
Lactuca sativa	TP; BP	20	4	7	Prechill	–	–
Lagenaria siceraria	BP; S	20<=>30	4	14	–	–	PP advisable

*The symbols '<=>' indicate alternating temperature regimes. 1st temperature: 16 h; 2nd temperature: 8 h

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Table 5A Part 1. Detailed methods for germination tests: agricultural and vegetable seeds (continued)

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendations for breaking dormancy	Additional directions	Additional advice
1	2	3	4	5	6	7	8
Lathyrus cicera	S	20	5	10	–	–	–
Lathyrus hirsutus	BP; S	20	7	14	–	–	–
Lathyrus sativus	BP; S	20	5	14	–	–	–
Lens culinaris	BP; S	20	5	10	Prechill	–	–
Lepidium sativum	TP	20<=>30; 20	4	10	Prechill	–	–
Lespedeza juncea	BP	20<=>35	7	21	–	–	–
Leucaena leucocephala	TP; BP	25	4	10	Cut seed	–	–
Linum usitatissimum	TP; BP	20<=>30; 20	3	7	Prechill	–	–
Listia bainesii	TP	20<=>30	7	21	–	–	–
Lolium ×hybridum	TP	20<=>30; 15<=>25; 20	5	10	KNO_3; prechill	–	–
Lolium multiflorum	TP	20<=>30; 15<=>25; 20	5	10	KNO_3; prechill	–	–
Lolium perenne	TP	20<=>30; 15<=>25; 20	5	10	KNO_3; prechill	–	–
Lolium rigidum	TP	20<=>30; 15<=>25	5	14	KNO_3; light; prechill at 5 to 10 °C for 7 d; if necessary prechill for 3 d and continue test at 15<=>25 °C for additional 4 d	–	–
Lotus corniculatus	TP; BP	20<=>30; 20	4	12	Prechill	–	–
Lotus tenuis	TP; BP	20<=>30; 20	4	12	Prechill	–	–
Lotus uliginosus	TP; BP	20<=>30; 20	4	12	Prechill	–	–
Luffa acutangula	BP; S	30	4	14	–	–	–
Luffa aegyptiaca	BP; S	20<=>30; 30	4	14	–	–	–
Lupinus albus	BP; S	20	5	10	Prechill	–	–
Lupinus angustifolius	BP; S	20	5	10	Prechill	–	–
Lupinus luteus	BP; S	20	10	21	Prechill	–	–
Macroptilium atropurpureum	TP	25	4	10	H_2SO_4	–	–
Macroptilium lathyroides	TP	25	4	10	H_2SO_4	–	–
Macrotyloma axillare	BP	25	4	10	H_2SO_4 or cut seed	–	–
Macrotyloma uniflorum	TP; S	20<=>30; 25	4	10	Cut seed	–	–
Medicago arabica	TP; BP	20	4	14	–	–	–
Medicago italica	TP; BP	20; 15	4	14	–	–	–
Medicago littoralis	TP	20	4	14	–	–	–
Medicago lupulina	TP; BP	20	4	10	Prechill	–	–

*The symbols '<=>' indicate alternating temperature regimes. 1st temperature: 16 h; 2nd temperature: 8 h

Table 5A Part 1. Detailed methods for germination tests: agricultural and vegetable seeds (continued)

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendations for breaking dormancy	Additional directions	Additional advice
1	2	3	4	5	6	7	8
Medicago orbicularis	TP; BP	20	4	10	Prechill	–	–
Medicago polymorpha	TP; BP	20	4	14	–	–	–
Medicago rugosa	TP; BP	20	4	14	–	–	–
Medicago sativa	TP; BP	20	4	10	Prechill	–	–
Medicago scutellata	TP; BP	20	4	14	–	–	–
Medicago truncatula	TP; BP	20	4	10	–	–	–
Melilotus albus	TP; BP	20	4	7	Prechill	–	–
Melilotus indicus	TP; BP	20	3	14	–	–	–
Melilotus officinalis	TP; BP	20	4	7	Prechill	–	–
Melinis minutiflora	TP	20<=>30	7	21	KNO_3; prechill	–	–
Momordica charantia	BP; S	20<=>30; 30	4	14	–	–	–
Mucuna pruriens	TP; S	20<=>30; 30	3	14	Cut seed	–	–
Nasturtium officinale	TP; BP	20<=>30	4	14	–	–	–
Neonotonia wightii	TP	20<=>30; 10<=>35	4	10	–	–	–
Nicotiana tabacum	TP	20<=>30	7	16	KNO_3	–	–
Ocimum basilicum	TP	20<=>30	4	14	KNO_3	–	–
Oenothera biennis	TP	20<=>30; 20	7	21	KNO_3	–	–
Onobrychis viciifolia	TP; BP; S	20<=>30; 20	4	14	Prechill	–	–
Origanum majorana	TP	20<=>30; 20	7	21	–	–	–
Origanum vulgare	TP	20<=>30; 20	7	21	–	–	–
Ornithopus compressus	TP	15	7	21	–	–	–
Ornithopus sativus	TP; BP	20	7	14	–	–	–
Oryza sativa	TP; BP; S	20<=>30; 25	5	14	Preheat (50 ±2 °C); soak in water or HNO_3 for 24 h	–	–
Panicum antidotale	TP	20<=>30	7	28	–	–	–
Panicum coloratum	TP	20<=>35	7	28	–	–	–
Panicum maximum	TP	20<=>30; 15<=>35	10	28	KNO_3; prechill	–	–
Panicum miliaceum	TP; BP	20<=>30; 25	3	7	–	–	–
Panicum virgatum	TP	15<=>30	7	28	KNO_3; prechill	–	–
Papaver somniferum	TP	20	5	10	Prechill	–	–
Pascopyrum smithii	TP	20<=>30; 15<=>25	7	28	KNO_3; prechill	–	–
Paspalum dilatatum	TP	20<=>35	7	28	KNO_3; light	–	–
Paspalum notatum	TP	20<=>35; 20<=>30	7	28	H_2SO_4; KNO_3	–	–
Paspalum plicatulum	TP	20<=>35	7	28	KNO_3; light	–	–

*The symbols '<=>' indicate alternating temperature regimes. 1st temperature: 16 h; 2nd temperature: 8 h

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Table 5A Part 1. Detailed methods for germination tests: agricultural and vegetable seeds (continued)

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendations for breaking dormancy	Additional directions	Additional advice
1	2	3	4	5	6	7	8
Paspalum scrobiculatum	TP	20⇐⇒30	7	20	KNO_3	–	–
Paspalum urvillei	TP	20⇐⇒35	7	21	KNO_3	–	–
Paspalum virgatum	TP	20⇐⇒35	7	28	KNO_3	–	–
Pastinaca sativa	TP; BP	20⇐⇒30	6	28	–	–	–
Pennisetum clandestinum	TP	20⇐⇒35; 20⇐⇒30	7	14	KNO_3; prechill	–	–
Pennisetum glaucum	TP; BP	20⇐⇒35; 20⇐⇒30	3	7	–	–	–
Petroselinum crispum	TP; BP	20⇐⇒30; 20	10	28	–	–	–
Phacelia tanacetifolia	TP; BP	20⇐⇒30; 20; 15	5	14	Prechill	No light	–
Phalaris aquatica	TP	20⇐⇒30; 20	7	21	KNO_3; prechill	–	–
Phalaris arundinacea	TP	20⇐⇒30	7	21	KNO_3; prechill	–	–
Phalaris canariensis	TP; BP	20⇐⇒30; 15⇐⇒25	7	21	KNO_3; prechill	–	–
Phaseolus coccineus	BP; S	20⇐⇒30; 20	5	9	–	–	–
Phaseolus lunatus	BP; S	20⇐⇒30; 25	5	9	–	–	–
Phaseolus vulgaris	BP; TPS; S	20⇐⇒30; 25; 20	5	9	–	–	–
Phleum nodosum	TP	20⇐⇒30; 15⇐⇒25	7	10	KNO_3; prechill	–	–
Phleum pratense	TP	20⇐⇒30; 15⇐⇒25	7	10	KNO_3; prechill	–	–
Physalis pubescens	TP	20⇐⇒30	7	28	KNO_3	–	–
Pimpinella anisum	TP; BP	20⇐⇒30	7	21	–	–	–
Piptatherum miliaceum	S	15	7	42	Prechill	–	–
Pisum sativum	BP; TPS; S	20	5	8	–	–	–
Plantago lanceolata	TP; BP	20⇐⇒30; 20	4–7	21	–	–	–
Poa annua	TP	20⇐⇒30; 15⇐⇒25	7	21	KNO_3; prechill	–	–
Poa bulbosa	TP	15⇐⇒25	10	35	KNO_3	–	–
Poa compressa	TP	15⇐⇒25; 10⇐⇒30	10	28	KNO_3; prechill	–	–
Poa nemoralis	TP	20⇐⇒30; 15⇐⇒25; 10⇐⇒30	10	21	KNO_3; prechill	–	–
Poa palustris	TP	20⇐⇒30; 15⇐⇒25; 10⇐⇒30	10	21	KNO_3; prechill	–	–
Poa pratensis	TP	20⇐⇒30; 15⇐⇒25; 10⇐⇒30	10	21	KNO_3; prechill	–	–
Poa secunda	TP	20⇐⇒30; 15⇐⇒25; 10⇐⇒30	7	28	KNO_3; prechill	–	–
Poa trivialis	TP	20⇐⇒30; 15⇐⇒25	7	21	KNO_3; prechill	–	–

*The symbols '<=>' indicate alternating temperature regimes. 1st temperature: 16 h; 2nd temperature: 8 h

Table 5A Part 1. Detailed methods for germination tests: agricultural and vegetable seeds (continued)

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendations for breaking dormancy	Additional directions	Additional advice
1	2	3	4	5	6	7	8
Portulaca oleracea	TP; BP	20<=>30	5	14	Prechill	–	–
Psathyrostachys juncea	TP	20<=>30	5	14	Prechill	–	–
Pseudoroegneria spicata	TP	20<=>30; 15<=>25	7	21	KNO_3; prechill	–	–
Psophocarpus tetragonolobus	BP; S	20<=>30; 30	4	14	–	–	–
Pueraria lobata	BP	20<=>30	5	14	–	–	–
Pueraria phaseoloides	TP	25	4	10	H_2SO_4	–	–
Raphanus sativus	TP; BP; S	20<=>30; 20	4	10	Prechill	–	–
Rheum rhaponticum	TP	20<=>30	7	21	–	–	–
Ricinus communis	BP; S	20<=>30	7	14	–	–	–
Rosmarinus officinalis	TP	20<=>30; 20	7	28	–	–	–
Rumex acetosa	TP	20<=>30	3	14	Prechill	–	–
Sanguisorba minor	TP; BP	20<=>30; 20	7	28	–	–	–
Satureja hortensis	TP	20<=>30	5	21	–	–	–
Schizachyrium scoparium	TP	20<=>30	7	28	KNO_3; prechill	–	–
Scorzonera hispanica	TP; BP; S	20<=>30; 20	4	8	Prechill	–	–
Secale cereale	TP; BP; S	20	4	7	GA_3; prechill	–	–
Securigera varia	TP; BP	20	7	14	–	–	–
Sesamum indicum	TP	20<=>30	3	6	–	–	–
Setaria italica	TP; BP	20<=>30	4	10	–	–	–
Setaria sphacelata	TP	20<=>35	7	21	KNO_3	–	–
Sinapis alba	BP; TP	20<=>30; 20	3	7	Prechill	–	–
Solanum lycopersicum	TP; BP; S	20<=>30	5	14	KNO_3	–	–
Solanum (sect. *Lycopersicon*) spp.	TP; BP; S	20<=>30	5	14	KNO_3	–	–
Solanum (sect. *Lycopersicon*) hybrids	TP; BP; S	20<=>30	5	14	KNO_3	–	–
Solanum melongena	TP; BP; S	20<=>30	7	14	–	–	–
Solanum nigrum	TP	20<=>30	7	14	–	–	–
Solanum tuberosum	TP	20<=>30	3	14	Imbibe in 1.5 % GA_3 for 24 h	–	–
Sorghastrum nutans	TP	20<=>30	7	28	KNO_3; prechill	–	–
Sorghum ×almum	TP; BP	20<=>35; 20<=>30	5	21	Prechill	–	–
Sorghum bicolor	TP; BP	20<=>30; 25	4	10	Prechill	–	–
Sorghum bicolor × *S. sudanense*	TP; BP	20<=>30	4	10	Prechill	–	–
Sorghum halepense	TP; BP	20<=>35; 20<=>30	7	35	–	–	–

*The symbols '<=>' indicate alternating temperature regimes. 1st temperature: 16 h; 2nd temperature: 8 h

Chapter 5: The germination test

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Table 5A Part 1. Detailed methods for germination tests: agricultural and vegetable seeds (continued)

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendations for breaking dormancy	Additional directions	Additional advice
1	2	3	4	5	6	7	8
Sorghum sudanense	TP; BP	20<=>30	4	10	Prechill	–	–
Spergula arvensis	TP	20	4	10	–	–	–
Spinacia oleracea	TP; BP	15; 10	7	21	Prechill	–	–
Stylosanthes guianensis	TP	20<=>35; 20<=>30	4	10	H_2SO_4	–	–
Stylosanthes hamata	TP	20<=>35; 10<=>35	4	10	Cut seed	–	–
Stylosanthes humilis	TP	20<=>30; 10<=>35	2	5	Cut seed	–	–
Stylosanthes scabra	TP	20<=>35	4	10	Cut seed	–	–
Taraxacum officinale	TP	20<=>30; 20	7	21	–	–	–
Tetragonia tetragonoides	BP; S	20<=>30; 20	7	35	Remove pulp; soak in water for 24 h	–	–
Thymus vulgaris	TP	20<=>30; 20	7	21	–	–	–
Tragopogon porrifolius	TP; BP	20	5	10	Prechill	–	–
Trifolium alexandrinum	TP; BP	20	3	7	–	–	–
Trifolium campestre	TP; BP	20	4	14	–	–	–
Trifolium dubium	TP; BP	20	5	14	Prechill	–	–
Trifolium fragiferum	TP; BP	20	3	7	–	–	–
Trifolium glomeratum	TP; BP	20	4	10	–	–	–
Trifolium hirtum	TP; BP	20	4	10	–	–	–
Trifolium hybridum	TP; BP	20	4	10	Sealed polythene envelope; prechill	–	–
Trifolium incarnatum	TP; BP	20	4	7	Sealed polythene envelope; prechill	–	–
Trifolium lappaceum	TP; BP	20	3	7	Prechill	–	–
Trifolium michelianum	TP	20; 15	4	10	Prechill	–	–
Trifolium pratense	TP; BP	20	4	10	Prechill	–	–
Trifolium repens	TP; BP	20	4	10	Sealed polythene envelope; prechill	–	–
Trifolium resupinatum	TP; BP	20	4	7	–	–	–
Trifolium semipilosum	BP; S	20; 15	3	7	–	–	–
Trifolium squarrosum	TP; BP	20; 15	4	14	Prechill	–	–
Trifolium subterraneum	TP; BP	20; 15	4	14	–	No light	–
Trifolium vesiculosum	TP; BP	20; 15	4	10	–	–	–
Trigonella foenum-graecum	TP; BP	20<=>30; 20	5	14	–	–	–
Trisetum flavescens	TP	20<=>30	7	21	KNO_3; prechill	–	–
×*Triticosecale*	TP; BP; S	20	4	8	Preheat at 30 to 35 °C; GA_3; prechill	–	–
Triticum aestivum	TP; BP; S	20	4	8	Preheat at 30 to 35 °C; GA_3; prechill	–	–
Triticum dicoccon	TP; BP; S	20	4	8	Preheat at 30 to 35 °C; GA_3; prechill	–	–
Triticum durum	TP; BP; S	20	4	8	Preheat at 30 to 35 °C; GA_3; prechill	–	–

*The symbols '<=>' indicate alternating temperature regimes. 1st temperature: 16 h; 2nd temperature: 8 h

Table 5A Part 1. Detailed methods for germination tests: agricultural and vegetable seeds (continued)

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendations for breaking dormancy	Additional directions	Additional advice
1	2	3	4	5	6	7	8
Triticum spelta	BP; S	20	4	8	Preheat at 30 to 35 °C; GA_3; prechill	–	–
Urochloa mosambicensis	TP	20<=>35	7	21	GA_3; KNO_3	–	–
Valerianella locusta	TP; BP	20; 15	7	28	GA_3; prechill	–	–
Vicia benghalensis	BP	20	5	10	–	–	–
Vicia ervilia	BP; S	20	5	8	–	–	–
Vicia faba	BP; S; O	20	4	14	Prechill	–	–
Vicia narbonensis	BP; S	20	5	10	–	–	–
Vicia pannonica	BP; S	20	5	10	Prechill	–	–
Vicia sativa	BP; S	20	5	14	Prechill	–	–
Vicia villosa	BP; S	20	5	14	Prechill	–	–
Vigna angularis	BP; S	20<=>30	4	10	–	–	–
Vigna marina	BP	20<=>30	4	8	–	–	–
Vigna mungo	BP; S	20<=>30; 25; 20	4	7	–	–	–
Vigna radiata	BP;S	20<=>30; 25	5	7	–	–	–
Vigna subterranea	BP; S	20<=>30; 30; 25	5	10	–	–	–
Vigna unguiculata	BP; S	20<=>30; 25	5	8	–	–	–
Zea mays	BP; TPS; S	20<=>30; 25; 20	4	7	–	–	–
Zoysia japonica	TP	20<=>35	10	28	KNO_3	–	–

*The symbols '<=>' indicate alternating temperature regimes. 1st temperature: 16 h; 2nd temperature: 8 h

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Table 5A Part 2. Detailed methods for germination tests: Tree and shrub seeds

For certain species, 'double tests' (with and without prechilling) are mandatory (see column 7). Methods and procedures in brackets are less desirable.

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendation for breaking dormancy	Additional directions	Additional advice
1	2	3	4	5	6	7	8
Abies alba, *Abies balsamea,* *Abies cilicica,* *Abies firma,* *Abies fraseri,* *Abies homolepis,* *Abies lasiocarpa,* *Abies magnifica,* *Abies numidica,* *Abies sachalinensis*	TP	20⇔30	7	28	Prechill 21 d	–	–
Abies amabilis, *Abies cephalonica,* *Abies concolor,* *Abies grandis,* *Abies nordmanniana,* *Abies pinsapo,* *Abies procera,* *Abies veitchii*	TP	20⇔30	7	28	–	Double test: no prechill and prechill 21 d	–
Acacia spp.	TP	20⇔30; (20)	7	21	1. Pierce seed; or chip or file off fragment of testa at cotyledon end; then soak for 3 h 2. (Soak seed for 1 h in concentrated H_2SO_4, then wash seed thoroughly in running water)	–	–
Acer campestre	–	–	–	–	–	Use TTZ	–
Acer negundo, *Acer platanoides,* *Acer pseudoplatanus,* *Acer saccharum*	S; TP	20	7	21	Prechill 2 months. It is advantageous to remove pericarp before testing. Newly harvested undried seeds are usually more dormant than dried and/or stored	–	–
Acer palmatum	S; TP	20	7	21	Prechill 4 months. It is advantageous to remove pericarp before testing	–	TTZ (or EET) advisable

*The symbols '⇔' indicate alternating temperature regimes. 1st temperature: 16 h; 2nd temperature: 8 h

Table 5A Part 2. Detailed methods for germination tests: Tree and shrub seeds (continued)

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendation for breaking dormancy	Additional directions	Additional advice
1	2	3	4	5	6	7	8
Acer rubrum, *Acer saccharinum*	S; (TP)	20	7	21	–	–	–
Aesculus hippocastanum	TS; (S)	20⇔30; (20)	7	21	Soak in water for 48 h; cut off scar end of seed. Do not remove testa from sown portion. Fresh nuts may require prechill	–	–
Ailanthus altissima	TP	20⇔30	7	21	Removal of pericarp after soaking for 24 h in water may speed up germination	–	–
Alnus cordata, *Alnus glutinosa*, *Alnus incana*, *Alnus rubra*	TP	20⇔30	7	21	–	–	–
Amorpha fruticosa	TP	20⇔30	10	28	Light	–	–
Berberis aquifolium	–	–	–	–	–	Use TTZ	–
Betula papyrifera	TP	20⇔30	7	21	–	–	–
Betula pendula	TP	20⇔30	7	21	–	Double test: no prechill and prechill 21 d.	Testing by weighed replicates also allowed (Chapter 13)
Betula pubescens	TP	20⇔30	7	21	–	–	Testing by weighed replicates also allowed (Chapter 13)
Calocedrus decurrens	TP	20⇔30	7	28	Prechill 28 d	–	For deeply dormant seed TTZ (or EET) advisable
Caragana arborescens	TP	20⇔30	7	21	Pierce seed; or chip or file off fragment of testa from cotyledon end; then soak in water for 3 h	–	–
Carica papaya	S	20⇔30	12	28	Soak in water for 16 h; soak in GA_3 0.05 % for 16 h	–	–
Carpinus betulus	S	20	14	42	Incubate in moist substrate for 1 month at 20 °C followed by 4 months at 1 to 5 °C	–	TTZ advisable

*The symbols '⇔' indicate alternating temperature regimes. 1st temperature: 16 h; 2nd temperature: 8 h

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Chapter 5: The germination test

Table 5A Part 2. Detailed methods for germination tests: Tree and shrub seeds (continued)

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendation for breaking dormancy	Additional directions	Additional advice
1	2	3	4	5	6	7	8
Castanea sativa	TS; (S)	20⇔30	7	21	Soak in water for 48 h; cut off at scar end and remove testa	–	–
Catalpa spp.	TP	20⇔30	7	21	–	–	–
Cedrela spp.	TP	20⇔30	7	28	–	–	–
Cedrus atlantica, Cedrus deodora, Cedrus libani	TP	20; (20⇔30)	7	21	Prechill 21 d	–	–
Chamaecyparis lawsoniana	TP	20; (20⇔30)	7	28	–	–	–
Chamaecyparis nootkatensis	TP	20; (20⇔30)	7	28	Prechill 21 d	–	–
Chamaecyparis obtusa	TP	20⇔30	7	21	–	–	–
Chamaecyparis pisifera	TP	20⇔30	7	21	–	–	–
Chamaecyparis thyoides	TP	20	7	28	Prechill 90 d	–	TTZ advisable
Cornus sanguinea	–	–	–	–	–	Use TTZ	–
Corylus avellana	S	20; 20⇔30	14	35	Remove pericarp and prechill 2 months	–	TTZ advisable
Corymbia spp.	–	–	–	–	–	All *Corymbia* spp. tested by weighed replicates method (see Ch. 13, Table 13A)	–
Cotoneaster spp.	–	–	–	–	–	Use TTZ	–
Crataegus monogyna	S	20⇔30	7	28	Incubate in moist substrate 3 months at 25 °C followed by 9 months prechill	–	TTZ advisable
Cryptomeria japonica	TP	20⇔30	7	28	–	–	–
Cupressus arizonica	TP	20⇔30	7	28	–	Double test: no prechill and prechill 21 d	–
Cupressus macrocarpa	TP	20⇔30	14	35	–	–	–
Cupressus sempervirens	TP	20	7	28	–	–	–
Cydonia oblonga	–	–	–	–	–	Use TTZ	–

*The symbols '⇔' indicate alternating temperature regimes. 1st temperature: 16 h; 2nd temperature: 8 h

Table 5A Part 2. Detailed methods for germination tests: Tree and shrub seeds (continued)

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendation for breaking dormancy	Additional directions	Additional advice
1	2	3	4	5	6	7	8
Cytisus scoparius	TP	20⇔30	7	28	Pierce seed; or chip or file off fragment of testa at cotyledon end; then soak in water for 3 h	–	–
Elaeagnus angustifolia	–	–	–	–	–	Use TTZ	–
Eucalyptus spp.	–	–	–	–	–	All *Eucalyptus* spp. tested by weighed replicates method (see Ch. 13, Table 13A)	–
Euonymus europaeus	TP	20⇔30	7	28	Prechill 45 d	–	TTZ advisable
Fagus sylvatica	TP	5	–	–	Duration of the test depends on dormancy, and in an extreme case could require about 24 weeks	–	For deeply dormant seed TTZ advisable
Fraxinus spp.	TP	20⇔30	14	56	Pretreat seed 2 months at 20 °C followed by 7 months at 1 to 5 °C	–	TTZ (or EET) advisable
Ginkgo biloba	TP; BP	20⇔30; 20	10	30	Remove seed coat	–	–
Gleditsia triacanthos	TP	20	7	21	1. Pierce seed; or chip or file off fragment of testa at cotyledon end; then soak in water for 6 h 2. (Soak whole seed in concentrated H_2SO_4 for as long as necessary to pit surface of testa, then wash thoroughly in running water)	–	–
Ilex aquifolium	–	–	–	–	–	Use TTZ	–
Juniperus communis	TP; S	20	14	28	Prechill 90 d	–	TTZ advisable
Juniperus scopulorum	TP; S	15	14	42	Pretreat 60 d at 20 °C followed by 40 d at 1 to 5 °C	–	TTZ advisable
Juniperus virginiana	TP; S	15	14	28	Pretreat 60 d at 20 °C followed by 45 d at 1 to 5 °C	–	TTZ advisable
Koelreuteria paniculata	–	–	–	–	–	Use TTZ	–

*The symbols '⇔' indicate alternating temperature regimes. 1st temperature: 16 h; 2nd temperature: 8 h

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Chapter 5: The germination test

Table 5A Part 2. Detailed methods for germination tests: Tree and shrub seeds (continued)

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendation for breaking dormancy	Additional directions	Additional advice
1	2	3	4	5	6	7	8
Laburnum alpinum, *Laburnum anagyroides*	TP	20⇔30	7	21	1. Pierce seed; or chip or file off fragment of testa at cotyledon end; then soak in water for 3 h 2. (Soak whole seed in concentrated H_2SO_4 for as long as necessary to pit surface of testa, then wash thoroughly in running water)	–	–
Larix decidua, *Larix ×eurolepis*, *Larix gmelinii*, *Larix laricina*, *Larix sibirica*	TP	20⇔30	7	21	–	–	–
Larix kaempferi, *Larix occidentalis*	TP	20⇔30	7	21	–	Double test: no prechill and prechill 21 d	–
Ligustrum vulgare	–	–	–	–	–	Use TTZ	–
Liquidambar styraciflua	TP	20⇔30	7	21	Sensitive to drying in test	–	–
Liriodendron tulipifera	TP	20⇔30	7	28	Prechill 60 d	–	TTZ advisable
Malus spp. (*except M. sylvestris, M. sargentii*)	–	–	–	–	–	Use TTZ (or EET)	–
Malus sargentii, *Malus sylvestris*	–	–	–	–	–	Use TTZ	–
Malva sylvestris	TP	20⇔30; 20	7	21	–	–	–
Morus spp.	TP	20⇔30	14	28	–	–	–
Nothofagus obliqua	TP	20⇔30	7	28	–	Double test: no prechill and prechill 28 d	–
Nothofagus procera	TP	20⇔30	7	28	–	–	–

*The symbols '⇔' indicate alternating temperature regimes. 1st temperature: 16 h; 2nd temperature: 8 h

Table 5A Part 2. Detailed methods for germination tests: Tree and shrub seeds (continued)

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendation for breaking dormancy	Additional directions	Additional advice
1	2	3	4	5	6	7	8
Picea abies, *Picea engelmannii*, *Picea koyamai*, *Picea mariana*, *Picea omorika*, *Picea orientalis*, *Picea polita*, *Picea pungens*, *Picea rubens*	TP	20⇔30	7	21	–	–	–
Picea glauca, *Picea glehnii*, *Picea jezoensis*, *Picea sitchensis*	TP	20⇔30	7	21	–	Double test: no prechill and prechill 21 d	–
Pinus albicaulis	TP	20⇔30	7	28	Prechill 28 d	–	–
Pinus aristata	TP	20⇔30	7	14	–	–	–
Pinus banksiana	TP	20⇔30	7	14	–	–	–
Pinus bruita	TP	20	7	28	–	–	–
Pinus canariensis	TP	20	7	28	–	–	–
Pinus caribaea	TP	20⇔30	7	21	–	–	–
Pinus cembra	S	20⇔30	7	28	Prechill 6–9 months	–	TTZ (or EET) advisable
Pinus cembroides	S	20	7	28	Prechill 21 d	–	–
Pinus clausa	TP; (TS)	20	7	21	–	–	Sensitive to excess moisture
Pinus contorta	TP	20⇔30	7	21	–	Double test: no prechill and prechill 21 d	–
Pinus coulteri	S	20⇔30	7	28	Prechill 60–90 d	–	TTZ (or EET) advisable
Pinus densiflora	TP	20⇔30	7	21	Prechill 14 d	–	–
Pinus echinata	TP	20⇔30	7	28	–	–	–
Pinus edulis	TP	20⇔30	7	28	Light for 16 h or more	–	–
Pinus elliottii	TP	20⇔30; 22	7	28	–	Double test: no prechill and prechill 14 d	–
Pinus flexilis	TP	20⇔30	7	21	Prechill 21 d	–	–

*The symbols '⇔' indicate alternating temperature regimes. 1st temperature: 16 h; 2nd temperature: 8 h

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Table 5A Part 2. Detailed methods for germination tests: Tree and shrub seeds (continued)

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendation for breaking dormancy	Additional directions	Additional advice
1	2	3	4	5	6	7	8
Pinus glabra	TP	20⇔30	7	21	Prechill 21 d	–	–
Pinus halepensis	TP	20	7	28	–	–	–
Pinus heldreichii	TP	20⇔30	7	28	Prechill 42 d	–	TTZ (or EET) advisable
Pinus jeffreyi	TP; (S)	20⇔30	7	28	Prechill 28 d	–	For deeply dormant seeds use TTZ (or EET)
Pinus kesiya	TP	20⇔30	7	21	–	–	–
Pinus koraiensis	S	20⇔30	7	28	Pretreat 2 months at 25 °C followed by 3 months at 1 to 5 °C	–	TTZ (or EET) advisable
Pinus lambertiana	TP; (S)	20⇔30	7	28	Prechill 60–90 d	–	TTZ (or EET) advisable
Pinus merkusii	TP	20⇔30	7	21	–	–	–
Pinus monticola	TP	20⇔30	7	28	Prechill 60–90 d	–	TTZ (or EET) advisable
Pinus mugo	TP	20⇔30	7	21	–	–	–
Pinus muricata	TP	20⇔30	7	21	–	–	–
Pinus nigra	TP	20⇔30	7	21	–	–	Seedlings may be sufficiently developed to finalise test at a 14-day intermediate count
Pinus oocarpa	TP	20⇔30	7	21	–	–	–
Pinus palustris	S; (TP)	20	7	21	–	–	–
Pinus parviflora	TP; (S)	20⇔30	7	28	Prechill 6–9 months		TTZ (or EET) advisable
Pinus patula	TP	20; (20⇔30)	7	21	–	–	–
Pinus peuce	TP; (S)	20⇔30	7	28	Prechill 6 months	–	TTZ (or EET) advisable
Pinus pinaster	TP	20	7	35	–	Double test: no prechill and prechill 28 d. Light for max. 16 h per day	For deeply dormant seed TTZ advisable
Pinus pinea	TP	20	7	28	Soak in water for 24 h	–	–

*The symbols '⇔' indicate alternating temperature regimes. 1st temperature: 16 h; 2nd temperature: 8 h

Table 5A Part 2. Detailed methods for germination tests: Tree and shrub seeds (continued)

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendation for breaking dormancy	Additional directions	Additional advice
1	2	3	4	5	6	7	8
Pinus ponderosa	TP	20⇔30	7	21	–	Double test: no prechill and prechill 28 d	–
Pinus pumila	S	20⇔30	7	21	Prechill 4 months	–	TTZ advisable
Pinus radiata	TP	20	7	28	–	–	–
Pinus resinosa	TP	20⇔30; (25)	7	14	–	–	–
Pinus rigida	TP	20⇔30	7	14	–	–	–
Pinus strobus	TP	20⇔30; 20	7	28	–	Double test: no prechill and prechill 28 d	For deeply dormant seed TTZ advisable
Pinus sylvestris	TP	20⇔30; (20)	7	21	Eastern and Mediterranean provenances may require prechill 21 d	–	–
Pinus tabuliformis	TP	20⇔30	7	14	–	–	–
Pinus taeda	TP	20⇔30; 22	7	28	–	Double test: no prechill and prechill 28 d	–
Pinus taiwanensis	TP	20⇔30	7	21	–	–	–
Pinus thunbergii	TP	20⇔30	7	21	–	–	–
Pinus virginiana	TP	20⇔30	7	21	–	–	–
Pinus wallichiana (*P. excelsa*)	TP	20⇔30	7	28	–	–	–
Platanus spp.	TP	20⇔30	7	21	–	–	–
Platycladus orientalis	TP	20	7	21	–	–	–
Populus spp.	TP	20⇔30	3	10	–	–	–
Prunus avium, *Prunus padus*, *Prunus serotina*	S	20⇔30; (20)	7	28	Prechill 3–4 months	–	TTZ (or EET) advisable
Pseudotsuga menziesii	TP	20⇔30	7	21	–	Double test: no prechill and prechill 21 d	–
Pyrus spp.	S	20⇔30	7	28	Prechill 3–4 months	–	TTZ (or EET) advisable
Quercus spp.	TS; (S)	20	7	28	Soak seed in water for 48 h, cut off at scar end of seed and remove pericarp	–	–

*The symbols '⇔' indicate alternating temperature regimes. 1st temperature: 16 h; 2nd temperature: 8 h

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Chapter 5: The germination test

Table 5A Part 2. Detailed methods for germination tests: Tree and shrub seeds (continued)

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendation for breaking dormancy	Additional directions	Additional advice
1	2	3	4	5	6	7	8
Robinia pseudoacacia	TP	20⇔30	7	14	1. Pierce seed; or chip or file off fragment of testa at cotyledon end; then soak in water for 3 h 2. (Soak whole seed in concentrated H_2SO_4 for as long as necessary to pit surface of testa, then wash thoroughly in running water)	–	–
Rosa spp. (except *R. multiflora*)	S	20	35	70	Prechill for 12 months	–	TTZ advisable
Rosa multiflora	T	10⇔30	7	28	Prechill 28 d	–	TTZ advisable
Salix spp.	TP	20⇔30	7	14	–	–	–
Sequoia sempervirens	TP	20⇔30	7	21	–	–	–
Sequoiadendron giganteum	TP	20⇔30	7	28	–	–	–
Sorbus spp.	S	20⇔30	7	28	Prechill 4 months	–	TTZ advisable
Spartium junceum	TP	20	7	14	Pierce seed; or chip or file off fragment of testa at cotyledon end; then soak in water for 3 h	–	–
Styphnolobium japonica	–	–	–	–	–	Use TTZ	–
Syringa reflexa	TP	20	7	21	–	Double test: no prechill and prechill 27 d	–
Syringa villosa	TP	20⇔30	7	21	–	–	–
Syringa vulgaris	TP	20	7	21	–	–	–
Taxodium distichum	S	20⇔30 (20)	7	28	Prechill 30 d	–	For deeply dormant seeds TTZ advisable
Taxus spp.	S	20⇔30	7	28	Prechill 9 months	–	TTZ advisable
Tectona grandis	S	30	14	28	Soak in water and allow to dry for 3 d. Repeat this 6 times	–	–
Thuja occidentalis	TP	20⇔30	7	21	–	–	–
Thuja plicata	TP	20⇔30	7	21	–	–	–
Tilia cordata, *Tilia platyphyllos*	S	20⇔30	7	28	Prechill 6–9 months	–	TTZ (or EET) advisable

*The symbols '⇔' indicate alternating temperature regimes. 1st temperature: 16 h; 2nd temperature: 8 h

Table 5A Part 2. Detailed methods for germination tests: Tree and shrub seeds (continued)

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendation for breaking dormancy	Additional directions	Additional advice
1	2	3	4	5	6	7	8
Tsuga canadensis	TP	15	7	28	Prechill 28 d	–	–
Tsuga heterophylla	TP	20	7	35	–	Double test: no prechill and prechill 21 d	–
Ulmus americana, Ulmus parviflora, Ulmus pumila	TP	20⇔30; (20)	7	14	Pericarp may be removed	–	–
Viburnum opulus	–	–	–	–	–	Use TTZ	–
Zelkova serrata	TP	10⇔30	7	28	–	Double test: no prechill and prechill 14 d	–

*The symbols '⇔' indicate alternating temperature regimes. 1st temperature: 16 h; 2nd temperature: 8 h

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Table 5A Part 3. Detailed methods for germination tests: Flower, spice, herb and medicinal species

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendations for breaking dormancy
1	2	3	4	5	6
Abutilon ×hybridum	TP; BP	20⇔30; 20	5–7	21	–
Achillea clavennae	TP; BP	20⇔30; 20	5	14	Light
Achillea filipendulina	TP; BP	20⇔30; 20	5	14	Light
Achillea ptarmica	TP; BP	20⇔30; 20	5	14	Light
Achillea umbellata	TP; BP	20⇔30; 20	5	14	Light
Adonis vernalis	TP; BP	15; 10	7–14	35	KNO_3; prechill
Ageratum houstonianum	TP	20⇔30; 20	3–5	14	–
Agrimonia eupatoria	TP	20⇔30	7–14	60	Soak in water for 24 h; chip or file off fragment of testa
Alcea rosea	TP; BP	20⇔30; 20	4–7	21	Pierce seed; or chip or file off fragment of testa at cotyledon end
Althaea hybrids	TP; BP	20⇔30; 20	4–7	21	Pierce seed; or chip or file off fragment of testa at cotyledon end
Althaea officinalis	TP; BP	20⇔30; 20	4–7	21	–
Alyssum argenteum	TP	20⇔30; 20; 15	4–7	21	KNO_3; prechill
Alyssum montanum	TP	20⇔30; 20; 15	4–7	21	KNO_3; prechill
Amaranthus caudatus	TP	20⇔30; 20	4–5	14	KNO_3; prechill
Amaranthus cruentus	TP	20⇔30; 20	4–5	14	KNO_3; prechill
Amaranthus hybridus	TP	20⇔30; 20	4–5	14	KNO_3; prechill
Amaranthus tricolor	TP	20⇔30; 20	4–5	14	KNO_3; prechill
Amberboa moschata	TP; BP	20⇔30; 20; 15	4–7	21	Prechill
Ammobium alatum	TP; BP	20⇔30; 20	5–7	14	–
Anagallis arvensis	TP	20⇔30; 15	7–10	21	KNO_3; prechill
Anchusa azurea	TP; BP	20⇔30; 20	5–7	21	–
Anchusa capensis	TP; BP	20⇔30; 15	5–7	21	–
Anemone coronaria	TP	20; 15	7–14	28	Prechill
Anemone pulsatilla	TP	20; 15	7–14	28	Prechill
Anemone sylvestris	TP	20; 15	7–14	28	Prechill
Angelica archangelica	TP; BP	20⇔30	7–10	28	Prechill; light
Antirrhinum majus	TP	20⇔30; 20	5–7	21	KNO_3; prechill
Aquilegia alpina	TP; BP	20⇔30; 15	7–14	28	Prechill; light
Aquilegia canadensis	TP; BP	20⇔30; 15	7–14	28	Prechill; light
Aquilegia chrysantha	TP; BP	20⇔30; 15	7–14	28	Prechill; light
Aquilegia ×cultorum	TP; BP	20⇔30; 15	7–14	28	Prechill; light
Aquilegia vulgaris	TP; BP	20⇔30; 15	7–14	28	Prechill; light
Arabis alpina	TP	20⇔30; 15	5–7	21	KNO_3; prechill
Arabis ×arendsii	TP	20⇔30; 15	5–7	21	KNO_3; prechill

*The symbols '⇔' indicate alternating temperature regimes. 1st temperature: 16 h; 2nd temperature: 8 h

Table 5A Part 3. Detailed methods for germination tests: Flower, spice, herb and medicinal species (continued)

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendations for breaking dormancy
1	2	3	4	5	6
Arabis blepharophylla	TP	20⇔30; 15	5–7	21	KNO_3; prechill
Arabis caucasica	TP	20⇔30; 15	5–7	21	KNO_3; prechill
Arabis procurrens	TP	20⇔30; 15	5–7	21	KNO_3; prechill
Arabis scopoliana	TP	20⇔30; 15	5–7	21	KNO_3; prechill
Arctotis stoechadifolia	TP; BP	20⇔30; 20; 15	7	21	Light
Armeria maritima	TP; BP	20⇔30; 15	4–7	21	KNO_3
Artemisia absinthium	TP	20⇔30	4–7	21	–
Artemisia dracunculus	TP	20⇔30	4–7	21	–
Artemisia maritima	TP	20⇔30	4–7	21	–
Artemisia vulgaris	TP	20⇔30	4–7	21	–
Asclepias tuberosa	TP	20⇔30	7	28	–
Asparagus aethiopicus	TP; BP; S	20⇔30; 20	7–14	35	Soak in water for 24 h
Asparagus plumosus	TP; BP; S	20⇔30; 20	7–14	35	Soak in water for 24 h
Aster alpinus	TP	20⇔30; 20	3–5	14	Prechill
Aster amellus	TP	20⇔30; 20	3–5	14	Prechill
Aster dumosus	TP	20⇔30; 20	3–5	14	Prechill
Aubrieta deltoidea	TP	20; 15; 10	7	21	Prechill
Aurinia saxatilis	TP	20⇔30; 20; 15	4–7	21	KNO_3; prechill
Bassia scoparia	TP; BP	20⇔30; 20	3–5	14	GA_3; prechill
Begonia Semperflorens-Cultorum Group	TP	20⇔30; 20	7–14	21	Prechill
Begonia ×tuberhybrida	TP	20⇔30; 20	7–14	21	Prechill
Bellis perennis	TP	20⇔30; 20	4–7	14	Prechill
Brachyscome iberidifolia	TP	20⇔30; 15	4–7	14	–
Briza maxima	TP	20⇔30; 20	4–7	21	Prechill
Browallia viscosa	TP; BP	20⇔30; 20	7	21	–
Brunnera macrophylla	TP; BP	20⇔30; 20	7	21	–
Calceolaria ×herbeohybrida	TP	20⇔30; 15	7	21	KNO_3; prechill
Calceolaria polyrrhiza	TP	20⇔30; 15	7	21	KNO_3; prechill
Calendula officinalis	TP; BP	20⇔30; 20	4–7	14	Prechill; KNO_3; light
Callistephus chinensis	TP	20⇔30; 20	4–7	14	Light
Campanula carpatica	TP; BP	20⇔30; 20	4–7	21	Prechill; light
Campanula fragilis	TP; BP	20⇔30; 20	4–7	21	Prechill; light
Campanula garganica	TP; BP	20⇔30; 20	4–7	21	Prechill; light
Campanula glomerata	TP; BP	20⇔30; 20	4–7	21	Prechill; light
Campanula lactiflora	TP; BP	20⇔30; 20	4–7	21	Prechill; light

*The symbols '⇔' indicate alternating temperature regimes. 1st temperature: 16 h; 2nd temperature: 8 h

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Table 5A Part 3. Detailed methods for germination tests: Flower, spice, herb and medicinal species (continued)

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendations for breaking dormancy
1	2	3	4	5	6
Campanula medium	TP; BP	20⇔30; 20	4–7	21	Prechill; light
Campanula persicifolia	TP; BP	20⇔30; 20	4–7	21	Prechill; light
Campanula portenschlagiana	TP; BP	20⇔30; 20	4–7	21	Prechill; light
Campanula pyramidalis	TP; BP	20⇔30; 20	4–7	21	Prechill; light
Campanula rapunculus	TP; BP	20⇔30; 20	4–7	21	Prechill; light
Celosia argentea	TP	20⇔30; 20	3–5	14	Prechill
Centaurea benedicta	TP; BP; S	20⇔30	7	21	Prechill
Centaurea cyanus	TP; BP	20⇔30; 20; 15	4–7	21	Prechill; light
Centaurea gymnocarpa	TP; BP	20⇔30; 20; 15	4–7	21	Prechill; light
Centaurea imperialis	TP; BP	20⇔30; 20; 15	4–7	21	Prechill; light
Centaurea macrocephala	TP; BP	20⇔30; 20; 15	4–7	21	Prechill; light
Centaurea montana	TP; BP	20⇔30; 20; 15	4–7	21	Prechill; light
Centaurea ragusina	TP; BP	20⇔30; 20; 15	4–7	21	Prechill; light
Cerastium tomentosum	TP; BP	20⇔30; 20	4–7	21	KNO_3
Chelidonium majus	TP	20⇔30	7–14	28	Prechill
Chrysanthemum indicum	TP; BP	20⇔30; 20	4–7	21	Prechill; light
Clarkia amoena	TP; BP	20⇔30; 15	4–7	14	Prechill; light
Clarkia pulchella	TP	20⇔30; 15	3–5	14	Prechill; light
Clarkia unguiculata	TP	20⇔30; 15	3–5	14	Prechill; light
Cleome hassleriana	TP	20⇔30; 20	7	28	KNO_3
Cobaea scandens	TP; BP	20⇔30; 20	4–7	21	–
Coix lacrymajobi	BP	20⇔30	7–10	21	–
Coleostephus multicaulis	TP; BP	20⇔30; 20	4–7	21	Prechill; light
Consolida ajacis	TP; BP	20; 15; 10	7–10	21	Prechill
Consolida regalis	TP; BP	20; 15; 10	7–10	21	Prechill
Convolvulus tricolor	TP; BP	20⇔30; 20	4–7	14	Pierce seed; or chip or file off fragment of testa
Coreopsis basalis	TP; BP	20⇔30; 20	4–7	14	KNO_3; prechill; light
Coreopsis lanceolata	TP; BP	20⇔30; 20	4–7	14	KNO_3; prechill; light
Coreopsis maritima	TP; BP	20⇔30; 20	4–7	14	KNO_3; prechill; light
Coreopsis tinctoria	TP	20⇔30; 20	4–7	14	KNO_3; prechill
Cosmos bipinnatus	TP; BP	20⇔30; 20	3–5	14	KNO_3; prechill; light
Cosmos sulphureus	TP; BP	20⇔30; 20	3–5	14	KNO_3; prechill; light
Cyclamen persicum	TP; BP; S	20; 15	14–21	35	KNO_3; soak in water for 24 h
Cymbalaria muralis	TP	15; 10	4–7	21	Prechill
Cynoglossum amabile	TP; BP	20⇔30; 20	4–7	14	KNO_3; prechill; light

*The symbols '⇔' indicate alternating temperature regimes. 1st temperature: 16 h; 2nd temperature: 8 h

Table 5A Part 3. Detailed methods for germination tests: Flower, spice, herb and medicinal species (continued)

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendations for breaking dormancy
1	2	3	4	5	6
Dahlia pinnata	TP; BP	20⇔30; 20; 15	4–7	21	Prechill
Datura metel	TP; BP; S	20⇔30; 20	5–7	21	File hard seeds; prechill
Datura stramonium	TP; BP; S	20⇔30; 20	5–7	21	File hard seeds; prechill
Delphinium ×belladonna	TP; BP	20⇔15; 10	7–10	21	Prechill; light
Delphinium cardinale	TP; BP	20; 15; 10	7–10	21	Prechill
Delphinium ×cultorum	TP; BP	20; 15; 10	7–10	21	Prechill; light
Delphinium formosum	TP; BP	20⇔15; 10	7–10	21	Prechill; light
Delphinium grandiflorum	TP; BP	20; 15; 10	7–10	21	Prechill; light
Dianthus barbatus	TP; BP	20⇔30; 20	4–7	14	Prechill
Dianthus caryophyllus	TP; BP	20⇔30; 20	4–7	14	Prechill
Dianthus chinensis	TP; BP	20⇔30; 20	4–7	14	Prechill
Dianthus deltoides	TP; BP	20⇔30; 20	4–7	14	Prechill
Dianthus plumarius	TP; BP	20⇔30; 20	4–7	14	Prechill
Digitalis lanata	TP	20⇔30; 20	4–7	14	Prechill
Digitalis purpurea	TP	20⇔30; 20	4–7	14	Prechill
Dimorphotheca pluvialis	TP; BP	20⇔30; 15	4–7	14	KNO_3; prechill; light
Dimorphotheca tragus	TP; BP	20⇔30; 20; 15	4–7	14	KNO_3; prechill; light
Doronicum orientale	TP	20⇔30; 20	4–7	21	KNO_3; prechill
Dorotheanthus bellidiformis	TP; BP	20; 15	5–7	35	KNO_3; prechill
Echinacea purpurea	TP; BP	20⇔30; 20	4–7	21	Prechill; light
Echinops ritro	TP; BP	20⇔30	7–14	21	–
Echium candicans	TP; BP	20⇔30; 20	4–7	14	–
Echium plantagineum	TP; BP	20⇔30; 20	4–7	14	–
Erigeron speciosus	TP	20⇔30; 20	7	28	–
Erysimum cheiri	TP	20⇔30; 20; 15	4–5	14	KNO_3; prechill; light
Erysimum ×marshallii	TP	20⇔30; 20; 15	4–7	14	KNO_3; prechill
Eschscholzia californica	TP; BP	15; 10	4–7	14	KNO_3
Fatsia japonica	TP	20⇔30; 20	7–14	28	–
Freesia refracta	TP; BP	20; 15	7–10	35	Pierce seed; or chip or file off fragment of testa; prechill
Gaillardia aristata	TP; BP	20⇔30; 20	4–7	21	Prechill; light
Gaillardia pulchella	TP; BP	20⇔30; 20	4–7	21	Prechill; light
Galega officinalis	TP; BP	20⇔30; 20	3–5	14	Soak in water for 24 h
Galeopsis segetum	TP; BP	20⇔30; 20	7	21	Prechill; file hard seeds
Gazania rigens	TP; BP	20⇔30; 15	4–7	21	Prechill
Gentiana acaulis	TP	20⇔30; 20	7–14	28	Prechill

*The symbols '⇔' indicate alternating temperature regimes. 1st temperature: 16 h; 2nd temperature: 8 h

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Table 5A Part 3. Detailed methods for germination tests: Flower, spice, herb and medicinal species (continued)

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendations for breaking dormancy
1	2	3	4	5	6
Geranium hybrids	TP; BP	20⇔30	7	28	Pierce seed; chip or file off fragment of testa
Gerbera jamesonii	TP	20⇔30; 20	4–7	14	–
Geum coccineum	TP; BP	20⇔30; 20	7–10	21	Light
Geum quellyon	TP; BP	20⇔30; 20	7–10	21	Light
Gilia tricolor	TP; BP	20⇔30; 15	4–7	14	–
Glandularia canadensis	TP	20⇔30; 15	7–10	28	KNO_3; prechill
Glebionis carinata	TP; BP	20⇔30; 15	4–7	21	Prechill; light
Glebionis coronaria	TP; BP	20⇔30; 15	4–7	21	Prechill; light
Glebionis segetum	TP; BP	20⇔30; 15	4–7	21	Prechill
Gomphrena globosa	TP; BP	20⇔30; 20	4–7	14	KNO_3
Goniolimon tataricum	TP; BP	15; 10	5–7	21	Soak in water for 24 h
Grevillea robusta	TP	20⇔30	7–10	28	KNO_3; prechill
Gypsophila elegans	TP; BP	20; 15	4–7	14	Light
Gypsophila paniculata	TP; BP	20; 15	4–7	14	Light
Gypsophila repens	TP; BP	20; 15	4–7	14	Light
Helenium autumnale	TP; BP	20⇔30; 20	5	14	–
Helianthemum nummularium	TP; BP	20⇔30; 20	5–7	28	KNO_3
Helianthus debilis	TP; BP; S	20⇔30; 20	3–5	14	Prechill
Heliopsis helianthoides	TP; BP	20⇔30	4–7	21	Soak in water for 24 h; KNO_3
Heliotropium arborescens	TP	20⇔30; 20	7	21	–
Hesperis matronalis	TP	20⇔30; 20	4–7	14	KNO_3; prechill
Heteranthemis viscidehirta	TP; BP	20⇔30; 20	4–7	21	Prechill
Heuchera sanguinea	TP	20⇔30; 20	7	21	KNO_3; prechill
Hibiscus trionum	TP; BP	20⇔30	4–7	21	–
Hippeastrum hybrids	TP; BP	20⇔30	7–10	28	–
Hypericum perforatum	TP	20⇔30; 20	4–7	21	–
Hyssopus officinalis	TP; BP	20⇔30; 20	4–7	14	Light
Iberis amara	TP; BP	20⇔30; 20; 15	4–7	14	KNO_3; prechill
Iberis gibraltarica	TP; BP	20⇔30; 20; 15	4–7	14	KNO_3; prechill
Iberis sempervirens	TP; BP	20⇔30; 20; 15	4–7	14	KNO_3; prechill
Iberis umbellate	TP; BP	20⇔30; 20; 15	4–7	14	KNO_3; prechill
Impatiens balsamina	TP; BP	20⇔30; 20	4–7	21	KNO_3; prechill; light
Impatiens walleriana	TP; BP	20⇔30; 20	4–7	21	KNO_3; prechill; light
Inula helenium	TP	20⇔30; 20	7–10	28	–
Ipomoea alba	TP; BP; S	20⇔30; 20	4–7	21	Pierce seed; or chip or file off fragment of testa

*The symbols '⇔' indicate alternating temperature regimes. 1st temperature: 16 h; 2nd temperature: 8 h

Table 5A Part 3. Detailed methods for germination tests: Flower, spice, herb and medicinal species (continued)

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendations for breaking dormancy
1	2	3	4	5	6
Ipomoea purpurea	TP; BP; S	20⇔30; 20	4–7	21	Pierce seed; or chip or file off fragment of testa
Ipomoea quamoclit	TP; BP; S	20⇔30; 20	4–7	21	Pierce seed; or chip or file off fragment of testa
Ipomoea tricolor	TP; BP; S	20⇔30; 20	4–7	21	Pierce seed; or chip or file off fragment of testa
Jacobaea maritima	TP	20⇔30; 20	4–7	21	Prechill
Kalanchoe blossfeldiana	TP	20⇔30; 20	7–14	21	–
Kalanchoe crenata	TP	20⇔30; 20	14	21	–
Kalanchoe globulifera	TP	20⇔30; 20	7–14	21	–
Kniphofia uvaria	TP	20⇔30	4–7	21	–
Lathyrus latifolius	TP; BP; S	20	7–10	21	Pierce seed; or chip or file off fragment of testa at cotyledon end; prechill
Lathyrus odoratus	TP; BP; S	20	5–7	14	Prechill
Lavandula angustifolia	TP; BP; S	20⇔30; 20	7–10	21	GA_3; prechill
Lavatera trimestris	TP; BP	20⇔30; 20	4–7	21	Prechill
Legousia speculum-veneris	TP; BP	20⇔30; 20	4–7	21	Prechill; light
Leontopodium nivale	TP	20⇔30; 20	5	14	Prechill
Leonurus cardiaca	TP	20⇔30	5–7	42	Prechill
Leucanthemum maximum	TP; BP	20⇔30; 20	4–7	21	Prechill; light
Leucanthemum vulgare	TP; BP	20⇔30; 20	4–7	21	Prechill; light
Levisticum officinale	TP; BP	20⇔30; 20	10	21	–
Liatris pycnostachya	TP	20⇔30	5–7	28	–
Liatris spicata	TP	20⇔30	5–7	28	–
Lilium regale	TP; S	20⇔30; 20	7	28	–
Limonium bellidifolium	TP; BP	15; 10	5–7	21	Soak in water for 24 h
Limonium bonduellei	TP; BP; S	20; 15	5–7	21	Soak in water for 24 h
Limonium gerberi	TP; BP	15; 10	5–7	21	Soak in water for 24 h
Limonium sinuatum	TP; BP; S	15; 10	5–7	21	Soak in water for 24 h
Linaria bipartita	TP	15; 10	4–7	21	Prechill
Linaria maroccana	TP	15; 10	4–7	21	Prechill
Linaria vulgaris	TP	15; 10	4–7	21	Prechill
Linum flavum	TP; BP	20⇔30; 15	4–7	21	KNO_3
Linum grandiflorum	TP; BP	20; 15; 10	4–7	21	KNO_3
Linum narbonense	TP; BP	20⇔30; 20; 15	4–7	21	KNO_3
Linum perenne	TP; BP	20; 15; 10	4–7	21	KNO_3
Lobelia cardinalis	TP	20⇔30; 20	7–14	21	KNO_3; prechill
Lobelia erinus	TP	20⇔30; 20	7–14	21	KNO_3; prechill
Lobularia maritima	TP	20⇔30; 20; 15	4–7	21	KNO_3; prechill

*The symbols '⇔' indicate alternating temperature regimes. 1st temperature: 16 h; 2nd temperature: 8 h

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Table 5A Part 3. Detailed methods for germination tests: Flower, spice, herb and medicinal species (continued)

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendations for breaking dormancy
1	2	3	4	5	6
Lomelosia caucasica	TP; BP	20⇔30; 20; 15	4–7	21	Prechill
Lonas annua	TP	20⇔30	4–5	14	–
Lunaria annua	TP; BP	20; 15	7	21	KNO_3; prechill
Lupinus hartwegii	TP; BP; S	20⇔30; 20	4–7	21	Pierce seed; or file fragment off testa at cotyledon end; prechill
Lupinus hybrids	TP; BP; S	20⇔30; 20	4–7	21	–
Lupinus nanus	TP; BP; S	20⇔30; 20	4–7	21	–
Lupinus polyphyllus	TP; BP; S	20⇔30; 20	4–7	21	–
Malcolmia maritima	TP	20⇔30; 20; 15	4–5	14	KNO_3; prechill; light
Malope trifida	TP; BP	20⇔30; 20	4–7	14	Prechill
Marrubium vulgare	TP	20⇔30	5–7	21	Prechill
Matricaria chamomilla	TP	20⇔30; 20	4–7	14	Prechill
Matthiola incana	TP	20⇔30; 20	4–7	14	KNO_3; prechill
Matthiola longipetala	TP	20⇔30; 20; 15	4–7	14	KNO_3; prechill
Melissa officinalis	TP	20⇔30; 20	4–7	21	Prechill
Mentha ×piperita	TP	20⇔30	7–14	21	KNO_3; prechill
Mimosa pudica	TP; BP	20⇔30; 20	4–7	28	Soak in water for 24 h
Mimulus cardinalis	TP	20⇔30; 20	4–7	21	Prechill
Mimulus cupreus	TP	20⇔30; 20	4–7	21	Prechill
Mimulus ×hybridus	TP	20⇔30; 20	4–7	21	Prechill
Mimulus luteus	TP	20⇔30; 20	4–7	21	Prechill
Mirabilis jalapa	TP; BP; S	20⇔30; 20	4–7	14	Prechill; light
Moluccella laevis	TP; BP	20⇔30; 20	5–7	21	Prechill; light
Myosotis hybrids	TP; BP	20⇔30; 20; 15	5–7	21	Prechill; light
Myosotis scorpioides	TP; BP	20⇔30; 20; 15	5–7	21	Prechill; light
Myosotis sylvatica	TP; BP	20⇔30; 20; 15	5–7	21	Prechill; light
Nemesia strumosa	TP; BP	20; 15	5–7	21	Prechill; light
Nemesia versicolor	TP; BP	20; 15	5–7	21	Prechill; light
Nemophila maculata	TP; BP	15; 10	5–7	21	Prechill
Nemophila menziesii	TP; BP	15; 10	5–7	21	Prechill
Nepeta cataria	TP; BP	20⇔30; 20	7–14	28	Prechill
Nicotiana alata	TP	20⇔30; 20	5–7	14	KNO_3
Nicotiana ×sanderae	TP	20⇔30; 20	5–7	14	KNO_3
Nicotiana suaveolens	TP	20⇔30; 20	5–7	14	KNO_3
Nierembergia hippomanica	TP	20⇔30; 20	5–7	21	–
Nigella damascena	TP; BP	20⇔30; 20; 15	7–10	21	KNO_3; prechill; 15 °C dark for 14 d then 20⇔30 °C

*The symbols '⇔' indicate alternating temperature regimes. 1st temperature: 16 h; 2nd temperature: 8 h

Table 5A Part 3. Detailed methods for germination tests: Flower, spice, herb and medicinal species (continued)

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendations for breaking dormancy
1	2	3	4	5	6
Nigella hispanica	TP; BP	20⇔30; 20; 15	7–10	21	KNO_3; prechill; 15 °C dark for 14 d then 20⇔30 °C
Nigella sativa	TP; BP	20⇔30; 20	7–10	21	KNO_3; prechill
Oenothera macrocarpa	TP; BP	20⇔30; 20	4–7	21	KNO_3
Osteospermum ecklonis	TP; BP	20⇔30; 20	4–7	14	KNO_3; prechill; light
Papaver alpinum	TP	15; 10	4–7	14	KNO_3
Papaver glaucum	TP	15; 10	4–7	14	KNO_3; light
Papaver nudicaule	TP	15; 10	4–7	14	KNO_3; light
Papaver orientale	TP	20⇔30; 20	4–7	14	KNO_3; prechill
Papaver rhoeas	TP	20⇔30; 20; 15	4–7	14	KNO_3; prechill; light
Pelargonium Zonale Group	TP; BP	20⇔30; 20	7	28	Pierce seed; or file off fragment of testa
Penstemon barbatus	TP	20⇔30; 15	7	21	Prechill
Penstemon hartwegii	TP	20⇔30; 15	7	21	Prechill
Penstemon hybrids	TP	20⇔30; 15	7	21	Prechill
Pericallis cruenta	TP	20⇔30; 20	4–7	21	Prechill
Perilla frutescens	TP; BP	20⇔30; 20	5–7	21	Prechill
Petunia ×atkinsiana	TP	20⇔30; 20	5–7	14	KNO_3; prechill
Phacelia campanularia	TP; BP	15; 10	3–5	21	KNO_3; prechill
Phlox drummondii	TP; BP	20⇔30; 20; 15	5–7	21	KNO_3; prechill
Phlox paniculata	TP; BP	20; 15	5–7	21	KNO_3; prechill
Phlox subulata	TP; BP	20; 15	5–7	21	KNO_3; prechill
Pholistoma auritum	TP; BP	15; 10	5–7	21	Prechill
Physalis alkekengi	TP	20⇔30	4–7	28	KNO_3; prechill; light
Pimpinella major	TP; BP	20⇔30	7–10	21	Prechill
Pimpinella saxifraga	TP; BP	20⇔30	5–7	21	–
Plectocephalus americana	TP; BP	20⇔30; 20; 15	4–7	21	Soak in water for 24 h; prechill; light
Plectranthus scutellarioides	TP; BP	20⇔30; 20	5–7	21	Light
Portulaca grandiflora	TP; BP	20⇔30; 20	4–7	14	KNO_3; prechill; light
Primula auricula	TP	20⇔30; 20; 15	7–14	28	KNO_3; prechill
Primula denticulata	TP	20⇔30; 20; 15	7–14	28	KNO_3; prechill
Primula elatior	TP	20⇔30; 20; 15	7–14	28	KNO_3; prechill
Primula japonica	TP	20⇔30; 20; 15	7–14	28	KNO_3; prechill
Primula ×kewensis	TP	20⇔30; 20; 15	7–14	28	KNO_3; prechill
Primula malacoides	TP	20⇔30; 20; 15	7–14	28	KNO_3; prechill
Primula obconica	TP	20⇔30; 20; 15	7–14	28	KNO_3; prechill
Primula praenitens	TP	20⇔30; 20; 15	7–14	28	KNO_3; prechill

*The symbols '⇔' indicate alternating temperature regimes. 1st temperature: 16 h; 2nd temperature: 8 h

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Table 5A Part 3. Detailed methods for germination tests: Flower, spice, herb and medicinal species (continued)

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendations for breaking dormancy
1	2	3	4	5	6
Primula veris	TP	20⇔30; 20; 15	7–14	28	KNO_3; prechill
Primula vulgaris	TP	20⇔30; 20; 15	7–14	28	KNO_3; prechill
Psephellus dealbatus	TP; BP	20⇔30; 20; 15	4–7	21	Prechill; light
Psylliostachys suworowii	TP; BP	15; 10	5–7	21	Soak in water for 24 h
Ranunculus asiaticus	TP; S	20; 15	7–14	28	–
Reseda odorata	TP; BP	20⇔30; 15	4–7	14	Light
Rheum palmatum	TP; BP	20⇔30; 20	7	21	–
Rhodanthe humboldtiana	TP; BP	20⇔30; 15	7–14	21	Prechill
Rhodanthe manglesii	TP; BP	20⇔30; 15	7–14	21	Prechill
Rhodanthe chlorocephala	TP; BP	20⇔30; 15	7–14	21	Prechill
Rudbeckia fulgida	TP; BP	20⇔30; 20	4–7	21	Prechill; light
Rudbeckia hirta	TP; BP	20⇔30; 20	4–7	21	Prechill; light
Ruta graveolens	TP; BP	20⇔30; 20	7	28	Prechill
Saintpaulia ionantha	TP	20⇔30; 20	7–14	28	–
Salpiglossis sinuata	TP; BP	20⇔30; 20	4–7	21	KNO_3; prechill; light
Salvia coccinea	TP	20⇔30; 20	4–7	21	Prechill
Salvia farinacea	TP	20⇔30; 20	4–7	21	Prechill
Salvia officinalis	TP	20⇔30; 20	4–7	21	Prechill
Salvia patens	TP	20⇔30; 20	4–7	21	Prechill
Salvia pratensis	TP	20⇔30; 20	4–7	21	Prechill
Salvia sclarea	TP; BP	20⇔30; 20	4–7	21	Prechill
Salvia splendens	TP	20⇔30; 20	4–7	21	Prechill
Salvia viridis	TP	20⇔30; 20	4–7	21	Prechill
Sanvitalia procumbens	TP; BP	20⇔30; 20	3–5	14	Prechill
Saponaria calabrica	TP; BP	15; 10	4–7	21	Prechill; light
Saponaria ocymoides	TP; BP	15; 10	4–7	21	Prechill; light
Saponaria officinalis	TP; BP	15; 10	4–7	21	Prechill; light
Scabiosa atropurpurea	TP; BP	20⇔30; 20	4–7	21	Prechill
Schefflera elegantissima	TP; BP	20⇔30	7–14	28	–
Schizanthus pinnatus	TP; BP	15; 10	4–7	14	Prechill
Senecio elegans	TP	20⇔30; 20	4–7	21	Prechill
Silene chalcedonica	TP	20⇔30; 20	5–10	21	Light
Silene coronaria	TP	20⇔30	5–10	21	–
Silene pendula	TP; BP	20⇔30; 20	7–14	28	KNO_3
Silybum marianum	TP; BP	20⇔30; 20	5–7	21	Prechill

*The symbols '⇔' indicate alternating temperature regimes. 1st temperature: 16 h; 2nd temperature: 8 h

Table 5A Part 3. Detailed methods for germination tests: Flower, spice, herb and medicinal species (continued)

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendations for breaking dormancy
1	2	3	4	5	6
Sinningia speciosa	TP	20⇔30; 20	7–14	28	Prechill
Solanum pseudocapsicum	TP; BP	20⇔30; 20	5–7	28	KNO_3; light
Solanum giganteum	TP; BP	20⇔30; 20	5–7	28	KNO_3; light
Solanum laciniatum	TP	20⇔30; 20	5–7	28	KNO_3
Solanum marginatum	TP; BP	20⇔30; 20	5–7	28	KNO_3; light
Stachys macrantha	TP	20	7	14	–
Tagetes erecta	TP; BP	20⇔30; 20	3–5	14	Light
Tagetes patula	TP; BP	20⇔30; 20	3–5	14	Light
Tagetes tenuifolia	TP; BP	20⇔30; 20	3–5	14	Light
Tanacetum achilleifolium	TP; BP	20⇔30; 15	4–7	21	Prechill; light
Tanacetum cinerariifolium	TP; BP	20⇔30; 20	4–7	21	Prechill
Tanacetum coccineum	TP; BP	20⇔30; 15	4–7	21	KNO_3; prechill; light
Tanacetum parthenium	TP; BP	20⇔30; 20	4–7	21	Prechill; light
Thunbergia alata	TP; BP	20⇔30; 20	4–7	21	–
Thymus serpyllum	TP; BP	20⇔30; 20; 15	7	21	Light
Torenia fournieri	TP	20⇔30	5–7	14	KNO_3
Tripleurospermum maritimum	TP	20⇔30; 20	4–7	14	Prechill
Tripleurospermum inodorum	TP	20⇔30; 20	4–7	14	Prechill
Tropaeolum majus	TP; BP; S	20⇔30; 20; 15	4–7	21	Prechill
Tropaeolum peltophorum	TP; BP; S	20; 15	4–7	21	Prechill
Tropaeolum peregrinum	TP; BP; S	20; 15	4–7	21	Prechill
Vaccaria hispanica	TP; BP	15⇔10	4–7	21	Prechill; light
Valeriana officinalis	TP	20⇔30; 20	5–7	21	Prechill
Verbascum densiflorum	TP	20⇔30	4–7	21	Prechill
Verbascum phlomoides	TP	20⇔30	4–7	21	Prechill
Verbascum thapsus	TP	20⇔30	4–7	21	Prechill
Verbena bonariensis	TP	20⇔30; 15	7–10	28	KNO_3; prechill
Verbena Hybrida Group	TP	20⇔30; 20; 15	7–10	28	KNO_3; prechill
Verbena rigida	TP	20⇔30; 15	7–10	28	KNO_3; prechill
Vinca minor	TP	20⇔30; 20	4–7	14	–
Viola cornuta	TP	20⇔30; 20	4–7	21	KNO_3; prechill
Viola odorata	TP	20; 10	4–7	21	KNO_3; prechill
Viola tricolor	TP	20⇔30; 20	4–7	21	KNO_3; prechill
Xeranthemum annuum	TP; BP	20⇔30; 20	4–7	14	–
Xerochrysum bracteatum	TP; BP	20⇔30; 15	4–7	14	KNO_3; prechill; light

*The symbols '⇔' indicate alternating temperature regimes. 1st temperature: 16 h; 2nd temperature: 8 h

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Table 5A Part 3. Detailed methods for germination tests: Flower, spice, herb and medicinal species (continued)

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendations for breaking dormancy
1	2	3	4	5	6
Zinnia elegans	TP; BP	20⇔30; 20	3–5	10	Prechill; light
Zinnia haageana	TP; BP	20⇔30; 20	3–5	10	Prechill; light

*The symbols '⇔' indicate alternating temperature regimes. 1st temperature: 16 h; 2nd temperature: 8 h

5.11 Tolerance tables

Table 5B gives the maximum tolerated differences between the highest and lowest germination percentages of the replicates of a germination test, allowing only for random sampling variation at a probability of 0.025.

To determine whether a test is reliable, calculate the average germination percentage over all replicates, to the nearest whole number. If necessary, in tests of 400 or 200 seeds, four or two replicates, respectively, of 100 seeds each can be formed by combining the subreplicates of 50 or 25 seeds which were closest together in the germinator. In tests of 100 seeds, two replicates of 50 seeds each can be formed by combining the subreplicates of 25 seeds which were closest together in the germinator, and multiplying the results of each of the two replicates by 2 to obtain an average germination percentage.

Locate the average germination percentage in the appropriate part of the table for the number of seeds tested, and read off the tolerance in the adjoining column. If the difference between the highest and lowest replicates does not exceed this tolerance, the test is reliable.

The tolerances for tests with 400 and 200 seeds are extracted from Table G1, columns D and L respectively, in Miles (1963). The tolerances for tests with 100 seeds are calculated in accordance to Miles (1963).

Tables 5C–5E give the tolerances for percentages of normal seedlings, abnormal seedlings, dead seeds, hard seeds, fresh seeds or any combination of these, when tests are made on the same or a different submitted sample in the same laboratory. For two tests, use Table 5C, for three, Table 5D, and for four, Table 5E.

Table 5F gives the tolerances for percentages of normal seedlings, abnormal seedlings, dead seeds, hard seeds, fresh seeds or any combination of these, when tests are made on the same or a different submitted sample in two different laboratories. For tolerances between results of more than two laboratories or on tests of less than 400 seeds, see the Germination tolerances calculator in the Germination Committee Toolbox on the ISTA website.

To determine whether tests are compatible, calculate the average of the test results to the nearest whole number. Locate this in the appropriate part of the table for the number of seeds tested, and read off the tolerance in the adjoining column. If the difference between the highest and lowest results of the tests does not exceed the tolerance, the tests are compatible.

The sources for tolerances are as follows:

- tests with 2 × 400 seeds: extracted from Table G2, column L, in Miles (1963);
- tests with 2 × 200 and 2 × 100 seeds: derived from Miles (1963);
- tests with 3 × 400 seeds: extracted from Table G2, column H, in Miles (1963);
- tests with 3 × 200 and 3 × 100 seeds: derived from Miles (1963);
- tests with 4 × 400 seeds: extracted from Table G2, column D, in Miles (1963);
- tests with 4 × 200 and 4 × 100 seeds: derived from Miles (1963).

Miles, S. R. (1963). Handbook of Tolerances and of Measures of Precision for Seed Testing. *Proceedings of the International Seed Testing Association*, **28** (3).

Table 5B. Tolerances between highest and lowest germination percentages of replicates in one germination test (two-way test at the 2.5 % significance level)

Table 5B Part 1. 4 replicates of 100 seeds

Average germination percentage of test		Tolerance
51–100 %	0–50 %	
99	2	5
98	3	6
97	4	7
96	5	8
95	6	9
93–94	7–8	10
91–92	9–10	11
89–90	11–12	12
87–88	13–14	13
84–86	15–17	14
81–83	18–20	15
78–80	21–23	16
73–77	24–28	17
67–72	29–34	18
56–66	35–45	19
51–55	46–50	20

Table 5B Part 2. 2 replicates of 100 seeds

Average germination percentage of test		Tolerance
51–100 %	0–50 %	
99	2	4
98	3	5
96–97	4–5	6
95	6	7
93–94	7–8	8
90–92	9–11	9
88–89	12–13	10
84–87	14–17	11
81–83	18–20	12
76–80	21–25	13
69–75	26–32	14
55–68	33–46	15
51–54	47–50	16

Table 5B Part 3. 2 replicates of 50 seeds

Average germination percentage of test		Tolerance
51–100 %	0–50 %	
99	2	5
98	3	7
97	4	8
96	5	9
95	6	10
94	7	11
92–93	8–9	12
90–91	10–11	13
89	12	14
86–88	13–15	15
84–85	16–17	16
81–83	18–20	17
78–80	21–23	18
74–77	24–27	19
70–73	28–31	20
63–69	32–38	21
51–62	39–50	22

Table 5C. Tolerances between results of two tests on the same or a different submitted sample when tests are made in the same laboratory (two-way test at the 2.5 % significance level)

Table 5C Part 1. 2 tests of 400 seeds

Average germination percentage of 2 tests		Tolerance
51–100 %	0–50 %	
98–99	2–3	2
95–97	4–6	3
91–94	7–10	4
85–90	11–16	5
77–84	17–24	6
60–76	25–41	7
51–59	42–50	8

Table 5C Part 2. 2 tests of 200 seeds

Average germination percentage of 2 tests		Tolerance
51–100 %	0–50 %	
99	2	2
98	3	3
96–97	4–5	4
94–95	6–7	5
91–93	8–10	6
87–90	11–14	7
82–86	15–19	8
75–81	20–26	9
64–74	27–37	10
51–63	38–50	11

Table 5C Part 3. 2 tests of 100 seeds

Average germination percentage of 2 tests		Tolerance
51–100 %	0–50 %	
99	2	4
98	3	5
96–97	4–5	6
95	6	7
93–94	7–8	8
90–92	9–11	9
88–89	12–13	10
84–87	14–17	11
81–83	18–20	12
76–80	21–25	13
69–75	26–32	14
55–68	33–46	15
51–54	47–50	16

Table 5D. Tolerances between results of three tests on the same or a different submitted sample when tests are made in the same laboratory (two-way test at the 2.5 % significance level)

Table 5D Part 1. 3 tests of 400 seeds

Average germination percentage of 3 tests		Tolerance
51–100 %	0–50 %	
99	2	2
97–98	3–4	3
94–96	5–7	4
90–93	8–11	5
85–89	12–16	6
78–84	17–23	7
66–77	24–35	8
51–65	36–50	9

Table 5D Part 2. 3 tests of 200 seeds

Average germination percentage of 3 tests		Tolerance
51–100 %	0–50 %	
99	2	3
97–98	3–4	4
96	5	5
94–95	6–7	6
91–93	8–10	7
88–90	11–13	8
84–87	14–17	9
79–83	18–22	10
72–78	23–29	11
60–71	30–41	12
51–59	42–50	13

Table 5D Part 3. 3 tests of 100 seeds

Average germination percentage of 3 tests		Tolerance
51–100 %	0–50 %	
99	2	4
98	3	5
97	4	6
96	5	7
95	6	8
93–94	7–8	9
91–92	9–10	10
89–90	11–12	11
87–88	13–14	12
84–86	15–17	13
81–83	18–20	14
77–80	21–24	15
71–76	25–30	16
64–70	31–37	17
51–63	38–50	18

Table 5E. Tolerances between results of four tests on the same or a different submitted sample when tests are made in the same laboratory (two-way test at the 2.5 % significance level)

Table 5E Part 1. 4 tests of 400 seeds

Average germination percentage of 4 tests		Tolerance
51–100 %	0–50 %	
99	2	2
97–98	3–4	3
95–96	5–6	4
92–94	7–9	5
88–91	10–13	6
82–87	14–19	7
74–81	20–27	8
60–73	28–41	9
51–59	42–50	10

Table 5E Part 2. 4 tests of 200 seeds

Average germination percentage of 4 tests		Tolerance
51–100 %	0–50 %	
99	2	3
98	3	4
97	4	5
95–96	5–6	6
93–94	7–8	7
90–92	9–11	8
87–89	12–14	9
83–86	15–18	10
78–82	19–23	11
72–77	24–29	12
61–71	30–40	13
51–60	41–50	14

Table 5E Part 3. 4 tests of 100 seeds

Average germination percentage of 4 tests		Tolerance
51–100 %	0–50 %	
99	2	5
98	3	6
97	4	7
96	5	8
95	6	9
93–94	7–8	10
91–92	9–10	11
89–90	11–12	12
87–88	13–14	13
84–86	15–17	14
81–83	18–20	15
78–80	21–23	16
73–77	24–28	17
68–72	29–33	18
56–67	34–45	19
51–55	46–50	20

Table 5F. Tolerances between results of two tests made in different laboratories on the same or different samples from the same seed lot (two-way test at 5 % significance level) on 400 seed tests. Updated by ISTA Statistics Technical Committee, based on Miles (1963) Table G5, column C, 400 seed tests.

Average germination percentage of 2 tests		Tolerance
51–100 %	0–50 %	
99	2	2
98	3	3
96–97	4–5	4
94–95	6–7	5
91–93	8–10	6
88–90	11–13	7
84–87	14–17	8
79–83	18–22	9
74–78	23–27	10
68–73	28–33	11
60–67	34–41	12
51–59	42–50	13

Chapter 6: Biochemical test for viability: the topographical tetrazolium test

6.1 Object

The objects of biochemical tests are:
a) To make a quick estimate of the viability of seed samples in general and those showing dormancy in particular.
b) In the case of particular samples which at the end of a germination test reveal a high percentage of dormant seeds (5.6.5), to determine the viability of individual dormant seeds or the viability of a working sample.

6.2 Definition

The Topographical Tetrazolium Test is a biochemical test that may be used to make a rapid assessment of seed viability: when seeds have to be sown shortly after harvest; in seeds with deep dormancy; in seeds showing slow germination; or in cases where a very quick estimate of germination potential is required. It can also be used: to determine the viability of individual seeds at the end of a germination test, especially where dormancy is suspected (see 5.6.3); to detect the presence of sprouting and various types of harvesting and/or processing damage (heat damage, mechanical damage, insect damage); and to solve problems encountered in a germination test, e.g. when reasons for abnormals are not clear, treatment with pesticides is suspected, etc.

A viable seed should show staining in all those tissues whose viability is necessary for normal seedling development. Depending on the species, small unstained areas in some parts of these tissues can be accepted. For the purposes of the test, a viable seed should show by its biochemical activity the potential to produce a normal seedling. A non-viable seed shows deficiencies and/or abnormalities of such a nature as to prevent its development into a normal seedling.

The test is valid for all species for which a method is described in Table 6A. If the result is to be reported on an ISTA Certificate, the test must be carried out strictly in accordance with the methods described in this Chapter.

The tetrazolium test is a versatile tool, which may have applications other than in the issue of an ISTA Certificate. Further information on such applications may be found in the *Handbook on Tetrazolium Testing* and in the *ISTA Working Sheets on Tetrazolium Testing*.

6.3 General principles

In the topographical tetrazolium test a colourless solution of 2,3,5-triphenyl tetrazolium chloride or bromide is used as an indicator to reveal the reduction processes which take place within living cells. The indicator is imbibed by the seed. Within the seed tissues it interacts with the reduction processes of living cells and accepts hydrogen from the dehydrogenases. By hydrogenation of the 2,3,5-triphenyl tetrazolium chloride a red, stable and non-diffusible substance, triphenyl formazan, is produced in living cells. This makes it possible to distinguish the red-coloured living parts of seeds from the colourless dead ones.

In addition to completely stained viable seeds and completely unstained non-viable seeds, partially stained seeds may occur. Varying proportions of necrotic tissue are found in different zones of these partially stained seeds. The position and size of the necrotic areas, and not necessarily the intensity of the colour, determine whether such seeds are classified as viable or non-viable. However, colour differences along with tissue soundness are to be considered decisive mainly to the extent that they permit recognition and location of sound, weak or dead tissue.

6.4 Reagent

6.4.1 Tetrazolium solution

An aqueous solution of 2,3,5-triphenyl tetrazolium chloride or bromide of pH 6.5–7.5 is used. The concentration normally used should be 1.0 %; however, lower or higher percentages are permissible. Distilled/deionised water should be used in the preparation, and the tetrazolium solution should have a pH of between 6.5 and 7.5. If necessary to ensure the pH is within the required range, a phosphate buffer, as described in 6.4.2, should be used.

When using buffer the correct amount of tetrazolium salt (either chloride or bromide) is dissolved in the buffer to obtain a solution of the correct concentration, e.g. 1 g tetrazolium salt per 100 mL buffer gives a 1 % solution.

The tetrazolium solution can be stored in the dark at 5–10 °C for up to one year.

6.4.2 Buffer solution

To achieve the correct pH range it may be necessary to prepare the tetrazolium solution in phosphate buffer solution.

The buffer solution should be made up as follows, using distilled/deionised water.

Prepare two solutions:

Solution 1: dissolve 9.078 g KH_2PO_4 in 1000 mL distilled/deionised water

Solution 2: dissolve 9.472 g Na_2HPO_4 in 1000 mL distilled/deionised water, or dissolve 11.876 g $Na_2HPO_4 \cdot 2H_2O$ in 1000 mL distilled/deionised water

Mix two parts of solution 1 with three parts of solution 2 and check the pH, which must be between 6.5 and 7.5.

6.5 Procedures

6.5.1 Working sample

A full test must be carried out on four replicates of 100 pure seeds drawn at random from either the pure seed fraction of a purity test carried out as prescribed in Chapter 3, or from a representative fraction of the submitted sample. The pure seed fraction must be mixed thoroughly and due care should be taken when drawing the seeds to ensure that there is no selection of seeds that would cause biased results (see Chapter 5 for methods of counting seeds). A test may also be carried out on individual ungerminated seeds that are found at the end of a germination test.

6.5.2 Preparation and treatment of the seed

The seeds must be prepared in order to facilitate penetration of the tetrazolium solution.

6.5.2.1 Premoistening the seed

Premoistening is a necessary preliminary step to staining for some species and a highly recommended one for others. Imbibed seeds are generally less fragile than dry seeds and can be cut or punctured more readily. In addition, staining of premoistened seed is more even in colour and this facilitates evaluation. If the seed coat hampers imbibition, the coat must be punctured (e.g. *Fabaceae*). The minimum premoistening period is indicated in Table 6A. If a higher (40 ±2 °C) or lower temperature is used than that recommended, then the premoistening period must be adjusted accordingly, and any variation in premoistening time or temperature must be reported on the ISTA Certificate.

6.5.2.1.1 Slow moistening

The seed is allowed to imbibe on top of or between paper according to the method used for germination testing (see Table 5A). The technique should be used for those species that are inclined to fracture if immersed directly in water. Old and dry seeds of many species may also benefit from slow moistening.

In some species, slow moistening will not result in full imbibition and a further period of soaking in water will be necessary.

6.5.2.1.2 Soaking in water

Seeds should be fully immersed in water and left until completely imbibed. If the soaking period is more than 24 h, the water should be changed.

If the percentage of hard seeds of the *Fabaceae* is to be determined for the purpose of issuing an ISTA Certificate, the seed should be soaked in water at 20 °C for 22 h. Other procedures may lead to excessive variability in results.

6.5.2.2 Exposure of tissues prior to staining

See Figure 6.1 for details.

In many species (see Table 6A) it is necessary to expose the tissues prior to staining to allow easier penetration of the tetrazolium solution and to facilitate evaluation. Tissues that must be critically examined to establish the viability of a seed are considered to be ‘essential’ tissues while those that are less essential for this diagnosis are ‘non-essential’. Procedures for exposing the internal tissues have been standardised so that unavoidable injuries caused by the preparation technique can be easily recognised as such during the evaluation.

Seed coats can be opened or removed using a variety of different preparation techniques as described below. Once prepared, seeds should be kept moist until the whole replicate has been completed. Only then should the replicate be immersed in the tetrazolium solution.

During premoistening some kinds of seeds produce sticky mucilage that hampers further preparation. The mucilage can be reduced either by drying the surface, rubbing the seeds in cloth or between sheets of paper, or by soaking the seeds in a 1–2 % solution of aluminium potassium

sulphate ($AlK(SO_4)_2 \cdot 12H_2O$) for 5 min after premoistening for the appropriate period.

6.5.2.2.1 Piercing the seed

Premoistened or hard seeds should be pierced at a non-essential part of the seed using a needle or sharp scalpel.

6.5.2.2.2 Longitudinal cutting

- For all cereals and grass seeds the size of *Festuca* spp. or larger, a longitudinal cut should be made through the middle of the embryonic axis and approximately three quarters of the length of the endosperm.
- For seeds of dicotyledonous species without endosperm and with a straight embryo a longitudinal cut should be made through the middle of the distal half of the cotyledons, leaving the embryo axis uncut.
- In seeds where there is an embryo surrounded by living tissue, a longitudinal cut can be safely made alongside the embryo.

6.5.2.2.3 Transverse cutting

Transverse cutting is made through non-essential tissue using scalpels, razor blades, dog nail clippers or similar devices.

- Grass seeds: make a transverse cut immediately above the embryo and immerse the embryo end in the tetrazolium solution.
- Dicotyledonous seeds with a straight embryo and without endosperm: cut off and discard a fragment of one third to two fifths of the distal end of the cotyledons.
- Coniferous seeds: cut a small fraction from both ends, big enough to ensure that the embryo cavity is opened without causing major injury to the embryo.

6.5.2.2.4 Transverse incision

A transverse incision may be used as a substitute for a transverse cut and is the preferred method for small grass seeds the size of *Agrostis*, *Phleum* and *Poa*.

6.5.2.2.5 Excision of the embryo

Embryo excision may be used for *Hordeum*, *Secale* and *Triticum*.

The embryo is excised with a dissecting lancet that is thrust through the endosperm just above the scutellum and a little off centre and then twisted slightly so that the endosperm bursts lengthwise. The embryo (with scutellum) becomes loosened from the endosperm and can be picked up and transferred to the tetrazolium solution.

6.5.2.2.6 Removal of the seed coat

When cutting techniques are inappropriate, the whole seed coat (and any other covering tissues) must be removed. If the outer coverings of the seed are hard, as in nuts and drupes (stone fruits) they can be split open or cracked either when the seed is dry or after premoistening, care being taken to avoid embryo damage. Leathery seed coats can be removed after premoistening by slitting them carefully with a sharp scalpel or dissecting needle and peeling them off.

6.5.2.3 Low pressure

The low pressure method utilises subatmospheric pressure to quickly infiltrate seed tissues with tetrazolium solution.

The dry seeds are prepared as described in Table 6A, placed in a 1 % tetrazolium solution and degassed to a subatmospheric pressure of about 18662 Pa (140 Torr) for 10 min. The pressure is then increased slowly for 1 min to normal atmospheric level. This treatment is repeated three times.

6.5.3 Staining

The prepared seeds or embryos should be completely immersed in tetrazolium solution. Small seeds, which are difficult to handle, may be premoistened and prepared on a strip of paper, which is then folded or rolled up and immersed in the tetrazolium solution.

The solution should not be exposed to direct light as this brings about a reduction of the tetrazolium salt. Table 6A gives details of optimum temperatures and staining times.

Staining temperatures used may deviate from those given in Table 6A, but must be in the range of 20–40 °C. If the optimum staining temperature of 30 °C is not used, then suitable adjustments in staining duration must be made, as an increase/decrease of 5 °C from the optimum of 30 °C reduces/increases staining time by one half. Staining periods should not be taken as absolute, because they may vary according to the condition of the seed. As experience is gained it may be possible to make evaluation at an earlier or later stage of staining. The staining period may be prolonged if the seeds are incompletely stained in order to verify if the lack of staining is due to slow uptake of tetrazolium salt rather than an indication of defects within the seed. However, over staining should be avoided as this may hide differential staining patterns, which are indicative of weak seed and specific damage such as that caused by frost.

For some species trace amounts of fungicides or antibiotics may be added to the tetrazolium solution to avoid the development of a frothy solution with a dark precipitate.

At the end of the staining period the solution is decanted and the seed rinsed with water and examined.

6.5.4 Evaluation

The main purpose of the tetrazolium test is to distinguish viable and non-viable seeds.

Each seed is examined and evaluated as viable or non-viable on the basis of the staining patterns and tissue soundness revealed. Procedures for preparation, treatment and evaluation of each approved species are given in 6.5.2.1, Table 6A and Figures 6.1–6.3.

Whether a seed is rated viable or non-viable is derived directly from the importance of the different seed tissues responsible for the emergence and development of a normal seedling, which is species specific. Viable seeds are those that show the potential to produce normal seedlings. Such seeds stain completely, or if only partly stained, the staining patterns indicate that the essential structures are viable.

Non-viable seeds are those that do not meet these requirements and in addition include seeds that reveal uncharacteristic colouring and/or flaccid essential structures. Seeds with obviously abnormal development of the embryo or other essential structures must be regarded as non-viable whether stained or not. Rudimentary embryos of coniferous seeds are non-viable.

Hard seeds are seeds with water-impermeable seed coats (e.g. *Fabaceae*) and remain hard even after premoistening. If the viability of these seeds needs to be determined, follow the instructions in Table 6A Column 8.

In order to evaluate seeds properly, it is necessary to expose the embryo and other essential structures. Appropriate light and magnification are indispensable for proper examination. Most seeds contain essential and non-essential tissues. Essential structures are the meristems and all structures recognised as necessary for the development of normal seedlings. Well-developed and differentiated seeds may have the ability to repair small necroses. In this case, superficial necrosis, of limited extent, may be tolerated even within essential tissue. Careful evaluation may also make it possible to distinguish different categories of viable and non-viable seeds.

Viability, as measured by the tetrazolium test, is a distinct and unique quality characteristic of a resting seed. Viability is clearly independent of realisation in a germination test. However, there will be no significant difference between viability and germination percentages only in the case where a seed:

– is not dormant nor hard-seeded or has been properly pre-treated for breaking dormancy and hard-seededness;
– is not infected or has been properly disinfected;
– has not been sprayed in the field nor dressed during processing or fumigated during storage with harmful chemicals;
– has not sprouted;
– has not been deteriorated during germination tests of normal or extended duration;
– has been germinated under optimal conditions.

6.6 Calculation, expression of results and tolerances

In testing a sample, the number of seeds considered viable is determined in each replicate. To check the reliability of a test result, the average percentage of the replicates is calculated to the nearest whole number and compared with Table 6B. The result is considered reliable, if the difference between the highest and the lowest replicate does not exceed the tolerance indicated. Maximum tolerated ranges for replicate differences are the same as for germination tests.

To decide whether two tests, which were performed independently in the same laboratory are compatible, use Table 6C. When the two tests were performed in different laboratories, use Table 6D. For both situations the average percentage viability of the two tests is calculated. The tests are compatible if the difference between the two results does not exceed the tolerance indicated for the calculated average in the respective table.

6.7 Reporting results

The result of a tetrazolium test must be reported under 'Other determinations' as follows:

- The statement 'Tetrazolium test: … % of seeds were viable' must be entered.
- In cases where the testing procedure deviates from that prescribed in Table 6A, any deviating procedure must also be reported. The only variations permitted from procedures given in Table 6A are for premoistening time, tetrazolium concentration, staining temperature or staining time. Precise prescriptions about the limitation of the variations are given in 6.5.
- If individual seeds are tested at the end of the germination test, the result must be reported in accordance with 1.5.2.6 and 5.9.

In addition, in the case of species of *Fabaceae*, one of the following, and only one, must be reported:

either (in cases where the viability percentage of the hard seed is not determined): 'Tetrazolium test: ... % of seeds were viable, ... % of hard seeds found in the test.'

or (in cases where the viability percentage of the hard seed is determined): 'Tetrazolium test: ... % of seeds were viable, ... % of hard seeds included in the percentage of viable seed'

At the discretion of the seed testing laboratory, further information may be reported, e.g. percentage of seeds that were empty, with larvae, broken or decayed.

6.8 Standard procedures for tetrazolium testing

Table 6A contains procedures as follows:

Column 1: Species Where methods are described for a group of species, only those species specifically listed in Table 2A Part 1 may be considered to be covered.

Column 2: Pretreatment Preparation of dry seeds, or premoistening at 20 °C in water (W), or between wet paper (BP), or in sand (S). In the case of two possible pretreatments in Table 6A Part 1, they are separated by a semicolon.

Column 3: Preparation before staining In some cases two different preparation methods can be used. In some cases the low-pressure method can be used to facilitate the infiltration of seed tissues with tetrazolium (TZ) solution.

Column 4: Staining solution Concentration of the tetrazolium solution (percentage).

Column 5: Optimum staining time Optimum staining time in hours based on a temperature of 30 ±2 °C.

Column 6: Preparation for evaluation Preparation for evaluation and tissue to be observed.

Column 7: Permitted non-viable tissue Normally all seeds with a completely stained embryo and those with unstained, flaccid and/or necrotic parts as noted in column 7 are viable. The area of tissue mentioned is the maximum area of unstained, flaccid and/or necrotic tissue permitted to evaluate a seed as viable. For some species the true endosperm, perisperm and/or gametophyte tissue must also be completely stained. For evaluation note that the whole seed structure has to be taken into account, so if a portion is removed during preparation before staining, it is considered as fully stained or as a part of the maximum area that can be unstained.

Column 8: Remarks Additional information.

Table 6A Part 1. Standard procedures for tetrazolium testing: agricultural and horticultural seeds

Species	Pretreatment: type/ minimum time (h)	Preparation before staining	Staining solution (%)	Optimum staining time (h)	Preparation for evaluation	Permitted non-viable tissue	Remarks
1	2	3	4	5	6	7	8
Agropyron spp.	BP/16; W/3	Remove glumes, cut transversely near embryo	1	18	Observe external embryo surface	⅓ radicle	–
	BP/16; W/3	Cut longitudinally through embryo and ¾ of endosperm	1	2	Observe cut surface	⅓ radicle	–
Agrostis spp.	BP/16; W/2	Pierce near embryo	1	18	Remove lemma to expose embryo	⅓ radicle	–
	BP/16; W/2	Transverse incision	1	18	Remove lemma to expose embryo	⅓ radicle	–
Allium spp.	W/18	Cut off thin slice at linear side of seed and longitudinally ⅔ into endosperm near middle of seed between radicle and cotyledons	1	18	Cut longitudinally from flat side through endosperm to expose embryo	None, including endosperm, except small superficial necrosis on outer part of endosperm, not in connection with embryo cavity	–
Alopecurus spp.	BP/18; W/2	Remove glumes, cut transversely near embryo	1	18	Observe external embryo surface	⅓ radicle	–
	BP/18; W/2	Cut longitudinally through embryo and ¾ of endosperm	1	18	Observe external embryo surface	⅓ radicle	–
Anthoxanthum spp.	BP/18	Remove glumes, cut transversely near embryo	1	18	Observe external embryo surface	⅓ radicle	–
Arctium spp.	W/18	Cut longitudinally through seed coat; open wide and extract embryo	1	6	Observe embryo	None	–
Arrhenatherum spp.	BP/16; W/3	Remove glumes, cut transversely near embryo	1	18	Observe external embryo surface	⅓ radicle	–
	BP/16; W/3	Cut longitudinally through embryo and ¾ of endosperm	1	2	Observe cut surface	⅓ radicle	–

Table 6A Part 1. Standard procedures for tetrazolium testing: agricultural and horticultural seeds (cont.)

Species	Pretreatment: type/ minimum time (h)	Preparation before staining	Staining solution (%)	Optimum staining time (h)	Preparation for evaluation	Permitted non-viable tissue	Remarks
1	2	3	4	5	6	7	8
Avena spp.	Remove glumes before premoistening BP/18; W/18	Cut seeds transversely near embryo	1	18	Extract embryo and observe embryo surface including back of scutellum*	Root area except one root initial, ⅓ of extremities of scutellum	*Unstained tissue at centre of scutellum is indicative of heat damage
	Remove glumes before premoistening BP/18; W/18	Cut longitudinally through embryo and ¾ of endosperm	1	2	Observe external embryo surface, cut surface, back of scutellum*	Root area except one root initial, ⅓ of extremities of scutellum	*Unstained tissue at centre of scutellum is indicative of heat damage
Brachiaria spp.	BP/18; W/6	Remove glumes, cut transversely near embryo	1	18	Observe external embryo surface	⅓ radicle	–
	BP/18; W/6	Cut longitudinally through embryo and ¾ of endosperm	1	18	Observe cut surface	⅓ radicle	–
Brassica spp.	W/18	Incise seed coat crosswise at one of the outer cotyledons, avoid damaging hypocotyl or radicle. Remove seed coat by gently pressing	1	3	Observe embryo	⅓ radicle, measured from radicle tip, ⅓ superficial necrosis on the cotyledons not in connection with the hypocotyls	–
Bromus spp.	BP/16; W/3	Remove glumes, cut transversely near embryo	1	18	Observe external embryo surface	⅓ radicle	–
	BP/16; W/3	Cut longitudinally through embryo and ¾ of endosperm	1	2	Observe cut surface	⅓ radicle	–
Chloris gayana	Remove glumes before premoistening BP/16 at 10 °C; W/3	Cut transversely near embryo	1	6	Observe surface of embryo and scutellum	⅓ radicle, measured from radicle tip; in total ⅓ of extremities of scutellum	Empty florets without caryopses are non-viable

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Table 6A Part 1. Standard procedures for tetrazolium testing: agricultural and horticultural seeds (cont.)

Species	Pretreatment: type/ minimum time (h)	Preparation before staining	Staining solution (%)	Optimum staining time (h)	Preparation for evaluation	Permitted non-viable tissue	Remarks
1	2	3	4	5	6	7	8
Cucumis spp.	W/18	Cut off transversally a small part of seed at distal end. Cut lateral longitudinally through seed coat. Remove seed coat and thin inner skin	1	6	Observe embryo	⅓ radicle, measured from radicle tip, ½ of distal end of cotyledons	–
Cynosurus spp.	BP/16; W/3	Remove glumes, cut transversely near embryo	1	18	Observe external embryo surface	⅓ radicle	–
	BP/16; W/3	Cut longitudinally through embryo and ¾ of endosperm	1	2	Observe cut surface	⅓ radicle	–
Dactylis spp.	BP/18; W/2	Remove glumes, cut transversely near embryo	1	18	Observe external embryo surface	⅓ radicle	–
Deschampsia spp.	BP/18; W/2	Remove glumes, cut transversely near embryo	1	18	Observe external embryo surface	⅓ radicle	–
Elymus spp.	BP/16; W/3	Remove glumes, cut transversely near embryo	1	18	Observe external embryo surface	⅓ radicle	–
	BP/16; W/3	Cut longitudinally through embryo and ¾ of endosperm	1	2	Observe cut surface	⅓ radicle	–
Elytrigia spp.	BP/16; W/3	Remove glumes, cut transversely near embryo	1	18	Observe external embryo surface	⅓ radicle	–
	BP/16; W/3	Cut longitudinally through embryo and ¾ of endosperm	1	2	Observe cut surface	⅓ radicle	–
Eragrostis spp.	BP* at ≤ 7 °C/18	Cut transversely near embryo	1	18	Observe external embryo surface	⅓ radicle	*Temperature of ≤7 °C is needed to avoid germination

Table 6A Part 1. Standard procedures for tetrazolium testing: agricultural and horticultural seeds (cont.)

Species	Pretreatment: type/ minimum time (h)	Preparation before staining	Staining solution (%)	Optimum staining time (h)	Preparation for evaluation	Permitted non-viable tissue	Remarks
1	2	3	4	5	6	7	8
Festuca spp.	BP/16; W/3	Remove glumes, cut transversely near embryo	1	18	Observe external embryo surface	⅓ radicle	–
	BP/16; W/3	Cut longitudinally through embryo and ¾ of endosperm	1	2	Observe cut surface	⅓ radicle	–
Helianthus spp.	W/18	Remove pericarp and seed coats from the seed	1	3	Cut longitudinally through cotyledons and the radicle-hypocotyl axis. Observe both sides of the seed	⅓ radicle measured from radicle tip, ½ of distal end of cotyledons if superficial, ⅓ of distal end of cotyledons if pervading	–
Holcus spp.	BP/16; W/3	Remove glumes, cut transversely near embryo	1	18	Observe external embryo surface	⅓ radicle	–
	BP/16; W/3	Cut longitudinally through embryo and ¾ of endosperm	1	2	Observe cut surface	⅓ radicle	–
Hordeum vulgare	W/4	Excise embryo with scutellum	1	3	Observe external embryo surface, back of scutellum*	Root area except one root initial, ⅓ of extremities of scutellum	*Unstained tissue at centre of scutellum is indicative of heat damage
	W/18	Cut longitudinally through embryo and ¾ of endosperm	1	3	Observe external embryo surface, cut surface, back of scutellum*	Root area except one root initial, ⅓ of extremities of scutellum	*Unstained tissue at centre of scutellum is indicative of heat damage
Lactuca spp.	Prepare dry seed, cut longitudinally ¼ through distal end of fruit (achene). W/18	Expose the embryo by gently pressing the seed coat	1	3	Observe embryo	⅓ radicle, measured from radicle tip; ½ of distal end of cotyledons,if superficial; ⅓ at distal end, if pervading	–

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Table 6A Part 1. Standard procedures for tetrazolium testing: agricultural and horticultural seeds (cont.)

Species	Pretreatment: type/ minimum time (h)	Preparation before staining	Staining solution (%)	Optimum staining time (h)	Preparation for evaluation	Permitted non-viable tissue	Remarks
1	2	3	4	5	6	7	8
Lolium spp.	BP/16; W/3	Remove glumes, cut transversely near embryo	1	18	Observe external embryo surface	⅓ radicle	–
	BP/16; W/3	Cut longitudinally through embryo and ¾ of endosperm	1	2	Observe cut surface	⅓ radicle	–
Lotus spp.	W/18	Leave seed intact*	1	18	Remove seed coat to expose embryo	⅓ radicle, ⅓ distal area of cotyledons, ½ if superficial	*If the viability of hard seeds is to be determined, the seed coat can be incised at distal end of cotyledons and soaked (W/4)
Medicago spp.	W/18	Leave seed intact*	1	18	Remove seed coat to expose embryo	⅓ radicle, ⅓ distal area of cotyledons, ½ if superficial	*If the viability of hard seeds is to be determined, the seed coat can be incised at distal end of cotyledons and soaked (W/4)
Melilotus spp.	W/18	Leave seed intact*	1	18	Remove seed coat to expose embryo	⅓ radicle, ⅓ distal area of cotyledons, ½ if superficial	*If the viability of hard seeds is to be determined, the seed coat can be incised at distal end of cotyledons and soaked (W/4)
Ocimum spp.	W/18	Cut longitudinally along the side of the fruit and seed coat; open wide and extract embryo	1	4	Observe embryo	⅓ radicle, small superficial necrosis at distal end of cotyledons	If slime develops, soak seeds 15–20 min in 1 % alunite solution; blot gently with filter paper
Onobrychis spp.	W/18	Leave seed intact*	1	18	Remove seed coat to expose embryo	⅓ radicle, ⅓ distal area of cotyledons, ½ if superficial	*If the viability of hard seeds is to be determined, the seed coat can be incised at distal end of cotyledons and soaked (W/4)
Ornithopus spp.	W/18	Leave seed intact*	1	18	Remove seed coat to expose embryo	⅓ radicle, ⅓ distal area of cotyledons, ½ if superficial	*If the viability of hard seeds is to be determined; the seed coat can be incised at distal end of cotyledons and soaked (W/4)

Table 6A Part 1. Standard procedures for tetrazolium testing: agricultural and horticultural seeds (cont.)

Species	Pretreatment: type/ minimum time (h)	Preparation before staining	Staining solution (%)	Optimum staining time (h)	Preparation for evaluation	Permitted non-viable tissue	Remarks
1	2	3	4	5	6	7	8
Oryza sativa	W/18	Cut longitudinally through embryo and ¾ of endosperm*	1	2	Observe cut surfaces	⅔ radicle	*If necessary remove lemma
Panicum spp.	BP/18; W/6	Remove glumes, cut transversely near embryo	1	18	Expose embryo and observe external surface	⅓ radicle, ¼ distal parts of scutellum	Empty florets without caryopses are non-viable
	BP/18; W/6	Cut longitudinally through distal ½ of endosperm	1	18	Expose embryo and observe external surface	⅓ radicle, ¼ distal parts of scutellum	Empty florets without caryopses are non-viable
Pascopyrum spp.	BP/16; W/3	Remove glumes, cut transversely near embryo	1	18	Observe external embryo surface	⅓ radicle	–
	BP/16; W/3	Cut longitudinally through embryo and ¾ of endosperm	1	2	Observe cut surface	⅓ radicle	–
Phalaris spp.	BP/18; W/6	Remove glumes, cut transversely near embryo	1	18	Expose embryo and observe external surface	⅓ radicle, ¼ distal parts of scutellum	–
	BP/18; W/6	Cut longitudinally through distal ½ of endosperm	1	18	Expose embryo and observe external surface	⅓ radicle, ¼ distal parts of scutellum	–
Phleum spp.	BP/16; W/2	Pierce near embryo	1	18	Remove lemma to expose embryo	⅓ radicle	–
	BP/16; W/2	Transverse incision	1	18	Remove lemma to expose embryo	⅓ radicle	–
Poa spp.	BP/16; W/2	Pierce near embryo	1	18	Remove lemma to expose embryo	⅓ radicle	–
	BP/16; W/2	Incision	1	18	Remove lemma to expose embryo	⅓ radicle	–

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Table 6A Part 1. Standard procedures for tetrazolium testing: agricultural and horticultural seeds (cont.)

Species	Pretreatment: type/ minimum time (h)	Preparation before staining	Staining solution (%)	Optimum staining time (h)	Preparation for evaluation	Permitted non-viable tissue	Remarks
1	2	3	4	5	6	7	8
Pseudoroegneria spp.	BP/16; W/3	Remove glumes, cut transversely near embryo	1	18	Observe external embryo surface	⅓ radicle	–
	BP/16; W/3	Cut longitudinally through embryo and ¾ of endosperm	1	2	Observe cut surface	⅓ radicle	–
Secale cereale	W/4	Excise embryo with scutellum	1	3	Observe external embryo surface, back of scutellum*	Root area except one root initial, ⅓ of extremities of scutellum	*Unstained tissue at centre of scutellum is indicative of heat damage
	W/18	Cut longitudinally through embryo and ¾ of endosperm	1	3	Observe external embryo surface, cut surface, back of scutellum*	Root area except one root initial, ⅓ of extremities of scutellum	*Unstained tissue at centre of scutellum is indicative of heat damage
Setaria spp.	Remove lemma and palea before premoistening. W* at 7 °C/5	Cut transversely near embryo	1	16	Observe external embryo, cut longitudinally through embryo, cut surface	⅓ radicle measured from radicle tip, ¼ distal part of scutellum	*Temperature of 7 °C is needed to decrease sprouting during premoistening
Solanum (sect. *Lycopersicon*) spp. and hybrids	W/18	Cut between radicle and cotyledons ⅓ into endosperm	1	18	Cut the seed at flat side into two halves; observe cut surfaces	None	Sometimes 42 h staining gives clearer and darker staining. Size of embryo must be more than ½ normal size
Sorghum spp.	W* at 7 °C/18	Cut longitudinally through embryo and ¼ of endosperm	1	3	Observe cut surface	⅓ radicle measured from radicle tip	*Temperature of 7 °C is needed to decrease sprouting during premoistening
Trifolium spp.	W/18	Leave seed intact*	1	18	Remove seed coat to expose embryo	⅓ radicle, ⅓ distal area of cotyledons, ½ if superficial	*If the viability of hard seeds is to be determined, the seed coat can be in cised at distal end of cotyledons and soaked (W/4)
Trisetum spp.	BP/18; W/2	Remove glumes, cut transversely near embryo	1	18	Observe external embryo surface	⅓ radicle	–

Table 6A Part 1. Standard procedures for tetrazolium testing: agricultural and horticultural seeds (cont.)

Species	Pretreatment: type/ minimum time (h)	Preparation before staining	Staining solution (%)	Optimum staining time (h)	Preparation for evaluation	Permitted non-viable tissue	Remarks
1	2	3	4	5	6	7	8
×*Triticosecale*	W/4	Excise embryo with scutellum	1	3	Observe external embryo surface, back of scutellum*	Root area except one root initial, ⅓ of extremities of scutellum	*Unstained tissue at centre of scutellum is indicative of heat damage
	W/18	Cut longitudinally through embryo and ¾ of endosperm	1	3	Observe external embryo surface, cut surface, back of scutellum*	Root area except one root initial, ⅓ of extremities of scutellum	*Unstained tissue at centre of scutellum is indicative of heat damage
Triticum spp.	W/4	Excise embryo with scutellum	1	3	Observe external embryo surface, back of scutellum*	Root area except one root initial, ⅓ of extremities of scutellum	*Unstained tissue at centre of scutellum is indicative of heat damage
	W/ 18	Cut longitudinally through embryo and ¾ of endosperm	1	3	Observe external embryo surface, cut surface, back of scutellum*	Root area except one root initial, ⅓ of extremities of scutellum	*Unstained tissue at centre of scutellum is indicative of heat damage
Zea mays	W/18	Cut longitudinally through embryo and ¾ of endosperm	1	2	Observe cut surfaces*	Primary root, ⅓ extremities of scutellum	*Unstained tissue at centre of scutellum is indicative of heat damage

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Table 6A Part 2. Standard procedures for tetrazolium testing: tree and shrub seeds

Species	Pretreatment: type/min. time (h)	Preparation before staining	Staining solution (%)	Optimum staining time (h)	Preparation for evaluation	Permitted non-viable tissue	Remarks
1	2	3	4	5	6	7	8
Abies spp.	Prepare the dry seeds or W/18	Cut transversely at both ends, to open embryo cavity. Treat TZ-imbibed seeds with low pressure (optional)	1	18	Cut longitudinally through endosperm and expose embryo; remove seed coat	None, except small superficial necrosis on outer part of endosperm, not in connection with embryo cavity	Old and dry seeds may give more consistent results if imbibed for 48 h
	W/18	Cut longitudinally beside embryo	1	12	Expose embryo; remove seed coat	None, except small superficial necrosis on outer part of endosperm, not in connection with embryo cavity	Old and dry seeds may give more consistent results if imbibed for 48 h
Acer campestre	W/18	Cut pericarp along 3 sides except at the connecting link between the 2 fruits; remove pericarp. Cut small piece of seed coat and resoak for 3 h. Remove seed coat	1	18	–	Radicle tip	Old and dry seeds may give more consistent results with prechill. BP; S 14 days at 3–5 °C
Acer ginnala	W/18*	Cut ⅛ of the fruit from the wing's end	1	24	Extract embryo from pericarp and seed coat	Radicle tip, small necrosis at distal end of cotyledons	Old and dry seeds may give more consistent results with prechill. *Optional: S; BP. Prechill 10–14 days at 3–5 °C
	W/18*	Remove pericarp and slit through seed coat along edge of cotyledon	1	18	Split cotyledons apart to expose embryo axis	Radicle tip, small necrosis at distal end of cotyledons	Old and dry seeds may give more consistent results with prechill. *Optional: S; BP. Prechill 10–14 days at 3–5 °C

Table 6A Part 2. Standard procedures for tetrazolium testing: tree and shrub seeds (continued)

Species	Pretreatment: type/min. time (h)	Preparation before staining	Staining solution (%)	Optimum staining time (h)	Preparation for evaluation	Permitted non-viable tissue	Remarks
1	2	3	4	5	6	7	8
Acer palmatum	W/18*	Cut pericarp along 3 sides except at the connecting link between the 2 fruits; remove pericarp	1	18	Extract embryo from pericarp and seed coat	Radicle tip, small necrosis of cotyledons if superficial	Old and dry seeds may give more consistent results with prechill. *Optional: S; BP. Prechill 10–14 days at 3–5 °C
	W/18	Cut pericarp along 3 sides except at the connecting link between the 2 fruits; remove pericarp. Cut small piece of seed coat and resoak for a few hours. Remove seed coat	1	18	–	Radicle tip, small necrosis of cotyledons if superficial	Old and dry seeds may give more consistent results with prechill
Acer platanoides and *A. pseudoplatanus* only	W/18*	Remove pericarp. Cut small piece of seed coat and resoak for a few hours, remove seed coat	1	8	Observe embryo	Radicle tip, small necrosis of cotyledons if superficial except near radicle/hypocotyl	Old and dry seeds may give more consistent results with prechill. *Optional: S; BP. Prechill 10–14 days at 3–5 °C
Acer, all other species	Remove wings and soak W/18*	Remove pericarp, cut seed coat opposite radicle; resoak for about 3 h. Remove seed coat	1	18	Observe embryo	Radicle tip, small necrosis of cotyledons if superficial except near radicle/hypocotyl	Old and dry seeds may give more consistent results with prechill. *Optional: S; BP. Prechill 10–14 days at 3–5 °C
Amorpha fruticosa	W/24	Cut off ⅓ end of seed. Do not remove testa from lower portion	1	18	Remove seed coat	(None)	–

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Table 6A Part 2. Standard procedures for tetrazolium testing: tree and shrub seeds (continued)

Species	Pretreatment: type/min. time (h)	Preparation before staining	Staining solution (%)	Optimum staining time (h)	Preparation for evaluation	Permitted non-viable tissue	Remarks
1	2	3	4	5	6	7	8
Berberis spp.	W/18	Cut transversely ⅓ from distal end	1	18	Cut longitudinally through endosperm and expose embryo	None, including endosperm	–
	W/18	Cut longitudinally 2 pieces of endosperm; at least one cut should open embryo cavity	1	18	Cut longitudinally through endosperm and expose embryo	None, including endosperm	–
Calocedrus spp.	Prepare the dry seeds or W/18	Cut transversely at both ends to open embryo cavity. Treat TZ imbibed seeds with low pressure	1	18	Cut longitudinally through endosperm and expose embryo; remove seed coat	None, except small superficial necrosis on outer part of endosperm, not in connection with embryo cavity	Old and dry seeds may give more consistent results if imbibed for 48 h
	W/18	Cut longitudinally beside embryo	1	12	Expose embryo; remove seed coat	None, except small superficial necrosis on outer part of endosperm, not in connection with embryo cavity	Old and dry seeds may give more consistent results if imbibed for 48 h
Carpinus spp.	W/18*	Cut transversely ⅓ from distal end	1	18	Extract embryo from pericarp and seed coat	None	*Cutting before soaking can sometimes avoid preparation damage
Chamaecyparis spp.	Prepare the dry seeds or W/18	Cut transversely ⅓ from distal end to open embryo cavity	1	18	Cut longitudinally through endosperm and expose embryo, remove seed coat	None, including endosperm	–
	Prepare the dry seeds or W/18	Cut longitudinally beside embryo	1	18	Expose embryo; remove seed coat	None, including endosperm	–
Cornus mas	Prepare the dry seed or W/48	Cut transversely ⅓ from distal end to open embryo cavity	1	48*	Extract embryo	None, including endosperm as far as visible	*Low pressure can be helpful to shorten staining time to 18 h
Cornus spp.	Prepare the dry seed or W/48	Cut transversely ¼ from distal end	1	18	Extract embryo and endosperm	None, including endosperm	–

Table 6A Part 2. Standard procedures for tetrazolium testing: tree and shrub seeds (continued)

Species	Pretreatment: type/min. time (h)	Preparation before staining	Staining solution (%)	Optimum staining time (h)	Preparation for evaluation	Permitted non-viable tissue	Remarks
1	2	3	4	5	6	7	8
Corylus spp.	Crack the nuts. and soak W/18	Cut 1–2 mm of cotyledons at distal end, split longitudinally between them (should not fall to pieces)	1	18	Spread cotyledons apart and cut, especially through unstained parts	Radicle tip, superficial necrosis at distal end of cotyledons; centre of ventral side of cotyledon, if not exceeding ⅓ of diameter	'Hollow hearts' may disappear if nuts are moistened BP 7 days at 20 °C before cracking
Cotoneaster spp.	Prepare the dry seed or W/18	Cut transversely ⅓ from distal end	1	18	Extract embryo	Radicle tip, ⅓ distal area of cotyledons, ½ if superficial	–
Crataegus spp.	Prepare the dry seed or W/18*	Cut transversely ⅓ from distal end	1	18	Extract embryo	Radicle tip, ⅓ distal area of cotyledons, ½ if superficial	*Cutting before soaking can sometimes avoid preparation damage
Elaeagnus spp.	W/18	Cut transversely ⅓ from distal end, opposite to the stalk base, to open embryo cavity	1	18	Cut longitudinally through endosperm and expose embryo	Radicle tip, ⅓ distal area of cotyledons, ½ if superficial	–
	W/18	Cut longitudinally alongside embryo, expose embryo, imbibe 1 h in water, remove seed coat	1	18	Observe embryo	Radicle tip, ⅓ distal area of cotyledons, ½ if superficial	–
Euonymus spp.	W/18	Cut transversely ⅓ from distal end	1	18	Cut longitudinally through endosperm and expose embryo	None, including endosperm	–
	W/18	Cut longitudinally 2 pieces of endosperm; at least 1 cut should open embryo cavity	1	18	Cut longitudinally through endosperm and expose embryo	None, including endosperm	–
Fagus spp.	Remove pericarp of dry seeds* and W/18	Remove seed coat	1	18	Open cotyledons	Radicle tip, ⅓ distal area of cotyledons if superficial	*Pericarp of very dry seeds is easier to remove after soaking for a few hours

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Table 6A Part 2. Standard procedures for tetrazolium testing: tree and shrub seeds (continued)

Species	Pretreatment: type/min. time (h)	Preparation before staining	Staining solution (%)	Optimum staining time (h)	Preparation for evaluation	Permitted non-viable tissue	Remarks
1	2	3	4	5	6	7	8
Fraxinus spp.	Remove pericarp of dry seeds and soak W/18	Cut a small piece off longitudinally on both sides, to open embryo cavity	1	18*	Expose embryo by splitting endosperm into two halves	None, except small necrosis on endosperm away from embryo	*Freshly harvested seeds need only 8 h
Ginkgo biloba	Crack the dry seeds	Cut longitudinally through the middle of the endosperm to open embryo cavity	1	18	Open endosperm, expose embryo	None, including endosperm	–
Ilex spp.	W/18	Cut transversely ⅓ from distal end and cut longitudinally towards embryo	1	18	Expose embryo and endosperm	None, including endosperm	Use binocular as embryo is very small
	W/18	Cut longitudinally through seed coat and into endosperm	1	18	Expose embryo and endosperm	None, including endosperm	Use binocular as embryo is very small
Juniperus spp.	Prepare the dry seed or W/18*	Cut transversely ⅓ from distal end to open embryo cavity	1	18	Cut longitudinally through endosperm and expose embryo, remove seed coat	None, including endosperm	*If necessary remove seeds from surrounding structures
	W/18*	Cut longitudinally beside embryo	1	18	Expose embryo; remove seed coat	None, including endosperm	*If necessary remove seeds from surrounding structures
Koelreuteria spp.	Cut dry seeds at base of stalk and soak W/18	Remove pericarp, soak additionally for about 3 h. Remove seed coat	1	18	–	Radicle tip, ⅓ distal area of cotyledons; ½ if superficial	–
Ligustrum spp.	W/18	Cut transversely ¼ from distal end	1	18	Cut longitudinally through embryo and endosperm	None, including endosperm	–
	W/18	Cut longitudinally a piece of endosperm on both sides	1	18	Expose embryo; remove seed coat	None, including endosperm	–
Liriodendron spp.	W/18	Cut transversely opposite to wing's end a piece of pericarp and endosperm	1	18	Cut longitudinally through endosperm and expose embryo	None, including endosperm	–
	W/18	Cut longitudinally into endosperm	1	18	Expose embryo; remove seed coat	None, including endosperm	–

Table 6A Part 2. Standard procedures for tetrazolium testing: tree and shrub seeds (continued)

Species	Pretreatment: type/min. time (h)	Preparation before staining	Staining solution (%)	Optimum staining time (h)	Preparation for evaluation	Permitted non-viable tissue	Remarks
1	2	3	4	5	6	7	8
Malus spp.	W/18	Remove seed coat	1	18	Observe embryo	Radicle tip, ⅓ distal area of cotyledons, ½ if superficial	–
Malva spp.	W/18	Cut transversely a thin slice off from reverse side of the seed	1	18	Remove seed coat	None	Embryo can become brittle if swelling occurs quickly
*Pinus, hard-shelled species**	Crack the dry seeds or W/18	Cut transversely ⅓ from distal end of endosperm to open embryo cavity	1	18	Cut longitudinally through endosperm and expose embryo; remove seed coat	None, including endosperm, except small superficial necrosis on outer part of endosperm, not in connection with embryo cavity	Embryos shorter than ⅓ embryo cavity are non-viable *E.g. *Pinus cembra, Pinus coulteri, Pinus koraiensis*
*Pinus, thin-skinned species**	Prepare the dry seeds or W/18	Cut transversely ⅓ from distal end of endosperm to open embryo cavity	1	18	Extract embryo and endosperm from seed coat	None, including endosperm, except small superficial necrosis on outer part of endosperm, not in connection with embryo cavity	Embryos shorter than ⅓ embryo cavity are non-viable *E.g. *Pinus nigra, Pinus mugo*
	Prepare the dry seeds or W/18	Cut longitudinally beside embryo	1	18	Extract embryo and endosperm from seed coat	None, including endosperm, except small superficial necrosis on outer part of endosperm, not in connection with embryo cavity	Embryos shorter than ⅓ embryo cavity are non-viable
Prunus spp.*	Crack stones and soak W/18, change water if necessary (i.e. if smells of bitter almonds)	Remove seed coat **	1	18	Spread cotyledons apart	Radicle tip, ⅓ distal area of cotyledons if superficial	*Large-seeded species need longer staining time (24 h) ** Open cotyledons carefully in *Prunus persica, Prunus domestica*

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Table 6A Part 2. Standard procedures for tetrazolium testing: tree and shrub seeds (continued)

Species	Pretreatment: type/min. time (h)	Preparation before staining	Staining solution (%)	Optimum staining time (h)	Preparation for evaluation	Permitted non-viable tissue	Remarks
1	2	3	4	5	6	7	8
Pseudotsuga spp.	Prepare the dry seeds or W/18	Cut transversely ⅓ from distal end of endosperm to open embryo cavity	1	18	Cut longitudinally through endosperm and expose embryo; remove seed coat	None, except small superficial necrosis on endosperm at distal end	–
	Prepare the dry seed or W/18	Cut longitudinally beside embryo	1	12	Expose embryo; remove seed coat	None, except small superficial necrosis on endosperm at distal end	–
Pyrus spp.	W/18	Remove seed coat	1	18	Observe embryo	Radicle tip, ⅓ distal area of cotyledons, ½ if superficial	–
Rosa spp.	Prepare the dry seed or W/18*	Cut transversely ⅓ from distal end	1	18	Extract embryo	Radicle tip, ⅓ distal area of cotyledons, ½ if superficial	*Cutting before soaking can sometimes prevent preparation damage
Styphnolobium spp.	Prepare the dry seeds or W/24	Cut transversely a thin slice off from distal end	1	18	Remove seed coat	Radicle tip, ½ distal area of cotyledons	–
Sorbus spp.	W/18	Cut transversely ⅓ from distal end	1	18	Extract embryo	Radicle tip, ⅓ distal area of cotyledons, ½ if superficial	–
Taxodium distichum	Prepare the dry seed or W/18	Cut transversely ¼ at both ends to open embryo cavity	1	18	Cut longitudinally through endosperm and expose embryo; remove seed coat	None, including endosperm	–
	Prepare the dry seed or W/18	Cut longitudinally beside embryo	1	18	Expose embryo; remove seed coat	None, including endosperm	–
Taxus spp.	W/18	Cut transversely ¼ from distal end (including a piece of endosperm)	1	24	Cut longitudinally through endosperm and expose embryo	None including endosperm	–
	W/18	Cut longitudinally beside embryo	1	24	Expose embryo; remove seed coat	None, including endosperm	–

Table 6A Part 2. Standard procedures for tetrazolium testing: tree and shrub seeds (continued)

Species	Pretreatment: type/min. time (h)	Preparation before staining	Staining solution (%)	Optimum staining time (h)	Preparation for evaluation	Permitted non-viable tissue	Remarks
1	2	3	4	5	6	7	8
Tilia spp.	Remove peri-carp, cut off stalk base and soak W/18	Remove seed coat	1	18	Open endosperm with a small incision and expose embryo	None, except small necrosis on endosperm at distal end, if superficial	–
Viburnum spp.	W/18	Cut seed coat along 3 sides (distal and long sides); remove seed coat	1	18	Cut longitudinally flat through endosperm and expose embryo, begin at the region of embryo	None, except small necrosis on endosperm opposite to the embryo	–

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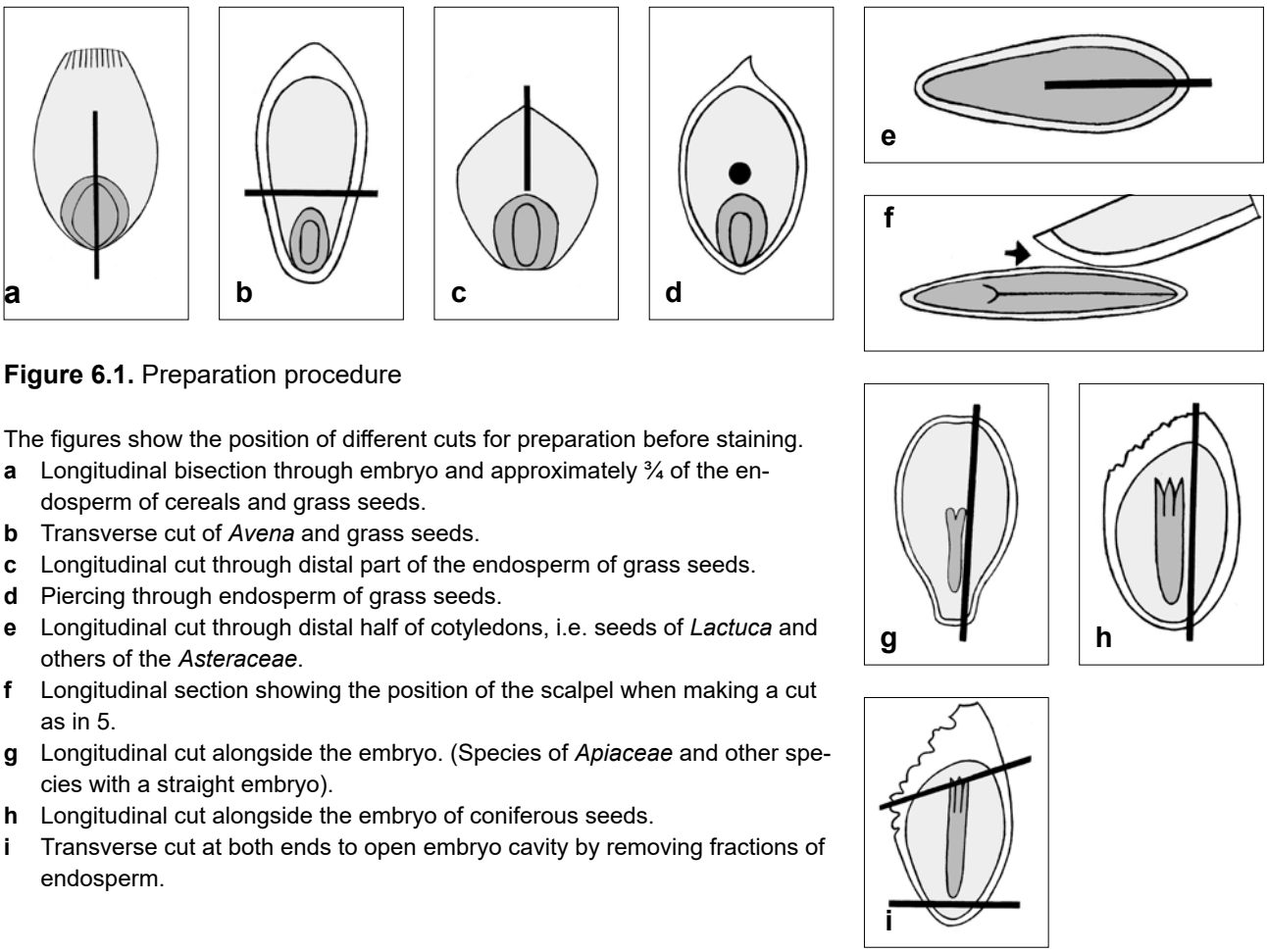
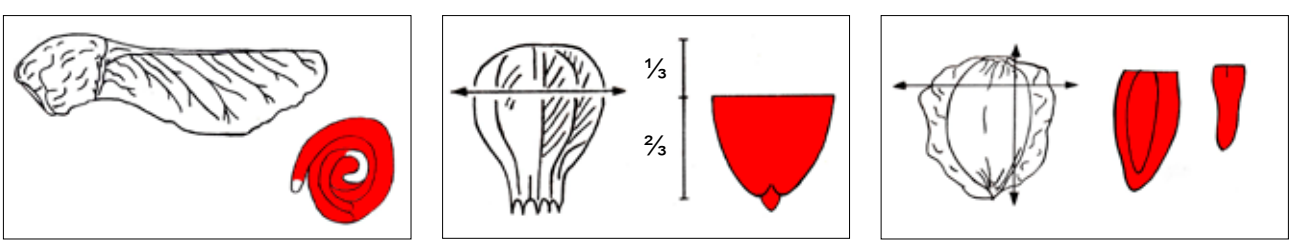


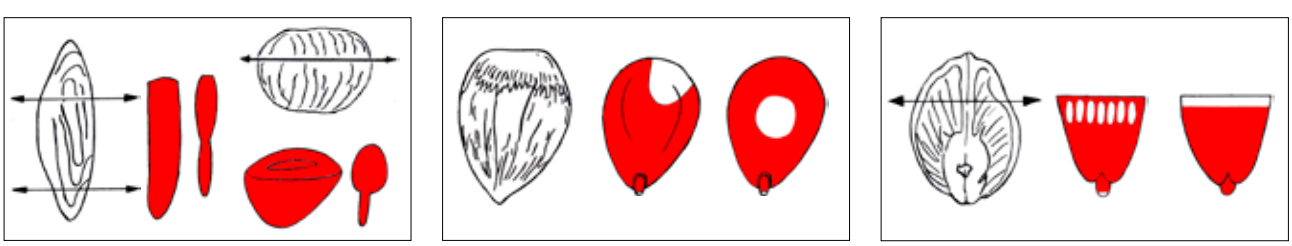
Figure 6.1. Preparation procedure

The figures show the position of different cuts for preparation before staining.

- **a** Longitudinal bisection through embryo and approximately ¾ of the endosperm of cereals and grass seeds.
- **b** Transverse cut of *Avena* and grass seeds.
- **c** Longitudinal cut through distal part of the endosperm of grass seeds.
- **d** Piercing through endosperm of grass seeds.
- **e** Longitudinal cut through distal half of cotyledons, i.e. seeds of *Lactuca* and others of the *Asteraceae*.
- **f** Longitudinal section showing the position of the scalpel when making a cut as in 5.
- **g** Longitudinal cut alongside the embryo. (Species of *Apiaceae* and other species with a straight embryo).
- **h** Longitudinal cut alongside the embryo of coniferous seeds.
- **i** Transverse cut at both ends to open embryo cavity by removing fractions of endosperm.

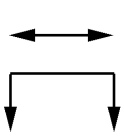


Acer *Carpinus* *Chamaecyparis thyoides*



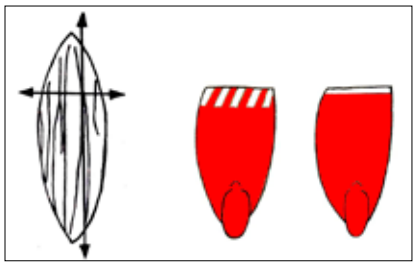
Cornus *Corylus* *Cotoneaster, Crataegus, Rosa* and *Sorbus*

Figure 6.2. Preparation and evaluation procedure for tree and shrub seeds.
All examples shown are viable seeds (continued on following page).

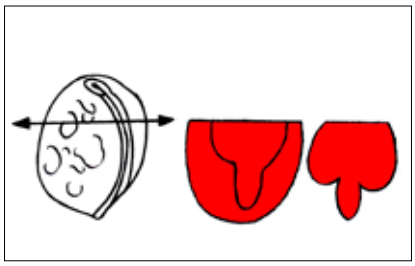


Position of a cut going all through the tissue.

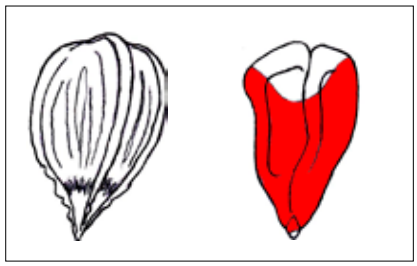
Position of a cut going into the endosperm.



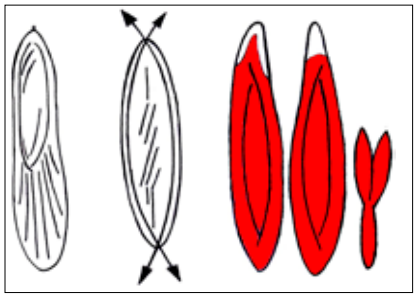
Elaeagnus



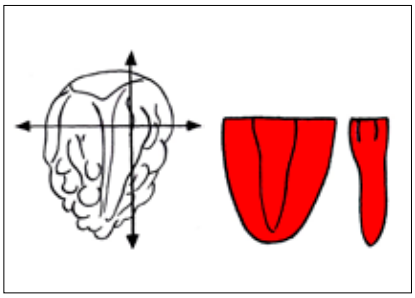
Euonymus



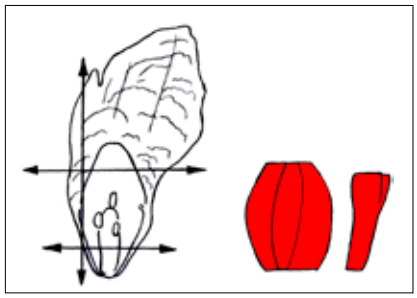
Fagus



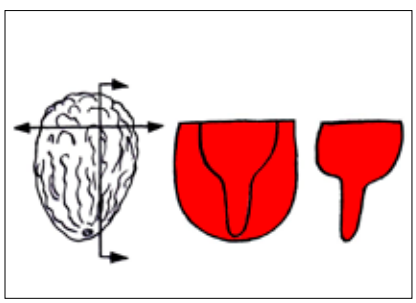
Fraxinus



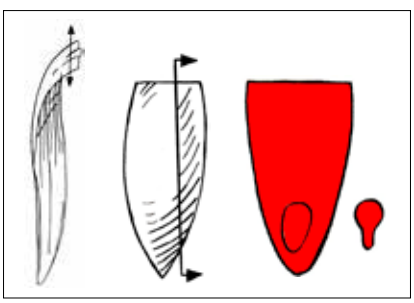
Juniperus



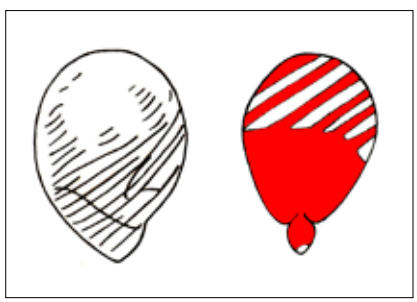
Libocedrus



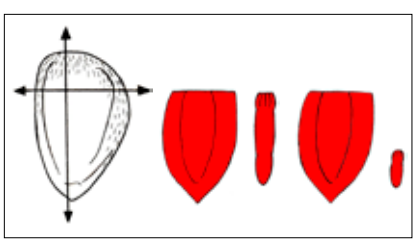
Ligustrum



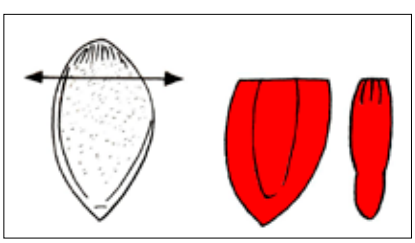
Liriodendron tulipifera



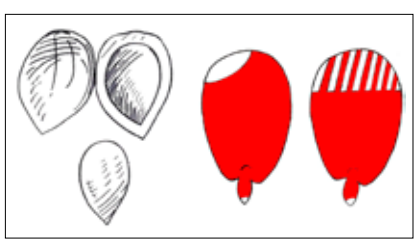
Malus and *Pyrus*



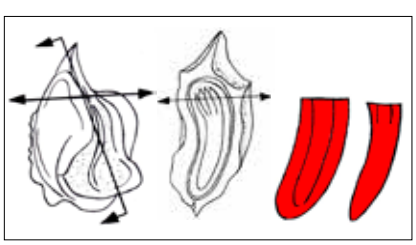
Pinus (hard-shelled)



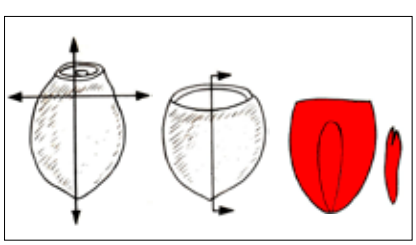
Pinus (thin-skinned)



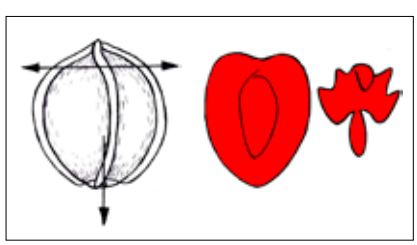
Prunus



Taxodium distichum

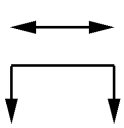


Taxus



Tilia

Figure 6.2. (Cont.) Preparation and evaluation procedure for tree and shrub seeds.
All examples shown are viable seeds.



Position of a cut going all through the tissue.

Position of a cut going into the endosperm.

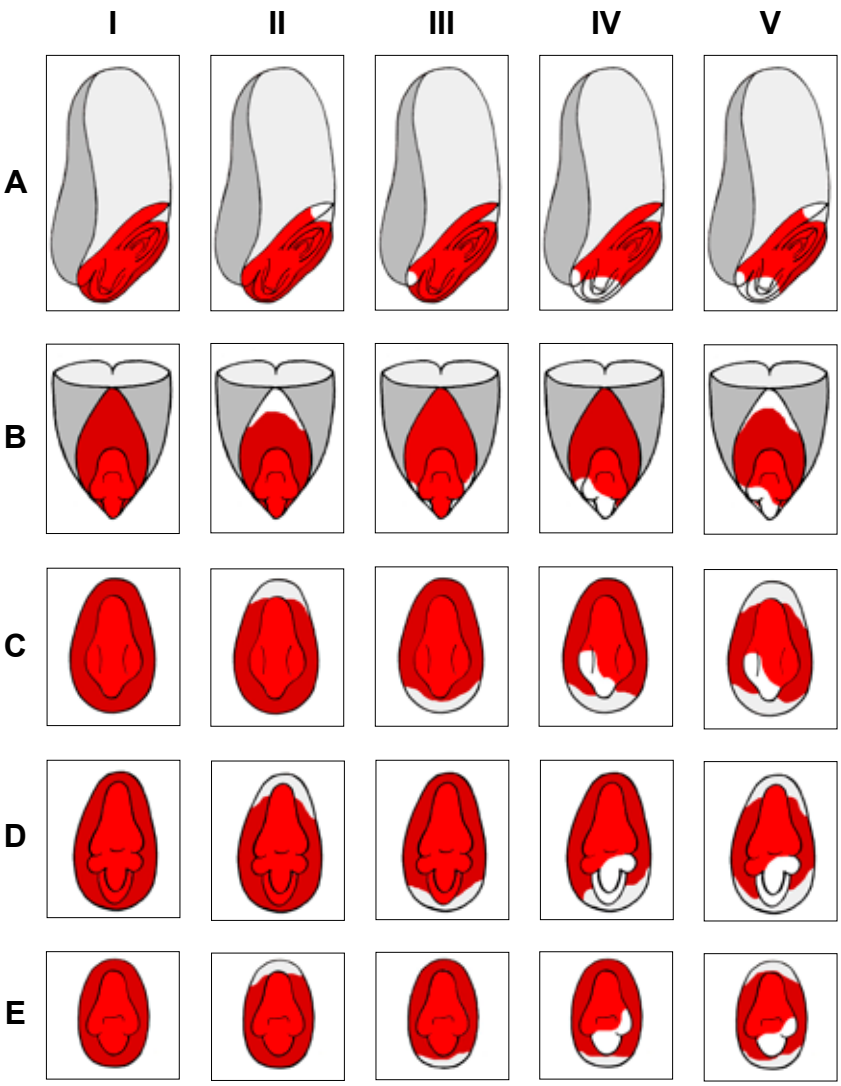


Figure 6.3a. Evaluation guide for cereals: viable seeds.

The figures in column **I** are all completely stained and viable. Columns **II–V** show the maximum area of unstained, flaccid or necrotic tissue permitted in viable seeds.

A The figures are representative for *Triticum, ×Triticosecale, Secale, Hordeum* and *Avena* when prepared by bisection or bisected for evaluation
B *Avena* prepared by transverse cutting
C *Hordeum* prepared by the excised embryo method
D *Secale* prepared by the excised embryo method
E *Triticum* and *×Triticosecale* prepared by the excised embryo method

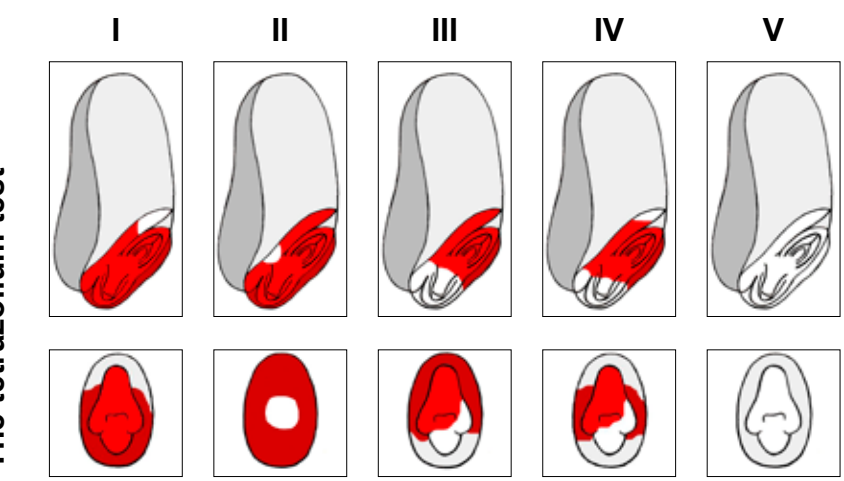


Figure 6.3b. Evaluation guide for cereals: non-viable seeds.

A Figures are representative for *Triticum, ×Triticosecale, Secale, Hordeum* and *Avena* when prepared by bisection or bisected for evaluation
B *Triticum* prepared by excised embryo method
B II: *Triticum* scutellum seen from back

6.9 Tolerance tables

Table 6B indicates the maximum range (i.e. difference between highest and lowest) in percentage of viable seeds tolerable between replicates, allowing for random sampling variation only at 0.025 probability. To find the maximum tolerated range in any case calculate the average percentage, to the nearest whole number, of the four replicates: if necessary, form 100-seed replicates by combining the subreplicates of 50 or 25 seeds which were closest together in the incubator. Locate the average in column 1 or 2 of the table and read off the maximum tolerated range opposite in column 3.

The tolerances are based on Table G1, column D, in Miles (1963).

In **Table 6C**, the tolerances take into account the experimental error within a laboratory as described in the TEZ Committee Report 1998–2001 and are not extracted from Miles (1963).

In **Table 6D**, the tolerances take into account the experimental error between the laboratories as described in the TEZ Committee Report 1998–2001 and are not extracted from Miles (1963).

Miles, S.R. (1963). Handbook of Tolerances and of Measures of Precision for Seed Testing. *Proceedings of the International Seed Testing Association*, **28** (3), 644.

Table 6B. Maximum tolerated range between four replicates of 100 seeds in one tetrazolium test (two-way test at 2.5 % significance level)

Average viability (%)		Maximum range
1	2	3
99	2	5
98	3	6
97	4	7
96	5	8
95	6	9
93–94	7–8	10
91–92	9–10	11
89–90	11–12	12
87–88	13–14	13
84–86	15–17	14
81–83	18–20	15
78–80	21–23	16
73–77	24–28	17
67–72	29–34	18
56–66	35–45	19
51–55	46–50	20

Table 6C. Tolerances for tetrazolium viability tests on the same or a different submitted sample when tests are made in the same laboratory each on 400 seeds (two-way test at 2.5 % significance level)

Average viability (%)		Maximum range
1	2	3
98–99	2–3	2
96–97	4–5	3
93–95	6–8	4
89–92	9–12	5
83–88	13–18	6
75–82	19–26	7
58–74	27–43	8
51–57	44–50	9

Table 6D. Tolerances for tetrazolium viability tests on two different submitted samples in different laboratories each on 400 seeds (one-way test at 5 % significance level)

Average viability (%)		Maximum range
1	2	3
99	2	4
98	3	5
97	4	6
95–96	5–6	7
93–94	7–8	8
91–92	9–10	9
89–90	11–12	10
86–88	13–15	11
82–85	16–19	12
78–81	20–23	13
73–77	24–28	14
65–72	29–36	15
51–64	37–50	16

Chapter 7: Seed health testing

7.1 Object

The object of a seed health test is to determine the health status of a seed sample, and by inference that of the seed lot.

Health testing of seed is important for four reasons:

a) Seed-borne inoculum may give rise to progressive disease development in the field and reduce the commercial value of the crop.
b) Imported seed lots may introduce diseases into new regions. Tests to meet quarantine requirements may therefore be necessary.
c) Seed health testing may elucidate seedling evaluation and causes of poor germination or field establishment and thus supplement germination testing.
d) Seed health test results can/may indicate the necessity to carry out/perform seed lot treatment(s) in order to eradicate seed-borne pathogens or to reduce the risk of disease transmission.

7.2 Definitions

7.2.1 Seed health

Health of seed refers primarily to the presence or absence of disease-causing organisms, such as fungi, bacteria and viruses, and animal pests, including nematodes and insects, but physiological conditions such as trace element deficiency may be involved.

7.2.2 Pretreatment

Any physical or chemical laboratory treatment of the working sample preceding incubation, given solely to facilitate testing.

7.2.3 Seed treatment

See 2.2.12. For seed health testing, a seed lot may be treated for the purpose of controlling plant pathogens or insect pests, or correcting trace element deficiencies.

7.2.4 ISTA Seed Health Method Validation Programme

Before publication in the *International Rules for Seed Testing*, the ISTA seed health testing methods (new or equivalent) are validated. The principles and factors which should be considered in the validation of methods for the detection of seed-borne pathogens are described in the *ISTA Handbook of Method Validation for the Detection of Seed-borne Pathogens*.

7.3 General principles

Seed health testing should be performed using methods and equipment which have been tested to ensure they are fit for purpose. Different methods of testing are available, varying in sensitivity and reproducibility and in the amount of training and equipment required. The method used will depend on the pathogen or condition to be investigated, the species of the seed, and the purpose of the test. Selection of the method and evaluation of the results requires knowledge and experience of the methods available. The presence or absence of disease organisms, pests and deleterious physiological conditions specified by the sender is estimated as accurately as the method used permits.

7.4 Procedures

7.4.1 Working sample

The entire submitted sample, or a proportion of it, depending on the test method, may be used as a working sample. The sample should be packaged and submitted in a manner which will not alter its seed health status.

Exceptionally, a submitted sample larger than that prescribed in 2.8 may be required and in such cases the sampler must be instructed accordingly.

When a portion of the submitted sample is required as a working sample, the reduction must be carried out in accordance with 2.5.2, taking appropriate precautions to avoid cross-contamination.

Normally the working sample must not be less than that specified in the method description.

Replicates containing a specified number of seeds, if required, must be taken at random from a subsample after thorough mixing.

7.4.2 Seed treatment

Test results may be influenced by treatment applied to the seed lot. Seed health tests on treated seeds will generally deliver unreliable test results caused by masking or inhibition of the growth of the target organism. Individual Method Sheets will determine whether the testing of treated seeds is acceptable.

7.4.3 Sample storage

The microflora of seed, in the lot or the sample, may change considerably during storage in conditions in which seed viability is satisfactorily maintained. The selection of the appropriate storage conditions must take into account the optimal storage temperature and container in order to maintain sample integrity.

Abundant development of saprophytic moulds including 'storage fungi' in tests can be an indication that the seed is not of good quality due to unfavourable harvesting, processing or storage conditions, or to ageing. Some fungi (such as *Rhizopus* spp.) spread rapidly over tests on blotters and may rot originally healthy seedlings or may interfere with outgrowth of the pathogen from the plated infected seeds. Pretreatment as described in the specific method may be advisable.

7.4.4 Specific directions

Specific seed health testing methods are published online on the ISTA web site at:

www.seedtest.org/seedhealthmethods

Seed health methods are normally based on one host, and one pathogen, but multi-pathogen methods may be included. Before publication, all seed health test methods must be validated through the ISTA Seed Health Method Validation Programme. Methods validated in this way at the time of printing are listed in Table 7A. Additions, updates and deletions to this list can be found on the ISTA web site (www.seedtest.org/seedhealthmethods). The definitive list is held by the ISTA Secretariat. It is the responsibility of the laboratory using the method to consult this list.

7.5 Calculation and expression of results

Results are expressed either qualitatively or quantitatively as specified in the individual prescribed methods.

7.6 Reporting results

The results of a test for seed health must be reported under 'Other determinations' as follows:

- either qualitative or quantitative results, as specified in the individual methods;
- negative and positive results, as specified in the individual methods;
- the scientific name of the pathogen detected;
- the percentage of infected seeds;
- the method used, including any pretreatment (7.2.2);
- the size of the sample or fraction examined;
- any additional permitted procedure used.

The absence of a statement concerning the health condition of the seed does not necessarily imply that the health condition is satisfactory.

Table 7A. ISTA official seed health testing methods

7-001a: Detection of *Alternaria dauci* in *Daucus carota* (Carrot) seed by blotter method
Host: *Daucus carota* L.
Pathogen(s): *Alternaria dauci* (J.G.Kühn) J.J.Groves & Skolko, syn. *A. porri* f.sp. *dauci* (J.G.Kühn) Neerg., syn. *A. carotae* (Ellis & Langlois) Stevenson & Wellman
Date approved: 2012
Review due: 2017

7-001b: Detection of *Alternaria dauci* in *Daucus carota* (Carrot) seed by malt agar method
Host: *Daucus carota* L.
Pathogen(s): *Alternaria dauci* (J.G.Kühn) J.J.Groves & Skolko, syn. *A. porri* f.sp. *dauci* (J.G.Kühn) Neerg., syn. *A. carotae* (Ellis & Langlois) Stevenson & Wellman
Date approved: 2012
Review due: 2017

7-002a: Detection of *Alternaria radicina* in *Daucus carota* (Carrot) seed by blotter method
Host: *Daucus carota* L.
Pathogen(s): *Alternaria radicina* Meier, Drechsler & E.D.Eddy, syn. *Stemphylium radicinum* (Meier, Drechsler & E.D.Eddy) Neergaard
Date approved: 2012
Review due: 2017

7-002b: Detection of *Alternaria radicina* in *Daucus carota* (Carrot) seed by malt agar method
Host: *Daucus carota* L.
Pathogen(s): *Alternaria radicina* Meier, Drechsler & E.D.Eddy, syn. *Stemphylium radicinum* (Meier, Drechsler & E.D.Eddy) Neergaard
Date approved: 2012
Review due: 2017

7-003: Detection of *Botrytis cinerea* in *Helianthus annuus* (Sunflower) seed
Host: *Helianthus annuus* L.
Pathogen(s): *Botrytis cinerea* Pers. ex Pers. (Perfect state *Botryotinia fuckeliana* (de Bary) Whetzel, syn. *Sclerotinia fuckeliana* (de Bary) Fuckel.)
Date approved: 2011
Review due: 2016

7-004: Detection of *Leptosphaeria maculans* and *Plenodomus biglobosus* on *Brassica* spp. seed
Host: *Brassica* spp.
Pathogen(s): *Leptosphaeria maculans* (Tode ex Fr.) Ces. & de Not (previously *Phoma lingam*) or *Plenodomus biglobosus* (Shoemaker & H. Brun) (previously *Leptosphaeria biglobosa*)
Date approved: 2017
Review due: 2022

7-005: Detection of *Ascochyta pisi* in *Pisum sativum* (Pea) seed
Host: *Pisum sativum* L.s.l.
Pathogen(s): *Ascochyta pisi* Lib.
Date approved: 2011
Review due: 2016

7-006: Detection of *Colletotrichum lindemuthianum* in *Phaseolus vulgaris* (Bean) seed
Host: *Phaseolus vulgaris* L.
Pathogen(s): *Colletotrichum lindemuthianum* (Sacc. & Magn.) Briosi & Cav.
Date approved: 2011
Review due: 2016

7-007: Detection of *Alternaria linicola*, *Botrytis cinerea* and *Colletotrichum lini* in *Linum usitatissimum* (Flax) seed
Host: *Linum usitatissimum* L.
Pathogen(s): *Alternaria linicola* J.W.Groves & Skolko; *Botrytis cinerea* Pers. ex Pers. (Perfect state *Botryotinia fuckeliana* (de Bary) Whetzel, syn. *Sclerotinia fuckeliana* (de Bary) Fuckel.); *Colletotrichum lini* (Westerd.) Tochinai, syn. *C. linicola* Pethybr. & Laff.
Date approved: 2012
Review due: 2017

7-008: Detection of *Caloscypha fulgens* in *Picea engelmannii* and *P. glauca* (Spruce) seed
Host: *Picea engelmannii* Parry ex Engelm.; *Picea glauca* (Moench) Voss
Pathogen(s): *Caloscypha fulgens* (Pers.) Boud. (Imperfect state *Geniculodendron pyriforme* Salt)
Date approved: 2011
Review due: 2016

Table 7A. ISTA official seed health testing methods (cont.)

7-009: Detection of *Gibberella circinata* on *Pinus* spp. (Pine) and *Pseudotsuga menziesii* (Douglas-fir) seed
Host: *Pinus spp.; Pseudotsuga menziesii (Mirb.) Franco*
Pathogen(s): *Gibberella circinata* Nirenberg & O'Donnell (Imperfect state *Fusarium circinatum* Nirenberg & O'Donnell, syn. *F. subglutinans* f. sp. *pini* Hepting, syn. *F. lateritium* f. sp. *pini* Hepting)
Date approved: 2011
Review due: 2016

7-010: Detection of *Drechslera oryzae* in *Oryza sativa* (Rice) seed
Host: *Oryza sativa* L.
Pathogen(s): *Drechslera oryzae* (Breda de Haan) Subram. & Jain, syn. *Bipolaris oryzae* (Breda de Haan) Shoem., syn. *Helminthosporium oryzae* Breda de Haan (Perfect state *Cochliobolus miyabeanus* (Ito & Kurib.) Drechsler ex Dastur, syn. *Ophiobolus miyabeanus* Ito & Kuribayashi)
Date approved: 2011
Review due: 2016

7-011: Detection of *Pyricularia oryzae* in *Oryza sativa* (Rice) seed
Host: *Oryza sativa* L.
Pathogen(s): *Magnaporthe grisea* (Hebert) Barr (Imperfect state *Pyricularia oryzae* Cavara, syn. *P. grisea*)
Date approved: 2011
Review due: 2016

7-012: Detection of *Alternaria padwickii* in *Oryza sativa* (Rice) seed
Host: *Oryza sativa* L.
Pathogen(s): *Alternaria padwickii* (Ganguly) M.B.Ellis, syn. *Trichoconis padwickii* Ganguly, syn. *Trichoconiella padwickii* (Ganguly) Jain
Date approved: 2011
Review due: 2016

7-013a: Detection of *Ustilago nuda* in *Hordeum vulgare* (Barley) seed by embryo extraction
Host: *Hordeum vulgare* L.
Pathogen(s): *Ustilago nuda* (Jens.) Rostr.
Date approved: 2011
Review due: 2016

7-013b: Detection of *Ustilago nuda* in *Hordeum vulgare* (Barley) seed by dehulling and embryo extraction
Host: *Hordeum vulgare* L.
Pathogen(s): *Ustilago nuda* (Jens.) Rostr.
Date approved: 2011
Review due: 2016

7-014: Detection of *Stagonospora nodorum* in *Triticum aestivum* (Wheat) seed
Host: *Triticum aestivum* L.
Pathogen(s): *Stagonospora nodorum* Berk., syn. *Septoria nodorum* Berk. (Perfect state *Leptosphaeria nodorum* Mailer)
Date approved: 2011
Review due: 2016

7-015: Detection of *Epichloë coenophiala* in *Festuca* spp. (Fescue) and of *Neotyphodium lolii* in *Lolium* spp. (Ryegrass) seed
Host: *Festuca* spp., *Lolium* spp.
Pathogen(s): *Epichloë coenophiala* (Morgan-Jones & W. Gams) C.W. Bacon & Schardl; *Neotyphodium lolii* (Latch, M.J.Chr. & Samuels) Glenn, C.W.Bacon & Hanlin
Date approved: 2012
Review due: 2017

7-016: Detection of *Phomopsis* complex in *Glycine max* (Soybean, Soya bean) seed
Host: *Glycine max* (L.) Merr.
Pathogen(s): *Phomopsis longicolla* Hobbs, *Diaporthe phaseolorum* var. *sojae* (Lehm.) Wehm. (Imperfect state *P. phaseoli* (Desm.) Sacc., syn. *P. sojae* Lehmann); *Diaporthe phaseolorum* (Cke. & Ell.) Sacc. f. sp. *caulivora* (DPC), syn. *D. phaseolorum* var. *caulivora* Athow & Caldwell
Date approved: 2012
Review due: 2017

7-017: (Replaced by 7-007)

7-018: (Replaced by 7-007)

7-019a: Detection of *Xanthomonas campestris* pv. *campestris* on *Brassica* spp. seed
Host: *Brassica* spp.
Pathogen(s): *Xanthomonas campestris* pv. *campestris* (Pammel) Dowson
Date approved: 2014
Review due: 2019

Table 7A. ISTA official seed health testing methods (cont.)

7-019b: Detection of *Xanthomonas campestris* pv. *campestris* in disinfested/disinfected *Brassica* spp. seed
Host: *Brassica* spp.
Pathogen(s): *Xanthomonas campestris* pv. *campestris* (Pammel) Dowson
Date approved: 2012
Review due: 2017

7-020: Detection of *Xanthomonas hortorum* pv. *carotae* in *Daucus carota* (Carrot) seed
Host: *Daucus carota* L.
Pathogen(s): *Xanthomonas hortorum* pv. *carotae* (Kendrick) Vauterin, Hoste, Kersters & Swings, syn. *X. campestris* pv. *carotae* (Kend) Dye
Date approved: 2010
Review due: 2015

7-021: Detection of *Xanthomonas axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* in *Phaseolus vulgaris* (Bean) seed
Host: *Phaseolus vulgaris* L.
Pathogen(s): *Xanthomonas axonopodis* pv. *phaseoli* (Smith) Vauterin, Hoste, Kersters & Swings, syn. *X. campestris* pv. *phaseoli* (Smith) Dye; *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* Vauterin, Hoste, Kersters & Swings, syn. *X. campestris pv. phaseoli var. fuscans* (Burkholder) Starr & Burkholder
Date approved: 2011
Review due: 2016

7-022: Detection of *Microdochium nivale* and *M. majus* in *Triticum* spp. (Wheat) seed
Host: *Triticum* spp.
Pathogen(s): *Microdochium nivale* Samuels & Hallett, syn. *Fusarium nivale* (Fr.) Rabenh. (Perfect state *Monographella nivalis* (Schaff.) Müller); *M. majus* (Wollenw.) Glynn & S.G.Edwards, syn. *M. nivale* var. *majus* (Wollenw.) Samuels & I.C.Hallett
Date approved: 2012
Review due: 2017

7-023: Detection of *Pseudomonas savastanoi* pv. *phaseolicola* in *Phaseolus vulgaris* (Bean) seed
Host: *Phaseolus vulgaris* L.
Pathogen(s): *Pseudomonas savastanoi* pv. *phaseolicola* (Burkh.) Gardan, Bollet, Abu, Ghorrah, Grimont & Grimont, syn. *P. syringae* pv. *phaseolicola* (Burkh.) Young, Dye & Wilkie
Date approved: 2012
Review due: 2017

7-024: Detection of *Pea early browning virus* and *Pea seed-borne mosaic virus* in *Pisum sativum* (Pea) seed
Host: *Pisum sativum* L.s.l.
Pathogen(s): *Pea early browning virus* (PEBV) and *Pea seed-borne mosaic virus* (PSbMV)
Date approved: 2012
Review due: 2017

7-025: Detection of *Aphelenchoides besseyi* in *Oryza sativa* (Rice) seed
Host: *Oryza sativa* L.
Pathogen(s): *Aphelenchoides besseyi* Christie
Date approved: 2013
Review due: 2018

7-026: Detection of *Squash mosaic virus*, *Cucumber green mottle mosaic virus* and *Melon necrotic spot virus* in cucurbit seed
Host: Cucurbits
Pathogen(s): *Squash mosaic virus* (SqMV); *Cucumber green mottle mosaic virus* (CGMMV); *Melon necrotic spot virus* (MNSV)
Date approved: 2014
Review due: 2019

7-027: Detection of *Pyrenophora teres* and *P. graminea* on *Hordeum vulgare* (Barley) seed
Host: *Hordeum vulgare* L.
Pathogen(s): *Pyrenophora teres* Drechsler (Imperfect state *Drechslera teres* (Sacc.) Shoem.); *Pyrenophora. graminea* Ito & Kurib. (Imperfect state *D. graminea* (Rabenh. Ex Schlecht.) Shoem.)
Date approved: 2011
Review due: 2016

7-028: Detection of infectious *Tobacco mosaic virus* and *Tomato mosaic virus* in *Solanum lycopersicum* (Tomato) seed by the local lesion assay (indexing) on *Nicotiana tabacum* plants
Host: *Solanum lycopersicum* L.
Pathogen(s): *Tobacco mosaic virus* (TMV); *Tomato mosaic virus* (ToMV)
Date approved: 2012
Review due: 2017

7-029: Detection of *Pseudomonas syringae* pv. *pisi* in *Pisum sativum* (Pea) seed
Host: *Pisum sativum* L.s.l.
Pathogen(s): *Pseudomonas syringae* pv. *pisi* (Sack.) Young, Dye & Wilkie
Date approved: 2012
Review due: 2017

Table 7A. ISTA official seed health testing methods (cont.)

7-030: Detection of *Acidovorax valerianellae* in *Valerianella locusta* (corn salad) seed
Host: *Valerianella locusta* (L.) Laterr.
Pathogen(s): *Acidovorax valerianellae* sp. nov.
Date approved: 2014
Review due: 2019

7-031: Filtration method for detection of *Ditylenchus dipsaci* on *Medicago sativa*; *Ditylenchus dipsaci* and *Ditylenchus gigas* on *Vicia faba*
Host: *Medicago sativa* L. and *Vicia faba* L.
Pathogen(s): *Ditylenchus dipsaci* Kuhn, 1857; *Ditylenchus gigas* n. sp.
Date approved: 2017
Review due: 2022

7-032: Detection of *Verticillium dahliae* on *Spinacia oleracea* (spinach) seed
Host: *Spinacia oleracea* L.
Pathogen(s): *Verticillium dahliae* Kleb.
Date approved: 2017
Review due: 2022

Chapter 8: Species and variety testing

8.1 Object

The object of species and variety verification is to determine the extent that the submitted sample conforms to the species or variety as requested by the applicant, using methods not permissible in a purity test according to Chapter 3.

8.2 Definitions

8.2.1 Authentic standard sample

An authentic standard sample is a seed sample of a known species or variety or a sample with a known specific trait. It is recommended that this sample is of a known origin, e.g. a certified reference sample or a sample taken by an official or another person who can ensure the sample identity and characteristics. This sample will be used for obtaining enzymatic, protein or DNA profiles.

8.2.2 Standard reference

A standard reference is a valid descriptive attribute of a species or variety, e.g. zygosity, or an isozyme, protein or DNA banding pattern produced by gel electrophoresis or similar techniques, or an allelic profile or nucleotide sequence or a molecular weight standard (MWS) for protein or DNA. This trait should be obtained by a validated method and should be from an authentic standard sample or obtained from a reliable source as for MWS.

8.2.3 Allele

An allele is one of several alternate forms of a DNA sequence that may occur at a particular gene or other specific location within an organism's genome.

8.2.4 Microsatellite

A microsatellite is a repetitive DNA element, also known as a simple sequence repeat (SSR), consisting of a short, tandemly repeated motif of one to a few DNA subunits (nucleotides). For example, CTGCTGCTGCTGCTGCTGCTG is a microsatellite with a "CTG" repeat motif. A given microsatellite at a particular location within an organism's genome may vary in size when examined in different individuals due to differences in the number of times the motif is repeated.

8.2.5 Semi-performance-based approach

The semi-performance-based approach (SPBA) is an approach to testing in which individual laboratories can choose some components of the test method, as long as those components have been validated as fit for purpose and comply with given performance standards, while one or more other components of the test method are prescribed.

8.2.6 Allele profile

An allele profile is the combination of alleles determined for a specific set of DNA markers examined within a sample, individual or variety. It is sometimes referred to as a DNA 'fingerprint'.

8.3 General principles

8.3.1 Field of application

The determination of a species or variety is valid only if the species or variety is stated by the applicant and an authentic standard sample of the species or variety is available for comparison to ensure the certainty of the determination. The traits compared may be morphological, physiological, cytological or chemical.

8.3.2 Testing principles

The determination is carried out, depending on the species or variety in question on seeds, seedlings or more mature plants grown in a laboratory, a glasshouse, a growth chamber or a field plot.

The working sample will be compared with the authentic standard sample. Whenever possible, the working sample and the authentic standard sample must be handled in the same way, e.g. in field plots they must be grown contemporaneously, near each other and in identical environmental conditions, and the evaluation must be done at the same stage of development.

When a standard reference is used in a test, the interpretation of the result is done by comparing the traits of

the seeds, seedlings or plants of the working sample with the standard reference.

In the case of species or variety that are sufficiently uniform in one or more traits (e.g. in self-pollinated species), the conformity of the working sample with an authentic standard sample can be determined and if possible, the degree of conformity may be quantified. If the species or variety is not sufficiently uniform (e.g. in cross-pollinated species), the proportion of any obvious off-types is calculated and the conformity of the working sample is expressed.

8.3.3 Semi-performance-based approach for DNA-based testing

The technologies associated with DNA analysis are continuously evolving, and an assortment of instrumentation and procedures exist that may yield equivalent results. Individual laboratories have invested in varied instrumentation according to their circumstances, and it is not practical to require standardised use of specific technologies. Therefore, in order to establish a harmonised approach that both provides guidance to laboratories and facilitates processes for laboratories seeking accreditation for these types of tests, an SPBA has been instituted. Specific molecular markers are prescribed, but the analytical procedures used to interrogate those markers are at the discretion of individual laboratories, so long as those procedures have been evaluated as fit for purpose, and the end result meets acceptable standards as set by ISTA.

8.4 Personnel and equipment

Determinations must be made by a specialist familiar with the morphological, physiological, biomolecular or other traits of seeds. The specialist must possess specific knowledge of procedures, apparatus and equipment required for determining species and variety. It may be necessary to consult the international scientific literature, official government documents, other laboratories or other resources for guidance.

Appropriate facilities and equipment must be available as specified in detail in 8.8 for testing species and variety as follows:

In the laboratory: apparatus and reagents for morphological, physiological, cytological or bio-molecular examinations, chemical tests and germination of seeds as appropriate;

In glasshouses and growth chambers: provision of controlled environmental conditions adequate to induce the development of the trait;

In field plots: climatic, soil and cultural conditions to permit normal development of the trait to be tested and sufficient protection against pests and diseases.

8.5 Procedures

8.5.1 Submitted sample

The testing laboratory must ensure that the size of the submitted sample is sufficient to perform the tests as requested by the applicant.

The guiding values for the size of the submitted sample for tests covered by this chapter are shown in Table 8A.

Depending on the method and the degree of precision required, more seeds or fewer seeds than the amount listed above may be necessary.

Table 8A. Sample sizes for the species and variety test

	Laboratory only (g)	Field plot and laboratory (g)
Glycine, Lupinus, Phaseolus, Pisum, Vicia, Zea and species of other genera with seeds of similar size	1000	2000
Avena, Hordeum, Secale, Triticum and species of other genera with seeds of similar size	500	1000
Beta and species of other genera with seeds of similar size	250	500
All smaller seeded species	100	250

8.5.2 Working sample

The size of the working sample and the number and size of replicates will depend on the object, the method to be used and the degree of precision as requested by the applicant. If technically possible and justified, replicates should be tested to improve the reliability of the test result. Preparation of the working sample and the replicates must be done according to the procedures described under 2.5.2.

8.5.3 Examination of seeds

There may be different procedures for examining seeds.

For testing morphological traits, the seeds must be examined with the aid of a suitable magnifying apparatus when necessary. For testing colour traits, the seeds may be examined under full daylight or light of limited spectrum, e.g. ultra-violet. For testing chemical traits, the seeds must be treated with the appropriate reagent and the reaction of each seed must be noted. For testing bio-molecular traits, DNA, RNA, protein or other specific metabolic products are extracted from the seeds and the traits may be detected, elucidated and quantified.

Standardised methods for examining seeds listed under 8.8 are applicable to both objects according to 8.1. For the application of performance approved methods see 8.2.3.

8.5.4 Examination of seedlings

The seeds must be germinated on an appropriate medium. When the seedlings have reached a suitable stage of development, they are examined in whole or in part, with or without further treatment. For testing biomolecular traits, DNA, RNA, protein or other specific metabolic products are extracted from the seedlings and the traits may be detected, elucidated and quantified. In bioassays, seeds may be treated before germination or the seedlings may be treated to induce the expression of the traits if present.

Standardised methods for examining seedlings listed under 8.9 are applicable to both objects according to 8.1. For the application of performance approved methods see 8.2.3.

8.5.5 Examination of plants in glasshouse or growth chamber

The seeds must be sown in suitable containers and maintained in environmental conditions necessary for the development of the traits. When the plants have reached a suitable stage of development, the traits must be observed on each plant and noted. For testing bio-molecular traits, DNA, RNA, protein or other specific metabolic products are extracted from the plants and the traits may be detected, elucidated and quantified. In bioassays, seeds may be treated before germination or the seedlings or plants may be treated directly to induce the expression of the traits if present.

For the application of performance approved methods see 8.2.3.

8.5.6 Examination of plants in field plots

When plants are tested in field plots, each working sample must be sown in at least two replicate plots. As insurance against failure, the replicates should be situated in different fields or different parts of the same field. The plots may be of any convenient size that will provide enough plants for the determination to be of the accuracy required. If the seed is sown *in situ*, it must be sown in rows, mechanically if possible. Spacing between rows and between plants must be sufficient to allow development of the traits. Both transplanting and thinning are possible sources of error and the sowing rate must be adjusted to produce approximately the same number of plants in the plots produced from the working sample and the authentic standard sample. When absolutely necessary, thinning or transplanting of seedlings from elsewhere into the plot is permitted.

Observations must be made during the whole growing period, but particularly at the times indicated in 8.10. Plants showing the traits must be counted and recorded.

When practical, either an actual count or an estimate of the number of plants in the plot must be made, preferably at the time the plants are examined.

Standardised methods for examining plants listed under 8.10 are applicable to both objects according to 8.1. For the application of performance approved methods see 8.2.3.

8.6 Calculation and expression of results

The calculation and expression of results depends on the object, the method used, the testing plan and whether a qualitative or quantitative result is requested by the applicant. The mean and other statistics may be calculated and reported when results of replicates are within the range of expected variability. Methods for determining tolerances may be found in the *ISTA Handbook of Variety Testing* (Electrophoresis Testing) as well as in the *ISTA Handbook on Statistics in Seed Testing* (Appendix II). In the case of verification of species and variety, the determined proportion of other species, other varieties or aberrant (e.g. fatuoid oats, speltoid wheat) is calculated and expressed.

8.6.1 Examination of individual seeds, seedlings or plants

Whenever possible, the number of divergent seeds, seedlings or plants or those with the trait under test must be calculated as a percentage of the number of seeds, seedlings or plants examined.

When testing seedlings, the result is expressed as the proportion of the number of normal seedlings (as defined in Chapter 5). If the applicant requests reporting in a different way, it must be in addition to the above.

When testing plants in field plots in rows without wide spacing, it may be difficult to estimate the total number of plants examined per plot. The result may be expressed as the number of divergent plants or plants with the trait under test produced by the mass of seed sown.

8.6.2 Tests for traits of bulk samples

For bulk samples, tests may be done by measuring traits that do not allow a reference to individual seeds, seedlings or plants. There are various principles for calculation and expression of test results of such measurements. The result must be expressed as agreed with the applicant.

8.7 Reporting results

Results must be reported under 'Other determinations', and in addition the following information must be given:

a) the request of the applicant;
b) the trait(s) and the method(s) used;
c) the kind of preparation of the working sample (e.g. the whole working sample excluding the inert matter or only the pure seed fraction, washing);
d) whether an authentic standard sample or a standard reference was used; if a standard reference was used, its origin must be indicated;
e) the number of seeds, seedlings or plants examined. When it is difficult to determine the total number of plants examined in field plots, the mass of seed sown must be reported.

8.7.1 Examination of individual seeds or seedlings

Suggested phrases for reporting divergent seeds or seedlings are as follows, depending upon the result:

a) if none was found: "The test performed revealed nothing to indicate that the species (and/or variety) stated by the applicant is incorrect."
b) if non-conforming seeds were found: "Out of … seeds examined, … seeds do not conform to the authentic standard sample of the species (and/or variety) stated by the applicant."
c) if non-conforming seedlings were found: "Out of .... seeds producing normal seedlings, … % do not conform to the authentic standard sample of the species (and/or variety) stated by the applicant."
d) if the total working sample was found to be of a species and/or variety other than that stated by the applicant: "The sample does not conform to the authentic standard sample of the species (and/or variety) stated by the applicant."

8.7.2 Field plot examinations

The results of a field plot examination must, whenever possible, be reported as a percentage of each other species, other variety or aberrant found. When the expression of the result as a percentage is not possible, appropriate comments regarding the conformity of the sample may be reported.

If nothing worthy of special comment was found the following statement is suggested: "The results of a field plot examination of this sample revealed nothing to indicate that the species (and/or variety) stated by the sender is (are) incorrect."

8.8 Conventional methods

8.8.1 Cereals

Morphological characters of cereal grains can be observed by direct visual examination or with suitable magnification.

In *Hordeum*, the most useful characters are shape of the grain, base of the lemma, colour, hairs in the ventral crease, opening of the ventral crease, rachilla hairs, denta-

tion of the lateral dorsal nerves, wrinkling of the lemma and palea, and shape and hairiness of the lodicules.

In *Avena* a useful character is grain colour, which may be white, yellowish grey or black.

In *Avena* and *Hordeum,* the colour of the grain under ultra-violet light is sometimes diagnostic.

Colour reaction in dilute phenol is a useful character, especially in *Triticum.* Soak the grains in distilled water overnight. Drain and place them on filter paper in petri dishes and add a few drops of approximately 1 % phenol. Classify the grains according to depth of colour. Varieties develop a characteristic brown colour varying from pale to very dark.

8.8.2 *Fabaceae* and *Poaceae*

In some species of *Fabaceae* (e.g. of *Pisum* and *Lupinus*) and species of *Poaceae* (e.g. *Festuca* spp.), diagnostic differences in colour, size and shape can be observed by direct visual examination under daylight or ultraviolet light.

In *Lupinus* spp., the presence or absence of alkaloids is a diagnostic feature. Soak the seeds singly in water (2.5–5.0 mL for each seed) for at least 2 h in transparent dishes or other suitable equipment. Scarify or pierce each seed with an appropriate tool (scalpel, needle, pliers) to remove hardseededness and to allow a release of alkaloids into the water. Soak the seeds for a further 5–24 h. Add 1–3 drops of freshly prepared 1 % Lugol's solution (1.0 g iodine + 2.0 g potassium iodide, made up with water to 100 mL) to each seed. A distinct brown-red precipitate indicates the presence of alkaloids. Doubtful cases can be easily resolved by adding further drops of the Lugol's solution. Evaluation can be done on either a white surface or a luminescent screen.

8.9 Protein-based methods

8.9.1 *Hordeum* (barley)

8.9.1.1 Principle

The standard reference method for verifying varieties of *Hordeum* is by polyacrylamide gel electrophoresis (PAGE). The alcohol-soluble proteins (hordeins) are extracted from the seeds and separated by PAGE at pH 3.2. The pattern of protein bands produced (electropherogram) is related to genetic constitution and can be considered as a 'fingerprint' of a variety. The 'fingerprints' can be used to identify unknown samples and mixtures, by single seed analysis.

As a guideline it is recommended that 100 seeds are used. Very precise estimates of varietal purity may require a larger sample. If a comparison is being made with a standard value, sequential testing using batches of 50 seeds can be undertaken in order to minimise the workload. A simple check on the identity of a single major constituent of a seed lot can be done using less than 50 seeds.

8.9.1.2 Apparatus and equipment

8.9.1.2.1 Apparatus

Any suitable vertical electrophoresis apparatus with a cooling system and power supply may be used.

8.9.1.2.2 Chemicals

All chemicals should be of 'Analytical Reagent' grade or equivalent.

- Acrylamide ('specially purified for electrophoresis')
- Bisacrylamide ('specially purified for electrophoresis')
- Urea
- Glacial acetic acid
- Glycine
- Ferrous sulphate
- Ascorbic acid
- Hydrogen peroxide (**or** ammonium persulphate and TEMED)
- Monothioglycerol (**or** 2-mercaptoethanol)
- Pyronine G (**or** methyl green)
- Trichloroacetic acid
- Ethanol
- 2-Chloroethanol
- PAGE Blue G-90 (**or** PAGE Blue 83)(**or** any reagent equivalent to the 'Coomassie Blue' series of dyes)

8.9.1.2.3 Solutions

a) Extraction solution: pyronine G (or methyl green) (0.05 %) in 2-chloroethanol (20 %) containing urea (18 %) and monothioglycerol (or 2-mercaptoethanol) (1 %)(keep cold or prepare fresh)

b) Stock tank buffer solution: glacial acetic acid (4 mL) and glycine (0.4 g), made up to 1 L with water; keep cold
c) Stock gel buffer solution: glacial acetic acid (20 mL) and glycine (1.0 g), made up to 1 L with water; keep cold
d) Staining solutions:
   – trichloroacetic acid (100 g) in 1 L of water
   – PAGE Blue G-90 (or PAGE Blue 83)(1 g) in ethanol (100 mL).

8.9.1.3 Procedure

8.9.1.3.1 Protein extraction

Single seeds are crushed with pliers and transferred to 1.5 mL polypropylene centrifuge tubes. Extraction solution (0.3 mL) is added, the contents of the tubes are thoroughly mixed and the tubes are allowed to stand overnight at room temperature. The tubes are centrifuged at 18 000 × *g* and the supernatants used for electrophoresis. Extracts can normally be stored at 4 °C for 3–4 days.

8.9.1.3.2 Gel preparation

Clean and dry gel cassettes are assembled, according to the design of the equipment. Treating the glass plates with silicon prior to assembly can facilitate subsequent removal of the gel. The gel cassettes can incorporate a plastic backing sheet (e.g. 'Gel Bond PAG', FMC Corporation). This supports the gel during subsequent operations. To make 100 mL of gel medium, stock gel buffer (approx 60 mL) is taken and acrylamide (10 g), bisacrylamide (0.4 g), urea (6 g), ascorbic acid (0.1 g), ferrous sulphate (0.005 g) are added. The solution is stirred and made up to 100 mL with stock gel buffer solution. Freshly prepared 0.6 % hydrogen peroxide solution (0.35 mL per 100 mL of gel medium) is added and mixed quickly and the gel poured. Note that the gel mixture can be cooled to near freezing prior to the addition of the peroxide. Polymerisation should be complete in 5–10 min. An acrylic 'comb' is placed in the top of the cassette, to make wells in the gel. The gel mixture should over-fill the cassette, or be overlaid with water, to ensure satisfactory polymerisation of the upper surface.

Note that as an alternative to the hydrogen peroxide catalyst, it is possible to use ammonium persulphate (0.1 mL of 10 % solution, freshly prepared) and TEMED (0.3 mL) added to the gel mixture prior to pouring the gel.

8.9.1.3.3 Electrophoresis

The acrylic comb is removed from the gel and the sample wells washed with tank buffer. The tank is filled with an appropriate volume of buffer (depending on the equipment used). Samples (10–20 µL) are loaded into the wells and the gel placed in the tank, ensuring that the sample wells are completely filled. Electrophoresis is carried out at 500 V (constant voltage) for twice the time taken for the pyronine G marker dye to leave the gel, or three times if methyl green is used as a tracking dye. Water should be circulated through the buffer tank to maintain the temperature at 15–20 °C.

8.9.1.3.4 Fixing and staining

The gel cassette is removed from the tank, opened and the gel placed in a plastic box containing 5–10 mL of 1 % PAGE G90 (or PAGE Blue 83) in 200 mL of 10 % trichloroacetic acid. Staining is complete in 1–2 days and destaining is not usually needed. Precipitated stain should be scraped from the surface of the gel. The gel is washed in water to enhance the stain and can then be examined or photographed. Any blue background in the gel is removed by washing in 10 % trichloroacetic acid. Gels can be stored in polythene bags at 4 °C for many months without deterioration.

8.9.1.4 Nomenclature of hordein bands

Hordein bands can be identified either by measuring their relative mobilities (Wrigley, C.W., Autran, J.C. & Bushuk, W. (1982). *Advances in Cereal Science and Technology,* 5, 211–259), by means of an electrophoretic formula (Konarev, V.B., Gavrilyuk, I.P., Gubareva, N.K. & Peneva, T.I. (1979). *Cereal Chemistry* **56**, 272–278) or by designation of patterns (Shewry, P.R., Pratt, H.M., Faulks, A.J., Parmar, S. & Miflin, B.J. (1979). *Journal of the National Institute of Agricultural Botany*, **15**, 5–40).

8.9.2 *Pisum* and *Lolium*

8.9.2.1 Principle

The standard reference method for the verifying varieties of *Pisum* and *Lolium* is by polyacrylamide gel electrophoresis (PAGE). Seed proteins are extracted from individual *Pisum* seeds or from a meal of *Lolium* seeds, treated with

SDS and separated using a discontinuous SDS-PAGE procedure. The pattern of protein bands found on the gel is characteristic of a variety.

As a guideline for *Pisum,* it is recommended that 100 individual seeds are used. Very precise estimates of varietal purity may require a larger sample. If a comparison is made with a standard value, sequential testing using batches of 50 seeds can be undertaken in order to minimise the workload. A simple check on the identity of a single major constituent of a seed lot can be done using less than 50 seeds.

For *Lolium*, a bulk sample of seeds is analysed. In most cases, whilst this will serve to verify seed lots, it will not permit the detection of admixtures of two or more varieties.

8.9.2.2 Apparatus and equipment

8.9.2.2.1 Apparatus

Any suitable vertical electrophoresis apparatus with a cooling system and power supply may be used.

8.9.2.2.2 Chemicals

All chemicals should be of 'Analytical Reagent' grade or equivalent.

- Acrylamide ('specially purified for electrophoresis')
- Bisacrylamide ('specially purified for electrophoresis') (BIS)
- Tris (Tris [hydroxymethyl] methylamine)
- Glycine
- Hydrochloric acid
- Sodium dodecyl sulphate (SDS)
- Glycerol
- 2-mercaptoethanol
- Dimethylformamide
- Ammonium persulphate (APS) (**or** riboflavin)
- NNN'N'-tetramethylethylenediamine (TEMED)
- Methanol
- Glacial acetic acid
- Trichloroacetic acid (TCA)
- PAGE Blue G-90 (**or** PAGE Blue 83), (**or** any reagent equivalent to the 'Coomassie Brilliant Blue' G or R series of dyes)
- Bromophenol Blue

8.9.2.2.3 Solutions

a) Stock gel buffer (main or resolving gel): 1 M Tris HCl, pH 8.8. 121.1 g of Tris is dissolved in about 750 mL of distilled water, adjusted to pH 8.8 with hydrochloric acid (use 1 M [approximately 90 mL concentrated HCl per L distilled water] solution added dropwise; this will require about 15 mL) and made up to 1 L. This can be stored at 4 °C.

b) Stock gel buffer (stacking gel): 1 M Tris HCl, pH 6.8. 30.3 g of Tris is dissolved in 200 mL of distilled water, adjusted to pH 6.8 with hydrochloric acid (use concentrated at first [approx. 8 mL, added dropwise], then 1 M solution) and made up to 250 mL. This can be stored at 4 °C.

c) Stock SDS solution: 10 g of SDS is dissolved in distilled water (this requires gentle stirring and heating) and made up to 100 mL. This should be stored at room temperature. If the SDS comes out of solution, it can be re-dissolved by gentle heating.

d) 1 % ammonium persulphate solution: 0.1 g of ammonium persulphate is dissolved in 10 mL of distilled water. This must be prepared freshly on each occasion, immediately prior to use.

e) Stock sample extraction buffer solution: To 12.5 mL of stacking gel buffer (see 8.9.2.2.3b) is added 20 mL glycerol, 24.1 mL of distilled water, 4 g of SDS and 12 mg bromophenol blue (optional). This is mixed and stored at room temperature. If the SDS comes out of solution, it can be re-dissolved by gentle heating.

f) Gel fixing solution: To 400 mL methanol, 100 mL glacial acetic is added and made up to 1 L with water. About 200 mL is needed per gel. (Note that it is possible to use TCA at a final concentration of 15–20 % [2.3 g] in place of glacial acetic acid).

g) Gel staining solution:
   (1) 15 % trichloroacetic acid (TCA) (375 g made up to 2.5 L with water)
   (2) 1 % PAGE Blue or equivalent in methanol (1 g in 100 mL of methanol)

   200 mL of (1) plus 10 mL of (2) is sufficient for staining one gel.

8.9.2.3 Procedure

8.9.2.3.1 *Pisum*

Finely ground *Pisum* cotyledon material is prepared from individual seeds using an electric blender. A pestle and mortar (or similar device) can be used if preferred. Diluted sample extraction buffer is prepared by diluting the stock sample extraction buffer (section 8.9.2.2.3e) in the ratio 17 buffer : 3 mercaptoethanol : 40 distilled water (make

up only a volume of the diluted extractant sufficient to be used within a day).

The finely ground seed meal is extracted with diluted sample extraction buffer in the ratio 40 mg/1.0 mL, using 1.5 mL polypropylene micro-centrifuge tubes. The samples are left for about 1 h at room temperature, resuspended using a vortex mixer and heated for 10 min in a boiling water bath. (A small slit can be made in the caps of the tubes to prevent build-up of pressure.) After cooling, the tubes are centrifuged at 18 000 × *g* for 5 min and the clarified supernatants used for electrophoresis.

8.9.2.3.2 *Lolium*

Seed meals for analysis are prepared by passing 0.5–2.0 g of seed through a hammer mill. If preferred, a rotor-type electric coffee grinder or other blender can be used. Diluted extraction buffer is prepared by diluting the stock sample extraction buffer (see 8.9.2.2.3e) in the ratio 17 buffer : 6 mercaptoethanol : 10 dimethylformamide : 17 distilled water (make up only a volume of this extractant sufficient to be used within a day).

The seed meal is extracted with diluted sample extraction buffer in the ratio of 80 mg/1.0 ml and subsequently treated exactly as above (see 8.9.2.3.1).

8.9.2.3.3 Gel preparation

The clean and dry gel cassettes are assembled according to the type of equipment in use. Note that if adhesive sealing tape is used in the system, it is advisable to prepare the cassettes at least one day in advance to allow the tape to 'age' and adhere more tightly. Many types of vertical electrophoresis equipment have been found to be suitable. It is strongly recommended that a gel of thickness of 1.5 mm or less is used, as this seems to give better results. The following instructions are for the preparation of a 12.5 % acrylamide main gel and a 5 % stacking gel.

8.9.2.3.3.1 Main (resolving) gel

To make 4 slab gels (180 × 140 × 1.5 mm), the following is required:

– 56.4 mL 1 M Tris pH 8.8 (see 8.9.2.2.3a)
– 86.25 mL gel solution (19.6 g acrylamide + 0.26 g BIS, made up to 90 mL with distilled water)

De-gas (in a Buchner flask) and then add:

– 3.75 mL 1 % APS (see 8.9.2.2.3d)
– 1.5 mL 10 % SDS (see 8.9.2.2.3c)
– 75 µL TEMED (full strength, straight out of the bottle).

After careful mixing (do not cause 'foaming') the gel is slowly poured. If appropriate to the type of equipment, a 25 mL disposable syringe and needle can be used to pour the gel mixture into the cassette. The gel should be poured to a height which leaves room for a 3–4 cm layer of stacking gel. The gel surface is carefully overlaid with a 1 cm layer of distilled water (or isopropanol) using a Pasteur pipette or syringe, and the gel is then left to polymerise (about 1 h).

Note that if de-gassing of the gel mixture is a problem, it is possible to eliminate this step and use a three times higher concentration of APS (i.e. 3.75 mL of a 3 % solution [0.3 g dissolved in 10 mL of distilled water]).

8.9.2.3.3.2 Stacking gel

The overlaid water (or isopropanol) is removed from the surface of the main gel with a Pasteur pipette and the gel surface is rinsed briefly with diluted stacking gel buffer (see 8.9.2.2.3b, stock buffer diluted 1:8) and then carefully drained and dried using filter paper.

To make sufficient stacking gel for 4 gels, as above, the following is required:

– 10 mL 1 M Tris buffer pH 6.8 (see 8.9.2.2.3b)
– 67.2 mL gel solution (4.0 g of acrylamide + 0.07 g BIS, made up to 67.2 mL with distilled water)

De-gas (in a Buchner flask) and then add:

– 3.0 mL 1 % APS (see 8.9.2.2.3d)
– 0.8 mL 10 % SDS (see 8.9.2.2.3c)
– 80 µL TEMED (full strength, straight out of the bottle).

The stacking gel is poured (using a syringe, as before, if appropriate) to the top of the gel cassette and an acrylic well-forming 'comb' is inserted, ensuring that no air-bubbles are trapped beneath the 'teeth'. The gel is allowed to polymerise (about 1 h). Again, de-gassing can be omitted if a higher concentration of APS is used. 3.0 mL of a 2 % solution (0.2 g in 10 mL of distilled water) is sufficient.

As an alternative polymerisation system for the stacking gel, it is possible to use a 0.008 % riboflavin solution (freshly prepared), in place of APS. Polymerisation should occur if the gels are left in the light, but it may be necessary to use an ultraviolet lamp. The precise volume to use should be determined by experimentation, to give a polymerisation time of 30–60 min. However, as a guide, about 7.5 mL of riboflavin should be used per 50 mL of stacking gel mixture.

8.9.2.3.4 Electrophoresis

The electrophoresis tank buffer (or running buffer) comprises 3.0 g tris, 14.1 g glycine and 1.0 g SDS made up to 1 L with distilled water (it may be necessary to warm the solution gently to dissolve the SDS). A sufficient volume to fill the electrophoresis apparatus in use (top and bottom chambers) should be freshly prepared.

The acrylic comb is removed from the stacking gel (with care; this gel is rather soft) and the resultant wells are washed and partially filled with tank buffer (as above). The samples are loaded into the wells, using a syringe. The gel thickness and the size of the wells largely determine the volume of extract which is loaded. As a guide, between 5 and 15 µL is appropriate in most cases. If required, bromophenol blue (5 µL of a 1 % aqueous solution containing 10 % glycerol) can be added to a few wells to act as a marker (this can also be incorporated into the sample extraction buffer, see 8.9.2.2.3e). If the gel cassette is sealed with adhesive tape, this is removed from the lower (bottom) side only. The wells are filled with tank buffer (as above), taking care not to disturb the samples. The gel is placed in the tank. Electrophoresis is carried out at 25 mA per gel until the tracking dye has migrated through the stacking gel and then at 45 mA per gel until the bromophenol blue is at the bottom of the gel. The temperature should be maintained at 15–20 °C, if possible, by circulating tap-water (or coolant) through the tank buffer.

8.9.2.3.5 Fixing and staining

Several different approaches can be used for fixing and staining the proteins. If results are not required very rapidly, then at the end of electrophoresis, the gel is removed from the tank, taken from the cassette and incubated in fixing solution (see 8.9.2.2.3f), with slow shaking, for at least 1 h. The gel is rinsed in distilled water (5 min), then stained by incubation (at least 2 h, usually overnight) in gel staining solution (see 8.9.2.2.3g). When properly stained, the gel is rinsed in distilled water for 2–3 h (TCA can be added if the background is very blue) and then sealed in a polythene bag for examination or photography. Gels can be stored for many months at 4 °C, if sealed properly.

For more rapid staining, the gel can be fixed and stained at a higher temperature (80 °C) in an oven for 30 min and then, following cooling, de-stained in a solution containing 10 % glacial acetic acid and 35 % ethanol for a further 30–60 min, with shaking.

8.9.2.4 Evaluation of results

This method is mostly used comparatively, i.e.: is the protein pattern of the sample identical to that of the authentic reference variety? It is also useful to include on each gel a sample of a known variety with a well-described and established protein banding pattern. This can serve as a quality standard for gels - if the banding pattern of the reference variety is clearly observed, then the gel can be analysed to provide useful information. In addition, gels can be 'calibrated' by the inclusion of standard proteins of known molecular weights on each gel, which allows the calculation of the molecular weights of protein bands of interest.

8.9.3 *Zea mays* (maize)

8.9.3.1 Principle

The standard reference method for the measuring hybrid purity and verifying varieties of *Zea mays* (maize) is by ultrathin-layer isoelectric focusing (UTLIEF). The alcohol-soluble proteins (zeins) or water soluble proteins are extracted from individual maize seeds and separated by isoelectric focusing (IEF) in ultrathin-layer gels. The pattern of protein bands found on the gel is characteristic for a variety or an inbred line. Also, it is generally possible to estimate the purity of hybrid samples by finding one or more zein bands in the male parent that are lacking in the female parent (and present in the hybrid). These bands can be used as marker bands for the verification of hybrids and as a means of estimating hybrid purity.

Ultrathin gels can be run at higher voltages with shorter running times, and stain more quickly than conventional gels.

8.9.3.2 Apparatus and equipment

8.9.3.2.1 Apparatus

Any suitable horizontal electrophoresis apparatus with a cooling system and high voltage power supply may be used.

8.9.3.2.2 Chemicals

All chemicals should be of 'Analytical Reagent' grade or equivalent.

– 2-Chloroethanol
– Acrylamide ('specially purified for electrophoresis')
– Bisacrylamide (BIS) ('specially purified for electrophoresis')
– Ampholytes: pH 2–4, pH 4–6, pH 5–8 and pH 4–9
– Ammonium peroxydisulphate (APS)
– N,N,N',N'-Tetramethylethylenediamine (TEMED)
– Urea
– L-Aspartic acid
– L-Glutamic acid
– L-Arginine (base)
– L-Lysine
– Ethylenediamine
– Trichloroacetic acid (TCA)
– Coomassie Brilliant Blue G 250 (**or** equivalent)
– Coomassie Brilliant Blue R 250 (**or** equivalent)
– Ethanol (96 %)
– Acetic acid (99 %)
– 'Gel-Slick' (**or** 'Repelsilane', **or** equivalent)

8.9.3.2.3 Solutions

a) Extraction solution: 30 % (v/v) 2-chloroethanol (30 mL 2-chloroethanol made up to 100 mL with distilled water). This solution can be stored for at least two weeks at room temperature. Optional extraction solution: distilled water.
b) Anode solution: L-aspartic acid (0.83 g) and L-glutamic acid (0.92 g) are dissolved in hot distilled water and diluted to 250 mL. This solution can be stored for two weeks at 4 °C.
c) Cathode solution: L-arginine (base) (1.18 g), L-lysine (0.91 g) and ethylenediamine (30.00 mL) are dissolved in distilled water and diluted to 250 mL. This solution can be stored for two weeks at 4 °C.
d) Stock gel solution: acrylamide (16.57 g) and bisacrylamide (0.43 g) are dissolved in distilled water and diluted to 250 mL. This solution can be stored for up to two weeks at 4 °C.
e) Gel fixing solution: trichloroacetic acid (TCA)(approx. 12 % (w/v) solution). Stock solution: 1 kg TCA dissolved in 450 mL distilled water. Before use, 120 mL stock solution is diluted with 880 mL distilled water, to give an approximately 12 % TCA solution. About 400 mL is needed for one gel, and the solution can be used up to three times.
f) Gel staining solution: Coomassie Blue R 250 (0.45 g), Coomassie Blue G 250 (1.35 g), acetic acid (330 mL) and ethanol (540 mL) are mixed and made up to 3000 mL with distilled water; 400 mL is sufficient for staining one gel.
g) Gel destaining solution - ethanol (750 mL) and acetic acid (125 mL), made up to 2500 ml with distilled water.

8.9.3.3 Procedure

8.9.3.3.1 Protein extraction

Either a single dry seed is bisected longitudinally and one half crushed to a fine 'semolina' by hand (with pliers or pestle and mortar), **or** a whole single seed is taken and crushed.

Approximately 50 mg of the seed meal is extracted with 0.2 mL of extraction solution (8.9.3.2.3a) in a microtitre plate **or** a microcentrifuge tube. The samples are left for about 1 h at 20 °C. After this time, the microtitre plate or microtube is treated with ultrasound for 30 s and then centrifuged at 2000 × *g* for 5 min. The clarified supernatant is used for electrophoresis. Frozen at –20 °C, the remaining extract will keep for up to 3 months.

8.9.3.3.2 Gel preparation

Gels are made up **either** directly between two thin glass plates **or** between a polyester carrier sheet held on glass and a glass plate. The plates or sheets must be treated before use, one (carrier) with a silylating reagent to make the gel adhere and one (cover) with 'Gel-Slick' (**or** equivalent) to prevent gel adhesion. Commercially available prepared gel carrier sheets (e.g. 'Gel-Bond') can also be used.

The clean and dry gel cassettes are assembled, according to the type of equipment in use. A gel thickness of 0.12 mm is recommended, which can be achieved by the use of a defined thickness of adhesive tape) as a spacer.

The following are taken and mixed:
– 50 mL stock gel solution (8.9.3.2.3d)
– 16 g urea
– 0.55 mL ampholytes (pH 2–4)
– 0.55 mL ampholytes (pH 4–6)
– 1.40 mL ampholytes (pH 5–8)
– 1.90 mL ampholytes (pH 4–9)

Optional gel solution:
– 50 mL stock gel solution (8.9.3.2.3d)
– 16 g urea
– 1.5 g taurin
– 2.90 mL ampholytes (pH 5–8)
– 1.50 mL ampholytes (pH 2–11)

Note: dissolve taurin first into the stock gel solution because of the slow solubility.

For polymerisation, 0.35 mL APS (20 % (w/v) solution *freshly prepared*) and 0.05 mL TEMED (full strength) are added carefully, to avoid introducing excessive amounts of air.

This will be sufficient for 10 gels of the dimensions 240 × 180 × 0.12 mm (one gel requires 6.5 mL). After careful mixing, the gel is poured onto the carrier plate/sheet, the cover plate is carefully lowered and the gel 'cassette' left to polymerise for at least 45 min. Gels not immediately used can be stored wrapped at 4 °C for at least one week.

8.9.3.3.3 Electrophoresis

The gel is placed on the pre-cooled (10 °C) cooling plate of the horizontal electrophoresis apparatus. To ensure better gel adhesion and cooling, a thin layer of water is placed between the gel and the plate. The electrode wicks are soaked in the appropriate solution (see 8.9.3.2.3b or 8.9.3.2.3c) and placed at either end of the gel. Samples (approx. 22 µL) are loaded in the applicator strip 0.5 cm below the bufferwick of the anode and focusing carried out at 2500 V, 15 mA, 40 W for about 70 min until completion (for one gel).

Notes:

a) Double-focusing is possible with this method and it is then necessary to place the cathode wick in the centre of the gel, with anode wicks at both ends.

b) The precise conditions and times required for focusing will vary depending on the dimensions of the gel, the type of maize hybrid, inbred line etc., and may need to be determined empirically.

8.9.3.3.4 Fixing and staining

At the end of the electrophoresis, the gel is removed and incubated in fixing solution (see 8.9.3.2.3e) with slow shaking, for at least 20 min. The gel is then stained by shaking in gel staining solution (see 8.9.3.2.3f) for 50 min. Destaining takes (solution 8.9.3.2.3g) about 15 min, followed by brief rinsing in distilled water. The gel is dried overnight at room temperature or in an oven at 70 °C for 20 min and can then be sealed with adhesive film. Gels can be stored for many years at room temperature.

8.9.3.4 Evaluation of results

At present, the method is best used to verify varieties in a comparative way, by examining whether or not the protein pattern of the sample is identical to that of the authentic reference variety, analysed at the same time.

For hybrid seed, it is possible to determine hybrid purity (selfing rate). It is assumed that both parental lines are homogeneous. When comparing the protein patterns of the female and male parents with the hybrid, one or more marker bands (present in the male only) need to be found in the hybrid (Fig. 8.1). Given the presence of such markers, hybrid purity can be determined by examining a suitable number of single seeds of a seed lot. Seeds with a protein pattern identical to the female parent are judged to be self-pollinations ('sibs'). Foreign pollinated seeds show a different pattern, mostly a protein band at an unexpected place in the variety pattern. Seeds with a different pattern may also occur if there is contamination with another variety.

With respect to the different types of hybrids, the evaluation is as follows:

a) single cross hybrid (Fig. 8.2): only one banding pattern is characteristic for the hybrid, with bands inherited from both male and female parents;

b) three-way cross hybrid (Fig. 8.3): The female parent is a cross, and so contains protein bands from two lines. Thus in the hybrid, there are two possible banding patterns (male band plus one of two female bands) which are characteristic. However, experience shows that most hybrids exhibit only one banding pattern;

c) Double cross hybrid (Fig. 8.4): Both parents used in hybrid production derive from single crosses, and therefore four different banding patterns can occur in the hybrid seeds.

The number of seeds to be tested for hybrid purity determination depends on the acceptable confidence intervals, established for each individual case. It is suggested that normally 200 single seeds are analysed, as a compromise between precision of results and working time needed (see Chapter 4, '*Handbook of Variety Testing – Electrophoresis Testing*', ISTA, 1992). For reports and issue of ISTA Certificates, analysis of 400 single seeds is required.

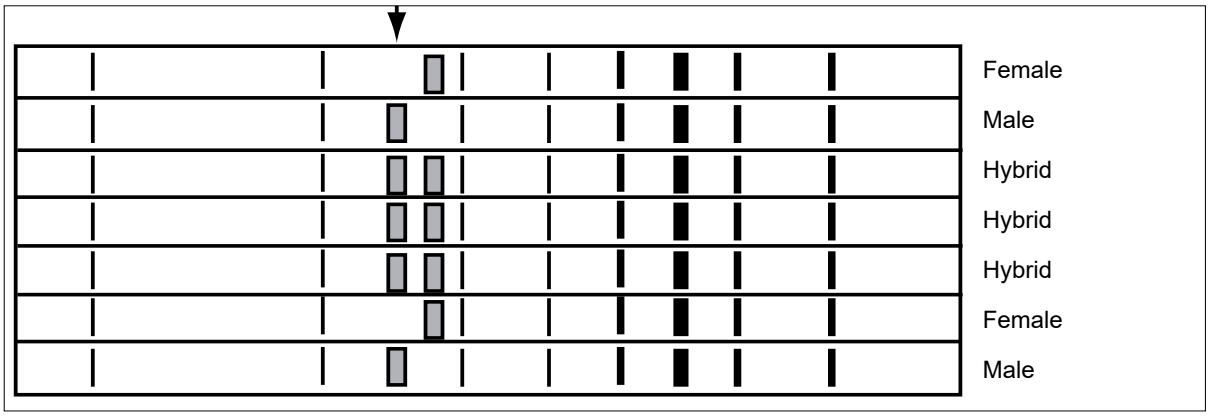


Figure 8.1. Evaluation of different hybrid types. Detection of a marker band (grey) present in the male parent line but not in the female.

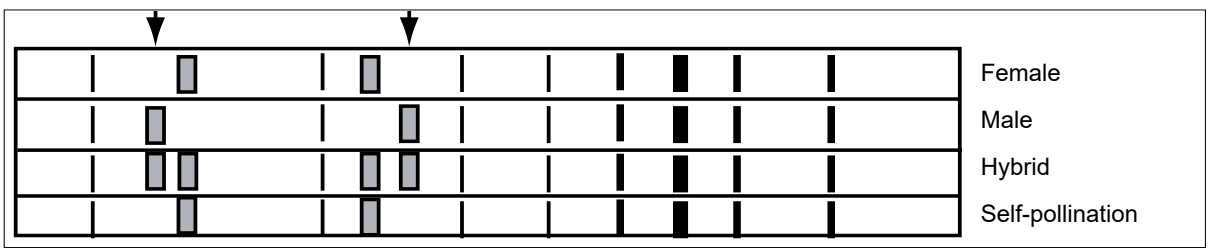


Figure 8.2. Evaluation of a single cross hybrid. Only one banding pattern characterises the hybrid; other patterns arise from self-pollination (same patterns as female) or from contamination.

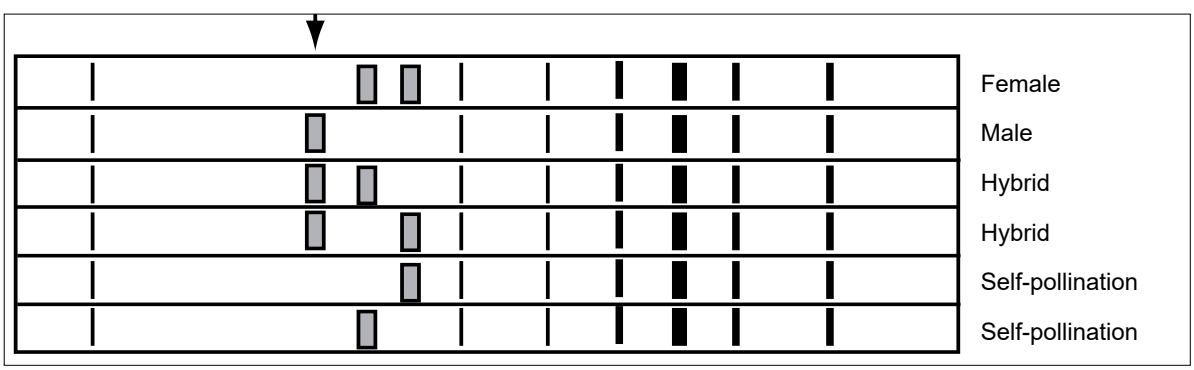


Figure 8.3. Evaluation of a three way cross hybrid. The female line is itself a cross, and so according to Mendelian rules, two hybrid banding patterns can occur (but most varieties show only one).

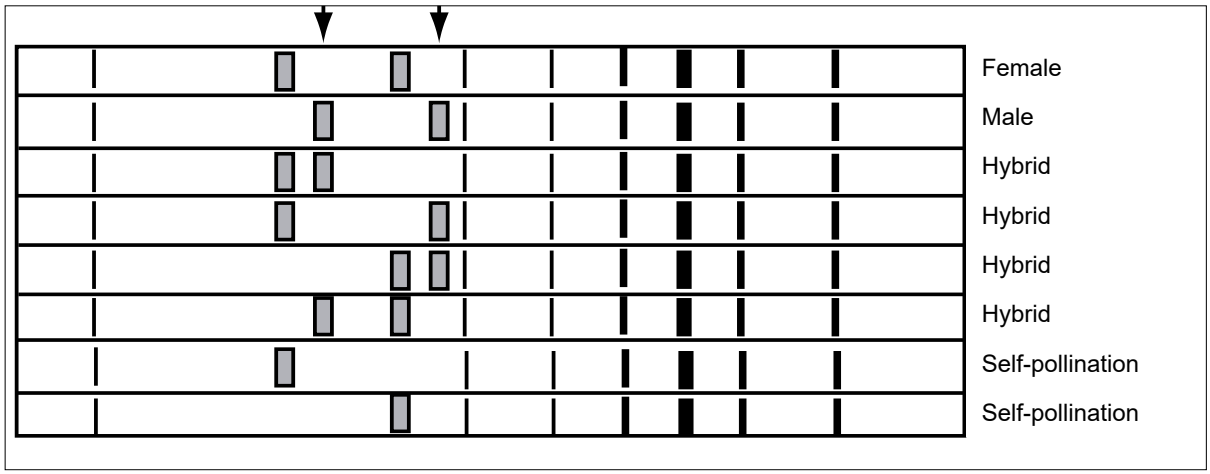


Figure 8.4. Evaluation of a double cross hybrid. Both parental lines derive from crosses and so according to Mendelian rules, four hybrid banding patterns can occur.

8.9.4 *Avena sativa* (oats)

8.9.4.1 Principle

The standard reference method for verifying varieties of *Avena sativa* (oats) is by polyacrylamide gel electrophoresis (PAGE). The urea/ethylene glycol-soluble proteins (primarily avenins) are extracted from seeds and separated by PAGE at pH 3.2. The pattern of protein bands can be considered as a 'fingerprint' of a variety. The 'fingerprints' can be used to identify (to verify the identity of) unknown samples and mixtures, by single seed analysis.

As a guideline, it is recommended that 100 seeds are used. Very precise estimates of varietal purity may require a larger sample. If a comparison is being made with a standard sample, sequential testing using subsamples of 50 seeds can be undertaken in order to minimise the workload. A simple check of the identity of a single major constituent of a seed lot can be done using less than 50 seeds.

8.9.4.2 Apparatus and equipment

8.9.4.2.1 Apparatus

Any suitable vertical electrophoresis apparatus with a cooling system and power supply may be used.

8.9.4.2.2 Chemicals

All chemicals should be of 'Analytical Reagent' grade or equivalent.

- Acrylamide ('specially purified for electrophoresis')
- Bisacrylamide ('specially purified for electrophoresis')
- Urea
- Glacial acetic acid
- Glycine
- Ferrous sulphate
- Ascorbic acid
- Hydrogen peroxide
- Pyronin G or Pyronin Y
- Trichloroacetic acid
- Ethylene glycol
- Methanol
- Coomassie Brilliant Blue G 250 or Serva Blue G (or equivalent)

8.9.4.2.3 Solutions

a) Extraction solution: Pyronin G (or Pyronin Y) (0.05 % (w/v) in 3M urea (18 % w/v) in a 75:25 (v/v) mixture of ethylene glycol and water (keep cold or prepare fresh).

b) Stock tank buffer solution: glacial acetic acid (4 mL) and glycine (0.4 g), made up to 1 L with water; keep cold.

c) Stock gel buffer solution: glacial acetic acid (20 mL) and glycine (1.0 g), made up to 1 L with water; keep cold.

d) Fixing and staining solution: Coomassie Blue G 250 or Serva Blue G (1 g) in methanol (250 mL) + 100 g trichloroacetic acid dissolved in 800 mL water.

8.9.4.3 Procedure

8.9.4.3.1 Protein extraction

The lemma and palea are removed, then samples are crushed with pliers and milled to a fine powder using a pestle and mortar (an electric blender may be used).The meal is extracted in 1.5 mL polypropylene centrifuge tubes. Extraction solution (8.9.4.2.3a)(0.1 mL per ground seed) is added, the contents of the tubes are thoroughly mixed with a vortex mixer and the tubes are allowed to stand for 2 h or overnight at room temperature. The tubes are centrifuged for 15 min at 14 000 × *g* and the supernatants used for electrophoresis.

8.9.4.3.2 Gel preparation

Clean and dry cassettes are assembled, according to the design of the equipment. Treating the glass plates with silicon prior to assembly can facilitate subsequent removal of the gel. The gel cassettes can incorporate a plastic backing sheet (e.g. 'Gel Bond PAG', FMC Corporation). This supports the gel during subsequent operations.

To make 2 slab gels (160 × 180 × 1.5 mm), the following is required:

stock gel buffer (approx. 60 mL) and the following added- acrylamide (12.5 g), bisacrylamide (0.4 g), urea (6.0 g), ascorbic acid (0.1 g), ferrous sulphate (0.005 g). The solution is stirred and made up to 100 mL with stock gel buffer solution. Freshly prepared 0.6 % (v/v) hydrogen peroxide solution (0.2 mL per 100 mL of gel solution) is added, mixed quickly and the gel poured. Note that the gel mixture can be cooled to near freezing prior to the addition of the peroxide. An acrylic 'comb' is placed in the top of the cassette, to make wells in the gel.

The gel mixture should over-fill the cassette, or be overlaid with water, to ensure satisfactory polymerisation of the upper surface. Polymerisation should be complete in 5–10 min.

8.9.4.3.3 Electrophoresis

The acrylic comb is removed from the gel and the sample wells filled with tank buffer. The tank is filled with an appropriate volume of buffer (depending on the equipment used). Samples (18 µL) are loaded into the wells and the gel cassettes are placed in the tank, ensuring that the sample wells are completely filled. Electrophoresis is carried out 10 min at 200 V and then at 500 V (constant voltage) for twice the time taken for the pyronine G marker dye to leave the gel. Water should be circulated through the buffer tank to maintain the temperature at 15–20 °C.

8.9.4.3.4 Fixing and staining

The gel cassette is removed from the tank, opened and the gel placed in a plastic box containing about 200 mL fixing and staining solution. Staining is complete in one day. When properly stained, the gel is rinsed in distilled water for 2–3 h, and can then be photographed. Any blue background in the gel is removed by rinsing in 10 % TCA solution.

8.9.4.4 Nomenclature of avenin bands

The method is best used comparatively, i.e., by comparing the avenin profile of an unknown sample with that of authentic reference samples extracted and analysed at the same time. There is no internationally agreed system of nomenclature for avenins, and bands are usually numbered sequentially or their relative mobilities measured.

8.9.5 *Helianthus annuus* (sunflower)

8.9.5.1 Principle

The standard reference method for the measuring hybrid purity and verifying varieties of *Helianthus annuus* (sunflower) is by ultrathin-layer isoelectric focusing (UTLIEF). The alcohol-soluble proteins are extracted from individual seeds and separated by IEF in ultrathin-layer gels. The pattern of protein bands found on the gel is characteristic for a variety or an inbred line. Also, it is generally possible to estimate the purity of hybrid samples by finding one or more bands in the male parent that are lacking in the female parent (and present in the hybrid). These bands can be used as marker bands for the verification of hybrids and as a means of estimating hybrid purity.

Ultrathin gels can be run at higher voltages with shorter running times, and stain more quickly than conventional gels.

8.9.5.2 Apparatus and equipment

8.9.5.2.1 Apparatus

Any suitable horizontal electrophoresis apparatus with a cooling system and high voltage power supply may be used.

8.9.5.2.2 Chemicals

All chemicals should be of 'Analytical Reagent' grade or equivalent.

- 2-Chloroethanol
- Acrylamide ('specially purified for electrophoresis')
- Bisacrylamide (BIS) ('specially purified for electrophoresis')
- Ampholytes: pH 5–8 and pH 2–11 (or "Seed Mix pH 5–8/2–11" from SINUS)
- Ammonium peroxydisulphate (APS)
- N,N,N',N'-Tetramethylethylenediamine (TEMED)
- Urea
- Taurin
- L-Aspartic acid
- L-Glutamic acid
- L-Arginine (base)
- L-Lysine
- Ethylenediamine
- Trichloroacetic acid (TCA)
- Coomassie Brilliant Blue G 250 (**or** equivalent)
- Coomassie Brilliant Blue R 250 (**or** equivalent)
- Ethanol (96 %)
- Acetic acid (99 %)
- 'Gel-Slick' (**or** 'Repelsilane', **or** equivalent)

8.9.5.2.3 Solutions

a) Extraction solution: 30 % (v/v) 2-chloroethanol, i.e. 30 mL 2-chloroethanol made up to 100 mL with distilled water. This solution can be stored at room temperature.

b) Anode solution: L-aspartic acid (0.83 g) and L-glutamic acid (0.92 g) are dissolved in hot distilled water and diluted to 250 mL. This solution can be stored for two weeks at 4 °C.

c) Cathode solution: L-arginine (base) (1.18 g), L-lysine (0.91 g) and ethylenediamine (30.00 mL) are dissolved in distilled water and diluted to 250 mL. This solution can be stored for two weeks at 4 °C.

d) Stock gel solution: Acrylamide (16.57 g) and bisacrylamide (0.43 g) are dissolved in distilled water and diluted to 250 mL. This solution can be stored for up to two weeks at 4 °C.

e) Gel fixing solution: trichloroacetic acid (TCA) (approx. 12 % (w/v) solution). Stock solution: 1 kg TCA dissolved in 450 mL distilled water. Before use, 120 mL stock solution is diluted with 880 mL distilled water, to give an approximately 12 % TCA solution. About 400 mL is needed for one gel, and the solution can be used up to three times.

f) Gel staining solution: Coomassie Blue R 250 (0.45 g), Coomassie Blue G 250 (1.35 g), acetic acid (330 mL) and ethanol (540 mL) are mixed and made up to 3000 mL with distilled water; 400 mL is sufficient for staining one gel.

g) Gel destaining solution: ethanol (750 mL) and acetic acid (125 mL), made up to 2500 mL with distilled water.

8.9.5.3 Procedure

8.9.5.3.1 Protein extraction

Either a single dry seed with or without seedcoat **or** one half is crushed to fine pieces by hand (with pliers or pestle and mortar).

The seed meal is extracted with 0.225 mL of extraction solution (8.9.5.2.3a) in a microtitre plate or a microcentrifuge tube. The samples are left for about 1 h at 20 °C. After this time, the microtitre plate or microtube is treated with ultrasound for 30 s and then centrifuged at 2000 × *g* for 5 min. The clarified supernatant is used for electrophoresis. Frozen at –20 °C, the remaining extract will keep for up to 3 months.

8.9.5.3.2 Gel preparation

Gels are made up **either** directly between two thin glass plates **or** between a polyester carrier sheet held on glass and a cover glass plate. The plates or sheets must be treated before use, one (carrier) with a silylating reagent to make the gel adhere and one (cover) with 'Gel-Slick' (**or** equivalent) to prevent gel adhesion. Commercially available pre-prepared gel carrier sheets can also be used.

The clean and dry gel cassettes are assembled, according to the type of equipment in use. A gel thickness of 0.12 mm is recommended, which can be achieved by the use of a defined thickness of adhesive tape as a spacer.

The following components are taken and mixed:
- 50 mL stock gel solution (8.9.3.2.3d)
- 16 g urea
- 1.5 g taurin
- 2.90 mL ampholytes (pH 5–8)
- 1.50 mL ampholytes (pH 2–11)

Note: dissolve taurin first into the stock gel solution because of the slow solubility.

Optional gel solution:
- 50 mL stock gel solution (8.9.3.2.3d)
- 16 g urea
- 0.55 mL ampholytes (pH 2–4)
- 0.55 mL ampholytes (pH 4–6)
- 1.40 mL ampholytes (pH 5–8)
- 1.90 mL ampholytes (pH 4–9)

For polymerisation, 0.35 mL APS (20 % (w/v) solution *freshly prepared*) and 0.05 mL TEMED (full strength) are added carefully, to avoid introducing excessive amounts of air.

This will be sufficient for 10 gels of dimensions 240 × 180 × 0.12 mm (one gel requires 6.5 mL). After careful mixing, the gel is poured onto the carrier plate/sheet, the cover plate is carefully lowered and the gel 'cassette' left to polymerise for at least 45 min. Gels not immediately used can be stored wrapped in cellophane at 4 °C for at least 14 days.

8.9.5.3.3 Electrophoresis

The gel is placed on the precooled (10 °C) cooling plate of the horizontal electrophoresis apparatus. To ensure better gel adhesion and cooling, a thin layer of water is placed between the gel and the plate. The electrode wicks are soaked in the appropriate solution (see 8.9.5.2.3b or 8.9.5.2.3c) and placed at either end of the gel. Samples (approx. 4 µL) are loaded in the applicator strip 0.5 cm below the bufferwick of the anode and focusing carried out at 2500 V, 15 mA, 40 W for about 70 min until completion (for one gel).

Notes:

a) Double-focusing is possible with this method, and it is then necessary to place the cathode wick in the centre of the gel, with anode wicks at both ends.

b) The precise conditions and times required for focusing will vary according to the dimensions of the gel and the type of sunflower, inbred line etc., and may need to be determined empirically.

8.9.5.3.4 Fixing and staining

At the end of the electrophoresis, the gel is removed and incubated in fixing solution (see 8.9.5.2.3e) with slow shaking, for at least 20 min. The gel is then stained by shaking in gel staining solution (see 8.9.5.2.3.f) for 45 min. Destaining takes (solution 8.9.5.2.3g) about 15 min, followed by brief rinsing in distilled water. The gel is dried overnight at room temperature and can then be sealed with adhesive film. Gels can be stored for many years at room temperature.

8.9.5.4 Evaluation of results

At present, the method is best used to verify varieties in a comparative way, by examining whether or not the protein pattern of the sample is identical to that of the authentic reference variety, analysed at the same time.

For hybrid seed, it is possible to determine hybrid purity (selfing rate). It is assumed that both parental lines are homogeneous. When comparing the protein patterns of the female and male parents with the hybrid, one or more marker bands (present in the male only) need to be found in the hybrid (8.9.3.4, Fig. 8.1). Given the presence of such markers, hybrid purity can be determined by examining a suitable number of single seeds of a seed lot. Seeds with a protein pattern identical to the female parent are judged to be self-pollinations ('sibs'). Foreign pollinated seeds show a different pattern, mostly a protein band at an unexpected place in the variety pattern. Seeds with a different pattern may also arise due to contamination with another variety.

The number of seeds to be tested for hybrid purity determination depends on the acceptable confidence intervals, established for each individual case. It is suggested that normally 192 single seeds are analysed, working with 2 × 96 titre plates, as a compromise between precision of results and working time needed (see Chapter 4, '*Handbook of Variety Testing – Electrophoresis Testing*', ISTA, 1992). For reports and issue of ISTA Certificates, analysis of 400 single seeds, working with 4 × 96 titre plates, is required.

8.9.6 *Triticum* (wheat)

8.9.6.1 Principle

The standard reference method for verifying varieties of *Triticum* is by acetic acid urea polyacrylamide gel electrophoresis (A-PAGE). The alcohol-soluble proteins (gliadins) are extracted from seeds and separated by A-PAGE at pH 3.2. The pattern of protein bands produced (electropherogram) is related to genetic constitution and can be considered as a 'fingerprint' of a variety. The 'fingerprints' can be used to identify unknown samples and mixtures, by single seed analysis.

8.9.6.2 Equipment

- Any suitable vertical electrophoresis system
- Cooling system
- Power supply
- Hood
- Mixer
- Centrifuge
- Shaker
- Transilluminator
- Oven or drying equipment (gel dryer or glass plates and cellophane sheets)

8.9.6.3 Chemicals

All chemicals must be of 'analytical reagent' grade or better (acrylamide and bisacrylamide specially purified for electrophoresis).

8.9.6.4 Sample preparation

Seeds can be ground, crushed or halved with pliers or a razor blade and transferred to microcentrifuge tubes (1.5 mL) or microtitre plates (200 µL).

8.9.6.5 Extraction

8.9.6.5.1 Extraction (option 1)

8.9.6.5.1.1 Solutions

a) Extraction solution
- Ethanol: 70 % prepared immediately before use
- Acetone: concentrated

b) Sample buffer
- Glycerol: 30 % w/v
- Urea: 6 M
- Acetic acid: 25 mM
- Pyronine G: 0.05 %

Add water to the final volume. Keep the solutions at room temperature.

8.9.6.5.1.2 Procedure

Add 70 % ethanol at 200 µL per seed or per 50–60 mg flour. When using microcentrifuge tubes, mix the samples with e.g. a vortex. With microtitre plates, mixing is not necessary. Leave the sample in the dark at room temperature for 1 h. Centrifuge, recover the clarified supernatant in 1.5 mL tubes, then add 1 mL acetone stored at room temperature. Proteins will precipitate in a few minutes (keep at 4 °C if not used). Centrifuge, discard the acetone, dry the pellet under the hood for 5 min. Add 150 µL of sample buffer. The extraction is finished in about 2 h.

Extracts can be stored at 4 °C for some weeks.

8.9.6.5.2 Extraction (option 2)

8.9.6.5.2.1 Solution
- 2-Chlorethanol: 25–30 %
- Pyronine G or methyl green: 0.05 %

Add water to the final volume. Keep the solution cold (4 °C).

8.9.6.5.2.2 Procedure

Add 150–200 µL extraction buffer. When using microcentrifuge tubes, mix the samples with e.g. a vortex. With microtitre plates, mixing is not necessary. Incubate the samples overnight at room temperature (approx. 20 °C).

If necessary, before loading the gel, centrifuge the samples at 13 000 rpm for 15 min.

Extracts can be stored at 4 °C for some days.

8.9.6.6 Gel preparation and buffer tank solutions

8.9.6.6.1 Gel preparation (option 1)

8.9.6.6.1.1 Gel mix
- Acrylamide: 12 % (from 40 % solution)
- Bisacrylamide: 0.4 % (from 2 % solution)
- Acetic acid: 0.75 %
- Urea: 12 %
- Ferrous sulphate: 0.0014 %
- Ascorbic acid: 0.1 %

Add water to final volume (for example 80 mL for 2 gels of 16 cm × 18 cm × 1.5 mm thick). Mix until complete dissolution.

8.9.6.6.1.2 Polymerisation starter
– Hydrogen peroxide: 100 vol, 0.001 % (v/v), final gel concentration.

Gel preparation should be done quickly because polymerisation is very rapid. Cooling the cassettes to 4 °C before filling with the gel mix helps to delay the polymerisation.

8.9.6.6.1.3 Buffer tank solutions
– Upper tank buffer: 700 mL water + 1 mL acetic acid (0.143 % v/v)
– Lower tank buffer: 4000 mL water + 10 mL acetic acid (0.25 % v/v)

8.9.6.6.2 Gel preparation (option 2)

8.9.6.6.2.1 Gel mix
– Acrylamide: 10 % final concentration (from solution or powder)
– Bisacrylamide: 0.4 % Final concentration (from solution or powder).

Or use a pre-prepared gel mix of acrylamide and bisacrylamide.

Note: The powder forms of acrylamide and bisacrylamide are much more readily inhaled, as they are very light and highly electrostatic, so the powder floats in the air as soon as the bottle is opened. Handle in a fume hood.

– Urea: 6 %
– Ferrous sulphate: 0.005 %
– Ascorbic acid: 0.005– 0.1 %

Add the following buffer: 0.1 % glycine (w/v), 2 % glacial acetic acid (v/v) and water to final volume. Mix until complete dissolution.

8.9.6.6.2.2 Polymerisation starter
– Hydrogen peroxide: 100 vol, 0.002–0.003 % (v/v), 30 %, final gel concentration.

Gel preparation should be done quickly because polymerisation is very rapid. Cooling the cassettes to 4 °C before filling with the gel mix helps to delay the polymerisation.

8.9.6.6.2.3 Buffer tank solution
Only one buffer: 0.4 % glacial acetic acid (v/v) + 0.04 % glycine (w/v) + water to final volume

8.9.6.7 Loading samples

5–20 µL, depending on the equipment used.
Loading can be performed using a syringe, a multichannel syringe, a pipette or a multichannel pipette.

8.9.6.8 Electrophoresis

8.9.6.8.1 Electrophoresis (option 1)

– Constant voltage: 500 V for the chamber.

Water should be circulated through the buffer tank to maintain the buffer temperature at 18 °C.

– Running time: 2 times the time required for the dye to leave the gel.

8.9.6.8.2 Electrophoresis (option 2)

– Constant current: 40 mA for each gel.

Water should be circulated through the buffer tank to maintain the buffer temperature at 10–20 °C.

– Running time: 2 times the time required for the dye to leave the gel.

8.9.6.9 Fixing and staining

8.9.6.9.1 One-step fixing and staining (option 1)

– Stock Coomassie: Coomassie Blue R 250 1 g/100 mL ethanol. Store this solution at 4 °C in a dark bottle.
– Fixing and staining solution: 2.5 % stock Coomassie Blue R 250 (v/v) + 6.25 % trichloroacetic acid (TCA) (w/v), water to 400 mL, or use a pre-prepared solution.

This solution is enough for 2 gels 16 × 18 cm × 1.5 mm thick. Shake overnight with orbital shaker. The solution can be used once only.

8.9.6.9.2 One-step fixing and staining (option 2)

- Stock Coomassie: 0.25 % (w/v) Coomassie Blue G 250 + 0.75 % (w/v) Coomassie Blue R 250 + water to complete volume, or use a pre-prepared solution.
- Fixing and staining solution: 8.3 % (w/v) trichloroacetic acid (TCA) + 5.8 (v/v) acetic acid + 12.5 % (v/v) ethanol + 2 % (v/v) stock Coomassie.

Staining is complete after 1 day, but at the earliest after 4 h. The solution can be used six times.

8.9.6.9.3 Two-step fixing and staining (option 3)

- Stock Coomassie: 0.25 % (w/v) Coomassie Blue G 250 + 0.25 % (w/v) Coomassie Blue R 250, complete volume with ethanol 100 %, or use a pre-prepared solution. Store the solution at 4 °C in a dark bottle.
- Fixing solution: 10 % TCA. Store at room temperature under hood.
- Staining solution: 20 % stock Coomassie (v/v) + 8 % acetic acid (v/v). Add water to complete volume. Store under hood at room temperature in a dark bottle.

1. Fix gels in TCA 10 % for 1 h. Gels can be saved in this solution for few days.
2. Stain the gels for approx. 3 h or overnight.

The fixing and staining solutions can be used six times.

8.9.6.10 Destaining

Destaining with tap water: rinse the gels 1–2 times (30 min each). For slow destaining, use a 10 % TCA solution.

8.9.6.11 Storage of the gels

Gels can be kept in either 10 % TCA solution or in a glycerol solution (3 %), and then dried between two cellophane sheets, photographed or scanned.

In polyethylene bags gels can be stored at 4 °C for months without deterioration. After drying, gels can be stored for years.

8.9.7 *Triticum* and ×*Triticosecale* (wheat and triticosecale)

8.9.7.1 Principle

The standard reference method for verifying varieties of *Triticum* and ×*Triticosecale* is by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Seed proteins are extracted from individual seeds, treated with SDS and separated using a discontinuous SDS-PAGE procedure. The pattern of protein bands found on the gel is characteristic of a variety.

As a guideline, it is recommended that 100 individual seeds are used. Very precise estimates of varietal purity may require a larger sample. If a comparison is being made with a standard value, sequential testing using batches of 50 seeds can be undertaken in order to minimise the workload. A simple check on the identity of a single major constituent of a seed lot can be done using less than 50 seeds.

8.9.7.2 Equipment

Any suitable vertical electrophoresis system may be used.

8.9.7.3 Chemicals

All chemicals must be of 'analytical reagent' grade or better (acrylamide and bisacrylamide specially purified for electrophoresis).

- Acrylamide 40 % solution
- Bisacrylamide 2 % solution
- Urea
- Glycine
- Ammonium persulphate (APS) and TEMED
- 2-Mercaptoethanol
- Sodium dodecyl sulphate (SDS) (10 % stock solution)
- Tris
- Pyronine G/bromophenol Blue
- Coomassie Blue R 250
- Coomassie Blue G 250
- Purified water

8.9.7.4 Sample preparation

Single seeds crushed with pliers or alternatively 50–70 mg of flour are transferred to 1.5 mL polypropylene centrifuge tubes or microtitre plates.

8.9.7.5 Extraction

8.9.7.5.1 Extraction buffer

– Urea: 4.5 M, 3 %
– 2-Mercaptoethanol: 10 % SDS

8.9.7.5.2 Extraction procedure

Add 150–500 µL (depending on the equipment used) of the extraction buffer and thoroughly mix the sample. Leave to stand overnight at room temperature. With microtitre plates, mixing is not necessary.

Heat the samples in a boiling water bath for 10 min and allow to cool. Before the gel is loaded, the tubes are centrifuged at 18 000 × *g*. With microtitre plates, heating is not necessary.

8.9.7.6 Gel preparation

Two gels, 16 × 18 cm, 1.5 mm thickness, depending on the equipment used.

8.9.7.6.1 Stacking gel

– Stacking gel: acrylamide 3 %, 0.125 M Tris-HCl, pH 6.8.
– Acrylamide 40 % solution: 1.5 mL
– Bisacrylamide 2 % solution: 0.43 mL

Or use a pre-prepared gel mix of acrylamide and bisacrylamide.

– Tris-HCl 1 M pH 6,8: 2.5 mL
– SDS 10 %: 0.16 mL
– Water: 14.87 mL

For polymerisation:
– APS 1 %: 0.75 mL
– TEMED: 20 µL

Add the reagents to a 19.46 mL of stacking gel solution.

8.9.7.6.2 Resolving gel

– Resolving gel: acrylamide 10 %, 0.375 M Tris-HCl, pH 8.8
– Acrylamide 40 % solution: 20 mL
– Bisacrylamide 2 % solution: 5.2 mL

Or use a pre-prepared gel mix of acrylamide and bisacrylamide.

– Tris-HCl 1 M pH 8.8: 30 mL
– SDS 10 %: 0.8 mL
– Water: 20.8 mL

For polymerisation:
– APS: 1 % 2 mL
– TEMED: 40 µL

Add the reagents to a 76.80 mL of resolving gel solution.

8.9.7.6.3 Tank buffer

– Tank buffer stock solution: tris 0.0250 M, glycine 0.187 M, SDS 1 %, pH 8.3
– Glycine: 141.1 g
– Tris: 30.0 g
– SDS: 10.0 g

Make up to 1000 mL with water. Dilute the stock solution 1:10 before use.

8.9.7.7 Loading samples

10–15 µL, depending on the equipment used. Loading can be performed using a syringe, a multichannel syringe, a pipette or a multichannel pipette.

8.9.7.8 Electrophoresis

Two gels, 16 × 18 cm, 1.5 mm thickness, depending on the equipment used.

– Stacking gel: constant voltage at 100 V (about 40 mA)
– Running gel: 80 mA (max. 400 mA)

Water should be circulated through the buffer tank to maintain the temperature at 15–20 °C.

Stop the run 40 min after the tracking dye has reached the bottom of the gel.

8.9.7.9 Fixing and staining

Fixing:
– TCA 15 %, approx. 30 min

Staining, solution A:
– Coomassie Blue G 250 0.25 g
– Coomassie Blue R 250 0.75 g

Made up to 100 mL with water, or use a pre-prepared solution.

Staining, solution B:
- TCA: 27.5 g
- Acetic acid: 32.5 mL
- Ethanol: 90 mL
- Solution A: 12.5 mL

Water to 400 mL. Stain overnight at room temperature.

8.9.7.10 Destaining

Destaining with tap water: rinse the gels 1–2 times (30 min each). For slow destaining, use a 10 % TCA solution.

8.9.7.11 Storage of the gels

Gels can be kept in either 10 % TCA solution or in a glycerol solution (3 %), and then dried between two cellophane sheets, photographed or scanned.

In polyethylene bags gels can be stored at 4 °C for months without deterioration. After drying gels can be stored for years.

8.10 DNA-based methods

8.10.1 Principles of DNA-based methods

DNA is extracted from seeds and a minimum number of microsatellite markers are amplified by the polymerase chain reaction (PCR). The amplified DNA fragments are separated according to size using electrophoresis and detected using an appropriate technique. Generally, electrophoresis and fragment detection are accomplished concurrently by the same instrument. Alleles are defined by length (number of base pairs). Allele profiles are characteristic of variety and can be used to identify unknown samples. Mixtures may be assessed by single seed analysis.

The procedure consists of several stages, including DNA extraction, PCR amplification, fragment separation and detection, and evaluation of results. Although recommended protocols are provided, in line with the principles of a SPBA, testing may be carried out using any suitable procedure for each stage of the process so long as the procedures have been validated as fit for purpose and the end result meets acceptable standards as set by ISTA. A core set of microsatellite markers is prescribed; its use is required for reports and issuance of ISTA Certificates. In some circumstances the prescribed set of markers may not be sufficient to provide unique allele profiles for all of the varieties that may be encountered. In such cases the analysis may be enhanced through the addition of recommended supplementary microsatellite markers, or other suitable microsatellites markers.

8.10.2 *Triticum* (wheat)

The standard reference DNA-based method for verifying varieties of Triticum is by analysis of a minimum of eight microsatellite markers. Verification of the identity of a single-constituent seed lot may be achieved using pooled seed samples or analysis of a small number of individual seeds. Estimates of varietal purity will require analysis of larger numbers of individual seeds; sample sizes of greater than 100 may be required for precise estimates.

8.10.2.1 Microsatellite markers

Table 8.5 contains prescribed microsatellite markers required for reports and issuance of ISTA Certificates.

Addition of 5′-tail sequences for labelling using a universal primer approach, or direct labelling through the addition of a fluorophore, are the only modifications permitted to PCR primers of the prescribed microsatellite markers.

Table 8.6 contains recommended supplementary microsatellite markers. Their use is optional.

8.10.2.2 Recommended DNA extraction protocol

The following procedure is suitable for extraction of DNA from individual seeds or pools of up to 10 seeds. Portions (400 mg) of finely ground bulk samples may also be used as starting material, in which case the initial pulverisation step may be omitted.

To make one litre of extraction buffer, the following are combined and mixed well:
- 200 mL 1 M Tris-HCl (pH 7.5)
- 16.8 g NaCl
- 50 mL 0.5 M EDTA (pH 8.0)
- 5 g SDS
- ultrapure water to 1 L final volume

For each kernel or pool of seeds to be extracted, a single grinding bead (such as a Qiagen 5 mm stainless steel bead) is placed in a 2 mL round-bottom microcentrifuge tube. Individual seeds are crushed with needle-nose pliers as they are placed into the tubes. The seeds are then

Table 8.5. Prescribed microsatellite markers and PCR primers for verification of wheat varieties

Marker	Forward primer	Reverse primer	Source
DuPw167	cggagcaaggacgatagg	caccacaccaatcaggaacc	[a]
DuPw217	cgaattacacttccttcttccg	cgagcgtgtctaacaagtgc	[a]
DuPw004	ggtctggtcggagaagaagc	tgggagcgtacgttgtatcc	[a]
DuPw115	tgtttcttcctcgcgtaacc	cctcgaatctcccagttatcg	[a]
DuPw205	atccagatcacaccaaacgg	cttccgcttcatcttcttgc	[a]
Xgwm155	caatcatttccccctccc	aatcattggaaatccatatgcc	[b]
Xgwm413	tgcttgtctagattgcttggg	gatcgtctcgtccttggca	[b]
Xgwm003	gcagcggcactggtacattt	aatatcgcatcactatccca	[b]

[a] Eujayl *et al*. (2002). Isolation of EST-derived microsatellite markers for genotyping the A and B genomes of wheat. *Theoretical and Applied Genetics* **104**, 399–407

[b] Röder *et al*. (1998). A microsatellite map of wheat. *Genetics* **149**, 2007–2023

Table 8.6. Recommended supplementary microsatellite markers and PCR primers for verification of wheat varieties

Marker	Forward primer	Reverse primer	Source
Xgwm372	aatagagccctgggactggg	gaaggacgacattccacctg	[b]
Xbarc347	gcgcacctctcctcaccttct	gcgaacatggaaatgaaaactatct	[a]
Xbarc184	ttcggtgatatcttttccccttga	ccgagttgactgtgtgggcttgctg	[a]
Xbarc074	gcgcttgccccttcaggcgag	cgcgggagaaccaccagtgacagagc	[a]
Xgwm052	ctatgaggcggaggttgaag	tgcggtgctcttccattt	[b]
Xgwm095	gatcaaacacacacccctcc	aatgcaaagtgaaaaacccg	[b]

[a] Song *et al*. (2005). Development and mapping of microsatellite (SSR) markers in wheat. *Theoretical and Applied Genetics* **110**, 550–560

[b] Röder *et al*. (1998). A microsatellite map of wheat. *Genetics* **149**, 2007–2023

pulverised for 1 min at 30 Hz in a mixer mill (such as a Qiagen TissueLyser II). The tubes are then tapped on the countertop to settle the contents and 1.25 mL extraction buffer is added to each, followed by agitation at 30 Hz for 30 seconds in a mixer mill. Solids are pelleted by centrifugation for 5 minutes at ~5800 × *g*.

For each extract, 750 µL of supernatant is transferred to a 1.5 mL microcentrifuge tube containing an equal volume of isopropanol. Mixing is accomplished through repeated inversion. Precipitated DNA is then pelleted by centrifugation for 2 min at ~ 4000 × *g*. The supernatant is poured off; the remaining liquid is collected at the tube bottom by brief centrifugation and then removed with a pipette.

Pellets are allowed to air-dry for about 15 min before resuspension in 200 µL ultrapure water, assisted by agitation for 15 seconds at 30 Hz in a mixer mill. Immediately prior to use in PCR, any remaining solids should be pelleted by centrifugation for at least 5 min at maximum speed in a microcentrifuge. Single-seed extracts can be used directly in PCR; for preparations from seed pools, a 1:10 dilution in ultrapure water should be used.

8.10.2.3 Recommended PCR procedures

The microsatellite markers comprising the prescribed and recommended supplementary marker sets were selected in part because they are compatible in multiplexed analyses; each set is amenable to amplification in a single PCR.

Fluorescent labelling can be accomplished using a universal primer approach (Oetting *et al*., Genomics. 1995 Dec 10;30(3):450–8) in which the M13 sequence 5′-CACGACGTTCTAAAACGAC-3′ is added to the 5′ end of each forward primer and a single fluorescently labelled M13 primer having the identical sequence is included in the reaction mixture. During PCR, this universal fluorescent primer hybridises with complementary sequences generated in early amplification cycles, resulting in the synthesis of fluorescent products for all of the microsatellite markers in the reaction.

Table 8.7. Recommended reaction composition for multiplexed PCR amplification of microsatellite markers for verification of wheat varieties

Amount per reaction (µL)	Component	Final concentration
X [a]	Ultrapure H_2O	
1	10 × PCR buffer [b]	1 ×
0.6 [c]	25 mM $MgCl_2$	1.5 mM
0.25 [d]	Each forward primer (2 µM stock)	0.05 µM
0.25	Each reverse primer (2 µM stock)	0.05 µM
0.25	Labelled M13 primer (2 µM)	0.05 µM
0.8	dNTP mix (2.5 mM each dNTP)	0.2 mM each
0.1	Taq DNA polymerase (5 U/µL)	0.05 U/µL

[a] Determined as the amount required to achieve a reaction volume of 9 µL (prior to addition of template DNA) and is dependent upon the total number of PCR primers included.
[b] Generally, the buffer should be as supplied with the DNA polymerase.
[c] Amount shown assumes the PCR buffer does not contain $MgCl_2$. If this is not the case, the amount of $MgCl_2$ added should be adjusted accordingly.
[d] This is a suggested starting point. Concentrations in multiplexed reactions may require adjustment (generally within a range of 0.03 µM to 0.10 µM) for some markers depending upon relative product intensities observed. For a given microsatellite marker, forward and reverse primers should be adjusted equally.

8.10.2.3.1 Reaction components

A master mix with all reaction components except the template DNA should be set up and aliquoted into reaction tubes or plate wells. Table 8.7 contains a list of reagents for a single 10 µL reaction.

When preparing a master mix, component quantities are determined by multiplying the amounts indicated per reaction by the number of samples to be tested, plus one extra to accommodate pipetting inaccuracies. The components should be combined in a microcentrifuge tube in the order listed. The mixture should be gently vortexed, briefly centrifuged to collect contents at the bottom of the tube and then distributed into reaction tubes or wells (9 µL each). Lastly, 1 µL template DNA (prepared as described in 8.10.2.3) is added to each, resulting in a final reaction volume of 10 µL.

8.10.2.3.2 Thermal cycling profile

The following thermal cycling profile (Table 8.8) has been used successfully with the prescribed and recommended supplementary microsatellite markers. The total number of cycles may require alteration based on product intensities achieved.

Table 8.8. Recommended thermal cycling profile for PCR amplification of microsatellite markers for verification of wheat varieties

Number of cycles	Temperature	Duration
1	94 °C	3 min
	58 °C	1 min
	72 °C	1 min
34	94 °C	30 s
	58 °C	30 s
	72 °C	30 s
1 (final extension)	72 °C	5 min
1 (hold)	20 °C	indefinitely

8.10.2.4 Evaluation of results

This method is best used to verify varieties in a comparative manner, i.e., to determine whether the allele profile of a sample is identical to that of an authentic reference variety. It can be useful, particularly in gel-based analysis systems, to include samples of known varieties with known allele profiles to assist in the determination of sample allele sizes.

Microsatellite variation may occur among seeds within a variety of wheat. If analyses are performed on individual seeds, reference profiles should be determined using a sufficient number of individual authentic reference variety seeds to ensure that variation within a variety is adequately represented. If analyses are performed on pooled samples, it is recommended that the reference profiles used should also be based upon pooled seeds of authentic reference varieties.

8.10.3 *Zea mays* (maize)

The standard reference DNA-based method for verifying varieties of maize is by analysis of a minimum of eight microsatellite markers. Verification of the identity of a single-constituent seed lot may be achieved using pooled seed samples or analysis of a small number of individual seeds. Estimates of varietal purity will require analysis of larger numbers of individual seeds; sample sizes of greater than 100 may be required for precise estimates.

8.10.3.1 Microsatellite markers

Use of the eight prescribed microsatellite markers (Table 8.9) is required for reports and issuance of ISTA Certificates. If these markers do not provide sufficient discrimination among the varieties for the purpose at hand, they may be supplemented with additional microsatellite markers of the laboratory's choosing. Addition of 5′-tail sequences for labelling, using a universal primer approach or direct labelling through the addition of a fluorophore, are the only modifications permitted to PCR primers of the prescribed microsatellite markers.

8.10.3.2 Recommended DNA extraction protocol

DNA can be extracted using column-based kits; manual procedures such as those based on CTAB are also adequate for extraction of DNA from maize seeds. The DNA quality can be verified by means of a 1 % agarose gel or spectrophotometry. The quantity of the DNA may also be verified on an agarose gel or by using fluorometry or spectrophotometry. Once the extraction procedure has been validated for the matrix, verification of DNA quality and quantity may not be necessary for all samples.

8.10.3.3 Recommended PCR procedures

The microsatellite markers set was selected based on performance in three comparative tests. The performance of these markers in multiplex PCR has not been investigated.

Fluorescent labelling can be accomplished using a universal primer approach (Oetting *et al*., 1995) in which the M13 sequence 5′-CACGACGTTCTAAAACGAC-3′ is added to the 5′ end of each forward primer and a single fluorescently labelled M13 primer having the identical sequence is included in the reaction mixture. During PCR, this universal fluorescent primer hybridises with complementary sequences generated in early amplification cycles, resulting in the synthesis of fluorescent products. Fluorescent labelling can also be achieved through the addition of fluorophores.

8.10.3.3.1 Reaction components

For each marker, a master mix with all reaction components except the template DNA should be set up and aliquoted into reaction tubes or plate wells. Reagents for a single 10 µL reaction are listed in Table 8.10.

Table 8.9. Prescribed microsatellite markers and PCR primers[a] for verification of maize varieties.

Marker	Forward primer	Reverse primer	Approximate allele size range (bp)
umc1545	gaaaactgcatcaacaacaagctg	attggttggttcttgcttccatta	66–81
umc1448	atcctctcatctttaggtccaccg	catatacagtctcttctggctgctca	165–180
umc1117	aattctagtcctgggtcggaactc	cgtggccgtggagtctactact	140–168
umc1061	agcaggagtacccatgaaagtcc	tatcacagcacgaagcgatagatg	99–108
phi109275	cggttcatgctagctctgc	gttgtggctgtggtggtg	123–138
phi102228	attccgacgcaatcaaca	ttcatctcctccaggagcctt	123–129
phi083	caaacatcagccagagacaaggac	attcatcgacgcgtcacagtctact	123–135
phi015	gcaacgtaccgtacctttccga	acgctgcattcaattaccgggaag	81–102

[a] Source: www.maizegdb.org/data_center/ssr.

Table 8.10. Recommended reaction composition for PCR amplification of microsatellite markers for verification of maize varieties.

Amount per reaction (µL)	Master mix components	Final concentration
1.95	ultrapure H_2O to complete 6 µL	–
1	10 × PCR buffer [a]	1 ×
1.2 [b]	25 mM $MgCl_2$	3 mM
0.25 [c]	forward primer (10 µM)	0.25 µM
0.25	reverse primer (10 µM)	0.25 µM
1.25	dNTP mix (10 mM)	0.125 mM
0.1	Taq DNA polymerase (5 U/µL)	0.05 U

[a] Generally, the buffer should be as supplied with the DNA polymerase.
[b] Amount shown assumes the PCR buffer does not contain $MgCl_2$. If this is not the case, the amount of $MgCl_2$ added should be adjusted accordingly.
[c] This is a suggested concentration. For a given microsatellite marker, forward and reverse primers should be adjusted equally.

When preparing a master mix, component quantities are determined by multiplying the amounts indicated per reaction by the number of samples to be tested, plus one extra to accommodate pipetting inaccuracies. The components should be combined in a microcentrifuge tube in the order listed. The mixture should be gently vortexed, briefly centrifuged to collect contents at the bottom of the tube and then distributed into reaction tubes or wells (6 µL each). Lastly, 4 µL (approximately 2.5 ng/µL) of template DNA is added to each reaction tube, resulting in a final reaction volume of 10 µL.

8.10.3.3.2 Thermal cycling profile

A "Touchdown" program, where the annealing temperature is decreased 1 °C per cycle from 64 °C until reaching 55 °C, is recommended. The thermal cycling profile presented in Table 8.11 has been used successfully with the prescribed markers in comparative tests, thus it is recommended for verification of maize varieties. The total number of cycles may require alteration based on product intensities achieved.

Table 8.11. Recommended thermal cycling profile for PCR amplification of microsatellite markers for verification of maize varieties.

Number of cycles	Temperature	Duration
1	94 °C	10 min
10	94 ºC	30 s
	64 °C (decreasing by 1 °C per cycle)	30 s
	72 °C	30 s
30	94 ºC	30 s
	55 °C	30 s
	72 °C	30 s
1 (final extension)	72 ºC	10 min
1 (hold)	10 °C	indefinitely

8.10.3.4 Evaluation of results

This method is best used to verify varieties in a comparative manner, i.e., to determine whether the allele profile of a sample is identical to that of an authentic reference variety. It can be useful, particularly in gel-based analysis systems, to include samples of known varieties with known allele profiles to assist in the determination of sample allele sizes.

If analyses are performed on individual seeds, reference profiles should be determined using a sufficient number of individual authentic reference variety seeds to ensure that variation within a variety is adequately represented. If analyses are performed on pooled samples, it is recommended that the reference profiles used should also be based upon pooled seeds of authentic reference varieties.

8.11 Examination of seedlings

8.11.1 Cereals

Certain varieties can be classified by the colour of their coleoptiles. Germinate the seeds on damp filter paper in dishes covered with glass plates. The colour of the coleoptile can vary from green to violet and is determined when the seedlings have reached a suitable stage of development. It can be intensified by moistening the filter paper with a 1 % solution of NaCl or HCl, or by illuminating the seedlings with ultra-violet light for 1–2 h before examination.

8.11.2 *Beta* spp.

Some varieties can be distinguished by seedling colour, which may be white, yellow, pale red, or red. Sow the seeds in damp sand in dishes placed in subdued daylight at room temperature. After seven days the seedlings are examined for hypocotyl colour. For sugar beet and white fodder beet, the proportion of white to pale-red seedlings gives some indication of the genuineness of the cultivar.

8.11.3 *Brassica* spp.

In swede and turnip, white-fleshed varieties can be distinguished from yellow-fleshed varieties by the colour of the cotyledons, which are lemon-coloured in white-fleshed varieties and orange-coloured in yellow-fleshed varieties. Germinate the seeds in darkness at 20<=>30 °C. After five days the cotyledons are transferred to Petri dishes containing alcohol (85–96 %) and placed on a white surface. After 4 h the colour is determined.

8.11.4 *Lolium* spp.

In most varieties of *Lolium multiflorum* the root traces of the majority of the seedlings show fluorescence under ultra-violet light, whereas in most varieties of *Lolium perenne* the root traces of the majority of the seedlings do not show fluorescence. The fluorescence test alone, however, is not always a sufficient basis for the identification of a species or a cultivar, because many of the varieties grown contain a certain number of plants which do not give the typical reaction for the species. Further, several of the hybrid forms between the two species may give an intermediate reaction.

For determination of the reaction of seedling root traces to ultra-violet light, the seeds are placed on suitable non-fluorescent white filter paper moistened with water for germination within the 20–30 °C range using either an alternating or constant 20 °C temperature, in darkness or weak light (not more than 250 lux), under conditions where no drying occurs; the seeds are spaced and arranged so as to prevent entwining of the roots and confusion of the fluorescent traces. For other germination conditions (pre-chilling etc.) see Chapter 5. The examination should be made only when the roots are sufficiently well developed, which may not be until the fourteenth, or in the case of dormant seed the eighteenth day. The seedlings are examined under ultra-violet light from a lamp transmitting radiations between 300 nm and 400 nm, with maximum radiation between 360 nm and 370 nm, and a trace of visible light. The examination must be made in such a manner that all seedlings, whose roots produce traces which fluorescence to any degree can be detected. Seedlings not showing fluorescent root traces should be pulled off the filter paper while under the ultra-violet light in order to detect fluorescence from roots which may have grown into the paper. The numbers of fluorescent and non-fluorescent seedlings and the number of normal seedlings are recorded for each replicate. The results should be reported in whole numbers.

Reference: Dales, H. (1953). Technique of the Gentner fluorescence test, a suggested modification. *Proc. Int. Seed Test. Ass.,* **18**, 263–266.

8.11.5 *Festuca* spp.

Festuca rubra L. and *Festuca ovina* L. can be distinguished in the same way as *Lolium* spp. The roots themselves show fluorescence in an atmosphere containing ammonia; those of *F. rubra* are yellow-green in ultra-violet light while the roots of *F. ovina* are bluish green.

The seeds are germinated using the method described for *Lolium* spp. in 8.9.4 with the count being made after 14 days or 21 days if development is not sufficient at 14 days. While under UV light the roots on the substratum are treated with ammonia gas produced from a plastic bottle ('spraying bottle') partially filled with a solution of ammonia in water. A weak solution (producing enough ammonia to smell) is adequate for this purpose.

Reference: Linder, H. (1958). Eine einfache und sichere Methode zur serienmässigen Unterscheidung der Spelzfrüchte von *Festuca rubra* und *ovina. Z.-landw. Vers. -u. Unters. Wes.,* **4**, 411–416.

8.12 Examination of plants in field plots

If the germination of the samples being sown varies significantly, the weight of seed sown should be adjusted accordingly to ensure that approximately equal numbers of plants are established in all plots of the same species.

If a seed drill is used, it must be carefully checked to ensure that it contains no seed of a previous sample before another sample is poured into it.

8.12.1 Cereals, legumes and oil plants

Each sample should be sown in plots of a convenient number of rows. Distances of 200–250 mm between rows for cereals and flax, and 400–500 mm for the other species listed below are recommended. Table 8B shows the optimum numbers of plants per metre of row.

Table 8B. Optimum number of plants per metre of row

Species	Number of plants
Linum	100
Cereals	60
Brassica	30
Vicia faba	10
Other *Vicia* spp.	30
Papaver	50
Pisum	30
Lupinus	30
Glycine	30

It is possible to distinguish between different varieties of the young plants of many species to the extent that mixtures of varieties or large admixtures of other varieties can be determined. However, it is during the time after earing (cereals) or the beginning of flowering (other species) that the most distinct differences between plants of individual varieties become apparent; consequently it is during these periods that each individual plant should be examined.

In certain species, admixture of other varieties can be determined by laboratory methods (8.5.3). Such admixtures should be removed and recorded prior to sowing the sample.

For further details of the current OECD Seed Schemes, see:

http://www.oecd.org/agriculture/code/seeds.htm

8.12.2 Herbage plants

Rows of about 15 m total length with a distance of 300–450 mm between the rows are recommended.

A spaced plant technique should be used where there is a possibility of distinguishing between two or more varieties by the study of single plants. Single plants are normally obtained by sowing the seeds separately in the laboratory or greenhouse. When the plants have grown to suitable size they are transplanted into field plots. Sowing of seed *in situ* may be possible under favourable conditions, in which case seedlings are thinned to single plants. The distance between plants should be at least 600 mm in both directions. For comparison, single plants of standard samples must also be planted out. The number of plants will depend on the replication and on the statistical treatment to be applied.

Dissimilarities in plants of different varieties become visible throughout the entire growing period. Consequently, the examination must extend over this entire period. However, it is the period from the beginning of flowering (clovers) or earing (grasses) to the conclusion of growth that offers the best opportunities for evaluating the samples. The plants must be inspected several times during this period.

For further details see report of the ISTA Variety Committee presented at the Washington Congress in 1971 (*Proc. Int. Seed Test. Ass.,* **37**, 441, 1972).

8.12.3 Root and other crops grown spaced in rows

Each plot should consist of two or more rows of sufficient total length to provide at least 400 plants for examination.

Examination must be carried out during the entire period of growth. In the case of root crops, however, the principal examination must be made at completion of growth, after the roots have been lifted and placed in rows, so that differences in shape and colour can be clearly seen.

Chapter 9: Determination of moisture content

9.0 Basic reference method for determination of moisture content

The basic reference method for the introduction of a new species and methods into the Rules is the low-constant-temperature oven method, i.e. 17 h at 103 °C. Comparative testing must be completed to validate that the moisture determination for the new species can be done accurately and reproducibly between laboratories using 17 h at 103 °C.

9.0.1 Test necessity for grinding

The necessity for grinding depends on factors such as seed size and seed coat permeability to water. However, if the seed size is too small to meet the requirements for fine grinding then testing for the effect of grinding is not necessary. Testing the effect of grinding is compulsory before a new species can be introduced into these Rules.

Characteristics of the seed, such as high moisture or an extremely hard seed coat, may prevent grinding. In these circumstances, breaking or cutting the seed into pieces no larger than 7 mm across is permissible.

9.0.2 Test for acceptance of the high-constant-temperature method

The test for acceptance of the high-constant-temperature method, i.e. 1, 2, 3 or 4 h at 130 °C, is not compulsory and is only required when a request for the inclusion of the high-constant-temperature method is made. It involves comparing the reference method with the high-constant-temperature method in a comparative test.

Further information and details on the procedures used to introduce new species are given in the current *ISTA Handbook on Moisture Determination*.

9.0.3 Test for acceptance of the low-constant-temperature method

The test for acceptance of the low-constant-temperature method, i.e. 17 h at 103 °C, is required where the high-constant-temperature method is the method in the Rules. It involves comparing the low-constant-temperature method with the high-constant-temperature method in a comparative test.

Further information and details on the procedures used to introduce new species are given in the current ISTA Handbook on Moisture Determination.

9.1 Determination of moisture content by the constant-temperature oven method

9.1.1 Object

The object is to determine the moisture content of seeds by an oven method for routine use.

9.1.2 Definition

The moisture content of a sample is the loss in weight when it is dried in accordance with the methods described in this chapter. It is expressed as a percentage of the weight of the original sample.

9.1.3 General principles

The methods prescribed are designed to reduce oxidation, decomposition or the loss of other volatile substances while ensuring the removal of as much moisture as possible.

9.1.4 Apparatus

The following apparatus is required, depending on the method used:

– a grinding mill;
– an electrically heated oven;
– containers;
– a desiccator;
– a balance;
– sieves;
– a cutting tool.

9.1.4.1 Grinding mill

The grinding mill must:
- be made of material which does not absorb moisture,
- be easy to clean and have as little dead space as possible,
- enable grinding to be carried out rapidly and uniformly, without appreciable development of heat and, as far as possible, without contact with the outside air,
- be adjustable so as to obtain particles of the dimensions indicated in 9.1.5.4.

9.1.4.2 Constant-temperature oven

The oven must be electrically heated, and capable of being controlled in such a way that during normal operation the temperature of the air and of the shelves is 103 or 130 °C in the area where the samples are being dried. The oven must have a heat capacity such that, when initially adjusted to a temperature of 103 or 130 °C, it can regain this temperature in less than 30 minutes after insertion of the maximum number of test samples that can be dried simultaneously.

The drying capacity of the oven must be determined using a species that requires high temperature and a drying time less than or equal to 2 h.

The ventilation must be such that after drying (2 h at 130 °C or 17 h at 103 °C), cooling and re-drying (1 h at 130 °C or 2 h at 103 °C) the maximum number of test portions, the results from the individual test portions do not differ by more than 0.15 % (for either temperature).

9.1.4.3 Containers

Containers must be metal dishes, non-corrodible under the test conditions, or, failing this, glass dishes, with lids and an effective surface area enabling the test sample to be distributed so as to give a mass per unit area of not more than 0.3 g/cm^2.

9.1.4.4 Desiccator

The desiccator should be fitted with a perforated metal plate to promote rapid cooling of the containers and must contain an effective desiccant.

9.1.4.5 Balance

The balance must be capable of weighing to an accuracy of at least ±0.001 g.

9.1.4.6 Sieves

Wire sieves with meshes of 0.50, 1.00, 2.00 and 4.00 mm are required.

9.1.4.7 Cutting tool

Where cutting is required according to Table 9A Parts 1 and 2, any suitable cutting instrument can be used, for example a knife, a scalpel or pruning secateurs.

9.1.5 Procedures

9.1.5.1 General directions and precautions

See Table 9A Parts 1 and 2 for directions for individual species.

The submitted sample (see 2.5.1.5–2.5.1.7 and 2.5.4.4) may be accepted for moisture determination only if it is in an intact, moisture-proof container (or, if issuing a Blue International Seed Sample Certificate, apparently moisture-proof) from which as much air as possible has been excluded.

The determination must be started as soon as possible after receipt. Prior to testing, the temperature of the sample must be equilibrated to that of the testing laboratory while the sample is still intact in the moisture-proof container.

During the determination, exposure of the sample to the atmosphere of the laboratory must be reduced to the absolute minimum, and, in the case of species that do not require grinding, no more than two minutes may elapse between the time the sampling of the submitted moisture sample begins and the time the replicates for the moisture test are weighed.

The remaining submitted sample after determination of moisture must be stored under controlled conditions in a moisture-proof container for a period defined by the laboratory, but long enough to ensure the possibility for re-testing in case of errors.

9.1.5.2 Working sample

The determination must be carried out in duplicate on two independently drawn working samples, each of the fol-

lowing weight, depending on the diameter of the containers used:
Diameter >5 cm and <8 cm: 4.5 ±0.5 g
Diameter ≥8 cm: 10.0 g ±1.0 g

For large-seeded tree and shrub seeds that have to be cut (see Table 9A Part 2), a different working sample size may be required. For cut seed, the working sample must be sufficient to draw two replicates of approximately 5 g each by cutting at least ten intact seeds (see 9.1.5.5).

Before the working sample is drawn, the submitted sample must be thoroughly mixed by one of the following methods:

either stir the sample in its container with a spoon,

or place the opening of the original container against the opening of a similar container and pour the seed back and forth between the two containers.

Take at minimum three subsamples with a spoon from different positions and combine them to form the subsample of the required size. The seed may not be exposed to the air during sample reduction for more than 30 s.

In the case of cutting or grinding, one working sample must be drawn for cutting or grinding and from the cut/ground material two replicates must be obtained.

9.1.5.3 Weighing

Weighing must be in accordance with 3.5.1 and must be in grams to at least three decimal places.

Containers and their lids are weighed before and after filling.

After weighing, containers should be covered with their lids to prevent possible contamination or loss of sample, if they are not placed directly into the oven.

Open containers and their lids are placed rapidly into an oven maintained at the required temperature for the species being tested (Table 9A Parts 1 and 2). The drying period begins at the time the oven returns to the required temperature after the placement of the containers. At the end of the prescribed period, containers have their lids replaced before cooling to ambient temperature in a desiccator.

After cooling, the containers, with lids and dried contents, are weighed.

9.1.5.4 Grinding

Large seeds and seeds with seed coats that impede water loss from the seeds must be ground before drying, unless their high oil content makes them difficult to grind or (particularly in seed such as *Linum* with oil of a high iodine number) liable to gain in weight through oxidation of the ground material.

If grinding is not possible, then cutting is allowed. See 9.1.5.5 for details.

It is obligatory to grind seed of a particular species if this is indicated in Table 9A Part 1 or Part 2.

The grinding mill should be adjusted so that particles of the required dimensions are obtained. For those species requiring fine grinding (Table 9A Part 1), at least 50 % of the ground material should pass through a wire sieve with meshes of 0.50 mm, and not more than 10 % should remain on a wire sieve with meshes of 1.00 mm. For those species requiring coarse grinding (Table 9A Parts 1 and 2), at least 50 % of the ground material should pass through a sieve with meshes of 4.00 mm, and not more than 55 % should pass through a wire sieve with meshes of 2.00 mm.

The total time of the grinding process must not exceed 2 min.

When using a grinder ensure that there is no contamination from one sample to the other.

9.1.5.5 Cutting

Large tree seeds (thousand-seed weight >200 g and if prescribed in Table 9A Part 2) and tree seeds with very hard seed coats, such as those of *Fabaceae*, and seeds with high oil contents should be cut into small pieces of less than 7 mm instead of being ground. The cutting must be carried out on a working sample from the submitted sample of at least ten intact seeds, to arrive at approximately 10 g (two replicates of approximately 5 g each).

The subsamples are quickly cut, recombined and mixed with a spoon prior to dividing into two replicates. The replicates are placed in weighed containers. Exposure to the atmosphere should not exceed 4 min.

9.1.5.6 Predrying

If the species is one for which grinding is necessary and the moisture content is higher than indicated in Table 9A Part 1, predrying is obligatory. Two subsamples, each weighing 25 ±1 g are placed in weighed containers. The two subsamples, in their containers, are then dried at 130 °C for 5 to 10 min, depending on the moisture con-

tent, to reduce the moisture content to below that required in Tables 9A Part 1. The partly dried material is then kept exposed in the laboratory for at least 2 h.

In the case of very moist seed of *Zea mays* (above 25 % moisture content) the seed is spread in a layer not deeper than 20 mm and dried at 65–75 °C for 2–5 h, depending on the initial water content. In the case of other species with a moisture content exceeding 30 %, samples should be dried overnight in a warm place.

After predrying, the subsamples are reweighed in their containers to determine the loss in weight. Immediately thereafter the two partly dried subsamples are separately ground. One working sample is drawn from each subsample. Drawing of the working sample should be in accordance with 9.1.5.2. The moisture is determined as prescribed in 9.1.5.3.

Predrying is not obligatory for any seeds that are cut (Table 9A Part 2).

9.1.5.7 Prescribed methods

a) The working sample, drawn according to 9.1.5.2, must be evenly distributed over the surface of the container.
b) Weigh the container and its cover before and after filling.
c) Place the container rapidly, on top of its cover or next to its cover, in an oven.
d) See Table 9A Parts 1 and 2 for additional details regarding grinding, temperature and duration per species.
e) Tolerances for the temperatures and durations are:
101–105 °C (low temperature): 17 ±1 h,
130–133 °C (high temperature): 1 h ±3 min, 2 h ±6 min or 4 h ±12 min.
f) The drying period begins at the time the oven returns to the required temperature.
g) At the end of the prescribed period, cover the container and place it in a desiccator to cool at ambient temperature.
h) After cooling, weigh the container with its cover and contents.

Table 9A Part 1. Details of methods for moisture determination: agricultural and vegetable seeds

The low-temperature method (low) or high temperature (high) method must be used as specified for the species in this Table.

Species	Grinding/cutting (9.1.5.4, 9.1.5.5)	Method to be used	Drying at high temperature (h)	Predrying requirement (9.1.5.6)
1	2	3	4	5
Agrostis spp.	No	High	1	–
Allium spp.	No	Low	–	–
Alopecurus pratensis	No	High	1	–
Anethum graveolens	No	High	1	–
Anthoxanthum odoratum	No	High	1	–
Anthriscus spp.	No	High	1	–
Apium graveolens	No	High	1	–
Arachis hypogaea	Cut	Low	–	To 17 % moisture content or less
Arrhenatherum spp.	No	High	1	–
Asparagus officinalis	No	High	1	–
Avena spp.	Coarse	High	2	To 17 % moisture content or less
Beta vulgaris	No	High	1	–
Brachiaria spp.	No	High	1	–
Brassica spp.	No	Low	–	–
Bromus spp.	No	High	1	–
Camelina sativa	No	Low	–	–
Cannabis sativa	No	High	1	–
Capsicum spp.	No	Low	–	–
Carum carvi	No	High	1	–
Cenchrus spp.	No	High	1	–

Table 9A Part 1. Details of methods for moisture determination: agricultural and vegetable seeds (continued)

Species	Grinding/cutting (9.1.5.4, 9.1.5.5)	Method to be used	Drying at high temperature (h)	Predrying requirement (9.1.5.6)
1	2	3	4	5
Chloris gayana	No	High	1	–
Cicer arietinum	Coarse	High	1	To 17 % moisture content or less
Cichorium spp.	No	High	1	–
Citrullus lanatus	Coarse	High	1	To 17 % moisture content or less
Cucumis spp.	No	High	1	–
Cucurbita spp.	No	High	1	–
Cuminum cyminum	No	High	1	–
Cynodon dactylon	No	High	1	–
Cynosurus cristatus	No	High	1	–
Dactylis glomerata	No	High	1	–
Daucus carota	No	High	1	–
Deschampsia spp.	No	High	1	–
Elytrigia spp.	No	High	1	–
Fagopyrum esculentum	Fine	High	2	To 17 % moisture content or less
Festuca spp.	No	High	1	–
Galega orientalis	No	High	1	–
Glycine max	Coarse	Low	–	To 12 % moisture content or less
Gossypium spp.	Fine	Low	–	To 17 % moisture content or less
Helianthus annuus	No	Low	–	–
Holcus lanatus	No	High	1	–
Hordeum vulgare	Fine	High	2	To 17 % moisture content or less
Lactuca sativa	No	High	1	–
Lathyrus spp.	Coarse	High	1	To 17 % moisture content or less
Lepidium sativum	No	High	1	–
Linum usitatissimum	No	Low	–	–
Lolium spp.	No	Low or high	2	–
Lotus spp.	No	High	1	–
Lupinus spp.	Coarse	High	1	To 17 % moisture content or less
Macroptilium atropurpureum	Coarse	High	1	To 17 % moisture content or less
Medicago spp.	No	High	1	–
Melilotus spp.	No	High	1	–
Nicotiana tabacum	No	High	1	–
Onobrychis viciifolia	No	High	1	–
Ornithopus sativus	No	High	1	–
Oryza sativa	Fine	High	2	To 13 % moisture content or less
Panicum spp.	No	High	2	–
Papaver somniferum	No	High	1	–
Paspalum spp.	No	High	1	–
Pastinaca sativa	No	High	1	–
Petroselinum crispum	No	High	1	–
Phacelia tanacetifolia	No	High	1	–
Phalaris spp.	No	High	1	–
Phaseolus spp.	Coarse	High	1	To 17 % moisture content or less
Phleum spp.	No	High	1	–
Pisum sativum	Coarse	High	1	To 17 % moisture content or less
Poa spp.	No	High	1	–
Raphanus sativus	No	Low	–	–
Ricinus communis	Cut	Low	–	To 17 % moisture content or less
Scorzonera hispanica	No	High	1	–
Secale cereale	Fine	High	2	To 17 % moisture content or less
Sesamum indicum	No	Low	–	–

Species	Grinding/cutting (9.1.5.4, 9.1.5.5)	Method to be used	Drying at high temperature (h)	Predrying requirement (9.1.5.6)
1	2	3	4	5
Setaria spp.	No	High	1	–
Sinapis spp.	No	Low	–	–
Solanum lycopersicum	No	High	1	–
Solanum melongena	No	Low	–	–
Sorghum spp.	Fine	High	2	To 17 % moisture content or less
Spinacia oleracea	No	High	1	–
Trifolium spp.	No	High	1	–
Trisetum flavescens	No	High	1	–
Triticum spp.	Fine	High	2	To 17 % moisture content or less
×*Triticosecale*	Fine	High	2	To 17 % moisture content or less
Valerianella locusta	No	High	1	–
Vicia spp.	Coarse	High	1	To 17 % moisture content or less
Vigna spp.	Coarse	High	1	To 17 % moisture content or less
Zea mays	Fine	High	4	To 17 % moisture content or less; see also 9.1.5.6

Table 9A Part 2. Details of methods for moisture determination: tree and shrub seeds

The low-temperature method must be used for all species in Table 9A Part 2.

Species	Grinding/cutting (9.1.5.4, 9.1.5.5)	Remarks
Abies spp. (TSW ≤200 g)	No	–
Abies spp. (TSW >200 g)	Cut	High oil content
Acacia spp.	Coarse	–
Acer spp.	Coarse	Because of heterogeneity
Aesculus hippocastanum	Cut	–
Ailanthus altissima	Coarse	–
Alnus spp.	No	–
Amorpha fruticosa	Coarse	Moved from Table 9A Part 1
Berberis aquifolium	No	–
Betula spp.	No	–
Calocedrus decurrens	Coarse	–
Caragana arborescens	Coarse	–
Carpinus betulus	Coarse	–
Castanea sativa	Cut	–
Catalpa spp.	Coarse	–
Cedrela spp.	No	–
Cedrus spp.	Cut	High oil content
Chamaecyparis spp.	No	–
Cornus spp. (TSW ≤200 g)	Coarse	Hard integument
Cornus spp. (TSW >200 g)	Coarse	–
Corylus avellana	Cut	–
Corymbia spp.	No	–
Cotoneaster spp.	No	–
Crataegus monogyna	Coarse	–
Cryptomeria japonica	No	–
Cupressus spp.	No	–
Cydonia oblonga	No	–
Cytisus scoparius	Coarse	–
Elaeagnus angustifolia	Coarse	–

Table 9A Part 2. Details of methods for moisture determination: tree and shrub seeds (continued)

Species	Grinding/cutting (9.1.5.4, 9.1.5.5)	Remarks
Eucalyptus spp.	No	–
Euonymus europaeus	Coarse	–
Fagus sylvatica	Cut	–
Fraxinus spp.	Coarse	–
Ginkgo biloba	Cut	–
Gleditsia triacanthos	Coarse	–
Ilex aquifolium	Coarse	–
Juniperus spp.	Coarse	–
Koelreuteria paniculata	Coarse	–
Laburnum spp.	Coarse	–
Larix spp.	No	–
Larix ×eurolepis	No	–
Ligustrum vulgare	Coarse	–
Liquidambar styraciflua	No	High oil content
Liriodendron tulipifera	Coarse	–
Malus spp. (except *M. sylvestris*)	No	–
Malus sylvestris	Coarse	–
Malva sylvestris	No	–
Morus spp.	No	–
Nothofagus spp.	No	–
Picea spp.	No	–
Pinus spp. (TSW ≤200 g)	No	–
Pinus spp. (TSW >200 g)	No	–
Platanus spp.	No	–
Populus spp.	No	–
Prunus spp.	Coarse	–
Pseudotsuga menziesii	No	–
Pyrus spp.	No	–
Quercus spp.	Cut	–
Robinia pseudoacacia	Coarse	–
Rosa spp.	No	–
Salix spp.	No	–
Sequoia sempervirens	No	–
Sequoiadendron giganteum	No	–
Styphnolobium japonicum	Coarse	–
Sorbus spp.	No	–
Spartium junceum	Coarse	–
Syringa spp.	No	–
Taxodium distichum	Cut	–
Taxus spp.	Coarse	–
Tectona grandis	Cut	–
Thuja spp.	No	–
Tilia spp. (TSW ≤200 g)	No	–
Tilia spp. (TSW >200 g)	Coarse	–
Tsuga spp.	No	–
Ulmus spp.	No	–
Viburnum opulus	Coarse	–
Zelkova serrata	No	–

9.1.6 Calculation and expression of results

9.1.6.1 Constant-temperature oven methods

The moisture content as a percentage by weight must be calculated to three decimal places for each replicate by means of the following formula:

$$\frac{Loss\ of\ weight}{Initial\ weight} \cdot 100 = \frac{M_2 - M_3}{M_2 - M_1} \cdot 100$$

Where

M_1 is the weight in grams (to a minimum of three decimal places) of the container and its cover,

M_2 is the weight in grams (to a minimum of three decimal places) of the container, its cover and its contents before drying, and

M_3 is the weight in grams (to a minimum of three decimal places) of the container, its cover and its contents after drying.

If the material is predried, the moisture content is calculated from the results obtained in the first (predrying) and second stages of the procedure. If S_1 is the moisture lost in the first stage, and S_2 is the moisture lost in the second stage, each calculated as above and expressed as a percentage, then the original moisture content of the sample calculated as a percentage is:

$$(S_1 + S_2) - \frac{S_1 \times S_2}{100}$$

9.1.6.2 Tolerances

The difference must be calculated to three decimal places and then rounded off to one decimal place. The maximum difference between the two replicates must not exceed 0.2 % after rounding from three to one decimal place. Otherwise, repeat the determination in duplicate. The reported result is the arithmetic mean of the results for two working samples (see 9.1.7). For tree and shrub species (Table 9A Part 2) it has been found impossible to meet a 0.2 % tolerance, and tolerances ranging from 0.3 to 2.5 % are prescribed. These are related to seed size and initial moisture content (Table 9B).

To use Table 9B, in column 1, select the relevant row depending on seed size. Then select the correct column (2, 3 or 4) depending on the initial moisture content of the sample.

Table 9B. Tolerance levels for differences between the two duplicate determinations of moisture content of tree and shrub seeds (significance level not defined).

Seed size	Average initial moisture content		
	<12 %	12–25 %	>25 %
1	2	3	4
Small: TSW <200 g	0.3 %	0.5 %	0.5 %
Large: TSW ≥200 g	0.4 %	0.8 %	2.5 %

(Source: F.T. Bonner (1984). Tolerance limits in measurement of tree seed moisture. *Seed Science and Technology* **12**, 789–794, 1984. [Table 3])

If the results of the duplicate determinations are out of tolerance, repeat the test beginning at 9.1.5.2. For repeated tests, report the result of the second test if its replicates are within tolerance. If the replicates of the second determination are out of tolerance as well, check if the averages of the two tests are in tolerance (0.2 % or Table 9B). If so, report this average. If replicates of both tests are out of tolerance and the average results of the repeat tests are out of tolerance discard the results, check the equipment, the laboratory procedure and start again.

9.1.7 Reporting of results

The result of a moisture content test must be reported in the space provided to the nearest 0.1 %.

The method must be reported (duration and temperature).

The following additional information must also be reported under ‘Other Determinations’:

– If germinating seeds were present in the sample, the following statement must be entered: ‘Germinating seeds were found in the submitted moisture sample.’
– If mouldy seeds were present in the sample, the following statement must be entered: ‘Mouldy seeds were found in the submitted moisture sample.’
– In the case of pelleted seeds (see Chapter 11), the following statement must be entered: ‘The seeds of the submitted moisture sample were pelleted, and the moisture content reported is the average of seed and pelleting materials.’
– For *Arachis hypogaea*, one of the following statements must be entered: ‘The submitted sample for moisture determination consisted of seeds in their pod’ or ‘The submitted sample for moisture determination consisted of seeds with the pod removed (shelled seeds)’.

9.2 Determination of moisture content by moisture meters

9.2.1 Calibration of moisture meters

9.2.1.1 Object

The object is to prepare check samples to be used for the calibration of moisture meters, and to check the calibration of moisture meters.

9.2.1.2 Definition

See 9.1.2.

9.2.1.3 General principles

The methods described are designed for the comparison of the results from moisture meters, with those obtained by the oven method (see 9.1). All moisture meters can be used, as long as the calibration requirements and the requirements of the determination are fulfilled.

Calibration must be carried out at least once every year.

A calibration report is required for each species analysed by means of a moisture meter.

A monitoring programme of the moisture meter must be implemented. Check samples must be measured on the moisture meter by using the normal procedure (9.2.2), and the moisture content must be determined once by using the oven method (9.1).

9.2.1.4 Apparatus

The following apparatus is necessary, depending on the method used:

Moisture meter:
- Where the moisture meter indicates the moisture content directly, the name of the selected species should be indicated clearly.
- Where the moisture meter does not indicate the moisture content directly, conversion table(s) should be available for each species tested. Where conversion tables are used the requirements regarding the scale interval (see 3) and the maximum permissible differences (see 9.2.1.6.3) apply to the results of the moisture content obtained from the conversion tables (expressed as a percentage) and not to the reading given on the conventional scale of the moisture meter.
- The scale interval should be such that moisture content can be read to at least one decimal place.
- The housing of the moisture meters must be robust and so constructed that the main components of the instrument are inaccessible and protected from dust and moisture.

Containers, airtight.

Sieves: appropriate for the species in question, to remove impurities from the check sample that might interfere with the measurement.

Grinder: where the operating manual of the electronic moisture meter specifies grinding, a subsample from the submitted sample must be ground. The fineness of the grinding must be according to the specific moisture meter manual. If it is not specified in the manual it should be according to 9.1.5.4.

Balance: appropriate for the meter in question (see 3.5.1).

See 9.1.4 for apparatus needed for the reference oven method.

9.2.1.5 Procedures

9.2.1.5.1 Precautions

The calibration of moisture meters may be affected by many variables, including species, variety, ripeness, humidity, temperature, and level of impurities.

The moisture meter and the samples should be equilibrated to the same temperature before the assessments are made. During the determination, exposure of the sample to the atmosphere of the laboratory should be reduced to the absolute minimum.

9.2.1.5.2 Calibration sample

Five samples should be obtained from each of a minimum of two varieties of the species for which the moisture meter is being calibrated. The samples from each variety should have a range of moisture contents evenly covering the required measurement range of the moisture meter being checked. If the full range is not available from natural samples, samples may be conditioned.

If there is evidence that varieties of a species give significantly different results, a calibration per variety, or group of varieties, is necessary.

The samples selected should be free of mustiness, fermentation and sprouted seed.

If samples contain impurities that might interfere with the measurement, they should be cleaned by hand, using sieves or a mechanical separator.

Calibration sample containers should be moisture proof and filled to at least two-thirds of their capacity. If the container is too full, the sample cannot be mixed thoroughly. If the container is not filled sufficiently there can be hygrometric exchanges between the seeds and the air that is present in the container, and this can result in a modification of the moisture content of the sample in the period prior to testing. The containers should be sealed and stored at 5 ±2 °C. The sealed containers must be moved to the room containing the moisture meter at least 24 h prior to use to ensure that the temperature of the seed has equilibrated with the temperature of the meter.

9.2.1.5.3 Working sample from calibration sample

Working samples should be drawn after thoroughly mixing them using one of the following methods:

either stir the sample in its container with a spoon,

or place the opening of the original container against the opening of a similar container and pour the seed back and forth between the two containers.

Each working sample must be drawn in such a manner that the sample is not exposed to the air for more than 30 s.

9.2.1.5.4 Weighing

Weighing, when required, should be in accordance with 3.5.1.

9.2.1.5.5 Prescribed methods

The moisture content of the calibration samples is assessed using the oven method (see 9.1), which is the reference method.

Three successive measurements are made on each calibration sample, using the moisture meter according to the manufacturer's instructions.

After each measurement, the sample tested is recombined with the calibration sample from which it was drawn. The calibration sample is then thoroughly mixed prior to drawing the next working sample (see 9.2.1.5.3). Where the determination is destructive, the measurements should be carried out on three independent working samples.

The moisture content of the calibration samples should be rechecked after the measurement, using the reference oven method (see 9.1).

9.2.1.6 Calculation and expression of results

9.2.1.6.1 Reference oven method

For each test sample two reference results are available: x_1, obtained before measuring the moisture content with the moisture meter, and x_2, obtained after measuring the moisture content with the moisture meter. The mean of these two values is taken as the true value (x_t) of the moisture content, provided that the difference between the readings is no greater than 0.3 %. If the difference is greater than 0.3 %, the calibration must be repeated.

9.2.1.6.2 Moisture meters

For each calibration sample three results are available (y_1, y_2, y_3).

Calculate the mean result y_x [$y_x = (y_1 + y_2 + y_3)/3$] and z_i (the difference of y_x from the true value x_t of the moisture content [see 9.2.1.6.1]): $z_i = y_x - x_t$.

9.2.1.6.3 Maximum permissible differences

A moisture meter is considered to be within calibration when z_i (the difference between y_x and the true value x_t) is lower than the following maximum permissible differences (Table 9C).

Table 9C. Permissible differences from the true value

True value (reference method)	Maximal permissible difference	
	Non-chaffy seeds	Chaffy seeds
<10.0 %	±0.4 %	±0.5 %
≥10.0 %	±0.04 × moisture content	±0.05 × moisture content

For the comparison the average result of the replicates after rounding to one decimal place must be used.

9.2.1.7 Calibration results

The results of calibrations must be recorded and retained for at least 6 years.

9.2.2 Determination of moisture content (moisture meters)

9.2.2.1 Object

The object is to determine the moisture content of specified species of seed using a calibrated moisture meter.

9.2.2.2 General principles

The moisture content of a sample of seed affects its physiochemical and electrical properties. These can be measured, and meters are available for the routine determinations of the moisture content.

9.2.2.3 Apparatus

The following apparatus is necessary, depending on the method used:
- moisture meter;
- containers, airtight;
- grinder;
- balance.

Details of this equipment are given in 9.2.1.4.

9.2.2.4 Procedures

9.2.2.4.1 Precautions

The submitted sample (see 2.5.1.5–2.5.1.7 and 2.5.4.4) may be accepted for moisture determination only if it is in an intact, moisture-proof container from which as much air as possible has been excluded.

The determination must be started as soon as possible after receipt. Prior to testing, the temperature of the sample must be equilibrated to that of the testing laboratory, while the sample is still intact in the moisture-proof container.

During the determination, exposure of the sample to the atmosphere of the laboratory must be reduced to the absolute minimum.

When the temperature of the sample is very different from the room temperature where the moisture meter is operated, there is a risk of condensation. Before testing, samples should therefore be equilibrated to the required room temperature.

9.2.2.4.2 Working sample

The determination must be carried out in duplicate on two independently drawn working samples each of the weight/volume required for the specified meter.

Before the working sample is drawn, the submitted sample must be thoroughly mixed by one of the following methods:

either stir the sample in its container with a spoon,

or place the opening of the original container against the opening of a similar container and pour the seed back and forth between the two containers.

Each working sample must be drawn in such a manner that the sample is not exposed to the air for more than 30 s.

9.2.2.4.3 Weighing

Weighing, when required, should be in accordance with 3.5.1.

9.2.2.5 Calculation and expression of results

The moisture content as a percentage by weight must be calculated to one decimal place by means of the following formula:

$$\frac{M_1 + M_2}{2}$$

Where M_1 and M_2 are the readings of replicates 1 and 2 from the meter.

9.2.2.6 Tolerances

The result is the arithmetic mean of the duplicate measurements, if the difference between the two does not exceed 0.2 %.

If the results of the duplicate measurements are out of tolerance, repeat the test. For repeated tests, report the result of the second test if its replicates are in tolerance. If the replicates of the second measurement of tolerance as well, check whether the averages of the two tests are in tolerance (0.2 % or Table 9B). If so, report this average. If replicates of both tests are out of tolerance and the average results of the repeat tests are out of tolerance, discard the

results, check the equipment and the laboratory procedure, and start again.

The reported result is rounded to one decimal place.

9.2.2.7 Reporting of moisture meter results

The result of a moisture content test must be reported in the space provided to the nearest 0.1 %.

The following additional information must also be reported under 'Other Determinations':

- The following statement must be entered: 'A moisture meter was used.'
- If germinating seeds were present in the sample, the following statement must be entered: 'Germinating seeds were found in the submitted moisture sample.'
- If mouldy seeds were present in the sample, the following statement must be entered: 'Mouldy seeds were found in the submitted moisture sample.'
- In the case of pelleted seeds (see Chapter 11), the following statement must be entered: 'The seeds of the submitted moisture sample were pelleted, and the moisture content reported is the average of seed and pelleting materials.'

9.2.2.8 Routine checking of moisture meter and oven moisture content results

Table 9D should be used when checking moisture meters against oven results.

For check samples, a maximum of 5 % may have a difference greater than the maximum permissible difference. If more than 5 % of the samples have differences greater than this, a new calibration is required (see 9.2.1).

Table 9D. Tolerance limits for differences between constant-temperature oven moisture measurements and moisture meter measurements.

Oven measurement average (average; %)	Tolerance
Chaffy seeds	
<10.9 %	0.5
11–12.9 %	0.6
13–14.9 %	0.7
15–16.9 %	0.8
17.0–18.0 %	0.9
Non-chaffy seeds	
<11.3 %	0.4
11.3–13.7 %	0.5
13.8–16.2 %	0.6
16.3–18.0 %	0.7

9.2.2.9 Checking of results from different moisture meters

Table 9E should be used when checking two moisture meters against each other.

Table 9E. Tolerance limits for differences between moisture determinations conducted using different moisture meters

Moisture content (average of 2 meters; %)	Tolerance
Chaffy seeds	
<10.5 %	1.0
10.5–11.4 %	1.1
11.5–12.4 %	1.2
12.5–13.4 %	1.3
13.5–14.4 %	1.4
14.5–15.4 %	1.5
15.5–16.4 %	1.6
16.5–17.4 %	1.7
17.5–18.0 %	1.8
Non-chaffy seeds	
<10.7 %	0.8
10.7–11.8 %	0.9
11.9–13.1 %	1.0
13.2–14.3 %	1.1
14.4–15.6 %	1.2
15.7–16.8 %	1.3
16.9–18.0 %	1.4

Chapter 10: Weight determination

10.1 Object

The object is to determine the weight per 1000 pure seeds of the sample as submitted.

10.2 Definition

The number of seeds in a weighed quantity of pure seed is counted, and the weight per 1000 calculated.

10.3 General principles

Only pure seeds are counted and weighed using the procedures outlined in Chapter 3: The Purity Analysis.

10.4 Apparatus

Seeds can be counted manually, or a suitable counting machine, or counting equipment for germination tests, i.e. vacuum planting head (5.5) may be used.

10.5 Procedure

Either the whole working sample (10.5.2),
or replicates of pure seed from the working sample,
or replicates from a representative fraction of the submitted sample (10.5.3)
must be used.

10.5.1 Working sample

The working sample must be the entire pure seed fraction of a purity analysis carried out in accordance with Chapter 3: The Purity Analysis of the *ISTA Rules*, or pure seed taken from a representative fraction of the submitted sample. A change of the moisture content of the working sample must be avoided as far as possible by storing the working samples before testing only for short periods and in moisture proof containers.

10.5.2 Counting the entire working sample by machine

Put the whole working sample through the machine, and read the number of seeds on the indicator. After counting, weigh the sample in grams to the same number of decimal places as in the purity analysis (3.5.1).

10.5.3 Counting replicates

From the working sample count out at random, by hand or with a germination counter, eight replicates, each of 100 seeds. Weigh each replicate in grams to the same number of decimal places as in the purity analysis (3.5.1), i.e.

Weight of working sample (g)	Decimal places (minimum)
Less than 1.0000	4
1.000–9.999	3
10.00–99.99	2
100.0–999.9	1
1000 or more	0

Calculate the variance, standard deviation and coefficient of variation as follows:

$$\text{Variance} = \frac{N\sum x^2 - (\sum x)^2}{N(N-1)}$$

where
x = weight of each replicate in grams
N = number of replicates
Σ = sum of
Standard deviation $s = \sqrt{\text{Variance}}$

$$\text{Coefficient of variation} = \frac{s}{\bar{x}} \times 100$$

where $\bar{x}$ = mean weight of 100 seeds

If the coefficient of variation does not exceed 6.0 for chaffy grass seeds, or 4.0 for other seeds, the result of the determination can be calculated. ISTA does not define grass seeds as a group.

If the coefficient of variation exceeds whichever of these limits is appropriate, count and weigh a further eight replicates and calculate the standard deviation for the 16 replicates. Discard any replicate that diverges from the mean by more than twice the standard deviation as calculated.

10.6 Calculation and expression of results

If counting is by machine, calculate the weight of 1000 seeds from the weight of the whole working sample.

If counting is by replicate, from the eight or more weights of 100-seed replicates, calculate the average weight of 1000 seeds.

The result must be expressed to the number of decimal places used in the determination (10.5.3).

10.7 Reporting results

The result of a weight determination test must be reported under 'Other determinations' to the number of decimal places used in the determination (10.5.3).

The method used ('Counting the entire working sample' or 'Counting replicates') and the result as calculated according to 10.6 must be reported under 'Other Determinations'.

Chapter 11: Testing coated seeds

11.1 Object

The object is to gain reproducible information as to the planting value of seeds coated in non-seed materials which have been applied in a way which makes positive identification of all individual seeds and inert matter as described in Chapter 3 impracticable without destroying the structure(s) presented for testing. For this purpose, techniques and definitions are prescribed where those described in the appropriate chapter are not directly applicable. A wide range of materials may be used to coat seeds as individuals in discrete units as in pellets or spaced in strips or sheets. However, treated seeds are not covered and should be tested according to the methods prescribed in other chapters. When specific instructions are not given, those in the appropriate chapter must be followed. Where reference is made to seed pellets the rules also apply to encrusted seed and seed granules, and where to seed tapes, to seed mats.

11.1.1 Definitions

Seed pellets More or less spherical units developed for precision sowing, usually incorporating a single seed with the size and shape of the seed no longer readily evident. The pellet, in addition to the pelleting material, may contain pesticides, dyes or other additives.

Encrusted seed Units more or less retaining the shape of the seed with the size and weight changed to a greater or lesser extent. The encrusting material may contain pesticides, fungicides, dyes or other additives.

Seed granules Units more or less cylindrical, including types with more than one seed joined together. The granule, in addition to the granulating material, may contain pesticides, dyes or other additives.

Seed tapes Narrow bands of material, such as paper or other degradable material, with seeds spaced randomly, in groups or in a single row.

Seed mats Broad sheets of material, such as paper or other degradable material, with seeds placed in rows, groups or at random throughout the sheets.

Seed treatment See 2.2.11. Seeds which have received seed treatment must still be tested according to the methods prescribed in other chapters.

Note: the numbering in this Chapter refers to the appropriate paragraphs of the other Chapters in the Rules, e.g. 11.3.2.1 cross references Chapter 11 to Chapter 3.2.1.

11.2 Sampling

11.2.5 Procedures

11.2.5.1 Procedures for sampling a seed lot

11.2.5.1.2 Sampling intensity

Sampling the lot of seed pellets should be done according to the intensity appropriate to the particular lot, as laid down in Chapter 2. Sampling the lot of seed tapes should be done by taking packets or (from reels) pieces of tape at random, analogously following the prescriptions of 2.5.1.2, provided that packets or reels containing up to 2 000 000 (20 units of 100 000) seeds may be combined as a basic unit and therefore are to be considered as one container.

11.2.5.1.3–1.6 Drawing and disposal of submitted sample

As submitted samples of coated seeds normally contain fewer seeds than corresponding samples of uncoated seeds, special care is necessary in drawing the sample to ensure that it is representative of the lot. Precautions are necessary to avoid damage to or change in the pellets or seed tape during drawing, handling and transport, and samples must be submitted in suitable containers.

11.2.5.2 Procedure for obtaining the working sample

For pelleted seeds use one of the dividers described in 2.5.2.2.1. However, the distance of fall must never exceed 250 mm. For seed tapes take pieces of tape at random, to provide sufficient seeds for the test.

11.2.5.2.1 Minimum size of working sample

Working samples must contain not less than the number of pellets or seeds indicated in column 3 of Tables 11A and 11B. If a smaller sample is used the actual number of pellets or seeds in the sample must be reported on the ISTA Certificate.

Table 11A. Sample sizes of pelleted seeds in number of pellets. **Note:** this table is a copy of Table 2B Part 1

Determinations	Submitted samples not less than	Working samples not less than
1	2	3
Purity analysis (including verification of species)	2500	2500
Weight determination	2500	Pure pellet fraction
Germination	2500	400
Determination of other seeds	10000	7500
Determination of other seeds (encrusted seeds and seed granules)	25000	25000
Size grading	5000	1000

Table 11B. Sample sizes of seed tapes in number of seeds. **Note:** this table is a copy of Table 2B Part 2

Determinations	Submitted samples not less than	Working samples not less than
1	2	3
Verification of species	300	100
Germination	2000	400
Purity analysis (if required)	2500	2500
Determination of other seeds	10000	7500

11.2.5.4 Conditions for issuing Orange International Seed Lot Certificates

11.2.5.4.1 Seed lot size

Providing there is satisfactory evidence that the lot is reasonably homogeneous, the maximum weight of lot may be as great as the maximum weight of lot for which sampling procedures are prescribed in Chapter 2, subject to the tolerance of 5 %.

The maximum number of seeds that a lot of seed pellets, seed granules, seed tapes or seed mats may contain is 1 000 000 000 (10 000 units of 100 000), except that the weight of the lot, including the coating material, may not exceed 42 000 kg (40 000 kg plus 5 %).

11.2.5.4.4 Submitted sample

Submitted samples must contain not less than the number of pellets or seeds indicated in column 2 of Tables 11A and 11B. If a smaller sample is used the following statement must be inserted on the certificate: 'The sample submitted contained only .... pellets (seeds) and is not in accordance with the International Rules for Seed Testing.'

11.3 Purity analysis

11.3.1 Object

A purity analysis in the strict sense (i.e. of the seeds inside the pellets and tapes) is not obligatory though, if requested by the applicant, a purity analysis on depelleted seeds or seed removed from tape may be carried out in accordance with Chapter 3 of the International Rules for Seed Testing (see also 11.3.5.3). Separations for pelleted seed are defined in 11.3.2 but for taped seed no separation is made.

11.3.2 Definitions for pelleted seed

11.3.2.1 Pure pellets

Pure pellets must include:

a) Entire pellets regardless of whether or not they contain seed,

b) Broken and damaged pellets in which more than half the surface of the seed is covered by pelleting material, except when it is obvious that either the seed is not of the species stated by the applicant (11.3.2.2.b), or there is no seed present (see 11.3.2.3.b).

11.3.2.2 Unpelleted seed

Unpelleted seed must include:

a) Free seeds of any species,

b) Broken pellets containing a seed that is recognisably not of the species stated by the applicant,

c) Broken pellets containing seed recognisable as being of the species stated by the applicant but not included in the pure pellets fraction.

11.3.2.3 Inert matter

Inert matter must include:

a) Loose pelleting material,

b) Broken pellets in which it is obvious that there is no seed,

c) Any other material defined as inert matter in 3.2.3 of Chapter 3.

11.3.3 General principles

The working sample of pellets is separated into the following three components: pure pellets, unpelleted seed and inert matter, and the percentage of each part is determined by weight.

All species of seed and each kind of inert matter present must be identified as far as possible and, if required for reporting, its percentage by weight determined.

11.3.4 Verification of species

In order to check that the seed in the pellets is largely of the species stated by the applicant, it is obligatory to remove the pelleting material from 100 pellets taken from the pure pellet fraction of the purity test and determine the species of each seed. The pelleting material may be washed off or removed in the dry state. Similarly 100 seeds must be removed from tapes and the identity of each seed determined.

For taped seed, depending on the material the tape is made of, strip off or dissolve away the tape so that 100 seeds can be examined. When the seeds in the tape are also pelleted, remove the pelleting material as indicated above.

11.3.5 Procedure

11.3.5.1 Working sample

For pellets the purity analysis must be made on a working sample taken from the submitted sample in accordance with 11.2.5.2. The size of the working sample must be that indicated in column 3 of Table 11A. The analysis may be made on one working sample of this number of pellets or on two subsamples of at least half this number each independently drawn. The working sample (or each subsample) must be weighed in grams to the minimum number of decimal places necessary to calculate the percentage of its component parts to one decimal place (see 3.5.1).

11.3.5.2 Separation

The working sample of pellets (or subsample) after weighing must be separated into its components as defined in 11.3.2.

11.3.5.3 Procedures for purity tests on depelleted seeds and seeds removed from tapes

When a purity test on depelleted seeds is to be undertaken at the request of the applicant, the working sample of not less than 2500 pellets is depelleted by shaking in fine mesh sieves immersed in water. A sieve of 1.00 mm mesh above a sieve of 0.5 mm is recommended. The pelleting material is dispersed in the water, and the remaining seed material is dried overnight in a warm dry place on moisture-absorbing material e.g. filter paper. After drying, the material must be subjected to a purity analysis in accordance with Chapter 3. The component parts (pure seed, other seeds and inert matter) must be reported as percentages of their total weight, ignoring the pelleting material. The percentage of pelleting material must be reported separately only on request.

When a purity test on seeds removed from tapes is requested, the tape material of the working sample with paper tapes is cautiously separated and stripped off. Water-soluble tape material is moistened until the seeds come free. When pelleted seeds are found in the tapes, follow the procedure in the paragraph above. The moistened seeds must be dried and the freed seed material must be subjected to a purity test as above. The component parts (pure seed, other seeds and inert matter) must be reported as percentages of their total weight ignoring the tape material.

The results of these tests are to be reported under ‘Other Determinations’ and endorsed “weight of .... material excluded”.

11.3.6 Calculation and expression of results

The percentage by weight of each of the component parts (11.3.3) must be calculated to one decimal place. The percentage of seed of any particular unpelleted species or of any particular kind of inert matter need not be calculated except as required by 3.7. Percentages must be based on the sum of the weights of the components, not on the original weight of the working sample, but the sum of the weights of the components must be compared to the original weight as a check against loss of material or other error. If a duplicate analysis is made of two half-working samples, the difference must not exceed the tolerance between duplicate analyses given in Table 3C, column 3 or 4. If the difference is in excess of the tolerance, employ the procedure laid down in 3.6.

11.3.7 Reporting results

The result of a purity test on coated seeds must be reported as follows:

- Following the species name, the words 'seed pellets', 'encrusted seeds', 'seed granules', 'seed tapes' or 'seed mats', as applicable, must be clearly entered.
- The results must be reported to one decimal place, and the percentage of all components must total 100 %. Components amounting to less than 0.05 % must be reported as 'Trace' or 'TR' (for 'Trace').
- In the case of pelleted seeds only, the percentages of pure pelleted seeds, inert matter and unpelleted seeds must be reported in the spaces provided for 'Pure seeds', 'Inert matter', and 'Other seeds', respectively.
- The name and number of the seeds of each species found in the examination of the 100 seeds removed from the pellets or tapes must be reported under 'Other determinations'.

Upon request, the following information may be reported under 'Other determinations' as follows:

- Purity test on depelleted seeds. The component parts (pure seed, other seeds and inert matter) may be reported as percentages of their total weight, ignoring the pelleting material.
  The percentage of pelleting material must be reported separately only on request. The result of this test is to be reported: 'weight of … material excluded".
- Purity of seeds removed from tapes. The component parts (pure seed, other seeds, and inert matter) may be reported as percentages of their total weight, ignoring the tape material. The result of this test is to be reported: 'weight of … material excluded'.

11.4 Determination of number of other seeds

11.4.1 Object

This determination to estimate the number of seeds of other species is carried out only at the request of the applicant .

11.4.2 Definitions

In determining the number of other seeds, the definition prescribed in 3.2 must be observed. Other seeds refer to species other than that of the pure seed as defined in 3.2.1.

11.4.3 General principles

The determination is made by a count of seeds of the species (or groups of species) designated by the applicant, and the result is expressed as a number of seeds found in the weight and approximate numbers of pellets examined or for tapes in the length of tape (or area of mat) examined.

11.4.5 Procedure

11.4.5.1 Working sample

The working sample must be not less than that prescribed in column 3 of Tables 11A and B. The working sample of pellets may be divided into two subsamples.

11.4.5.2 Determination

The pelleting material and/or tape material must be removed as described in 11.3.5.3, but drying is not obligatory. The working sample is searched either for seeds of all other species or of certain designated species, as required by the applicant.

11.4.6 Calculation and expression of results

The result is expressed as the number of seeds belonging to each designated species or category found in the actual weight and approximate number of pelleted seeds examined and for seed tapes the length of tape (or area of mat) examined. In addition the number per unit weight, per unit length or per unit area (e.g. per kilogram, per metre or per square metre) may be calculated. To decide whether two determinations, made in the same laboratory or in different laboratories are significantly different, use Table 4A. The two samples compared must be of approximately the same weight, length or area.

11.4.7 Reporting results

The result of a determination of other seeds by number on coated seeds must be reported as follows:

– Following the species name, the words 'seed pellets', 'encrusted seeds', 'seed granules', 'seed tapes' or 'seed mats', as applicable, must be clearly entered.
– Under 'Other determinations', the actual weight (or length of tape, or area of mat) and approximate number of pelleted seeds examined must be entered, together with the scientific name and number of seeds of each species sought and found in this weight, length or area.

Upon request, the result may in addition be expressed in some other way, such as number of seeds per kilogram, per metre or per square metre.

11.5 The germination test

11.5.1 Object

To determine the percentage by number of normal seedlings as defined in 5.2.4 of the kind of seed of which the sample purports to be, using pellets from the pure pellet fraction or tape without removing the seeds from the tape material. An additional germination test on pure seed taken out of the pellets or tape may be carried out at the request of the sender or as a check on a test of pellets or tapes, but care must be taken that the covering material is removed in such a way as not to affect the germination capacity of the seeds.

11.5.2 Definitions

Evaluation of seedlings as normal or abnormal must be in accordance with Chapter 5. A pellet is regarded as having germinated if it produces at least one normal seedling of the species stated by the sender. Seedlings that are obviously not of the species stated by the sender, even if normal for their species, are not included in the germination figure but their number must be reported separately.

11.5.3 General principles

Germination tests on pelleted seeds must be made with pellets from the pure pellet fraction of a purity test. The pellets must be placed on the substrate in the condition in which they are received (e.g. without rinsing or soaking). Germination tests on seed tapes are made on the tape without removing the seeds from the tape material or in any way pre-treating the tape.

11.5.4 Growing media

Paper, sand, organic growing media and in certain situations soil are permissible as substrates. For pelleted seed, the use of pleated paper is recommended. For seed tapes, a between paper method using upright rolled towels is recommended.

Specifications and characteristics of growing media for germination tests are indicated in Chapter 5, 5.4.

11.5.5 Apparatus

Types of germination apparatus and counting equipment should be used as described in 5.5.

11.5.6 Procedure

11.5.6.1 Working sample

The pure pellets must be well mixed and 400 pellets counted at random in replicates of 100. The working sample from seed tapes must consist of randomly taken pieces of tape to make up four replicates of at least 100 seeds each.

11.5.6.2 Test conditions

Methods, substrates, temperatures, light conditions and special treatments as described in Chapter 5 and prescribed for particular species in Table 5A should be used. Where substrates prescribed in Table 5A are found not to give satisfactory results, pleated paper should be used for pellets and a between paper method for tapes.

11.5.6.2.2 Moisture and aeration

The water supply may be varied according to the pelleting material and the kind of seed so as to achieve optimum conditions for germination. If pelleting material adheres to the cotyledons, water may be sprayed cautiously on to the seedlings at the time of counting.

11.5.6.3 Special treatments for breaking dormancy

When fresh or hard ungerminated seeds remain at the end of the test period (see 5.6.3 and 5.6.5.3), a retest may be made using one of the special treatments indicated in Table 5A.

11.5.6.4 Duration of the test

Extension beyond the period prescribed in 5.6.4 may be necessary. However, slow germination may be an indication that test conditions are not optimum and a germination test of seeds removed from the covering material may be made as a check.

11.5.6.5 Evaluation

Evaluation of seedlings as normal or abnormal must be in accordance with Chapter 5. Abnormality may on occasion be due to the pelleting or tape material and when this is suspected a retest must be carried out in sand, organic growing media or soil of good quality in accordance with 5.6.5.

Normal or abnormal seedlings that are obviously not of the species stated by the sender must be counted separately, and excluded from the calculation.

Pure pellets may not produce any seedlings at the end of the test period. These pellets “without seedlings” can be evaluated as:

Hard seeds: when ungerminated pellets include hard seeds (see 5.2.10)

Fresh seeds: when ungerminated pellets include fresh seeds (see 5.2.10)

Dead seeds: when ungerminated pellets include inert matter, no seed or ungerminated other seeds, not detected as such prior the germination test. They can also include dead seeds for the species stated.

11.5.6.6 Multiple seed structures

Multiple seed structures may occur in pellets or in tapes or more than one seed may be found in a pellet. In either case these must be tested as single seeds. The result of the test indicates the percentage of structures or pellets which have produced at least one normal seedling. Pellets or seeds in tapes producing two or more such seedlings are counted and their number recorded.

When pellets are tested for monogermity the numbers of pellets which have produced either one, two or more than two normal seedlings are determined in the germination test and each is expressed as a percentage of the total number of pellets producing at least one normal seedling.

11.5.7 Calculation and expression of results

Results are expressed as percentage by number. In addition, for taped seeds the total length of tape (or area of mat) used in the germination test is measured and the total number of normal seedlings is noted. From these data the number of normal seedlings per metre (or square metre) is calculated.

When seedlings that are not from the species stated by the applicant are found at the end of the germination test, their number must be counted and subtracted from the total of the five categories normal seedlings, abnormal seedlings, hard seeds, fresh seeds and dead seeds.

This new total must be taken as the basis for the calculation of the percentages using simple proportional calculation.

11.5.8 Reporting results

The result of a germination test on coated seeds must be reported as follows:
- Following the species name, the words 'seed pellets', 'encrusted seeds', 'seed granules', 'seed tapes' or 'seed mats', as applicable, must be clearly entered in the space provided.
- The percentage of pellets or seed in tapes with normal seedlings, with abnormal seedlings and without seedlings.
- The duration of the test.

The following additional information must also be reported under 'Other determinations':
- The method used for the germination test.
- For seed tapes or mats: the number of normal seedlings per metre of tape or square metre of mat.

Seedlings that are obviously not of the species stated by the applicant, even if otherwise normal, must not be included in the germination result, but their number must be reported separately under 'Other determinations'.

11.6 The tetrazolium test

11.6.1 Object

The objects are the same as defined in 6.1.

11.6.2 Definitions

The definitions are the same as described in 6.2.

11.6.3 General principles

The general principles are the same as described in 6.3.

11.6.4 Reagents

The reagents are the same as prescribed in 6.4.

11.6.5 Procedure

Coated seed units (seed pellets, encrusted seeds or seed granules): Four replicates of 100 coated seed units are washed to remove the coating mass. Depending on the consistency of the coating mass, it may be necessary to agitate, whilst soaking, to release seeds from the coating. The duration of the washing should not take longer than the premoistening period prescribed in Table 6A. The number of seeds determined in each replicate of 100 coated seed units (of the species stated by the applicant) must be reported as an average of all four replicates. If there are more than 100 seeds in each replicate of coated seed units, only 100 seeds per replicate will be used for tetrazolium testing. Coated units without seeds (e.g. empty pellets) are deemed to be non-viable seeds. The test procedure of the washed, uncoated seeds then continues with the premoistening or, if the total premoistening time is achieved, with the preparation for the staining step as prescribed in Table 6A.

Seed tapes: The number of seeds (of the species stated by the applicant) per metre must be detected and reported. To complete the test, 400 seeds must be extracted from the seed tape. The test procedure then continues with the premoistening step as prescribed in Table 6A.

Seed mats: The number of seeds (of the species stated by the applicant) per seed mat must be determined and reported (in large seed mats the number of seeds per square metre). To complete the test, 400 seeds must be extracted from the seed mats. The test procedure then continues with the premoistening step as prescribed in Table 6A.

11.6.6 Calculation, expression of results and tolerances

The same criteria are valid as prescribed in 6.6.

11.6.7 Reporting results

The result of a tetrazolium test on coated seeds must be reported as follows:
– Following the species name, the words 'seed pellets', 'encrusted seeds', 'seed granules', 'seed tapes' or 'seed mats', as applicable, must be clearly entered.

The following additional information must be reported under 'Other determinations':
– The statement 'Number of seeds (of the species stated by the applicant) included in 100 seed pellets' (or 'encrusted seeds', or 'seed granules');
– or the statement 'Number of seeds (of the species stated by the applicant) included in one metre of seed tape';
– or the statement 'Number of seeds (of the species stated by the applicant) included in one seed mat or in one square metre of seed mat'.
– The statement 'Tetrazolium test: …% were viable' must be entered.
– In cases where the testing procedure deviates from that prescribed in Table 6A, any deviating procedure must also be reported. The only areas where variations from procedures given in Table 6A are permitted are for premoistening time, tetrazolium concentration, staining temperature and staining time. For precise guidance about the limitation of the variations permitted, see 6.5.
– If individual seeds are tested at the end of the germination test, the result must be reported in accordance with 5.9.

In addition, in the case of species of *Fabaceae*, one of the following, and only one, must be reported:

either (in cases where the percentage of the viability of hard seed is not determined) 'Tetrazolium test: ...% of seeds were viable, ...% of hard seeds found in the test';

or (in cases where the percentage of the viability of hard seed is determined) 'Tetrazolium test: ...% of seeds were viable, ...% of hard seeds included in the percentage of viable seed'.

11.10 Weight determination and size grading of pelleted seed

Because of the technical requirements of precision drilling, weight determination or size grading may be necessary.

11.10.1 Object

The object is to determine the weight per 1000 pellets and/or size grading of the sample as submitted.

11.10.2 Principles

For a weight determination, the number of pellets in a weighed quantity of pure pellets is counted and the weight per 1000 calculated. For size determination, a sample of the size specified in Chapter 16 is screened as specified and the percentage of each screening fraction determined.

11.10.3 Apparatus

For weight determination, a suitable counting machine or counting equipment for germination tests may be used. For size determination, a suitable screening machine is used.

11.10.4–6 Procedure

For weight determination, follow the procedure prescribed in Chapter 10 sections 10.4 to 10.6. For size determination, follow the procedure prescribed in Chapter 16.

Chapter 12: Excised embryo test for viability

12.1 Object

The object of the excised embryo test is to determine promptly the viability of certain kinds of seeds which germinate slowly or show dormancy under the methods described in Chapter 5: The germination test of the ISTA Rules, to such an extent that a complete germination test (including pretreatment) requires more than 60 days.

12.2 Definitions

The test is not valid for previously germinated seeds and must not be applied to submitted samples which contain any dry germinated seeds. The result of the test may be reported on an ISTA Certificate only if the details of the method for the kind of seed concerned are described in the specific directions that follow.

12.3 General principles

Embryos are excised and incubated under prescribed conditions for 5 to 14 days. Viable embryos either remain firm and fresh or show evidence of growth (e.g. expansion, elongation or greening) or growth and differentiation (e.g. radicle and lateral root formation; and epicotyl and first leaf formation). Non-viable embryos show signs of decay.

12.4 Apparatus

Scalpels, dissecting needles, moist paper and other equipment that would be needed for a germination test are required.

12.5 Procedure

12.5.1 Working sample

The test is performed on 400 pure seeds. At least 425–450 seeds should be drawn from either the pure seed fraction of the purity test carried out as prescribed in Chapter 3, or from a representative fraction of the submitted sample to provide sufficient seed to replace those embryos that might be injured during the excision process.

The number of replicates (e.g. 4 × 100 or 8 × 50) is dependent on the embryo size and the container in which they are placed.

12.5.2 Preparation

Species requiring mechanical or chemical scarification of the seed coat must be given the appropriate treatment before soaking. The hard pericarp or endocarp surrounding some fruits must be removed.

12.5.3 Soaking

The seeds are soaked in tap water for 24–96 h, depending on rate of imbibition. The water temperature should not exceed 25 °C, and the water should be changed twice daily to retard the growth of fungi or bacteria and the accumulation of seed exudates.

12.5.4 Excision

Embryos are excised from soaked seed with the aid of a scalpel or razor blade and should be kept moist throughout the operation. Axenic (moderately ‘aseptic’) conditions are provided by cleaning the instruments and working area with a 70 % ethanol solution. Those seeds damaged by excision should be discarded and each replaced by one of the extra seeds of the working sample (12.5.1). When seeds in one of the following categories are found they should be included in the calculation of the percentage of seeds with viable embryos (see 12.6 below):

a) Empty fruits or embryoless seeds.
b) Fruits or seeds with embryos that have been seriously injured by insects or extraction and cleaning procedures.
c) Fruits or seeds with embryos that are badly discoloured, decayed or dead.
d) Embryos with badly deformed cotyledons.

12.5.5 Incubation

The embryos should be placed on moistened filter or blotting paper in Petri dishes, plastic boxes or the Jacobsen apparatus. They should be incubated at a constant 20–25 °C for up to 14 days with at least eight hours of light daily. The entire working sample should start incubation at the same time and decayed embryos, or those with visible fungal mycelia, should be discarded daily.

If heavy mould infection develops, a retest must be made. The seeds or fruits should be soaked in a 5 % sodium hypochlorite solution for 15 minutes and then washed thoroughly with water before excision.

12.5.6 Evaluation

Embryos mechanically damaged by excision can be distinguished from non-viable embryos by the localised discoloration of the tissue after 24 h incubation. Where excision damage makes evaluation difficult, a retest should be performed after further practice in the excision technique.

The following categories are considered viable:

a) embryos remaining firm, slightly enlarged and either white (e.g. most species), green (e.g. *Acer pseudoplatanus*) or yellow according to species.
b) embryos with one or more cotyledons exhibiting growth or greening.
c) developing embryos (which may grow into seedlings).
d) embryos of conifers that exhibit curvature of the hypocotyl.
e) embryos characterised by localised discoloration of injured tissue due to excision damage.

The following categories are considered non-viable:

a) embryos which rapidly develop severe mould and deteriorate or decay;
b) embryos exhibiting extreme brown or black discoloration, and off-grey colour or white, watery appearance.

12.6 Calculation and expression of results

The viability percentage is based on the total number of fruit or seeds tested, rather than on the number of embryos excised. Seeds in the five categories in 12.5.6 (1) a, b, c, d and e must be included in the total for each replicate. The final viability percentage is determined by dividing the number of viable embryos by the total number of seeds included in the test and multiplying by 100.

12.7 Reporting results

The result of an excised embryo test must be reported under 'Other determinations' as follows: 'Excised embryo test: …… % of seeds had viable embryos.'

Further details may be given at the discretion of the seed testing laboratory, e.g. percentages of seeds that were empty, insect-damaged or physically damaged.

12.8 Specific directions

12.8.1 *Acer* spp. excluding *A. negundo* and *A. palmatum*

Soak fruits in water for 24–48 h. Remove pericarp and wing. Resoak seeds for 24–72 h. Slit the testa in the region of the cotyledons opposite the radicle (by means of dissecting needle, manicure scissors or scalpel), and remove the testa. If the testa adheres to the embryo, resoak for 1–2 h after scraping off an area of testa.

12.8.2 *Euonymus* spp.

Soak fruit for 24 h and remove the pulpy arils. Soak seed in water for 48–72 h until fully imbibed. To excise the embryo, cut the seed coat and endosperm lengthwise and pull apart using a fingernail and scalpel. Remove the embryo with the scalpel blade.

12.8.3 *Fraxinus* spp.

Soak fruit for 24 h and remove wing and pericarp. Soak seed for 24–48 h until fully imbibed. To excise the embryo, hold the seed in place with the index fingernail. Cut through the seed coat and endosperm of the seed longitudinally, taking care not to damage the embryo. Resoak the seed for 24 h. The embryo may be removed by pulling apart the seed coat and endosperm with the fingernail and scalpel blade.

12.8.4 *Malus* spp. and *Pyrus* spp.

Soak the seeds in water for 72–96 h. Cut the hard outer seed coat and the inner seed coat longitudinally with a scalpel. The seed coats may then be removed by prying them apart with the fingernail of the index finger and a scalpel blade.

12.8.5 *Pinus monticola, P. peuce* and *P. strobus*

Soak the seed in water for 24–48 h. Hold the seed with the fingernail of the index finger and cut the seed coat and 'endosperm' (strictly megagametophyte) lengthwise from one end to the other and pry apart, exposing the embryo. The scalpel tip may be inserted into the megagametophyte, under the embryo, and the embryo may then be lifted out.

12.8.6 *Pinus cembra, P. coulteri, P. heldreichii, P. jeffreyi, P. koraiensis* and *P. parviflora*

Soak the seed in water for 48–72 h. Hold the seed between the index finger and thumb. Split the seed coat along the suture at the radicle end with a scalpel by tilting the tip of the blade into the seed and remove the seed coat. The 'endosperm' (strictly megagametophyte) containing the embryo should then be soaked in water until fully imbibed, split longitudinally with a scalpel and the embryo removed.

12.8.7 *Prunus* spp.

The seed must be removed from the stony endocarp by means of an instrument designed to permit breaking the stone without crushing the seeds, e.g. a nutcracker or vice.

a) *Prunus avium, P. besseyi, P. mahaleb, P. padus, P. serotina, P. virginiana* and other *Prunus* of similar size:
   Place scalpel tip into the suture of the stone at the radicle end. Tilt scalpel inwards and apply pressure until endocarp begins to split. Twist scalpel tip slightly opening endocarp. The endocarp may more readily be removed if softened by a 24–48 h water soak.
b) *Prunus armeniaca, P. persica* and other *Prunus* of similar size:
   Place the blade of a heavy scalpel in the ventral suture of the endocarp and strike the back of the blade with a hammer, opening the stone. Discard and replace seeds where the seed coat has been damaged by extraction.

The seed should be soaked in water for 48–96 h, depending on the rate of imbibition.

To excise the embryo, the seed should be held between the thumb and index finger, with the flat side facing upward and with the radicle tip pointed toward the palm of the hand. With a sharp instrument, make a small cut above the radicle tip and lift up this part of the seed coat. The seed coat must then be loosened on both edges of the seed. The seed must be held firmly to prevent the cotyledons from coming apart. In the process of extracting the embryo from the seed coat, the remnants of the nucellus and endosperm will also be removed.

12.8.8 *Pyrus* spp.: see *Malus* spp.

12.8.9 *Sorbus* spp.

Soak the seed in water for 24–48 h. Cut the seed coat longitudinally along one side. To excise the embryo, pry the seed coat apart with a fingernail and a scalpel.

12.8.10 *Tilia* spp.

Remove pericarp either dry (*T. cordata*) or after overnight soaking (*T. platyphyllos*). Soak or re-soak overnight. Remove testa and piece of endosperm covering the cotyledon tip. Gently squeeze the sides to split open the endosperm and reveal the radicle and hypocotyl. If the upper part of the endosperm cannot be lifted off easily on the needle blade, remove part of it carefully without damaging the embryo and replace the seed in water. The edges of the cotyledons may be embedded in the endosperm and a re-soak for a few hours can make extraction easier.

Chapter 13: Testing seeds by weighed replicates

13.1 Object

The object of the weighed replicate test is to determine the maximum germination potential of a seed lot. This can be used to compare the quality of different seed lots and also estimate field planting value.

13.2 Definitions

The definitions given in Chapter 5: The Germination Test of the ISTA Rules, to define germination, normal and abnormal seedlings, etc. also apply to Chapter 13.

13.3 General principles

For weighed replicate tests, the aim is to test a weight of material containing approximately 400 seed units. The actual weight of seed tested is a much smaller fraction of the lot than the total amount normally tested in purity and germination tests. Extreme care must therefore be taken to ensure that truly representative submitted and working samples are drawn. Because of the difficulties of carrying out a purity analysis, when testing by weighed replicates a purity test is not normally performed unless requested by the applicant. Nevertheless, the full size of the working sample for purity analysis specified in Table 2A must still be examined for authentication of species and removal of readily identifiable seeds of other species. The name and number of such other seeds found, together with the weight examined, must be reported.

In cases where determination of other seeds by number is requested, the requirements of Chapter 4 apply.

Four replicates of the prescribed weight are drawn from the working sample by an approved sampling method. The replicates are planted on or in the substrate, and germinated under the temperature conditions and for the length of time prescribed in Tables 13A and 13B; only the numbers of normal and abnormal seedlings produced are recorded. The result is reported as the number of normal seedlings produced by the weight of seed material examined.

The weighed replicate test is restricted to the tree species listed in Table 13A and non-tree species listed in Table 13B. In these species, measurements of purity percentage, thousand pure seed weight and/or germination percentage are impossible or impractical.

The reasons for this are varied, for example:

- a purity test may be impossible, owing to the seed and inert matter being indistinguishable by eye alone, e.g. most *Eucalyptus*;
- a purity test may be impractical, because although the seed and inert matter are just about distinguishable, the inert matter constitutes such a large proportion of the seed lot that a purity test is too costly to perform in relation to the value of the seed, e.g. some *Eucalyptus* and most *Betula*;
- the majority of the seed lots may have high percentages of empty seed, making it likely that the unequal distribution of full and empty seed between germination replicates will bias the number of potential germinants before the germination test has been started, e.g. most *Eucalyptus*, *Betula* and *Chloris*;
- any combination of the above.

13.4 Apparatus

Suitable germination media, materials and equipment as defined in Chapter 5 should also be used for testing in Chapter 13.

13.5 Procedure

13.5.1 Submitted and working samples

The minimum weights of the submitted and working samples must be those prescribed in Table 2A. Samples must be drawn in accordance with the methods referred to in 2.5.

13.5.2 Physical examination of the working sample

For *Eucalyptus* and *Betula* the whole working sample must be examined in order to determine that the seeds are of the species stated by the sender and in order to identify as far as possible any other seeds contaminating the seed lot.

13.5.3 Obtaining the weighed replicates

The weight of material to be tested in each replicate is given in Tables 13A and 13B, column 6. These weights have been derived from pure seed data so as to give approximately 100 pure seeds per replicate. If it appears that there are too few or too many seed units in each replicate, the procedure in 13.5.4 should be followed.

The working sample should be subsampled by an approved method (e.g. hand-halving, spoon, mechanical divider) to obtain four replicates of approximately the weight required, to be weighed to the accuracy described in 3.5.1.

13.5.4 Germination tests

The substrates, temperatures, light conditions and special treatments for overcoming dormancy must be the same as those permitted in Chapter 5. The conditions to be used for individual species are prescribed in Tables 13A and 13B. Where prechill and no prechill tests are prescribed, eight weighed replicates should be drawn as described in 13.5.3 above and four selected at random for the prechill test. If time and/or space permits, all eight replicates should be set to germinate at the same time.

The material in each replicate should be spread uniformly on the appropriate moist substrate. If during the preparation of the replicates, or at any other time during the course of the germination test it is clear that the number of seed units is significantly less than 100 per replicate, then the test must be repeated using replicates of greater weight. If, on the other hand, it appears that the number of seed units per replicate is significantly greater than the desired 100 seeds, then each replicate may be split into two or more parts and spread evenly between the appropriate number of substrates. Each part of the replicate should be carefully identified, kept close together and assessed as though they were one replicate (see 5.8). The duration of the test and the day of first assessment for individual species are given in Table 13A. Seedlings should be evaluated in accordance with 5.2.5–5.2.8.

At the termination of the test, no attempt need be made to categorise the remaining seeds into empty, hard, fresh and ungerminated. However, if the germination of the seeds is slow and uneven, and if the analyst has any other reason for suspecting that dormancy is present, the test should be repeated after a suitable treatment (see 5.6.3).

13.6 Calculation and expression of results

The result for the no prechill test is obtained by adding together the four individual replicate no prechill results. It is expressed as the number of normal seedlings in the total weight of seed tested.

The prechill test results are calculated and expressed similarly.

To check the reliability of a test result, the sum of the numbers of seeds germinated in the four replicates is calculated and compared with Table 13C.

13.7 Reporting results

The result of a weighed replicates test must be reported in the space provided as follows:

– The result of the purity test (if requested), in the spaces provided for purity tests.
– ‘N’ must be entered in all the spaces provided for reporting the percentages of the components of the germination tests.

The following additional information must also be reported under ‘Other determinations’:
– average weight of four replicates;
– average number of normal seedlings in four replicates;
– number of normal seedlings per kilogram;
– other information as specified in 1.5.2.6 and 5.9.

Upon request, other seeds found to be present in the weighed replicates may be reported, giving the scientific name(s) and number(s) of seeds found.

13.8 Tables of germination methods for specific species

Tables 13A and B indicate permissible substrates, temperatures, duration, weight of replicate and additional directions, including recommended special treatments for dormant samples, for tree species (Table 13A) and non-tree species (Table 13B).

For certain species indicated in column 7, a ‘double test’ (with and without prechilling) is mandatory.

Results of the tests can only be relied upon if the difference between the highest and lowest total replicate count is within acceptable limits. The tolerances to be used for tests on weighed replicates are to be found in Table 13C.

Table 13A. Germination methods for tree species

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Weight of replicate (g)	Additional directions incl. recommendations for breaking dormancy
1	2	3	4	5	6	7
Betula pendula	TP	20⇔30	7	21	0.10	Double test: no prechill and prechill 21 d at 4 °C
Betula pubescens	TP	20⇔30	7	21	0.10	
Corymbia citriodora	TS	25	5	14	0.50	
Corymbia ficifolia	TP	20	5	14	1.00	
Corymbia maculata	TP	25	5	14	0.50	
Eucalyptus astringens	TP	20	5	15	0.50	
Eucalyptus botryoides	TP	25	5	15	0.10	
Eucalyptus bridgesiana	TP	25	5	14	0.25	
Eucalyptus camaldulensis	TP	30	3	14	0.10	
Eucalyptus cinerea	TP	30	3	14	0.25	
Eucalyptus cladocalyx	TP	20	5	14	0.50	
Eucalyptus cloeziana	TS	25	14	21	0.50	
Eucalyptus cypellocarpa	TP	25	5	14	0.25	
Eucalyptus dalrympleana	TP	25	5	14	0.25	
Eucalyptus deanei	TP	20	5	21	0.10	
Eucalyptus deglupta	TS	35	5	14	0.10	
Eucalyptus delegatensis	TP	20	3	14	0.50	Prechill 28 d at 3–5 °C
Eucalyptus elata	TP	15	10	21	0.50	
Eucalyptus fastigata	TP	15	10	21	0.50	
Eucalyptus glaucescens	TP	20	7	21	0.50	Double test: no prechill and prechill 21 d at 3–5 °C
Eucalyptus globulus	TP	25	5	14	1.00	
Eucalyptus grandis	TP	25 (20⇔30)	5	14	0.10	
Eucalyptus gunnii	TP	20	7	28	0.10	
Eucalyptus largiflorens	TP	35	3	14	0.10	
Eucalyptus leucoxylon	TP	25	5	14	0.25	
Eucalyptus macrorrhyncha	TP	15	10	28	0.50	
Eucalyptus mannifera	TP	25	5	14	0.10	
Eucalyptus melliodora	TP	25	5	14	0.25	
Eucalyptus microtheca	TS	30	3	14	0.10	
Eucalyptus moluccana	TP	30	3	14	0.25	
Eucalyptus muelleriana	TP	15	10	21	1.00	
Eucalyptus nitens	TP	20	7	21	0.25	Double test: no prechill and prechill 21 d at 3–5 °C
Eucalyptus pauciflora	TP	15	10	21	1.00	
Eucalyptus pilularis	TP	25	5	14	1.00	
Eucalyptus polybractea	TP	15	10	21	0.10	
Eucalyptus radiata	TP	20	5	14	0.50	
Eucalyptus regnans	TP	15	10	21	0.25	
Eucalyptus resinifera	TS	25	5	21	0.25	
Eucalyptus robusta	TP	20	7	14	0.10	
Eucalyptus rudis	TP	35	3	14	0.10	
Eucalyptus saligna	TP	25	5	14	0.10	
Eucalyptus sideroxylon	TP	20	5	14	0.25	
Eucalyptus sieberi	TP	25	5	14	0.50	
Eucalyptus smithii	TP	20	5	14	0.25	
Eucalyptus tereticornis	TP	30	3	14	0.10	
Eucalyptus viminalis	TP	25	5	14	0.25	

TP = top of paper; TS = top of sand

*The symbols '⇔' indicate alternating temperature regimes. 1st temperature: 16 hours; 2nd temperature: 8 hours

Table 13B. Germination methods for non-tree species

Species	Sub-strate	Temperature* (°C)	First count (d)	Final count (d)	Weight of replicate (g)	Additional directions incl.recommendations for breaking dormancy
1	2	3	4	5	6	7
Chloris gayana	TP	20⇔35; 20⇔30	5	14	0.25	KNO_3; light; prechill

*The symbols '⇔' indicate alternating temperature regimes. 1st temperature: 16 h; 2nd temperature: 8 h

13.9 Tolerance tables

Table 13C, based on the Poisson distribution, indicates the maximum range (i.e. maximum difference between the highest and the lowest) in germination data tolerable between weighed replicates, allowing for random variation at 0.05 probability. To find the maximum tolerated range, calculate the sum of the numbers of normal seedlings in the four replicates. Locate the sum in column 1 of the table and read off the maximum tolerated range in column 2.

Table 13C. Maximum tolerated range between replicates

Number of normal seedlings in the total weight of seeds tested	Maximum range
1	2
> 0–6	4
> 7–10	6
> 11–14	8
> 15–18	9
> 19–22	11
> 23–26	12
> 27–30	13
> 31–38	14
> 39–50	15
> 51–56	16
> 57–62	17
> 63–70	18
> 71–82	19
> 83–90	20
> 91–102	21
>103–112	22
>113–122	23
>123–134	24
>135–146	25
>147–160	26
>161–174	27
>175–188	28
>189–202	29
>203–216	30
>217–230	31
>231–244	32
>245–256	33
>257–270	34
>271–288	35
>289–302	36
>303–321	37
>322–338	38
>339–358	39
>359–378	40
>379–402	41
>403–420	42
>421–438	43
>439–460	44
>460	45

Chapter 14: X-ray test

14.1 Object

The objects of X-radiography are:
– to provide a quick, non-destructive method of differentiating between filled, empty, insect-damaged and physically damaged seed from the morphological characteristics evident on an X-radiograph;
- to create a permanent photographic record of the proportions of filled, empty, insect-damaged and physically damaged seeds in a sample.

Further information on the X-ray test may be found in the *ISTA Tree and Shrub Seed Handbook*.

14.2 Definitions

14.2.1 Radiograph

A radiograph is an image on photosensitive film or paper that is formed when an object is placed between the film or paper and an X-ray source. Photographic processing converts a latent image to one that is visible.

14.2.2 X-rays

X-rays are electromagnetic waves in the electromagnetic spectrum travelling at the speed of light, but with variable wavelengths ($^{1}/_{10\,000}$ to $^{1}/_{100\,000}$ of that of light). High-energy (shorter wavelength) X-rays are more suitable for large and/or dense objects. Low-energy (longer wavelength) X-rays are suitable for small objects such as seeds.

14.3 General principles

Seeds are placed between a low-energy X-ray source and photosensitive film or paper. The various types of seed tissue absorb the X-rays to varying extents, depending on their thickness and/or density. The sensitive photographic emulsion is excited to varying degrees, depending on the amount of radiation it receives, thus creating a latent image. When the film or paper is processed, a visible image of varying shades of light and dark is formed. Several factors can affect the quality of the X-ray image.

The voltage, measured in kilovolts (kV), is the measure of potential between the electrodes within the X-ray tube. An increase in voltage will produce shorter-wavelength X-rays. The voltage affects the contrast of the image; a lower voltage improves the resolution, while a higher voltage reduces the density difference.

The electric current applied to the tube is measured in milliamperes (mA). Increasing the current increases the number of X-rays produced in a given time. The current influences the density, but not the contrast of the image. A high current will overexpose (darken) the image.

The exposure time is the time during which the specimen is exposed to X-rays for making the radiograph. There is an interaction between exposure time and current, so exposures should be expressed in milliampere-seconds (mAs) or milliampere-minutes (mA · min). Changing the exposure time alters the density of the image. To retain the same image quality, any increase in exposure time requires a proportional decrease in current. For example, an exposure of 100 mAs obtained with a tube current of 5 mA and an exposure time of 20 s produces the same image density as an exposure made at 10 mA for 10 s.

The distance between the focal spot (or target) and the film surface is the focus-film distance (FFD). An increase in the FFD decreases the intensity of the radiation according to the inverse square law. Thus, doubling the FFD requires four times the exposure to achieve the same degree of image density on the film or paper.

The distance between the object and the film surface (OFD) affects the image quality. The greater the distance, the poorer the image, as details will be less distinct. If fine detail is necessary, the seeds may be placed directly on the film surface, although in routine work the film is usually kept in a carrier or envelope to make handling easier.

It is possible to use contrast agents that differentially permeate the subject, making some parts more radiographically dense than others in order to enhance certain characteristics of the image.

14.4 Apparatus

The following apparatus is necessary:
– X-ray machine;
– X-ray film or paper;
– developer for film or paper;
– holder for film;
– holder for seeds.

14.5 Procedures

14.5.1 Loading the film, preparing the seed and developing the image

1. Load film/paper in cassettes or holder, or use prepackaged film/paper.
2. The test is performed on four replicates of 100 seeds, each drawn at random from the pure seed fraction (they may be the same seeds as those used for the germination test).
3. Spread the seeds (with or without a holder) evenly on top of the film or paper.
4. Place lead letters or other X-ray-opaque marking devices on the film or paper to identify the sample.
5. Make the exposure. Individual X-ray machines will require different exposure time and voltage settings to produce the best image. Settings will also vary for different species. For the best results, a time-voltage-exposure series should be made whenever new material or a different machine is used.
6. Develop the film or paper. Paper is usually developed in instant processors, which produce a print within a few seconds. Film must be developed in a darkroom.

14.5.2 Evaluating the image

Seeds are classified according to the internal anatomy revealed by the radiograph as:

filled: fruit or seed containing all tissues essential for germination;
empty: fruit or seed containing less than 50 % of seed tissue;
insect-damaged: fruit or seed containing insects, insect larvae or frass, or showing other evidence of insect damage affecting the ability of the seed to germinate;
physically damaged: filled fruit or seed with the coat outline cracked or broken.

14.6 Calculations and expression of results

Results are expressed as percentages of filled, empty, insect-damaged, or physically damaged seeds.

14.7 Reporting results

The results of an X-ray test must be reported under 'Other determinations' as percentages of filled, empty, insect-damaged or physically damaged seeds, as follows:
'X-ray test results:
…… % filled;
…… % empty;
…… % insect-damaged;
…… % physically damaged'.

Chapter 15: Seed vigour testing

15.1 Object

The object of a seed vigour test is to provide information about the planting value in a wide range of environments and/or the storage potential of seed lots. The test provides additional information to the standard germination test (see Chapter 5) to assist in the differentiation of seed lots of acceptable germination.

15.2 Definitions

15.2.1 Seed vigour

Seed vigour is the sum of those properties that determine the activity and performance of seed lots of acceptable germination in a wide range of environments.

Seed vigour is not a single measurable property, but is a concept describing several characteristics associated with the following aspects of seed lot performance:

– rate and uniformity of seed germination and seedling growth;
– emergence ability of seeds under unfavourable environmental conditions;
– performance after storage, particularly the retention of the ability to germinate.

A vigorous seed lot is one that is potentially able to perform well even under environmental conditions which are not optimal for the species.

15.2.2 Seed vigour test

A seed vigour test is either a direct or an indirect analytical procedure to evaluate the vigour of a seed lot under standardised conditions.

a) Direct tests reproduce environmental stresses or other conditions in the laboratory, and the percentage and/or rate of seedling emergence are recorded.
b) Indirect tests measure other characteristics of the seed that have proved to be associated with some aspect of seedling performance.

15.2.3 Acceptable germination

A seed lot of acceptable germination is one which, in the absence of seed dormancy, has an acceptable standard germination level for that species.

15.2.4 Additional definitions

Seedling emergence the emergence through the soil or other planting medium of a young plant developing from the embryo of the seed

Seedling performance the ability of a seedling to emerge from the soil or other medium and develop into a normal plant

Total germinated seeds the sum of the proportion of seedlings classified as normal and abnormal at the end of a controlled deterioration (CD) germination test conducted under the conditions and within the period specified in Chapter 5, Table 5A

15.3 General principles

A vigour test assesses, either directly or indirectly, the physiological and physical basis of potential seed lot performance in a wide range of environments, and provides a more sensitive differentiation between seed lots of acceptable germination than does the germination test. Such information can be used to make informed decisions regarding the value of different seed lots.

Vigour tests are able to provide:

– a more sensitive index of seed quality than the standard germination test;
– a consistent ranking of seed lots of acceptable germination in terms of their potential physiological and physical quality;
– information on emergence and storage potential of seed lots to plan marketing strategy.

An important source of information on seed vigour is the current *ISTA Handbook of Vigour Test Methods*. Compilation of this handbook is continuing as vigour tests are added and as vigour testing methodology is standardised. See also: the current *Association of Official Seed Analysts Seed Vigor Testing Handbook* and the *Proceedings of the ISTA Seed Vigour Testing Seminar* held in Copenhagen, Denmark in 1995.

Vigour test methods are species specific and require suitable equipment, the use of control samples and experience of the analyst. The expectation that a seed analyst can infrequently analyse an isolated sample to establish a level of vigour is unrealistic. Uniformity can be best achieved by working for a period of time alongside another analyst experienced in the use of the method. Training of analysts may be more important than the exact agreement in details of procedure.

The following ISTA vigour tests have completed validation:

Conductivity test: *Cicer arietinum* (Kabuli type), *Glycine max*, *Phaseolus vulgaris*, *Pisum sativum* (garden peas only, excluding petit-pois varieties), *Raphanus sativus*

Accelerated ageing test: *Glycine max*

Controlled deterioration test: *Brassica* spp.

Radicle emergence test: *Zea mays*, *Brassica napus* (oilseed rape, Argentine canola), *Raphanus sativus*

Tetrazolium vigour test: *Glycine max*

Detailed methods are given in 15.8.

15.4 Apparatus

Permissible apparatus, substrates, temperatures, seed moisture contents, test duration and additional directions are provided in 15.8.

15.5 Procedures

15.5.1 Working sample

The required number of seeds and replicates (see 15.8) must be taken at random from the pure seed fraction (see 3.2.1) of the sample.

15.5.2 General directions

Different vigour test methods (direct and indirect) are described under three general categories: physiological, biochemical and ageing tests. The tests in 15.8 have been rigorously evaluated through recognised protocols, including extensive comparative testing and many comparisons of seed lot performance for the species listed.

15.5.3 Test conditions

Methodology for each test is prescriptive, and no other methodology may be used if an ISTA Certificate is issued.

15.5.4 Control samples

All vigour tests require rigid control of test conditions and, where specified, should include a control seed sample to provide internal quality control of vigour test uniformity. Variability in control seed sample results provides an indication of slight fluctuations in test conditions (e.g. changes in temperature or seed moisture) which can significantly affect the reliability of results. Specific guidelines for the seed lot selection, storage and handling of control samples are described in the *ISTA Handbook of Vigour Test Methods*.

15.6 Calculation and expression of results

Results are expressed in different formats for different vigour tests, as shown in 15.8.

15.7 Reporting of results

The result is reported on an ISTA Certificate under 'Other Determinations', using the procedure described in 15.8.

The results must be accompanied by a statement of the test method used, including specific variables (time, temperature, seed moisture) when appropriate.

15.8 Detailed methods

15.8.1 Conductivity test

15.8.1.1 Principle

Measurement of the electrical conductivity of leachates provides an assessment of the extent of electrolyte leakage from plant tissues. Conductivity measurement of the soak water in which a bulk sample of seeds has been steeped gives an estimate of seed vigour. Seed lots with high electrolyte leakage, i.e. high leachate conductivity, are considered to have low vigour, whilst those with low leakage (low conductivity) are considered to have high vigour.

15.8.1.2 Scope and field of application

The conductivity test offers a vigour test for *Cicer arietinum* (Kabuli type), *Glycine max*, *Phaseolus vulgaris, Pisum sativum* (garden peas only) and *Raphanus sativus* which relates to the field emergence potential of seed lots. The test does not apply to field peas or the so-called 'petit-pois' varieties of garden peas (*Pisum sativum*).

Submitted seed lots may have been treated with fungicide. Various sources of fungicide preparations with different purity levels are commercially available and some fungicides may possess additives that may significantly alter conductivity results. Thus, caution must be exercised when using the conductivity test for treated seeds.

15.8.1.3 Apparatus

Conductivity meter: a direct-reading meter using AC or DC current, with a dip cell that has a cell constant of 1.0, is suitable. The meter specifications should include a conductivity range of 0–1999 µS cm^{-1}, a resolution of at least 0.1 µS cm^{-1}, an accuracy of ±1 % and a temperature range of 20–25 °C.

Containers: as specified in Table 15 A, the base diameter must provide adequate water depth to immerse all the seeds and the dip cell. Cleanliness is important, and all containers must be washed thoroughly and rinsed twice with deionised or distilled water before use.

Water: deionised water or distilled water should be used. The conductivity of the deionised or distilled water must be measured and must not exceed 5 µS cm^{-1} at 20 °C. The water used for testing must be at 20 ±2 °C before use.

Germinator, incubator or walk-in room: a constant temperature of 20 ±2 °C is required.

Moisture content test facilities: moisture content tests are conducted according to Chapter 9.

15.8.1.4 Preparation of the sample

Adjust seed moisture content as follows if specified in Table 15A.

Determine the moisture content of the submitted sample according to Chapter 9. If the moisture content is below 10.0 % or above 14.0 %, it must be adjusted to between 10.0 and 14.0 %, although it is not necessary for the moisture content of all samples to be the same within this range. To adjust the seed moisture content, mix the fraction of pure seed thoroughly and draw randomly a subsample of at least 200 seeds. In the case of a moisture content below 10.0 %, raise the moisture content by placing each weighed subsample between moist cloths (paper towels) until it reaches a weight equivalent to a moisture content between 10.0 and 14.0 %. Experience indicates that to raise the moisture content of pea seeds with an initial moisture content of 7 % to a moisture content of 10.0 or 14.0 % takes approximately 3 or 7 h, respectively. These times should be taken as a guide only, as the actual times will depend on the extent to which the cloths surrounding the seeds have been moistened.

In the case of a moisture content above 14.0 %, reduce the moisture content by placing the weighed subsample in an oven at 30 °C until it reaches a weight equivalent to a moisture content between 10.0 and 14.0 %. Experience indicates that seeds with an initial moisture content of around 15 % take 1 h to reach 14.0 %, and 5–6 h to reach 10.0 %, when dried in this way. When the initial

Table 15A. Conditions for the conductivity test carried out on different species

Species	Containers to be used	Sample size	Seed moisture content	Water volume	Temperature	Soak time
15A.1						
Cicer arietinum (Kabuli type) *Glycine max* *Phaseolus vulgaris* *Pisum sativum* (garden peas only, excluding petit-pois varieties)	Erlenmeyer flasks or beakers, capacity 400–500 mL with a base diameter of 80 mm (±5 mm)	4 weighed replicates of 50 seeds	Adjust to 10–14 %	250 mL	20 °C	24 h
15A.2						
Raphanus sativus	Tubes 7–8 cm high with a diameter of 4 cm	4 weighed replicates of 100 seeds	No adjustment	40 mL	20 °C	17 h

seed moisture content is approximately 16 %, it takes 1–2 h drying to reach 14.0 %, and 8–10 h to reach 10.0 %.

The range of seed weights for the subsamples that will give moisture contents from 10.0 and 14.0 % can be calculated for both methods using the following equation:

Weight of subsample at 10.0 or 14.0 % mc =

$$\text{(initial weight)} \cdot \frac{(100 - \text{initial mc})}{(100 - \text{desired seed mc*})}$$

mc = moisture content

*The desired moisture content will be either 10.0 or 14.0 %.

When the subsample has reached a weight equivalent to a moisture content between 10.0 and 14.0 %, it should be sealed in a moisture-proof container, such as an aluminium foil packet or polythene bag, and held for 12–18 h at 5–10 °C to allow the moisture content to equilibrate throughout the seed.

15.8.1.5 Checking equipment and materials

15.8.1.5.1 Calibrating the dip cell

Prior to use, calibrate the conductivity meter using traceable standard solutions. At least two solutions should be used, one with a conductivity less than 100 µS cm^{-1} and one with a conductivity between 1000 and 1500 µS cm^{-1}. Note that calibration of the meter using these solutions is carried out at 25 °C, which is possible when using a meter with the specifications in 15.8.1.3. If the reading is incorrect, the calibration must be repeated and, if necessary, the meter adjusted or repaired.

Alternatively, calibrate the conductivity meter using a potassium chloride solution made up in the testing laboratory, if this can be achieved with the accuracy required. Dissolve 0.745 g of pure, dry analytical grade potassium chloride (dried at 150 °C for 1 h and cooled in a desiccator before weighing) in deionised water to make 1 L of 0.01 M KCl solution. In this solution, the meter should read between 1273 and 1278 µS cm^{-1} at 20 °C. If the reading is out of range, the calibration test should be repeated and, if necessary, the meter adjusted or repaired. Conductivity meters that are out of calibration must not be used for the conductivity test.

15.8.1.5.2 Checking the cleanliness of equipment

Each testing day, select at random 2 out of every 10 containers to be used, add the required volume (Table 15A) of deionised or distilled water of known conductivity and which has been maintained at 20 ±2 °C, and read the conductivity. If the conductivity of the water in the containers is higher than 5 µS cm^{-1}, thoroughly rewash the dip cell and all containers to be used that day in deionised or distilled water. Retest the conductivity of another specified volume of deionised or distilled water in a further 2 out of every 10 randomly selected containers. Repeat the process if necessary, until the readings are not higher than 5 µS cm^{-1}.

15.8.1.5.3 Checking the temperature

Proceed with conductivity testing only if the records for the temperature of the germinator, incubator or walk-in-room, and water show that the required temperature of 20 ±2 °C is being achieved.

15.8.1.6 Conductivity measurement

15.8.1.6.1 Preparing the test samples

Count four replicates of seeds as specified in Table 15A, each drawn at random from either the pure seed fraction directly or, if seed moisture content has been adjusted, from the subsample with the adjusted moisture content. Weigh the replicates to two decimal places (0.01 g).

15.8.1.6.2 Preparing the containers

For each sample to be tested, prepare four containers and add the required volume of water (Table 15A). Cover all containers to prevent contamination and equilibrate to 20 ±2 °C for 18–24 h prior to placing the seeds in the water. Include two control containers with each test run, containing only deionised or distilled water.

15.8.1.6.3 Soaking the seeds

Place each weighed replicate into a prepared container. Gently swirl each container to ensure that all seeds are completely immersed. Cover each container with, for example, aluminium foil or cling film, prior to placing at 20 ±2 °C for the required time (Table 15A). Label each container with the start time. The number of containers started at one time must not exceed the number of evaluations for conductivity that can be made within 15 minutes of the conclusion of the soak period (usually 10 to 12 containers).

15.8.1.6.4 Preparing for the conductivity readings

Turn on the conductivity meter prior to testing; note that instructions for each meter may specify a minimum warm-up period. Add sufficient deionised or distilled water to cover the conductivity cell to each of two containers for rinsing the conductivity cell between each measurement.

15.8.1.6.5 Measuring the conductivity of the solution

Measure the conductivity of the soak solution at the end of the soak period (Table 15A). Mix the leachate using one of the following methods:

a) Gently swirl the container (with seeds) for 10–15 s to ensure thorough mixing of the leachate, remove the covering of the container, and immerse the dip cell into the solution without filtration. Do not place the cell directly onto the seeds.

b) Stir the seeds and solution gently with a plastic spatula before measuring the conductivity as above. The spatula should be rinsed twice using water between each reading and dried on a clean paper towel.

c) Transfer the contents of the container to another container by pouring the seeds plus soak water into a nylon sieve. The cleanliness of the container used for transfer should have been checked before use (see section 15.8.1.5.2). Pass the soak water back over the seeds into the original container and immerse the dip cell in the solution. After measuring the conductivity of a subsample, rinse both the dip cell and the nylon sieve twice using water, and dry by blotting on a clean paper towel prior to testing the next subsample.

Once the leachate has been mixed, take several measurements of the conductivity until a stable value is obtained. If hard seeds are observed during testing, they should be removed after the conductivity test and their number recorded. They should then be surface dried and weighed, and their weight subtracted from the initial weight of the 50-seed replicate.

15.8.1.6.6 Accounting for the conductivity of the original water source

Measure the conductivity of one control container. Any increase in conductivity above 5 µS cm^{-1} indicates a potential problem with the cleanliness of the dip cell. Rewash the dip cell and measure the conductivity of the other control container. If this also indicates an increase in conductivity, there is a problem with the dip cell, and conductivity measurements cannot be made until this has been satisfactorily cleaned. Most conductivity meters provide instructions for cleaning the dip cell. Where the conductivity of the second control container does not show an increase above 5 µS cm^{-1}, this value, or the mean of the two controls if neither has increased, represents the background conductivity, which should be subtracted from the values already recorded for each replicate container.

15.8.1.7 Calculation and expression of results

The conductivity per gram of seed weight for each replicate is calculated after accounting for the background conductivity of the original water (see above), and the average of the four replicates provides the seed lot test result. Thus for each replicate:

$$\frac{\text{Conductivity reading (µS cm}^{-1}\text{) – background reading}}{\text{Weight of replicate (g)}} =$$

Conductivity (µS cm^{-1} g^{-1})

For species in Table 15A.1

If the mean conductivity of the four replicates differs by more than the tolerance value (see Table 15C) for that conductivity, the lot must be retested. If the second result is compatible with the first (i.e. the difference does not exceed the tolerance indicated in Table 15D), the average of the two tests must be reported.

When a test on a seed lot is repeated within a laboratory, the tolerance values that indicate acceptable repeatability are shown in Table 15D. Tolerances for conductivity tests completed on different submitted samples and in different laboratories, are shown in Table 15E.

For species in Table 15A.2

Calculate the variance, standard deviation and coefficient of variation as follows:

$$\text{Variance} = \frac{N\sum x^2 - (\sum x)^2}{N(N-1)}$$

where
x = conductivity of each replicate in µS cm^{-1} g^{-1}
N = number of replicates
Σ = sum of
Standard deviation $s = \sqrt{\text{Variance}}$

$$\text{Coefficient of variation} = \frac{s}{\bar{x}} \times 100$$

where $\bar{x}$ = mean conductivity of the sample

If the coefficient of variation does not exceed 9.0, the replicates are acceptable. If the coefficient of variation is greater than 9.0, the test must be repeated.

When two tests are performed in different laboratories: maximum tolerance value for two test results = mean conductivity reading × 0.3326

15.8.1.8 Reporting results

The result of a seed vigour test using the conductivity test method must be reported under 'Other determinations' as follows:

- The result must be expressed in µS cm^{-1} g^{-1} to the nearest 0.1 µS cm^{-1} g^{-1}.
- The seed moisture content before the test must be reported. Where the moisture content has been adjusted before the test, both the initial moisture content and the calculated moisture content after adjustment must be reported.
- The results must be accompanied by a statement of the specific variables used in the test (soaking time and temperature).

15.8.2 Accelerated ageing (AA) test for *Glycine max*

15.8.2.1 Principle

The accelerated ageing (AA) stress test exposes seeds for short periods to high temperature and high relative humidity (≈ 95 %). During the test, the seeds absorb moisture from the humid environment and the raised seed moisture content, along with the high temperature, causes rapid seed ageing. High vigour seed lots will withstand these extreme stress conditions and age more slowly than low vigour seed lots. Thus, after AA, high vigour lots retain a high germination, whilst that of low vigour lots is reduced.

15.8.2.2 Scope and field of application

The accelerated ageing test provides a vigour test for *Glycine max* which relates to both field emergence and storage potential.

Seeds to be aged should not be treated with fungicide(s) if possible. However, if seeds are marketed with fungicide treatment, treated seeds may be tested.

15.8.2.3 Apparatus

Balance: analytical balance capable of weighing to the nearest 0.001 g

Plastic AA box: A plastic box (11.0 × 11.0 × 3.5 cm, length × width × depth) with a lid, into which is placed a plastic or wire tray with a 10.0 × 10.0 × 3.0 cm (length × width × depth) mesh screen. The pore size of the mesh screen should be 1.16 ±0.01 mm × 1.63 ±0.01 mm, i.e. ~1.89 mm^2. These trays can be purchased commercially or constructed according to the guidelines provided by Elliot (1982), *Association of Official Seed Analysts Newsletter* **56 (3)**, 61–64.

Bottle-top dispensette: Volume range from 0–100 mL, for dispensing 40 mL water from a standard screw-neck bottle into plastic AA boxes, or a 50 mL graduated cylinder, if dispensette is not available.

Ageing chamber: An ageing chamber capable of maintaining a constant temperature of 41 ±0.3 °C must be used. A water-jacketed ageing chamber is recommended. Place a plastic or stainless steel pan in the base of the chamber and fill with water to maintain relative humidity during ageing. Laboratory room temperature must be controlled with air conditioning in tropical climates.

Water: deionised or distilled

Moisture content test facilities: Moisture content tests are conducted according to Chapter 9.

Germination test facilities: Germination tests are conducted according to Chapter 5.

15.8.2.4 Preparation of the sample

Determine the moisture content of the submitted sample according to Chapter 9. If the moisture content is below 10.0 % or above 14.0 %, it must be adjusted to between 10.0 and 14.0 %, although it is not necessary for the moisture content of all samples to be the same within this range. After adjustment of the seed moisture content, mix the fraction of pure seed thoroughly and draw randomly a subsample of at least 42 g.

In the case of a moisture content below 10.0 %, raise the moisture content by placing each weighed subsample between moist cloths (paper towels) or in a high humidity environment until it reaches a weight equivalent to a moisture content between 10.0 and 14.0 %.

In the case of a moisture content above 14.0 %, reduce the moisture content by placing the weighed subsample in an oven at 30 °C until it reaches a weight equivalent to a moisture content between 10.0 and 14.0 %.

The range of seed weights for the subsamples that will give moisture contents from 10.0 and 14.0 % can be calculated for both methods using the following equation:

Weight of subsample at 10.0 or 14.0 % mc =

$$(\text{initial weight}) \cdot \frac{(100 - \text{initial mc})}{(100 - \text{desired seed mc*})}$$

mc = moisture content
*The desired moisture content will be either 10.0 or 14.0 %.

When the subsample has reached a weight equivalent to a moisture content between 10.0 and 14.0 %, it should be sealed in a moisture-proof container, such as an aluminium foil packet or polythene bag, and held for 12–18 h at 5–10 °C, to allow the moisture content to equilibrate throughout the seed.

15.8.2.5 Checking equipment and materials

15.8.2.5.1 Checking the temperature in the ageing chamber

Precisely calibrate the temperature of the ageing chamber at 41 ±0.3 °C using a thermometer approved by the National Institute of Standards and Testing (NIST) for your country. Proceed with AA testing only if the records show that the required temperature of 41 ±0.3 °C has been achieved and maintained for at least two days.

15.8.2.5.2 Cleanliness of equipment

To prevent fungal contamination prior to each testing run, thoroughly wash the plastic AA boxes and screen trays in a 1 % (10 000 ppm) sodium hypochlorite solution, or wash in a commercial dish washer and dry after each use. The interior of the ageing chamber including trays should also be washed, at least twice a year, with a sodium hypochlorite solution.

15.8.2.6 Accelerated ageing procedure

15.8.2.6.1 Preparing the plastic AA boxes and seed sample

Place 40 ±1.0 mL of deionised or distilled water in each plastic AA box and insert a dry screen tray, being careful not to splash water onto the screen. Weigh a *Glycine max* seed sample of 42 ±0.5 g and place on the surface of the screen tray. Seeds should be only one layer deep to ensure even uptake of moisture from the humid environment. Place a lid (do not seal edges) on each plastic AA box. The plastic boxes with seeds should be placed on a shelf from the ageing chamber, allowing an air space of approximately 2.5 cm between plastic AA boxes to assure temperature uniformity. Two plastic AA boxes with 42 g of seeds in each should be used to obtain at least 200 seeds for testing of larger seeded *Glycine max* cultivars. Include one or more *Glycine max* control samples with each test.

15.8.2.6.2 Ageing the seed

Place the shelves holding the plastic AA boxes containing the seeds into the ageing chamber, being careful not to splash water onto the screen surface during handling. If several samples are tested in the same run they should all be placed in the ageing chamber at the same time and the door closed. Record the time and date when the plastic boxes with seeds are placed in the ageing chamber. The 72 h ageing period starts with the placement of the shelves into the ageing chamber. The ageing chamber door should not be opened during the 72 h ageing period. After the temperature recovers to 41 ±0.3 °C, it should be monitored continuously during ageing to be certain that it remains at that level. Shelves containing plastic AA boxes with seed should be removed from the ageing chamber at 72 h (±15 min).

When ageing tests are initiated for many seed lots in several ageing chambers on the same day, seed samples should be set up at approximately one or two hour intervals, between ageing chambers. This will allow adequate time for the germination tests for each group of samples to be set up immediately after the ageing period.

At the conclusion of the ageing period, but prior to setting up the germination test, immediately weigh the imbibed seed in the control sample. If the imbibed seed weight is lower than 52 g or higher than 55 g, the test results may not be accurate and the samples of that run should be retested.

15.8.2.6.3 Testing for germination

Set up a germination test using four 50-seed replicates for each sample within 1 h after removal from the ageing chamber. The testing conditions for the standard germination test for *Glycine max* seed are those outlined in Chapter 5. If 400 seeds per sample are required for the germination test, two subsamples of 42 g seeds must be aged in two plastic AA boxes.

15.8.2.7 Calculation and expression of results

Calculate the average AA germination percentage according to Chapter 5 by combining two of the 50-seed replicates to one 100-seed replicate. If the two 100-seed replicates differ by more than the maximum tolerance value for AA germination shown in Table 15F, the seed lot must be re-tested. If the second result is compatible with the first (i.e. the difference does not exceed the tolerance indicated in Table 15G), the average of the two tests must be reported.

When an AA test on the same seed lot is repeated in the same laboratory, the tolerance values that indicate acceptable maximum range between the two tests are shown in Table 15G. Tolerances for AA tests completed on different submitted samples and in different laboratories are shown in Table 15H.

15.8.2.8 Reporting results

The result of a seed vigour test using the AA method must be reported under 'Other determinations' as follows:

- Results are expressed as a percentage, calculated to the nearest whole number (5.8.1) of normal seedlings, abnormal seedlings, hard seeds, fresh seeds and dead seeds. If the result for any of these categories is found to be zero, it must be reported as '0'.
- The seed moisture content before the test must be reported. Where the moisture content has been adjusted before the test, both the initial moisture content and the calculated moisture content after adjustment must be reported.
- The results must be accompanied by a statement of the specific variables used in the test (seed weight per AA box both before and after ageing, ageing time and temperature).

15.8.3 Controlled deterioration test for *Brassica* spp.

15.8.3.1 Principle

The controlled deterioration (CD) test exposes seeds to a high temperature while at a specified and constant raised seed moisture content. These conditions cause seeds to deteriorate, or age, rapidly. The moisture content of a seed sample is raised before the seeds are placed at the raised temperature, thus ensuring that all samples tested are exposed to a predetermined degree of deterioration during the test. High vigour seeds retain a high germination after deterioration, while the germination of low vigour seeds is reduced.

15.8.3.2 Scope

The CD test provides a vigour test for *Brassica* species which relates to both field emergence and storage potential. This test has not been validated on treated seed. Seed treatments may affect the performance of the method.

15.8.3.3 Apparatus

Water bath: This must have a temperature range to include 45 °C and be accurate to ±0.5 °C. Alternatively, an incubator giving the same degree of accuracy could be used. A water bath maintains the required temperature more uniformly when a number of tests are being

conducted. If an incubator is used, care must be taken to ensure that there are no differences in temperature within it, especially when many tests are being conducted.

Analytical balance: capable of weighing to the nearest 0.0001 g

Aluminium foil packets: Suitable for holding 100 seeds in a single layer, with at least 3 cm space above the seeds after the packet is sealed. Packets approximately 5–6 cm deep and 7–10 cm wide are suitable. Packets must be impermeable to moisture once sealed. A range of packets are available, but example specifications are: paper (white kraft 60 g) covered by aluminium foil of 8 µm and polyethylene film of 40 µm.

Packet sealer: Any instrument capable of producing a watertight seal to the foil packets is suitable.

Refrigerator or cooled incubator: capable of maintaining 7 ±2 °C

Moisture content test facilities: Moisture content tests are conducted according to Chapter 9 of the ISTA Rules.

If filter paper method is used in 15.8.3.4.1:

Filter paper or germination paper: e.g. as used in the germination test

Containers: to hold seeds and filter or germination papers during the procedure of raising the seed moisture content. A range of dishes or containers may be suitable, e.g. 9 cm Petri dishes, germination boxes.

If added water, rolled method is used in 15.8.3.4.1:

Laboratory tube roller: capable of 30 revolutions per minute

Glass vials: with sealable top

Micropipettes: capacity and accuracy determined by the weight/volume of water to be added, as shown below:

Water weight (mg) or volume (µL)	Micropipette capacity (µL)	Accuracy (µL)
<200	200	1
>200	1000	5

If the CD germination test is used to assess deterioration in 15.8.3.4.3:

Germination test facilities: Germination tests are conducted using the methods and test conditions described in Chapter 5 of the ISTA Rules.

If the conductivity test is used to assess deterioration in 15.8.3.4.3:

Water: deionised water or distilled water as described in 15.8.1.3

Conductivity meter: as described in 15.8.1.3

Containers (beakers or flasks): the containers should have a base diameter of 50 mm (±5 mm) and provide adequate water depth to immerse all the seeds and the dip cell.

15.8.3.4 Controlled deterioration procedure

15.8.3.4.1 Raising and equilibration of seed moisture content

Determine the initial moisture content of the submitted sample according to Chapter 9 of the ISTA Rules. This is subsequently referred to as the initial seed moisture content (SMC).

Raise the SMC following one of the two alternative methods described below.

Filter paper method

To adjust the seed moisture content, mix the fraction of pure seed thoroughly and draw randomly four replicates of at least 100 seeds. Weigh each replicate to four decimal places. Raise the seed moisture content of each replicate to 20 %. The weight of seed at this moisture content is calculated as:

Weight of replicate at 20 % mc =

$$\text{(initial seed weight)} \cdot \frac{(100 - \text{initial seed mc})}{(100 - \text{desired seed mc*})}$$

*i.e. 80
mc = moisture content

Calculate the required weight to four decimal places. The acceptable required weight is then correct to three decimal places.

Place each of the four replicates to imbibe on a moist germination/filter paper, placed in a suitable container. There should be no free water on the surface of the paper. If 9 cm germination papers are used, 3–4 mL water per paper usually gives a moist but not wet paper. Use the same volume of water for a standard amount of paper on each test occasion.

Weigh seeds regularly to determine when they reach the required moisture content. Weighing must be accurate and correct to three decimal places. Seeds may begin to reach the required moisture content after 1.25–1.5 h depending on the seed lot, laboratory temperature and relative humidity.

Once seeds have reached the required weight, place each replicate immediately into an aluminium foil packet. The seeds can lose moisture rapidly at this stage, so speed is essential. Flatten the packets with the edge of the hand to remove air, and heat-seal the packets approximately 3 cm above the level of the seeds.

Place the sealed packets at 7 ±2 °C for 24 h.

Added water, rolled method

Draw a sample of approximately 500 seeds from the pure seed fraction and weigh to four decimal places. Calculate the required weight of the sample at 20 % moisture content to four decimal places as described for the filter paper method. The acceptable required weight is then correct to three decimal places.

The volume of water required to raise the seed moisture content of the sample to 20 % is calculated as:

Volume of water required (µL) =
Calculated weight of sample at 20 % mc – initial seed weight

Place the weighed seed sample into a glass vial, add the required volume of water correct to three decimal places and seal the glass vial. Place the glass vial on a tube roller and roll at 30 revolutions per minute and 8 ±2 °C overnight. Reweigh each sample to calculate the raised SMC and to ensure it is 20 ±0.5 % before packaging seeds into an aluminium foil packet. Flatten the packets with the edge of the hand to remove air, and heat-seal the packets approximately 3 cm above the level of the seeds.

15.8.3.4.2 Deterioration of the seed

Place the four replicate packets of each seed lot into a water bath at 45 °C for 24 h ±15 min. When the packets have been removed from the water bath, cool the seeds within the packets by placing the packets under cold running water for 5 min.

15.8.3.4.3 Testing for response to deterioration

Testing for the response to deterioration should be done within 30 min of removing the seeds from the water bath, using the deteriorated seed and either of the two following methods.

a) CD germination test

Set up a CD germination test using 100 seeds from each replicate packet. The seeds may be divided into subreplicates for the germination test. The germination conditions for a CD germination test are the same as those outlined for the standard germination test for *Brassica* spp. in Chapter 5 of the ISTA Rules.

b) Conductivity test

Set up a conductivity test following the general directions in 15.8.1.5 and 15.8.1.6.

Count four replicates of 100 seeds, each drawn at random from the deteriorated seed sample. Weigh the replicates to two decimal places (0.01 g).

Add the 4 weighed replicates of 100 seeds to containers holding 50 mL distilled/deionised water and imbibe for 16 h ±15 min at 20 ±2 °C.

Place each weighed replicate into a container holding 50 mL distilled/deionised water. Gently swirl each container to ensure that all seeds are completely immersed. Cover each container with, e.g. aluminium foil or cling film, prior to placing at 20 ±2 °C for 16 h. Label the first container/replicate of each sample with the start time. The number of containers started at one time must not exceed the number of evaluations for conductivity that can be made within 15 minutes of the conclusion of a 16 h soak period (usually 10 to 12 containers).

15.8.3.5 Calculation and expression of results

Express the results in accordance with the method used in 15.8.3.4.3.

a) CD germination test

The total germinated percentage (normal plus abnormal seedlings) and percentage of normal seedlings are noted in each replicate. The result of the CD test is calculated as the average of the four 100-seed replicates, as described for the standard germination test in Chapter 5. Both the total germinated percentage and the percentage of normal seedlings are reported.

b) Conductivity test after CD

Measure the conductivity of the leachate for each replicate at the end of the 16 h ±15 min soak period following the directions in 15.8.1.6.5.

The conductivity per gram of seed weight for each replicate is calculated after accounting for the background conductivity of the original water (see 15.8.1.6.6), and the average of the four replicates provides the seed lot test result. For each replicate:

$$\frac{\text{Conductivity reading } (\mu S\ cm^{-1}) - \text{background reading}}{\text{Weight of replicate (g)}} =$$

Conductivity ($\mu S\ cm^{-1}\ g^{-1}$)

Then calculate the variance, standard deviation and coefficient of variation as follows:

$$\text{Variance} = \frac{N\sum x^2 - (\sum x)^2}{N(N-1)}$$

where
x = conductivity of each replicate in $\mu S\ cm^{-1}\ g^{-1}$
N = number of replicates
Σ = sum of
Standard deviation $s = \sqrt{\text{Variance}}$

$$\text{Coefficient of variation} = \frac{s}{\bar{x}} \times 100$$

where $\bar{x}$ = mean conductivity of the sample

If the coefficient of variation does not exceed 10.0, the replicates are acceptable. If the coefficient of variation is greater than 10.0, the test must be repeated.

When two tests are performed in different laboratories: maximum tolerance value for two test results = mean conductivity reading × 0.3326

15.8.3.6 Reporting results

The result of a seed vigour test using the controlled deterioration test method must be reported under 'Other determinations' as follows for the two alternative methods of assessing deterioration in 15.8.3.4.3.

a) CD germination test

- Results are expressed as a percentage, calculated to the nearest whole number (5.8.1), and stated as 'Total germinated seeds (normal plus abnormal seedlings) … %' and 'Normal seedlings … %'. If the result for either of these is found to be zero, it must be reported as '0'.
- The results must be accompanied by a statement of the specific variables used in the test (method used to raise seed moisture content, raised seed moisture content, deterioration period and temperature).

b) Conductivity test after deterioration

- The result must be expressed in $\mu S\ cm^{-1}\ g^{-1}$ to the nearest 0.1 $\mu S\ cm^{-1}\ g^{-1}$.
- The results must be accompanied by a statement of the specific variables used during deterioration (method used to raise seed moisture content, raised seed moisture content, deterioration period and temperature) and in the conductivity test (soaking time and temperature).

15.8.4 Radicle emergence (RE) test

15.8.4.1 Principle

A slower rate of germination is an early physiological expression of seed ageing, the major cause of reduced vigour. The rate of germination of all validated species listed in 15.3 is accurately reflected in a single count of radicle emergence early in germination and this single count relates closely to other expressions of the rate of germination. High counts of radicle emergence early in germination are indicative of high seed vigour; low counts indicate low seed vigour.

15.8.4.2 Scope

The RE test provides a vigour test which relates to field emergence for the species listed in 15.3.

15.8.4.3 Apparatus

Paper growing media: as used in a germination test (Chapter 5.4.3.1) and specified in Table 15B.
Plastic bags or containers: to prevent drying out during the test.
Germination test facilities: to maintain the prescribed temperature (Table 15B).

15.8.4.4 Radicle emergence test procedure

15.8.4.4.1 Setting up the radicle emergence test

The test must be set up using the media and conditions described in Table 15B, following the normal procedure in your laboratory for a germination test using the prescribed medium. A control seed lot must be included with each test.

15.8.4.4.2 Temperature for the test

The radicle emergence test must be conducted at the temperature prescribed for the species in Table 15B. Temperature is the most important potential variable in the test, and each seed lot must be transferred to the test temperature within 15 minutes after being set to germinate. Monitoring of temperature is desirable and rotation of seed lots and replicates is advised at time intervals of 24 h.

15.8.4.4.3 Timing of radicle emergence counts

The timing of radicle emergence counts depends on the species (Table 15B).

15.8.4.5 Calculation and expression of results

Record the number of seeds that have produced a radicle for each replicate. The criterion for radicle emergence in each species is defined in Table 15B.

The number of seeds showing radicle emergence in each replicate is converted into a percentage for each replicate.

Calculate the average radicle emergence percentage. If necessary combine seed replicates to 100-seed replicates according to Chapter 5. Where two 100-seed replicates differ by more than the maximum tolerance value for radicle emergence shown in Table 15I, the seed lot must be re-tested. If the second test result is compatible with the first (i.e. the difference does not exceed the tolerance indicated in Table 15J), the average of the two tests must be reported.

15.8.4.6 Reporting results

The result of a seed vigour test using the radicle emergence test must be reported under 'Other Determinations' as follows:

– Results are reported as a percentage of seeds with emerged radicles calculated to the nearest whole number (5.8.1). If the result is found to be nil, it must be entered as '0'.
– The results must be accompanied by a statement of the temperature used for the test and the time of the radicle emergence counts in hours, e.g. 'Radicle emergence test 90 % with emerged radicles after 66 h at 20 °C.'

Table 15B. Specific conditions for the radicle emergence test procedures

Species	Germination medium	Replication	Germination temperature	Criterion of radicle emergence	Timing of radicle emergence count
Brassica napus (oilseed rape, Argentine canola)	Pleated papers	2 replicates of 100 seeds	20 ±1 °C	Appearance of a radicle after breaking through the seed coat. Seeds in which the seed coat has split, but no radicle has emerged, must not be included.	30 h ±15 min
Raphanus sativus	Top of paper	4 replicates of 50 seeds	20 ±1 °C	Production of 2 mm radicle	48 h ±15 min
Zea mays	Paper towels	8 replicates of 25 seeds	20 ±1 °C or 13 ±1 °C	Production of 2 mm radicle	66 h ±15 min at 20 ±1 °C 144 h ±1 h at 13 ±1 °C

15.8.5 Tetrazolium vigour test

15.8.5.1 Principle

The principle of using the topographical tetrazolium test to indicate vigour differences between seed lots is the same as when using the test to estimate seed viability (Chapter 6). The test uses a colourless solution of 2,3,5-triphenyl tetrazolium chloride as an indicator to reveal the reduction processes that occur during respiration in living cells, through the hydrogenation of the 2,3,5-triphenyl tetrazolium chloride by dehydrogenase enzymes. This results in the production of the red, stable and non-diffusible compound formazan in living cells. It is therefore possible to distinguish red-coloured living parts of the seed from dead ones, and assessment of detailed differences in the location, colour and intensity of the staining allows the identification of seeds that are either vigorous or non-vigorous.

15.8.5.2 Scope and field of application

The tetrazolium vigour test provides a vigour test for *Glycine max* which relates to field emergence.

15.8.5.3 Reagent

An aqueous solution of 2,3,5-triphenyl tetrazolium chloride salt is made up following the directions in 6.4.1. The concentration used is 0.1 %.

15.8.5.4 Procedures

15.8.5.4.1 Working samples

A test is carried out on two replicates of 100 pure seeds drawn at random from a representative sample of the submitted sample.

15.8.5.4.2 Preparation and treatment of the seed

Allow seeds to imbibe overnight for 16–18 h between rolled filter paper at 20 ±2 °C placed within sealed plastic bags to avoid evaporation. If imbibition is incomplete, seeds should be imbibed in water for 30–60 min at 20 ±2 °C to complete additional imbibition. Hard seeds may be present at the end of the imbibition period. These seeds must be incised at the cotyledonary area opposite the embryo. The hard seeds should then be imbibed overnight for 16–18 h between rolled filter paper at 20 ±2 °C.

15.8.5.4.3 Staining the seeds

Place the intact imbibed seeds in a 0.1 % 2,3,5-triphenyl tetrazolium chloride solution in the dark for 3 h at 35 ±2 °C. The seeds should be completely immersed in the staining solution.

15.8.5.4.4 Preparation of the seeds for evaluation

Decant the tetrazolium solution and rinse the seeds with water. The seeds should then be kept submerged in water during the evaluation to avoid dehydration and discoloration. Remove the seed coat by hand and expose the embryo by cutting carefully down the middle of the cotyledons, and the hypocotyl axis with a sharp blade.

15.8.5.4.5 Evaluation

The main aim of the tetrazolium vigour test is to identify vigorous and non-vigorous seeds.

Examine each seed and classify into different categories of vigorous seed (A, B or C) according to the colour, tissue turgidity and the location (extension and depth) of damaged areas on the seed (Fig. 15.1). Other staining patterns (Fig. 15.5) reveal non-vigorous seed.

Category A: completely turgid and stained seed of a normal pink colour (Fig. 15.2).

Category B: presence of minor area of red colour, unstained, flaccid or necrotic tissues with limited extension and superficial depth localised at any site of the seed (including embryo axis and joining area on the embryo axis and the cotyledons)(Figs 15.1, 15.3).

Category C: presence of major or multiple areas of red colour, unstained, flaccid or necrotic tissues extending from ⅓ to the whole of the cotyledon area at the distal end of the cotyledon(s), and a depth from ½ of the cotyledon to entire cotyledon (Figs 15.1, 15.4).

Other staining: non-vigorous seeds (Figs 15.5a-l).

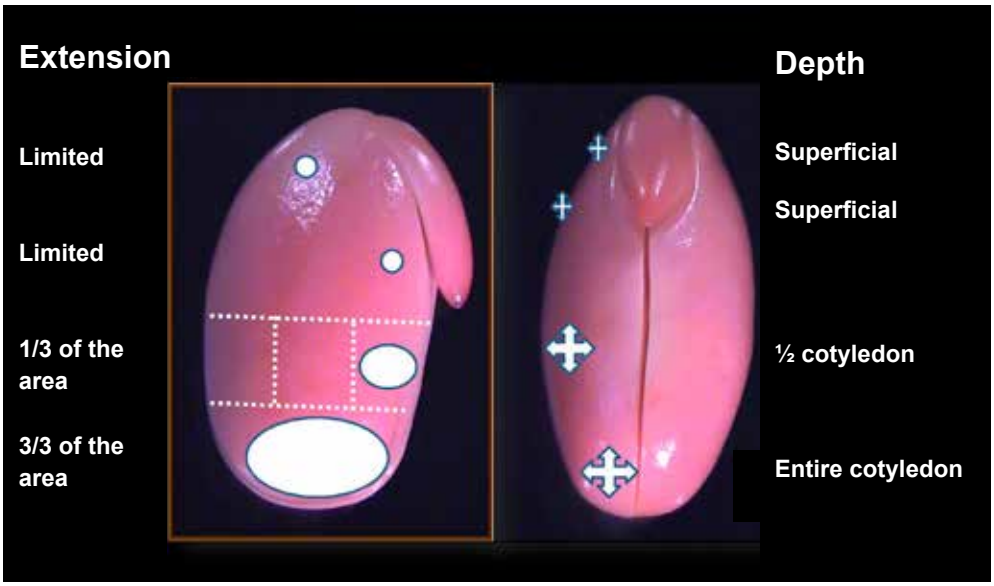


Figure 15.1. Definitions of damaged areas on the seed.

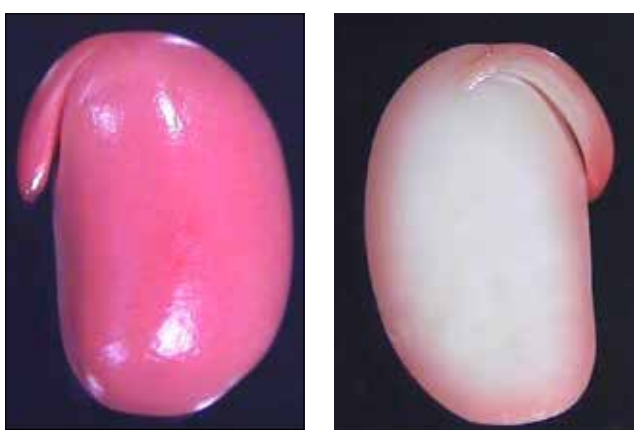


Figure 15.2. Vigorous seed, Category A. **a** External view shows uniform pink staining. **b** Internal view shows brilliant white surrounded by pink area.

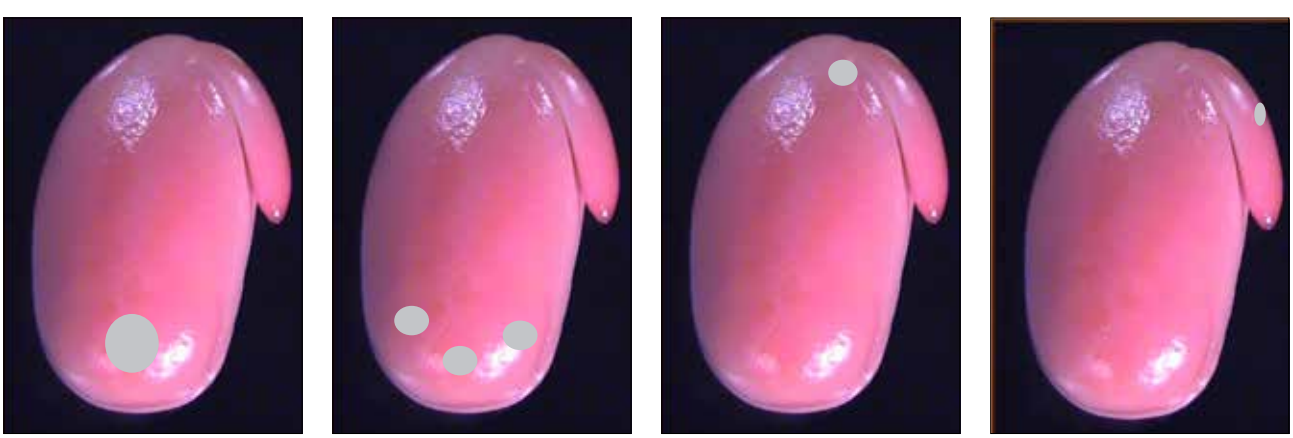


Figure 15.3. Vigorous seeds: Category B. The majority of the cotyledon is pink. Grey areas represent minor areas of red staining, unstained, flaccid or necrotic tissues with limited extension and superficial depth.

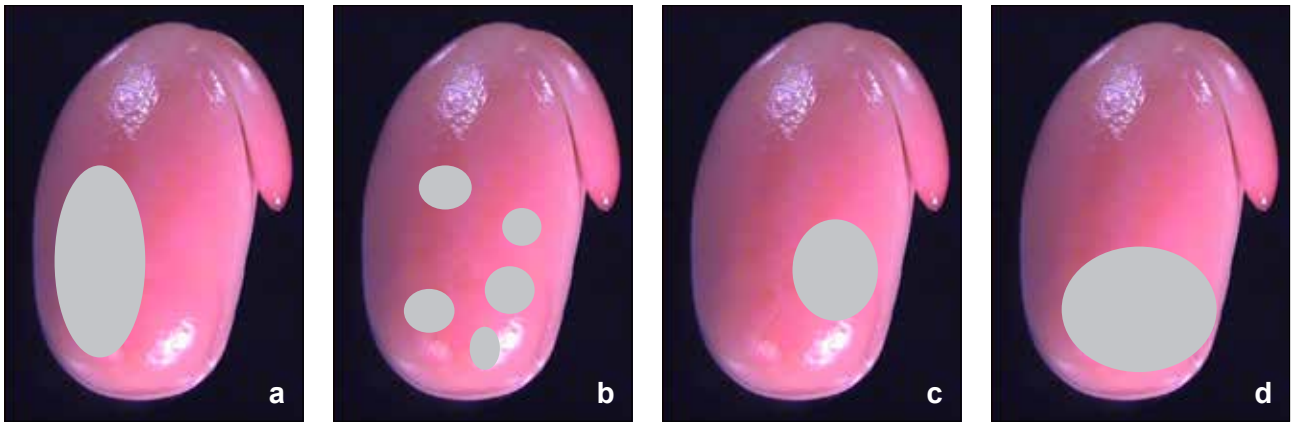


Figure 15.4. Vigorous seeds, Category C. Cotyledons mainly pink; cross-hatched areas represent major or multiple areas of red staining, unstained, flaccid or necrotic tissues with an extension of ⅓ of the cotyledon area (**a–c**) to 3/3 (**d**) of the cotyledon area at the distal end of the cotyledon(s), and a depth of ½ of the cotyledon to the entire cotyledon.

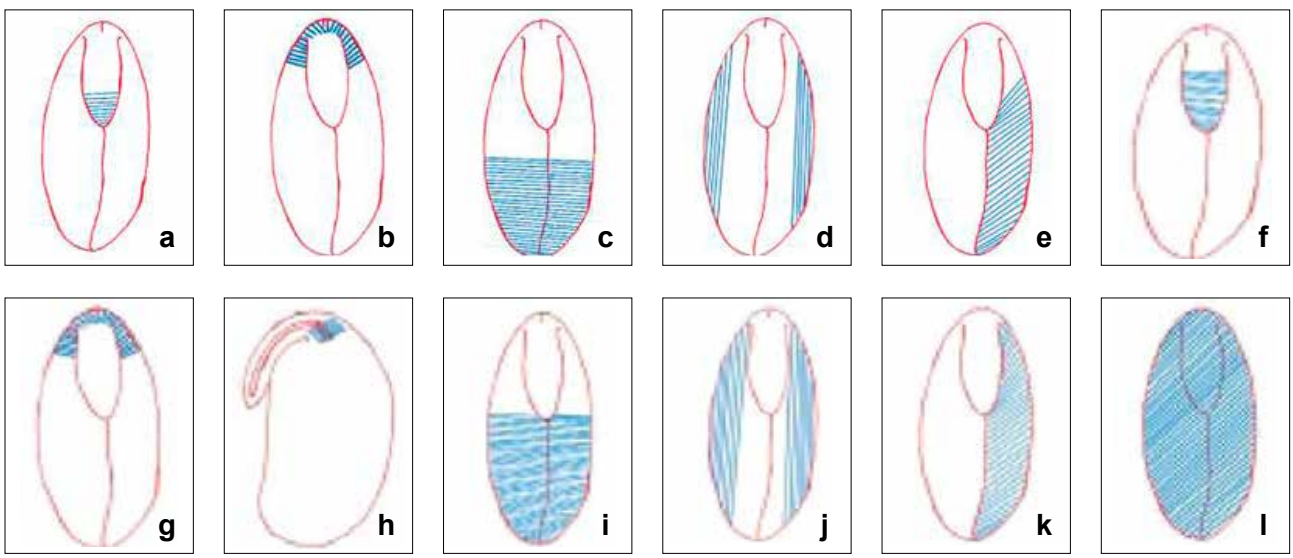


Figure 15.5. Non-vigorous seeds, other staining. **a** Radicle with tissues up to ⅓ deteriorated, unstained or lost. **b** Joining area between embryo axis and cotyledons with deteriorated red tissues. **c** Cotyledons with tissues up to ½ deteriorated, unstained or lost. **d** Cotyledons with tissues up to ¼ deep deteriorated or unstained. **e** Cotyledon with tissues up to ¾ deteriorated, unstained or lost. **f** Radicle with more than ⅓ of deteriorated, unstained or lost tissues. **g** Joining area embryo axis-cotyledons unstained. **h** plumule deteriorated or lost. **i** Cotyledons with more than ½ deteriorated, unstained or lost tissues. **j** Cotyledons with more than ¼ deep deterioration or unstained tissues. **k** Cotyledon with more than ¾ deteriorated, unstained or lost tissues. **l** Entire seed unstained.

15.8.5.5 Calculation, expression of results and tolerances

Calculate the vigour of each replicate as the sum of seeds in the three categories A, B and C and, express as a percentage of the whole sample. The mean of the two replicates is expressed as the TZ vigour (%). If the two 100-seed replicates differ by more than the tolerance value shown in Table 15K, the seed lot must be re-tested.

15.8.5.6 Reporting results

The result of a seed vigour test using the tetrazolium method must be reported under 'Other determinations'. Results are expressed as a percentage, calculated to the nearest whole number of vigorous seeds, e.g.: "Tetrazolium vigour tests using 0.1 % tetrazolium solution for 3 h at 35 °C: 90 % vigorous seeds."

15.9 Tolerance tables

Table 15C indicates the maximum range (i.e. difference between highest and lowest) in conductivity reading that is tolerable between replicates. To find the maximum tolerated range in any case, calculate the average conductivity from the four replicates. Locate the average in column 1 or 2 of the table and read off the maximum tolerated range in column 3.

The tolerances take into account the experimental error between laboratories participating in comparative tests completed by the Vigour Committee 1998–2001.

Table 15D indicates the maximum difference in conductivity readings that is tolerable between tests completed on the same sample in the same laboratory. To determine if the two tests are compatible, calculate the average of the two test results and locate this in columns 1 or 2 of the table. The tests are compatible if the difference between the conductivity readings in the two tests does not exceed the tolerance given in column 3.

The tolerances take into account the experimental error between laboratories participating in comparative tests completed by the Vigour Committee 1998–2001.

Table 15E gives the maximum difference in conductivity reading that is tolerable when tests are completed in different laboratories. To determine if the tests are compatible, calculate the average of the test results and locate this in columns 1 or 2 of the table. The tests are compatible if the difference between the conductivity readings does not exceed the tolerance given in column 3.

The tolerances take into account the experimental error between laboratories participating in comparative tests completed by the Vigour Committee 1998–2001.

Table 15F indicates the maximum range (i.e. difference between highest and lowest) in germination percentage tolerable between replicates in a germination test following accelerated ageing. To find the maximum tolerated range in any case, calculate the average percentage, to the nearest whole number, of the two replicates (form 100 seed replicates by combining two subreplicates of 50 seeds). Locate the average in column 1 or 2 of the table and read off the maximum tolerated range opposite in column 3.

Table 15G indicates the tolerances for the germination percentage after accelerated ageing when tests are made on the same sample in the same laboratory. To determine if the two tests are compatible, calculate the average percentage of the two test results to the nearest whole number and locate this in column 1 or 2 of the table. The tests are compatible if the difference between the percentage obtained in the two tests does not exceed the tolerance given in column 3.

Table 15H gives tolerances for the germination percentage after accelerated ageing when tests are made in different laboratories. To determine if tests are compatible, calculate the average percentage of the test results to the nearest whole number and locate this in columns 1 or 2 of the table. The tests are compatible if the difference between the percentages does not exceed the tolerance given in column 3.

Table 15I gives tolerances between highest and lowest radicle emergence of two replicates of 100 seeds in one radicle emergence test.

Table 15J gives tolerances between results of two radicle emergence tests of 200 seeds on the same or a different submitted sample when tests are made in the same laboratory.

Table 15K gives tolerances between highest and lowest vigour percentages of replicates in one tetrazolium vigour test for two replicates of 100 seeds.

Table 15C. Maximum tolerated range between four replicates within a conductivity test (5 % significance level).

Average conductivity (µS cm^{-1} g^{-1})		Maximum range (µS cm^{-1} g^{-1})
from	to	
1	2	3
10	10.9	3.1
11	11.9	3.3
12	12.9	3.6
13	13.9	3.8
14	14.9	4.1
15	15.9	4.3
16	16.9	4.6
17	17.9	4.8
18	18.9	5.1
19	19.9	5.3
20	20.9	5.5
21	21.9	5.8
22	22.9	6.0
23	23.9	6.3
24	24.9	6.5
25	25.9	6.8
26	26.9	7.0
27	27.9	7.3
28	28.9	7.5
29	29.9	7.8
30	30.9	8.0
31	31.9	8.3
32	32.9	8.5
33	33.9	8.8
34	34.9	9.0
35	35.9	9.3
36	36.9	9.5
37	37.9	9.8
38	38.9	10.0
39	39.9	10.3
40	40.9	10.5
41	41.9	10.8
42	42.9	11.0
43	43.9	11.3
44	44.9	11.5
45	45.9	11.8
46	46.9	12.0
47	47.9	12.3
48	48.9	12.5
49	49.9	12.8
50	50.9	13.0
51	51.9	13.3
52	52.9	13.5
53	53.9	13.8

Table 15D. Tolerances for two conductivity tests on the same submitted sample when tests are made in the same laboratory (two-way test at 5 % significance level).

Average conductivity (µS cm^{-1} g^{-1})		Maximum range (µS cm^{-1} g^{-1})
from	to	
1	2	3
10	10.9	2.0
11	11.9	2.1
12	12.9	2.3
13	13.9	2.4
14	14.9	2.5
15	15.9	2.7
16	16.9	2.8
17	17.9	3.0
18	18.9	3.1
19	19.9	3.2
20	20.9	3.4
21	21.9	3.5
22	22.9	3.7
23	23.9	3.8
24	24.9	4.0
25	25.9	4.1
26	26.9	4.2
27	27.9	4.4
28	28.9	4.5
29	29.9	4.7
30	30.9	4.8
31	31.9	4.9
32	32.9	5.1
33	33.9	5.2
34	34.9	5.4
35	35.9	5.5
36	36.9	5.6
37	37.9	5.8
38	38.9	5.9
39	39.9	6.1
40	40.9	6.2
41	41.9	6.4
42	42.9	6.5
43	43.9	6.6
44	44.9	6.8
45	45.9	6.9
46	46.9	7.1
47	47.9	7.2
48	48.9	7.3
49	49.9	7.5
50	50.9	7.6
51	51.9	7.8
52	52.9	7.9
53	53.9	8.0

Table 15E. Tolerances for conductivity tests on different submitted samples when tests are made in different laboratories (two-way test at 5 % significance level)

Average conductivity (µS cm^{-1} g^{-1})		Maximum range (µS cm^{-1} g^{-1})
from	to	
1	2	3
10	10.9	3.6
11	11.9	3.8
12	12.9	4.0
13	13.9	4.2
14	14.9	4.4
15	15.9	4.6
16	16.9	4.8
17	17.9	5.0
18	18.9	5.2
19	19.9	5.4
20	20.9	5.6
21	21.9	5.8
22	22.9	6.0
23	23.9	6.2
24	24.9	6.4
25	25.9	6.6
26	26.9	6.8
27	27.9	7.0
28	28.9	7.2
29	29.9	7.4
30	30.9	7.7
31	31.9	7.9
32	32.9	8.1
33	33.9	8.3
34	34.9	8.5
35	35.9	8.7
36	36.9	8.9
37	37.9	9.1
38	38.9	9.3
39	39.9	9.5
40	40.9	9.7
41	41.9	9.9
42	42.9	10.1
43	43.9	10.3
44	44.9	10.5
45	45.9	10.7
46	46.9	10.9
47	47.9	11.1
48	48.9	11.3
49	49.9	11.5
50	50.9	11.8
51	51.9	12.0
52	52.9	12.2
53	53.9	12.4

Table 15F. Maximum tolerated range between two replicates of 100 seeds in one accelerated ageing germination test (two way test at 2.5 % significance level). The tolerances are extracted from Table G1, column L, in Miles (1963)

Average germination percentage		Maximum range
from	to	
1	2	3
99	2	–*
98	3	–*
96–97	4–5	6
95	6	7
93–94	7–8	8
90–92	9–11	9
88–89	12–13	10
84–87	14–17	11
80–83	18–21	12
76–79	22–25	13
69–75	26–32	14
55–68	33–46	15
51–54	47–50	16

* cannot be tested

Table 15G. Tolerance for two accelerated ageing tests on the same submitted sample when tests are made in the same laboratory each on 200 seeds (two-way test at 5 % significance level).

Average germination percentage		Maximum range
from	to	
1	2	3
99	2	–*
98	3	–*
97	4	6
96	5	7
95	6	8
93–94	7–8	9
91–92	9–10	10
89–90	11–12	11
86–88	13–15	12
83–85	16–18	13
79–82	19–22	14
74–78	23–27	15
68–73	28–33	16
55–67	34–46	17
51–54	47–50	18

* cannot be tested

Table 15H. Tolerance for accelerated ageing tests on different submitted samples when tests are made in different laboratories each on 200 seeds (two-way test at 5 % significance level)

Average germination percentage		Maximum range
from	to	
1	2	3
99	2	–*
98	3	–*
97	4	–*
95–96	5–6	8
94	7	9
92–93	8–9	10
90–91	10–11	11
88–89	12–13	12
85–87	14–16	13
82–84	17–19	14
79–81	20–22	15
74–78	23–27	16
68–73	28–33	17
57–67	34–44	18
51–56	45–50	19

* cannot be tested

Table 15I. Tolerances between highest and lowest radicle emergence of two replicates of 100 seeds in one radicle emergence test (two-way test at the 2.5 % significance level). **Note:** this table is a copy of Table 5B Part 2

Average radicle emergence of test		Tolerance
51–100 %	0–50 %	
99	2	4
98	3	5
96–97	4–5	6
95	6	7
93–94	7–8	8
90–92	9–11	9
88–89	12–13	10
84–87	14–17	11
81–83	18–20	12
76–80	21–25	13
69–75	26–32	14
55–68	33–46	15
51–54	47–50	16

Table 15J. Tolerances between results of two radicle emergence tests of 200 seeds on the same or a different submitted sample when tests are made in the same laboratory (two-way test at the 2.5 % significance level). **Note:** this table is a copy of Table 5C Part 2

Average radicle emergence of 2 tests		Tolerance
51–100 %	0–50 %	
99	2	2
98	3	3
96–97	4–5	4
94–95	6–7	5
91–93	8–10	6
87–90	11–14	7
82–86	15–19	8
75–81	20–26	9
64–74	27–37	10
51–63	38–50	11

Table 15K. Tolerances between highest and lowest vigour percentages of replicates in one tetrazolium vigour test (two-way test at the 5.0 % significance level), 2 replicates of 100 seeds. Extracted from column K of Table G1, Miles, S. R. (1963), Handbook of Tolerances and of Measures of Precision for Seed Testing. *Proceedings of the International Seed Testing Association*, **28** (3)

Average vigour percentage of test		Tolerance
51–100 %	0–50 %	
99	2	3
98	3	4
96–97	4–5	5
95	6	6
92–94	7–9	7
90–91	10–11	8
86–89	12–15	9
82–85	16–19	10
77–81	20–24	11
70–76	25–31	12
55–69	32–46	13
51–54	47–50	14

Chapter 16: Rules for size and grading of seeds

16.1 For *Beta* seeds and pelleted seeds

The control of size grading is carried out on a sample weighing at least 250 g, or for pelleted seeds, a sample consisting of the number of seeds indicated in Table 2B Part 1. The sample must be sent to the testing laboratory in an airtight container. Two working samples of about 50 g (not less than 45 g and not more than 55 g) each are used. For pelleted seeds, two working samples of about 1000 seeds each are used. Each sample is subjected to a screening analysis.

The following round-hole screens must be used*:

– One screen with a hole diameter 0.25 mm smaller than the lower nominal value of the seed size,
– One set of screens which divides the stated seeds size range into quarter-millimetre fractions,
– One screen with a hole diameter 0.25 mm larger than the upper nominal value of the seed size.

The screening fractions (including the portion passing through the lowest screen) are weighed (two decimal places). The weights of the fractions are expressed as percentages (one decimal place) of the total weight. The average of the values for each of the two working samples represents the results of the analysis, provided that the difference between the sums of the percentages within the nominal grading limits does not exceed 1.5 %. If this tolerance is exceeded, a further sample of 50 g or 1000 pelleted seeds (and if necessary a fourth sample) must be analysed.

The result of a screening analysis test for size and grading of seeds must be reported under 'Other determinations' as the average of two screening analyses falling within the permitted tolerance limits.

* The 'Bonn' Screening apparatus, developed by the Institut für Landtechnik, Bonn, (Germany), with the requisite round-hole screens and automatic switch-gear for the regular interruption of the reciprocations may be used. The amplitude should be between ±45 and 50 mm. For pelleted seed the screening time is one minute and for unpelleted seed three minutes.

Chapter 17: Rules for the issue of Orange International Seed Lot Certificates on seed lots exceeding the maximum lot size prescribed in Table 2A being transported loose in bulk containers

17.1 Object

The object of these rules is to facilitate the trading of seeds loose in bulk containers thus allowing for full utilisation of the space available in the containers. At the same time they provide for maintaining the position of the Orange International Seed Lot Certificate as a trading document.

17.2 Field of application

These rules may only be applied to the species listed in 17.7 below.

17.3 Principle

Any number of whole seed lots (N = number of seed lots being combined) on which Orange International Seed Lot Certificates have already been issued may be combined loose in one or two sealable and identifiable bulk container(s) providing the results for the attributes reported on the existing Certificates are not significantly different from each other as determined by the tolerance tables included in 17.8. The destruction of the identity of the original lots and their combining in the bulk container must be supervised by a person authorised to carry out sampling for the issue of Orange International Seed Lot Certificates. The authorised person must be present during all steps of the combination of the seed lots including the sealing of the bulk container(s). On receipt of the necessary information the appropriate accredited laboratory will issue a new Orange International Seed Lot Certificate showing the weighted average of the results for the attributes reported on the Certificates representing the constituent lots and other information as designated in 17.6 below. Seed lots to be used to form the compound lot must be of the same cultivar. Germination tests of component lots must have germination completion dates within a period of not greater than 60 days.

17.4 Procedure

Lots of up to the maximum weight prescribed in Table 2A, which it is intended to place in the bulk container(s), must be officially sampled, sealed and tested according to the Rules and an Orange International Seed Lot Certificate issued for each of the lots. The results for each attribute reported on the Certificates must then be compared using the appropriate tolerance table included in this appendix and only those lots showing no significant difference for all reported attributes may be bulked. Calculate the straight average of the results of the lots to be combined. Then take the difference of the maximum and minimum values among all component lots ($R = X_{max} - X_{min}$). Now find the calculated straight average in 'The average of all lots' column. Read off the relevant maximum tolerated value (R_T) from the column (N = . . .) for the number of lots involved. Compare this maximum tolerated value (R_T) with R. If $R < R_T$ for all three attributes, then the lots are compatible and may be combined. If, however, $R > R_T$ for one or more attributes, the lots are incompatible and may not be combined. The purpose of this procedure is to avoid heterogeneity of compound seed lots. Only ISTA Certificate test results on samples obtained of the size prescribed by the Rules for all seed characteristics tested are to be used.

The accepted whole seed lots are then broken open and the seed placed loose in the bulk container(s) in the presence of an authorised person. The authorised person must then label and seal the container(s) and report to the accredited laboratory issuing the bulk container(s) Certificate the official identification of the container(s) and any other relevant facts such as the actual weights of seed put in the container(s). The identification of the bulk container(s) must be different from those used to identify the constituent lots.

Table 17.1. Example of the calculation of a weighted average

	Lot 1	Lot 2	Lot 3	Totals	Weighted average
Lot sizes (kg)	10 000	7 000	2 500	19 500	
Multiplication factor	0.513	0.359	0.128		
Germination (%)	91	87	92		
Proportional value	46.683	31.233	11.776	89.692	90 %
Purity (%)	99.4	99.1	99.3		
Proportional value	50.9922	35.5769	12.7104	99.2795	99.3 %
Other seed count	5	2	7		
Proportional value	2.565	0.718	0.896	4.179	4

17.5 Calculation and expression of results

The weighted average result for each attribute is calculated and reported with the degree of accuracy prescribed in the rules for that attribute. During calculation, use one decimal place beyond that reported and adjust following the normal rounding procedures. For the number count tests, all species recorded must be reported, and average figures below one must be reported as 1.

Weighted average calculation

Consider the weights of the individual component lots and sum these. Divide each in turn by the total and express to 3 decimal places; let this be called the multiplication factor for each lot. Consider the attribute measures individually for each lot in turn. Multiply each by the multiplication factor for each lot to obtain 'proportional values' for each attribute for each lot. Sum the proportional values for all lots for each attribute. Round each total to obtain a weighted average for each attribute applying rounding rules for place numbers (see Table 17.1).

17.6 Reporting results

The result of a weighted average test performed on seed lots, as described in Chapter 17, must be reported in the normal way, except that:

a) across the date of sampling, date sample received, date test concluded and test number boxes insert the statement: 'Seed loose in bulk container(s) – see under Other determinations.'

b) Under 'Other determinations', list the test number, date of sampling and date test concluded of all constituent lots together with the statement: 'The test results reported represent the weighted average of the results reported on these certificates which were not significantly different from each other.'

17.7 Species for which these rules apply

These rules apply only to species of the *Poaceae* and *Fabaceae* listed in Table 2A Part 1 with a maximum lot size in Table 2A of 10 000 kg.

17.8 Tolerance tables

Tolerance tables to be used for compatibility tests on ISTA certified seed lots to be combined to form compound lots exceeding 10 000 kg.

Table 17.2. Tolerances for purity percentages deviation of component lots at 1 % significance level

Average of all lots		Number of lots being blended								
		2	3	4	5	6	7	8	9	10
99.9	0.1	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
99.8	0.2	0.3	0.4	0.4	0.4	0.4	0.4	0.4	0.5	0.5
99.7	0.3	0.4	0.5	0.5	0.5	0.5	0.5	0.5	0.6	0.6
99.6	0.4	0.5	0.5	0.6	0.6	0.6	0.6	0.6	0.6	0.7
99.5	0.5	0.5	0.6	0.6	0.6	0.7	0.7	0.7	0.7	0.7
99.4	0.6	0.6	0.6	0.7	0.7	0.7	0.8	0.8	0.8	0.8
99.3	0.7	0.6	0.7	0.7	0.8	0.8	0.8	0.8	0.8	0.9
99.2	0.8	0.6	0.7	0.8	0.8	0.8	0.9	0.9	0.9	0.9
99.1	0.9	0.7	0.8	0.8	0.9	0.9	0.9	0.9	1.0	1.0
99.0	1.0	0.7	0.8	0.9	0.9	0.9	1.0	1.0	1.0	1.0
98.5–98.9	1.1–1.5	0.9	1.0	1.1	1.1	1.2	1.2	1.2	1.2	1.3
98.0–98.4	1.6–2.0	1.0	1.2	1.2	1.3	1.3	1.4	1.4	1.4	1.4
97.5–97.9	2.1–2.5	1.1	1.3	1.4	1.4	1.5	1.5	1.6	1.6	1.6
97.0–97.4	2.6–3.0	1.2	1.4	1.5	1.6	1.6	1.7	1.7	1.7	1.8
96.5–96.9	3.1–3.5	1.3	1.5	1.6	1.7	1.7	1.8	1.8	1.8	1.9
96.0–96.4	3.6–4.0	1.4	1.6	1.7	1.8	1.9	1.9	2.0	2.0	2.0
95.5–95.9	4.1–4.5	1.5	1.7	1.8	1.9	2.0	2.0	2.1	2.1	2.1
95.0–95.4	4.6–5.0	1.6	1.8	1.9	2.0	2.1	2.1	2.2	2.2	2.2
94.5–94.9	5.1–5.5	1.7	1.9	2.0	2.1	2.2	2.2	2.3	2.3	2.4
94.0–94.4	5.6–6.0	1.7	2.0	2.1	2.2	2.3	2.3	2.4	2.4	2.5
93.5–93.9	6.1–6.5	1.8	2.0	2.2	2.3	2.3	2.4	2.5	2.5	2.5
93.0–93.4	6.6–7.0	1.9	2.1	2.2	2.3	2.4	2.5	2.5	2.6	2.6
92.5–92.9	7.1–7.5	1.9	2.2	2.3	2.4	2.5	2.6	2.6	2.7	2.7
92.0–92.4	7.6–8.0	2.0	2.2	2.4	2.5	2.6	2.6	2.7	2.8	2.8
91.5–91.9	8.1–8.5	2.0	2.3	2.5	2.6	2.7	2.7	2.8	2.8	2.9
91.0–91.4	8.6–9.0	2.1	2.4	2.5	2.6	2.7	2.8	2.9	2.9	3.0
90.5–90.9	9.1–9.5	2.1	2.4	2.6	2.7	2.8	2.9	2.9	3.0	3.0
90.0–90.4	9.6–10.0	2.2	2.5	2.6	2.8	2.9	2.9	3.0	3.0	3.1
89.0–89.9	10.1–11.0	2.3	2.6	2.8	2.9	3.0	3.1	3.1	3.2	3.2
88.0–88.9	11.1–12.0	2.4	2.7	2.9	3.0	3.1	3.2	3.2	3.3	3.4
87.0–87.9	12.1–13.0	2.4	2.8	3.0	3.1	3.2	3.3	3.4	3.4	3.5
86.0–86.9	13.1–14.0	2.5	2.9	3.1	3.2	3.2	3.4	3.5	3.5	3.6
85.0–85.9	14.1–15.0	2.6	2.9	3.1	3.3	3.4	3.5	3.6	3.6	3.7
84.0–84.9	15.1–16.0	2.7	3.0	3.2	3.4	3.5	3.6	3.7	3.7	3.8
83.0–83.9	16.1–17.0	2.7	3.1	3.3	3.5	3.6	3.7	3.7	3.8	3.9
82.0–82.9	17.1–18.0	2.8	3.2	3.4	3.5	3.7	3.7	3.8	3.9	4.0
81.0–81.9	18.1–19.0	2.9	3.2	3.5	3.6	3.7	3.8	3.9	4.0	4.0
80.0–80.9	19.1–20.0	2.9	3.3	3.5	3.7	3.8	3.9	4.0	4.0	4.1
79.0–79.9	20.1–21.0	3.0	3.4	3.6	3.7	3.9	4.0	4.1	4.1	4.2
78.0–78.9	21.1–22.0	3.0	3.4	3.6	3.8	3.9	4.0	4.1	4.2	4.3
77.0–77.9	22.1–23.0	3.1	3.5	3.7	3.9	4.0	4.1	4.2	4.3	4.3
76.0–76.9	23.1–24.0	3.1	3.5	3.8	3.9	4.1	4.2	4.3	4.3	4.4
75.0–75.9	24.1–25.0	3.2	3.6	3.8	4.0	4.1	4.2	4.3	4.4	4.5
74.0–74.9	25.1–26.0	3.2	3.6	3.9	4.0	4.2	4.3	4.4	4.5	4.5
73.0–73.9	26.1–27.0	3.2	3.7	3.9	4.1	4.2	4.3	4.4	4.5	4.6
72.0–72.9	27.1–28.0	3.3	3.7	4.0	4.1	4.3	4.4	4.5	4.6	4.6
71.0–71.9	28.1–29.0	3.3	3.7	4.0	4.2	4.3	4.4	4.5	4.6	4.7
70.0–70.9	29.1–30.0	3.3	3.8	4.0	4.2	4.4	4.5	4.6	4.7	4.7
69.0–69.9	30.1–31.0	3.4	3.8	4.1	4.3	4.4	4.5	4.6	4.7	4.8
68.0–68.9	31.1–32.0	3.4	3.8	4.1	4.3	4.4	4.6	4.7	4.7	4.8
67.0–67.9	32.1–33.0	3.4	3.9	4.1	4.3	4.5	4.6	4.7	4.8	4.9
66.0–66.9	33.1–34.0	3.4	3.9	4.2	4.4	4.5	4.6	4.7	4.8	4.9
65.0–65.9	34.1–35.0	3.5	3.9	4.2	4.4	4.5	4.7	4.8	4.8	4.9
64.0–64.9	35.1–36.0	3.5	4.0	4.2	4.4	4.6	4.7	4.8	4.9	5.0
63.0–63.9	36.1–37.0	3.5	4.0	4.2	4.4	4.6	4.7	4.8	4.9	5.0
62.0–62.9	37.1–38.0	3.5	4.0	4.3	4.5	4.6	4.7	4.8	4.9	5.0
61.0–61.9	38.1–39.0	3.6	4.0	4.3	4.5	4.6	4.8	4.9	5.0	5.0
60.0–60.9	39.1–40.0	3.6	4.0	4.3	4.5	4.7	4.8	4.9	5.0	5.1
59.0–59.9	40.1–41.0	3.6	4.1	4.3	4.5	4.7	4.8	4.9	5.0	5.1
58.0–58.9	41.1–42.0	3.6	4.1	4.3	4.5	4.7	4.8	4.9	5.0	5.1
57.0–57.9	42.1–43.0	3.6	4.1	4.4	4.6	4.7	4.8	4.9	5.0	5.1
56.0–56.9	43.1–44.0	3.6	4.1	4.4	4.6	4.7	4.8	5.0	5.0	5.1
55.0–55.9	44.1–45.0	3.6	4.1	4.4	4.6	4.7	4.9	5.0	5.1	5.1
54.0–54.9	45.1–46.0	3.6	4.1	4.4	4.6	4.7	4.9	5.0	5.1	5.1
53.0–53.9	46.1–47.0	3.6	4.1	4.4	4.6	4.8	4.9	5.0	5.1	5.2
52.0–52.9	47.1–48.0	3.6	4.1	4.4	4.6	4.8	4.9	5.0	5.1	5.2
51.0–51.9	48.1–49.0	3.6	4.1	4.4	4.6	4.8	4.9	5.0	5.1	5.2
50.0–50.9	49.1–50.0	3.6	4.1	4.4	4.6	4.9	4.9	5.0	5.1	5.2

Table 17.3. Tolerances for germination percentages deviation of component lots at 1 % significance level

Average of all lots		Number of lots being blended								
		2	3	4	5	6	7	8	9	10
99	1	2	2	2	2	2	2	2	3	3
98	2	3	3	3	3	3	3	3	4	4
97	3	3	4	4	4	4	4	4	4	4
96	4	4	4	4	5	5	5	5	5	5
95	5	4	4	5	5	5	5	5	6	6
94	6	4	5	5	5	6	6	6	6	6
93	7	5	5	6	6	6	6	6	6	7
92	8	5	6	6	6	6	7	7	7	7
91	9	5	6	6	7	7	7	7	7	7
90	10	5	6	7	7	7	7	7	8	8
89	11	6	6	7	7	7	8	8	8	8
88	12	6	7	7	7	8	8	8	8	8
87	13	6	7	7	8	8	8	8	9	9
86	14	6	7	8	8	8	8	9	9	9
85	15	6	7	8	8	8	9	9	9	9
84	16	7	8	8	8	9	9	9	9	9
83	17	7	8	8	9	9	9	9	10	10
82	18	7	8	8	9	9	9	10	10	10
81	19	7	8	9	9	9	10	10	10	10
80	20	7	8	9	9	10	10	10	10	10
79	21	7	8	9	9	10	10	10	10	11
78	22	8	9	9	10	10	10	10	11	11
77	23	8	9	9	10	10	10	10	11	11
76	24	8	9	9	10	10	10	11	11	11
75	25	8	9	10	10	10	11	11	11	11

Average of all lots		Number of lots being blended								
		2	3	4	5	6	7	8	9	10
74	26	8	9	10	10	10	11	11	11	11
73	27	8	9	10	10	11	11	11	11	11
72	28	8	9	10	10	11	11	11	11	12
71	29	8	9	10	10	11	11	11	12	12
70	30	8	9	10	11	11	11	11	12	12
69	31	8	10	10	11	11	11	12	12	12
68	32	8	10	10	11	11	11	12	12	12
67	33	9	10	10	11	11	11	12	12	12
66	34	9	10	10	11	11	12	12	12	12
65	35	9	10	10	11	11	12	12	12	12
64	36	9	10	11	11	11	12	12	12	12
63	37	9	10	11	11	11	12	12	12	12
62	38	9	10	11	11	12	12	12	12	13
61	39	9	10	11	11	12	12	12	12	13
60	40	9	10	11	11	12	12	12	12	13
59	41	9	10	11	11	12	12	12	12	13
58	42	9	10	11	11	12	12	12	13	13
57	43	9	10	11	11	12	12	12	13	13
56	44	9	10	11	11	12	12	12	13	13
55	45	9	10	11	11	12	12	12	13	13
54	46	9	10	11	11	12	12	12	13	13
53	47	9	10	11	11	12	12	12	13	13
52	48	9	10	11	11	12	12	12	13	13
51	49	9	10	11	11	12	12	12	13	13
50	50	9	10	11	11	12	12	12	13	13

Table 17.4. Tolerances for other seed counts deviation of component lots at 1 % significance level

Average of all lots	Number of lots being blended								
	2	3	4	5	6	7	8	9	10
1	4	4	4	5	5	5	5	5	5
2	5	6	6	7	7	7	7	7	7
3	6	7	8	8	8	8	9	9	9
4	7	8	9	9	10	10	10	10	10
5	8	9	10	10	11	11	11	11	12
6	9	10	11	11	12	12	12	12	13
7	10	11	12	12	13	13	13	13	14
8	10	12	12	13	13	14	14	14	15
9	11	12	13	14	14	15	15	15	15
10	12	13	14	15	15	15	16	16	16
11	12	14	15	15	16	16	17	17	17
12	13	14	15	16	16	17	17	18	18
13	13	15	16	17	17	18	18	18	19
14	14	15	16	17	18	18	19	19	19
15	14	16	17	18	18	19	19	20	20
16	15	16	18	18	19	20	20	20	21
17	15	17	18	19	20	20	21	21	21
18	15	17	19	20	20	21	21	22	22
19	16	18	19	20	21	21	22	22	22
20	16	18	20	21	21	22	22	23	23
21	17	19	20	21	22	22	23	23	24
22	17	19	21	22	22	23	23	24	24
23	17	20	21	22	23	23	24	24	25
24	18	20	22	23	23	24	24	25	25
25	18	21	22	23	24	24	25	25	26
26	19	21	22	23	24	25	25	26	26
27	19	21	23	24	25	25	26	26	27
28	19	22	23	24	25	26	26	27	27
29	20	22	24	25	26	26	27	27	28
30	20	23	24	25	26	27	27	28	28
31	20	23	24	26	27	27	28	28	29
32	21	23	25	26	27	28	28	29	29
33	21	24	25	26	27	28	29	29	30

Average of all lots	Number of lots being blended								
	2	3	4	5	6	7	8	9	10
34	21	24	26	27	28	28	29	30	30
35	22	24	26	27	28	29	30	30	31
36	22	25	26	28	29	29	30	30	31
37	22	25	27	28	29	30	30	31	31
38	22	25	27	28	29	30	31	31	32
39	23	26	27	29	30	30	31	32	32
40	23	26	27	29	30	31	32	32	33
41	23	26	28	29	30	31	32	33	33
42	24	27	29	30	31	32	32	33	33
43	24	27	29	30	31	32	33	33	34
44	24	27	29	31	32	32	33	34	34
45	24	28	30	31	32	33	33	34	35
46	25	28	30	31	32	33	34	34	35
47	25	28	30	32	33	33	34	35	35
48	25	29	30	32	33	34	35	35	36
49	25	29	31	32	33	34	35	36	36
50	26	29	31	33	34	35	35	36	36
51	26	29	31	33	34	35	36	36	37
52	26	30	32	33	34	35	36	37	37
53	26	30	32	33	35	36	36	37	38
54	27	30	32	34	35	36	37	37	38
55	27	31	33	34	35	36	37	38	38
56	27	31	33	34	36	37	37	38	39
57	27	31	33	35	36	37	38	38	39
58	28	31	34	35	36	37	38	39	39
59	28	32	34	35	37	37	38	39	40
60	28	32	34	36	37	38	39	39	40
61	28	32	34	36	37	38	39	40	40
62	29	32	35	36	37	38	39	40	41
63	29	33	35	37	38	39	40	40	41
64	29	33	35	37	38	39	40	41	41
65	29	33	35	37	38	39	40	41	42

Chapter 18: Seed mixture analysis

18.1 Object

The objects of seed mixture analyses are:
– to determine the composition of a seed mixture as corresponding to the component declaration by using methods according to Chapter 3; and
– to provide pure seed of mixture components for further seed quality tests.

This chapter contains procedures to determine the seed mixture composition and details for testing seed quality of seed mixtures by other tests. When specific instructions are not given for other tests in this Chapter, then the appropriate Chapter of the ISTA Rules must be followed. The results of tests on seed mixture samples can only be reported on a Blue International Seed Sample Certificate (see 1.2.2, 1.5.2.20 and 18.8).

18.2 Definitions

Seed mixture is a quantity of seed that contains seed of two or more species by declaration of the applicant.

For issuing a Blue International Seed Sample Certificate for a seed mixture, at least two of the declared species must be listed in Table 2A of the ISTA Rules and it must not be difficult or impossible to distinguish between these two species (see 3.5.2.4). Seeds of declared species not listed in Table 2A belong to the other seed fraction (see 18.4).

Mixture composition is the composition of the seed mixture given in percentage of mixture components by weight or by number of seed or inert material.

Mixture component is a component of a seed mixture that contains seed of one or more species or inert material. A mixture component is:
– seed (including coated seed) of one species including seed of two or more varieties of the same species (see 3.2.1);
– seed of two or more species, if it is difficult or impossible to distinguish between the species (see 3.5.2.4);
or
– inert material according to the declaration of the mixture composition, e.g. in form of additives to improve sowability.

The component parts ‘other seed’ and ‘inert matter’ of a purity analysis according to Chapter 3 are not regarded as mixture components and are not part of the mixture composition.

If it is difficult or impossible to distinguish between coated seeds of different species, then the species are combined in one mixture component.

18.3 Sampling

As only Blue International Seed Sample Certificates can be issued for seed mixtures, sampling from the mixture lot is not covered by the ISTA Rules.

18.3.1 Size of the submitted sample

The submitted sample from a seed mixture must contain at least 25 000 seed units. The weight of the submitted sample can be determined using either method:
– ‘exact method’ on the basis of the declared mixture composition by using thousand seed weights (18.7) or the sample sizes given in Column 3 of Table 2A for the declared species (also see the Excel spreadsheet ‘Testing Seed Mixtures’ on the ISTA website in the Technical Committee documents to calculate submitted sample weights);
– ‘quick method’ using the greatest weight given in column 3 of Table 2A for a declared species of the seed mixture.

18.3.2 Sample reduction

For sample reduction, methods according to 2.5.2.2.1 may be used, but not the centrifugal divider (2.5.2.2.1c). The hand halving method (2.5.2.2.4) may only be used, if the other methods do not work and if this method is allowed for at least one of the declared species. Dimensions of equipment should be suitable for the largest seeds according to the declaration of the seed mixture.

18.4 Purity and composition analysis

Seeds of species not declared by the applicant as mixture component and seeds of declared species that are not listed in Table 2A are classified as ‘other seeds’. Inert material not declared by the applicant as mixture component is classified as ‘inert matter’. The pure seed assessment must follow the appropriate pure seed definition (PSD) of each species provided in Chapter 3.

18.4.1 Working sample

The purity analysis must be made on a working sample taken from the submitted sample in accordance with 18.3.2. The size of the working sample must be a weight estimated to contain at least 2 500 seed units.

The weight of the working sample can be determined using either method:

– 'exact method' on the basis of the declared mixture composition by using the thousand seed weights (18.7) or the sample sizes given in Column 4 of Table 2A for the declared species (also see the Excel spreadsheet 'Testing Seed Mixtures' on the ISTA website in the Technical Committee documents to calculate submitted sample weights).
– 'quick method' the greatest weight given in Column 4 of Table 2A for a declared species of the seed mixture.

18.4.2 Separation

The working sample is separated into the fractions 'pure seed', 'other seed', 'inert matter' and, if applicable, 'inert material according to declaration'.

The pure seed fraction includes seeds of those species that are declared by the applicant as mixture components and that are listed in Table 2A. The pure seed fraction is further separated into the declared species with methods according to 3.5.2. When it is difficult or impossible to distinguish between declared species (see 3.5.2.4), the species are combined into one mixture component.

If applicable, the inert material in the working sample is separated into 'inert material according to declaration' and 'inert matter' with methods according to 3.5.2. Each component part of the purity analysis and each mixture component must be weighed according to 3.5.1.

The uniform blowing method must not be used for seed mixtures, but the blower can be used as a tool to aid the purity analysis.

18.5 Determination of other seeds by number

The size of the working sample for a complete or limited test must be a weight estimated to contain 25 000 seed units, i.e. ten times the size of the purity working sample as determined according to 18.4.1.

18.6 Germination test, seed viability test, seed vigour test and other tests using replicates of 100 seeds

For mixture components representing 5 % or more of the seed mixture, according to the declared mixture composition, as many replicates of 100 pure seeds are tested as pure seeds are available from the purity test sample of 2 500 seeds. Maximum numbers of seeds per test are given in the appropriate Chapter. If fewer than 100 seeds are available, all seeds are used.

For mixture components representing less than 5 % of the seed mixture, according to the declared mixture composition, tests are not carried out except on the specific request of the applicant.

18.7 Weight determination

The thousand seed weight determination for mixture components does not follow Chapter 10 but is determined by counting the number and determining the weight of all pure seeds of a mixture component. The thousand seed weight is calculated using the weight and number of seeds of each component.

Note: If the applicant intends to use the reported results for verification of mixture composition, the thousand seed weight of all seed mixture components must be determined and reported.

18.8 Reporting results

The results of tests on seed mixtures can only be reported on a Blue International Seed Sample Certificate (see 1.2.2).

For the species tested, 'Seed mixture' together with the mixture composition according to the declaration of the applicant, must be entered.

18.8.1 Purity and component analysis

The results of the purity analysis are reported according to Chapter 3.

The actual weight of sample examined to the minimum number of decimal places indicated in Table 4.1 must be reported under 'Other determinations', i.e. 'Purity and composition analysis: … g of seed examined.'

The mixture composition is reported under 'Other determinations' in one of the following formats, as requested by the applicant:

1. The percentage by weight of the pure seeds of the mixture components using the total weight of the pure seed fraction. In addition, if applicable, the percentage by weight of the 'inert material according to declaration' referred to the sum of the weights of all mixture components (pure seeds and inert material according to declaration) must be given to one decimal place under 'Other determinations'.
2. The percentage by weight of mixture components, pure seeds or inert material according to declaration using the sum of the weights of the pure seed fraction and the declared inert material.
3. The percentage by number of the pure seeds of the mixture components using the total number of seeds of the pure seed fraction.

In addition, if applicable, the percentage by weight of the 'inert material according to declaration' using the sum of the weights of all mixture components must be given to one decimal place under 'Other determinations'.

18.8.2 Determination of other seeds by number

The results of a determination of other seeds by number on a seed mixture must be reported according to 4.7.

18.8.3 Germination, seed viability, seed vigour and other tests using replicates of 100 seeds

Test results are reported only for those species for which methods are given in the appropriate Chapter of the ISTA Rules. The results of these tests must be reported under 'Other determinations'.

Germination test results are not reported in the 'Germination' section of the certificate (an 'N' must be entered there), but under 'Other determinations'. When 100 or more seeds are tested, the percentage results of the test for each mixture component tested are reported to the nearest whole number. The number of seeds tested is also reported. Tolerances as described in the appropriate Chapters are applied to tests of 400, 300, 200 and 100 seeds.

When fewer than 100 seeds are tested, the actual number of seeds in each category (e.g. normal seedlings or viable seeds) is reported, together with the total number of seeds tested.

The method used in the test must be reported on the certificate according to the appropriate Chapter for each component species tested.

18.8.4 Weight determination

The results as calculated according to 18.7 must be reported under 'Other determinations'.

Chapter 19: Testing for seeds of genetically modified organisms

19.1 Object

The object of this chapter is to give guidelines to detect, quantify or confirm the presence of GMO seeds in seed lots.

These guidelines can be applied to testing adventitious presence (AP) of genetically modified organisms (GMOs) and GMO trait purity testing.

19.2 Definitions

19.2.1 Adventitious presence

Adventitious presence (AP) in seeds refers to the unintentional and incidental presence of foreign material in a seed lot. This may happen during production, harvesting, storage or marketing.

19.2.2 Analyte

An analyte is a substance or chemical constituent that is of interest in an analytical procedure.

19.2.3 Certified reference material

Certified reference material is reference material which has been characterised metrologically for a specific property by an official body. Such material is accompanied by a document attesting to the value of that property, its associated uncertainty and its metrological traceability.

19.2.4 Genetically modified organism

A genetically modified organism (GMO) is any living organism that possesses a novel combination of genetic material obtained through the use of modern biotechnology.

19.2.5 GMO event

A GMO event is a single transformation act that results in the integration of a new trait at a unique site in the plant genome, giving rise to a transgenic plant and subsequently incorporated into new varieties.

19.2.6 GMO trait

A GMO trait is a novel phenotypic character, added by genetic engineering to an organism and often derived from another species.

19.2.7 Limit of detection

The limit of detection is the smallest amount of target analyte that has been demonstrated to be detected with a given level of confidence. This limit must be verified by the laboratory.

19.2.8 Limit of quantification

The limit of quantification is the smallest amount of target analyte that has been demonstrated to be reliably measured with acceptable levels of accuracy and precision. This limit must be verified by the laboratory.

19.2.9 Performance-based approach

The performance-based approach (PBA) is an approach to testing in which individual laboratories can choose the test method, as long as the method has been validated as fit for purpose and complies to given performance standards.

19.2.10 Proficiency test

A proficiency test is a standardised test or series of tests that assesses the ability of a laboratory or an individual operator to carry out a particular method.

19.2.11 Seed bulk

The seed bulk is the whole working sample that is prepared at one time (e.g. grinding, DNA or protein extraction) and analysed (e.g. end-point PCR, ELISA, real-time PCR).

19.2.12 Seed group

A seed group is one of the portions of the working sample that is separately prepared (e.g. grinding, DNA or protein extraction) and analysed (e.g. end-point PCR, ELISA, real-time PCR) when using the group testing approach.

19.2.13 Transgenic

Transgenesis is the process of introducing a foreign genetic construct – called a transgene – into a living organism so that the organism will exhibit a new property and transmit that property to its offspring. The organisms and lines containing transgenes are referred to as transgenic. Cisgenesis occurs by the same process, but using genes from the same species.

19.2.14 Reference material

According to ISO Guide 30, reference material is: "material, sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process". It can also be classified according to its use, for instance "calibrants/calibrators" or "quality control materials".

19.3 General principles

The ISTA strategy regarding methods for the detection, identification and quantification of genetically modified seeds in conventional seed lots is available on the ISTA web site at www.seedtest.org/gmopp.

This chapter describes testing for adventitious presence of GM seeds and GMO trait purity. Currently there is no universal threshold for GM seeds in conventional seed lots, or of regulated GM seed in deregulated GM seed, or a specified level of GMO purity in a seed lot; the establishment of reliable methods for the detection, identification and quantification of GMO content is therefore very important. Different technologies, strategies and methods for GMO testing are continuously evolving and new methods being developed. The quality of these test results depends much more on methodology, equipment and training than in other classical seed testing methods. This makes the standardisation of GMO testing very difficult. The ISTA approach has targeted the uniformity in GMO testing results, not by the uniformity in testing methodology, but by using a performance-based approach (PBA). The PBA requires that laboratories demonstrate that the GMO detection, identification or quantification methods that they are using on seed samples for reporting results on ISTA Certificates meet acceptable standards set by ISTA. These standards include, among others, sampling, testing and reporting. In order for a laboratory to be recognised as ISTA accredited for GMO testing, it will need to ensure that documented evidence of validation and reliability of the laboratory is available to the ISTA auditors. The evidence must include:

– performance data based on seed samples for the event and species for which the laboratory is seeking ISTA accreditation, and
– participation in an ISTA GMO proficiency test including the specific event and species, if available.

This requirement will ensure the reliability of the analysis and the final test result reported on the ISTA Certificate. The PBA gives seed testing laboratories the choice to use different technological approaches, e.g. bioassays, protein-based methods and DNA-based methods.

For further information, see the *ISTA Principles and Conditions for Laboratory Accreditation under the Performance Based Approach* (see www.seedtest.org/accred-docs).

Generally, GMO tests that are used to assess GMO trait purity are identical to the tests used for testing for AP of GM seeds. However, there are differences in the testing steps as well as in the objectives. This chapter addresses these distinctions whenever they apply.

19.4 Procedure

Adventitious presence of GMO testing and GMO trait purity testing are "two sides of the same coin"; both applications make use of the same tests, and follow a very similar work flow (Fig. 1). The expected results differ in the two applications. In GMO AP testing, most of the time the expected outcome is "not detected" or a low estimate of the proportion of GMO present. In GMO trait purity testing, the expected result is the quantification of a high percentage of presence of the specified trait.

The methods used for these analyses can be classified and characterised in a number of ways. According to the level at which the analysis occurs, tests can be conducted at the DNA level (19.5.1), protein level (19.5.2) or organism level, as in bioassays (19.5.3).

The appropriate approach to GMO testing is chosen according to the question which the test is attempting to answer (see Fig. 1). A qualitative question, e.g. "Is there any GM seed in the sample?" can be answered by applying a qualitative test (see 19.5.1.2 and 19.5.2.2), while a quantitative question, e.g. "How much GM seed is there in a seed lot?" can be answered by using either a quantitative test (see 19.5.1.3) or a group-testing approach (Remund *et al.*, 2001), also known as the semi-quantitative method (which relies on qualitative tests of seed groups). Another classification that applies only to DNA-based methods is in relation to the specificity of the method, as described further in section 19.4.1.

Both AP GMO testing and GMO trait purity testing can be performed on individual seeds or on seed bulks, although each application will require a different sampling and testing scheme. Seed bulk testing is more common in AP GMO testing, where the detection target is a transgenic protein or a DNA segment. GMO trait purity tests are usually performed on a representative sample of individual seeds or seedlings, and target the GMO trait or, similarly, the protein or the DNA. However, when performed on seed bulks, the test is performed at the DNA level to detect the absence of transgenic DNA, and targets the uninterrupted insertion site (Battistini and Noli, 2009).

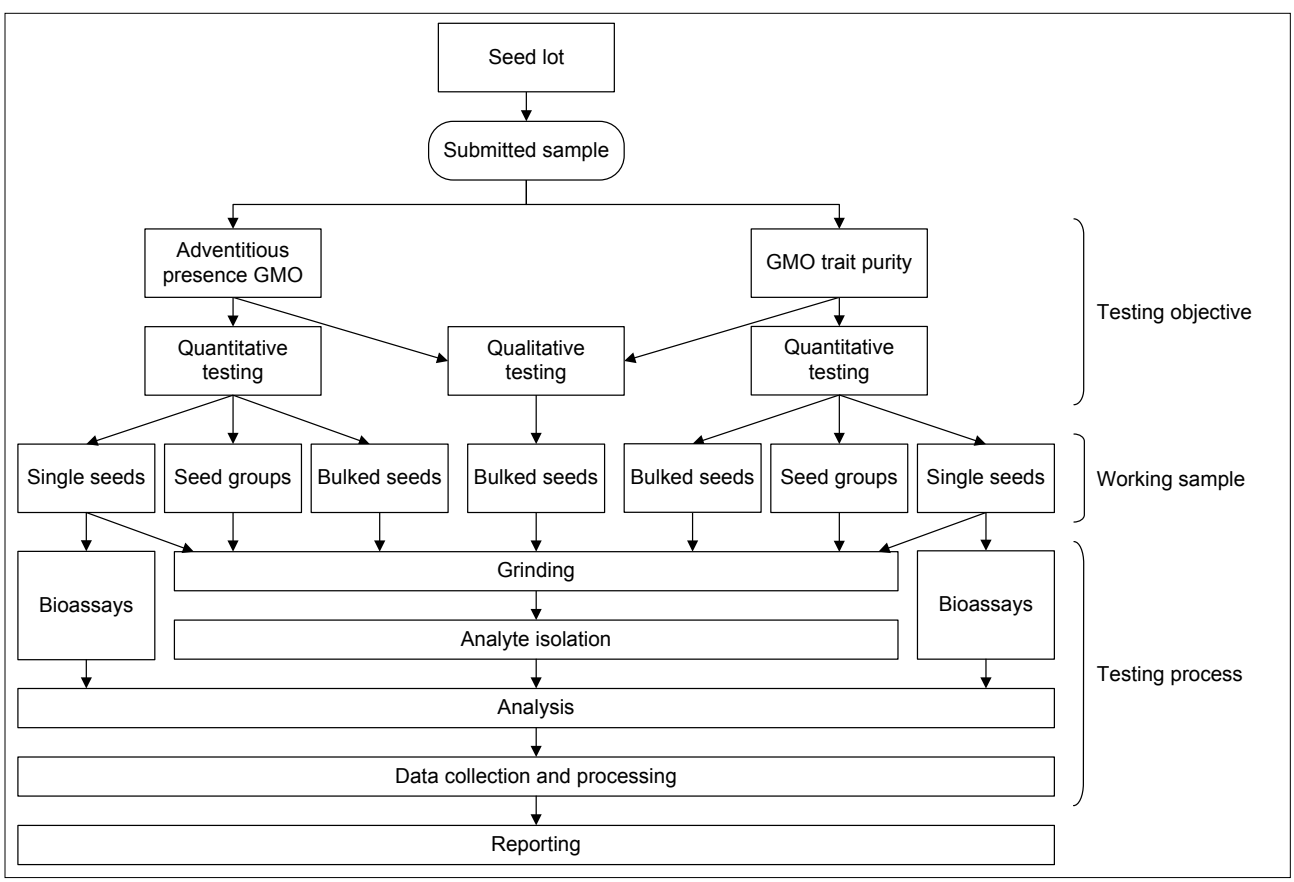


Figure 1. The different approaches to GMO testing and corresponding workflows.

19.4.1 Sample size

Chapter 2: Sampling gives definitions of various sample types, including primary, composite, submitted and working samples, as well as guidelines for obtaining seed lot samples that represent the properties of the seed lot. These definitions and guidelines apply also to GMO testing. The working sample is the portion of the submitted sample that is actually tested by the testing method (as defined in Chapter 2). The size of the working sample depends on given threshold requirements, the method capability and the degree of required statistical confidence, and can be determined using appropriate statistical tools (e.g. SeedCalc (19.6.3)). The sample submitted to the laboratory must therefore be at least the size of the working sample, but more realistically larger than the working sample. For more information regarding sampling, see Chapter 2.

The sizes of seed bulks and groups must be consistent with the performance of the analytical method in terms of limit of detection, in order to allow the detection of even one GM seed. For quantitative methods, if a laboratory aims at quantifying the presence of a single seed in the working sample then the size of the sample must be consistent with the limit of quantification.

19.4.2 Personnel and equipment

Many of the procedures used for GMO testing are composed of several stages (e.g. seed planting or grinding, DNA or protein extraction, detection of the target analyte, and reporting of results) which can be carried out by different personnel in the laboratory (see Figure 1). The laboratory must show that personnel are adequately trained in the procedures that they are carrying out, and that they understand the overall workflow of the procedures and their contribution to that workflow. Each part of the workflow and the equipment must be adequately validated, verified or calibrated before use.

Appropriate equipment and facilities must be provided for the use of the chosen methods. For biomolecular assays (DNA and protein), apparatus for grinding and analyte extraction are necessary, as well as equipment dedicated to the detection of the target analyte.

For DNA-based detection, it is important to prevent contamination, and the use of separate rooms for certain manipulations is preferred.

For protein-based detection, care must be taken to avoid degradation of the matrix and the extracted analyte.

For bioassays, care must be taken to ensure the provision of controlled germination conditions adequate to allow the expression of the trait.

19.4.3 Test conditions

Tests must be carried out under conditions of the ISTA Accreditation Standard quality framework. This includes, but is not limited to the following:

- Analysts involved in this testing must have the documented skills and training in the corresponding procedures.
- All equipment must be appropriate to the techniques used. Scheduled maintenance, verification, and calibration of the instrumentation used must be carried out.
- The spatial arrangements and organisation of the testing area must prevent contamination.
- Reagents of appropriate grade and certified reference materials (when available) must be used.
- Appropriate controls must be used to validate the testing results.

19.5 Testing approaches

19.5.1 DNA-based methods

19.5.1.1 General principles of DNA-based testing

DNA-based testing requires a series of steps which can be carried out by different laboratory personnel and which should all show evidence of validation and being fit for purpose for the testing being carried out. The steps are the following:

- examination of the seed sample;
- grinding of the seed to produce a homogenous matrix;
- subsampling and DNA extraction;
- DNA amplification;
- detection of the amplified DNA.

Because of the amplification step, it is important that the laboratory ensures adequate protection against contamination by seed dust, extracted DNA or amplified DNA for each tested sample. Appropriate control samples (e.g. environmental, blank or negative controls) must be used. If available, it is recommended to use certified reference materials.

In the case of methods using the polymerase chain reaction (PCR), several types of testing can be done that will differ in the level of selectivity and specificity.

- In GMO screening, primers are chosen that amplify individual genetic elements frequently found in a number of different GMO events. The detection of such targets suggests the presence of GMO, but does not represent by itself conclusive evidence.

– In construct-specific PCR, the primers are chosen such that the amplification target spans genetic elements not usually combined in nature, providing a strong indication of the presence of a GMO event that includes that construct.
– In event-specific testing, the primers are designed to detect the unique integration site of a specific transformation event. Thus, a positive result is indicative of the presence of that particular event.

Whatever the type of method selected and its origin, internally developed or publicly available, its performance must be evaluated according to the PBA requirements and following the procedures as directed by the ISTA GMO Committee.

19.5.1.2 End-point qualitative PCR

In end-point PCR, the standard steps of PCR are carried out, with detection of PCR products at the end of the process. This detection step can be the electrophoresis of the amplified DNA molecules on gel or the measurement of fluorescence associated with the PCR reaction. With electrophoresis, the test is scored as positive if a band of the appropriate size is observed on the gel, and negative if no band is observed. With fluorescence detection, the test is scored by comparison to the fluorescence measurement of appropriate positive and negative control samples.

19.5.1.3 Real-time PCR

During real-time PCR, DNA amplification activates fluorochromes attached to the primers or probes. This activation can be measured in real time and can give an estimate of the number of DNA molecules being amplified in each cycle.

DNA amplification can also be measured by activation of intercalating fluorescent dyes. In this case, special attention to false-positive results must be paid, since the activation of intercalating dyes can be associated with amplification of non-specific PCR products.

Real-time PCR can be qualitative or quantitative.

In qualitative real-time PCR tests, the test is scored positive if fluorescence above the defined baseline is detected before a given PCR cycle (usually set by amplification of a known GMO control DNA).

In quantitative real-time PCR tests, the assay is designed to quantify the target against a standard curve produced from reference material. The experimental set-up and reporting of results must follow accepted statistically sound methods such as those suggested in the GMO Handbook.

19.5.1.4 Other technologies

The descriptions in section 19.5.1 apply to technologies (primer and probe sets, methods and equipment used for amplification and detection as well as for quantification) that are widely used in laboratories carrying out GMO testing.

Other methods are currently being developed for use in GMO detection. Use of these methods can also be included in ISTA's PBA as long as the laboratory develops and maintains adequate validation data for the methods used.

19.5.2 Protein-based methods

19.5.2.1 General principles of protein-based testing

In order to detect single proteins in seeds, the seeds need to be ground and extracted with a suitable buffer. The detection of proteins using an immunoassay in a complex mixture such as that obtained by extraction of seed powder requires a number of precautions. The detectable protein content may vary due to the protein itself, the extraction process and buffer and the type of seed used. Particular difficulties are well known (e.g. oil content of oilseed rape, gossypol in cotton seeds, varietal differences, seed maturity, seed moisture) and the laboratory should have validated the extraction and detection methods for each seed matrix by spike and recovery tests (see GMO Method Handbook). Proteins are generally rapidly degraded. The extraction should be carried out at room temperature, or below, and after extraction the mixture should be used quickly or stored at low temperature. When using commercial lateral flow strip tests (19.5.2.2) or ELISA kits (19.5.2.3), it is important to refer to the assay conditions as defined by the test kit suppliers. Moreover, these assay conditions must be internally validated in the laboratory conditions, systematically include positive and negative controls in each test and follow the *ISTA Principles and Conditions for Laboratory Accreditation under the Performance Based Approach.* (see www.seedtest.org/accred-docs)

It is not recommended to use protein-based tests for quantification of GMO, as the variations in sample type (e.g. germplasm, seed maturity) and in extraction and detection methods can result in target protein content variation in the protein extract and cause difficulty in estimating the GMO content. Protein detection is not always event-specific, as several events may contain the same protein (e.g. NK603/MON88017; MON810/Bt11), but the careful use of multiple methods may allow a good indication of which event is being detected.

19.5.2.2 Lateral flow strip test

The lateral flow strip test consists of an immunoassay in which globulins or antibodies are immobilised on a capillary paper. The strip is dipped into the protein extract. The presence of the target protein (the antigen) is represented by the appearance of at least two bands, a negative result only by a control band. The result can be scored only if the control band is visible. A maximum time of reading must be defined to avoid false-positive scoring, due to unspecific staining which can occur after a long reaction time.

19.5.2.3 Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) is a sensitive immunoassay that uses an enzyme linked to an antibody or antigen as a marker for the detection of the specific trait protein through a colorimetric reaction.

19.5.3 Bioassays

19.5.3.1 General principles of bioassays

Bioassays are tests based on visual assessment of phenotypic effects of treatments on seeds or seedlings. The most common use of bioassays is to determine the presence of seed which carries herbicide-resistance traits. In this case the seeds or seedlings are exposed to herbicide, and the expected effect on the plant is lack of normal development when the seeds do not contain the herbicide-resistance trait. All seeds or plants that continue to germinate or grow normally are scored as positive for the GMO trait. The appropriate concentration of herbicide must be determined per crop and growth stage. It is important to consider that bioassays determine the presence of a GMO trait, but cannot determine the presence of any specific event, as in many crops multiple events exist with the same herbicide-resistant phenotype. Therefore, in such cases herbicide bioassays can only be used to screen for the presence of GMO, but cannot detect the presence of a particular event.

19.5.3.2 Scoring of GMO presence

Standardised methods of scoring and analysing the results for the herbicide testing should be in place. This should include statistical considerations of the numbers of seeds used and scored.

The result must take into consideration the germination percentage.

19.6 Calculation and expression of results

19.6.1 Consideration of the testing objective

The applicant must clearly state the specific testing objective, as this is critical in defining the testing approach and in calculating and expressing results. Possible testing objectives include:

– reporting the presence or absence of a GMO in the seed lot;
– estimating the proportion of the GMO present in the seed lot with the associated measurement uncertainty.

The methods described in 19.5 produce either qualitative, i.e., detected (GM trait observed) or not detected (GM trait not observed), or quantitative results. Both types of results can be statistically analysed to meet the testing objective, but the data analysis methods and associated calculation tools differ.

To assess for the presence of two or more stacked events in the same seed, testing individual seed is the appropriate approach. When seed are tested in bulk, the presence of stacked events cannot be demonstrated. However, some statistical tools such as the one proposed by ISTA in SeedCalc Stack9 can estimate the percentage of seeds that could have two or three stacked events.

19.6.2 Units of measurement

The calculation and expression of results depend on the testing objectives, testing methods and the associated units of measurement. The aim or request of the applicant will need to be carefully considered. In order to cope with the different objectives and circumstances where quantification of seeds with GMO traits is required, and in concordance with the PBA, it is acceptable to report quantitative test results using any one of the following units:

a) % in number of seeds: the estimate of the percentage of GM seeds in the seed lot. In addition to individual testing, the percentage in number of seeds is the unit to be used when a group testing approach is chosen; e.g. with SeedCalc (see 19.6.3).

b) % in mass of seeds: the estimate of the percentage of GMO content by mass. This unit should be used when a standard curve is prepared using certified reference material certified by % mass (g/kg).

c) % DNA copies: the estimate of the percentage of GMO content by number of copies. This unit should be used when a standard curve is prepared using certified reference material certified by % DNA copies.

All these three units are acceptable for preparing ISTA Certificates for reporting results by accredited laboratories. The acceptance of more than one unit can avoid raising the difficult question of converting factors. A simple mechanical conversion between units is complex or even impossible.

Whatever the unit used to express results, the resulting GM estimate should be methodologically meaningful, that is, a laboratory using quantitative real-time PCR should not report a value that is lower than its validated limit of quantification.

Moreover, in quantitative real-time PCR, results should be biologically meaningful. The lab should pay attention to results that are lower than 1 divided by the size of the working sample.

19.6.3 ISTA tools for calculation of results

Remund *et al.* (2001) and Laffont *et al.* (2005) provided statistical tools for qualitative and quantitative testing methods which are implemented in the SeedCalc MS Excel workbook (available on the ISTA web site).

19.7 Reporting results

The result of a genetically modified organism test must be reported under 'Other determinations' as follows:

– the request of the applicant;
– the name and scope (with reference to the target) of the method(s) used;
– a description of the working sample (e.g. pure seed fraction, inert matter present, other seeds present, washed seed);
– the number of seeds in the working sample;
– a description and the source of the reference material used (e.g. certified reference material, provider);
– the limit of detection of the method (when testing seed groups or seed bulk);
– the limit of quantification of the method (when testing seed bulk with a quantitative method)

19.7.1 Qualitative test results

Suggested phrases for reporting the detection of test targets depending upon the result are as follows:

a) If the test target(s) was(were) not detected: 'The test target was not detected.'

b) If the test target(s) was (were) detected: 'The test target was detected.'

19.7.2 Quantitative results obtained by multiple qualitative tests of individuals or groups of seeds or seedlings

Results should be reported relative to the percentage of seeds or seedlings showing the test target specified by the applicant. The total number of seeds tested, the number of groups, and the number of seeds per group must be reported. Suggested phrases for reporting such results depending upon the result are as follows:

a) If the test target(s) was (were) not detected: 'The test target(s) was (were) not detected.'

b) If the test target(s) was (were) detected: 'The % of seeds in the lot with the test target(s) was determined to be …%, with a 95 % confidence interval of […%, …%].'
or
'For the test target(s) specified by the applicant, the seed lot meets the specification of ...% (maximum or minimum) with …% confidence.'

If the results do not show evidence that the seed lot meets a given specification with some confidence, then the applicant will report the point estimate with the 95 % confidence interval.

19.7.3 Quantitative measurements of GMO in bulk samples

Results should be reported relative to the percentage of the test target specified by the applicant by mass or number of DNA copies. The testing plan (e.g. number of replicate seed samples, number of replicate flour samples per seed sample, number of extracts per flour sample, number of replicate measurements per extract) must be indicated.

Required phrases for reporting depending upon the results are as follows:

a) If the test target was not detected (no signal or below the limit of detection): 'The test target was not detected at a level above the limit of detection.'

b) If the test target was detected at a level above the limit of detection and below the limit of quantification: 'The test target was detected at a level below the limit of quantification of the method used.'

c) If seeds showing the test target were found at a level above the limit of quantification: 'The test target(s) percentage in the seed lot was determined to be …% by mass or number of copies, with a 95 % confidence interval of […%, …%]'
or
'For the test target(s) specified by the applicant, the seed lot meets the specification of ...% (maximum or minimum) by mass or number of copies with …% confidence.'

If the results do not show evidence that the seed lot meets a given specification with some confidence, then the applicant will report the point estimate with the 95 % confidence interval.

19.8 References

Battistini E. & Noli E. (2009). Real-time quantification of wild-type contaminants in glyphosate tolerant soybean. *BMC Biotechnology* **9**, 16.

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Remund, K.M., Dixon D.A., Wright D.L. & Holden L.R. (2001). Statistical considerations in seed purity testing for transgenic traits. *Seed Science Research* **11**, 101–119.

SeedCalc: http://www.seedtest.org/stats-tools (last verified 2014-11-10)