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Document OGM22-05

Rules Proposals for the International Rules for Seed Testing 2023 Edition

This document was prepared by the Technical Committees (TCOMs) and the Rules Committee of the Association and has been endorsed by the ISTA Executive Committee (ECOM). The proposals are submitted to the ISTA Ordinary General Meeting 2021 for voting by the nominated ISTA Designated Members on behalf of their respective Governments.

It is submitted to all ISTA Designated Authorities, ISTA Members and ISTA Observer Organizations for information two months prior to the ISTA Ordinary General Meeting 2022.

It contains proposed amendments and changes for the ISTA *International Rules for Seed Testing* and will be discussed and voted on at the Ordinary General Meeting 2022 to be held on 11 May, 2022, in Cairo, Egypt.

Introduction to the ISTA Rules Proposals to become effective 1 January 2023

The current version of the ISTA International Rules for Seed Testing (ISTA Rules) is the 2022 edition.

The ISTA Rules are only available electronically as a printable pdf file and are available for free download by ISTA members from the Ingenta website: http://www.ingentaconnect.com/content/ista/rules

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

For further information on the ISTA Rules, see: http://www.seedtest.org/rules

The effective dates are changed annually. The changes from the previous edition of the ISTA Rules can be displayed as yellow highlighted text as a 'layer' within the electronic copy with comments on what has changed. Previous Prefaces as a 'history of changes' are available on the ISTA website.

The ISTA Rules are the result of the work of the ISTA Technical Committees (TCOMs) with input from many different sources. Thanks go to all the Technical Committee members and the ISTA Secretariat for their help with the annual proposals.

The following Rules Proposals will be discussed at the ISTA Ordinary General Meeting in Cairo, Egypt on 11 May, 2022, and may be amended without changing the intent of the proposal. If the proposals are accepted by the membership, amendments will be issued, and they will become the 2023 edition of the ISTA Rules.

Please let us know about any problems with these proposals.

Many thanks.

Ernest Allen and Sue Alvarez

Chair and Vice-Chair of ISTA Rules Committee

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Key to text changes:

~~Deleted text~~

New text

New text in large blocks, not underlined for ease of reading

Any changes made after the proposals were published to the membership

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PART A. INTRODUCTION OF EDITORIAL CHANGES

A.1. Editorial corrections

This proposal clarifies the reporting authorities when determining the scientific names for pure and other seeds reported on ISTA certificates. The proposal also harmonizes the reporting between 3.7 and 4.7. The proposal is a response to an ISTA member's inquiry.

The following proposal has been developed by PUR Committee and approved by a unanimous vote of the Committee.

CURRENT VERSION	PROPOSED VERSION
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 2C (e.g. *Triticum aestivum* subsp. *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. *Elymus repens*).~~	**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 2C (e.g. *Triticum aestivum* subsp. *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 scientific name of every species of other seeds found. The scientific name used must be in accordance with the current ISTA List of Stabilised Plant Names, when applicable (e.g., *Elymus repens*). • 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.
4.7 Reporting results The result of a determination of other seeds by number must be reported under 'Other determinations' as follows: • ……. • The scientific name in accordance, where applicable, with the current ISTA List of Stabilised Plant Names (e.g. *Elymus repens*), and number of seeds of ~~each~~	**4.7 Reporting results** The result of a determination of other seeds by number must be reported under 'Other determinations' as follows: • ……. • The scientific name in accordance, where applicable, with the current ISTA List of Stabilised Plant Names (e.g. *Elymus*

~~species~~ found in this weight. If no other seeds are found, this must be indicated on the certificate.	*repens*), and number of seeds of ~~every~~ all the other species found in this weight. If no other seeds are found, this must be indicated on the certificate.

Editorial correction required for Table 8I if proposals for new methods in chapter 8 are approved during the OGM in 2022.

CURRENT VERSION	PROPOSED VERSION
Table 8~~I~~. Optimum number of plants per metre of row	**Table 8R.** Optimum number of plants per metre of row

Editorial correction required for 9.2.3.6. The words "are out" were not included in the last update of this section as intended. This edit corrects that error and improves sentence clarity.

CURRENT VERSION	PROPOSED VERSION
9.3.2.6 Tolerances …. 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…	**9.3.2.6 Tolerances** …. If the replicates of the second measurement are out 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…

Elytrigia spp. was not updated after approval of the latest version of the ISTA Stabilised list. This correction updates *Elytrigia spp.* to *Elymus spp.*

Current: Table 9A Part 1. Details of methods for moisture determination: agricultural and vegetable …

Species	Grinding/cutting (9.2.5.4, 9.2.5.5)	Method to be used	Drying at high temperature (h)	Predrying …
1	2	3	4	5
~~*Elytrigia spp.*~~	No	High	1	--

Proposed: Table 9A Part 1. Details of methods for moisture determination: agricultural and vegetable …

Species	Grinding/cutting (9.2.5.4, 9.2.5.5)	Method to be used	Drying at high temperature (h)	Predrying …
1	2	3	4	5
Elymus spp.	No	High	1	--

Editorial corrections made throughout Chapter 19 including renumbering of paragraphs, punctuation, and word substitutions that do not modify the intent.

CURRENT VERSION	PROPOSED VERSION
~~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	**19.2.1 Genetically modified organism** A genetically modified organism (GMO) is a living organism that possesses a novel combination of genetic material obtained through the use of modern biotechnology
~~19.2.13~~ **Transgenic** Transgenesis is the process of introducing a foreign genetic construct – called a transgene – into a living organism…	**19.2.2 Transgenic** Transgenesis is the process of introducing a foreign genetic construct – called a transgene – into a living organism…
~~19.2.5~~ **GMO event** A GMO event is a single transformation act that results in the integration of transgenic or cisgenic material…	**19.2.3 GMO event** A GMO event is a single transformation act that results in the integration of transgenic or cisgenic material…
~~19.2.6~~ **GMO trait** A GMO trait is a novel phenotypic character, added by genetic engineering to…	**19.2.4 GMO trait** A GMO trait is a novel phenotypic character, added by genetic engineering to…
~~19.2.1~~ **Adventitious presence** Adventitious presence (AP) in seeds refers to the unintentional and incidental presence of genetically modified material in a seed lot. …	**19.2.5 Adventitious presence** Adventitious presence (AP) in seeds refers to the unintentional and incidental presence of genetically modified material in a seed lot. …
~~19.2.7~~ **Limit of detection** The limit of detection is the smallest	**19.2.10 Limit of detection** The limit of detection is the smallest

amount of target analyte that has been…	amount of target analyte that has been…
~~19.2.8~~ **Limit of quantification**	**19.2.12 Limit of quantification**
The limit of quantification is the smallest amount of target analyte that has been…	The limit of quantification is the smallest amount of target analyte that has been…
~~19.2.14~~ **Reference material**	**19.2.18 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”.	Reference material is: “a 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” (ISO Guide 30). It can also be classified according to its use, for instance, “calibrants/calibrators” or “quality control materials”.
~~19.2.3~~ **Certified reference material**	**19.2.19 Certified reference material**
Certified reference material is reference material which has been…	Certified reference material is reference material which has been…
19.5.1.1 General principles of DNA-based testing	**19.5.1.1 General principles of DNA-based testing**
… 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 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 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 GMO events that include that construct. – In event-specific testing, the primers are designed to detect the unique integration site of a specific transformation event.	– 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 GMO events that include 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.	Thus, a positive result is indicative of the presence of that particular event

A new reference was added.

Link for SeedCalc download on ISTA website updated.

CURRENT VERSION	PROPOSED VERSION
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). **19.8 References** **…** 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~~)	**19.6.3 ISTA tools for calculation of results** Remund *et al.* (2001, 2020) 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). **19.8 References** **…** 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. Remund, K.M., Noli, E., Bates, E., Perri, E., Haldemann, C., Mathis, R. and J-L. Laffont (2020). Designing GMO testing plans and analyzing associated results. Seed Testing International, 160. SeedCalc: https://www.seedtest.org/en/statistical-tools-for-seed-testing-_content---1--3449.html (last verified 2021-11-20).

ACCEPTED BY VOTE	RESULT
26	PASS

Part B. New species and changes to species names

B.1.1 Addition of new species to Table 2C

None.

B.1.2 Changes to the ISTA Stabilised List

None this year.

Process Update: To ensure harmonization between the Stabilised List and Chapters within the rules, any species added to Chapter tables will be added to the Stabilised List concurrently.

The next revision of the ISTA Stabilised List will be discussed at the 2025 ISTA Congress and voted on during the 2025 OGM.

Part C. Rules changes and new methods requiring a vote

Chapter 1: ISTA Certificates

C.1.1 Adding eCertificates to the ISTA Rules

ISTA will begin offering optional electronic seed analysis certificates in 2023. The electronic certificates will be available for use by accredited member laboratories of ISTA. The addition of electronic certificates will require edits to Chapter 1.

The following proposal was developed by the ISTA Rules Chair with input from the Secretariat and the ECOM.

CURRENT VERSION	PROPOSED VERSION

Chapter 1: ISTA Certificates	**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.	**1.1 Object** The object is to prescribe rules for the issue of a) electronic (eCertificates) and b) paper 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 Definitions** Blank paper ISTA Certificates and access to blank electronic ISTA Certificates for seed analysis is controlled by ISTA. Both blank paper, and access to blank electronic certificates are only provided to accredited laboratories (see 1.2.6) for reporting the results of tests. The completed certificates are the property of ISTA and may only be issued under the authority of ISTA. …………….
1.2.4 Duplicate certificate A duplicate certificate is an exact printed copy, not a photocopy, of a completed original ISTA Certificate, marked DUPLICATE.	**1.2.4 Duplicate certificate** A duplicate paper certificate is an exact printed copy, not a photocopy, of a completed original ISTA Certificate, marked DUPLICATE. ISTA eCertificates may be viewed with appropriate access credentials. Therefore, there are no duplicate certificates with eCertificates, only originals.
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. …………….	**1.3 Conditions for issuance of ISTA Certificates** ISTA Certificates must be issued only on paper forms obtained from the ISTA Secretariat or generated as electronic Certificates through the ISTA website using valid access credentials obtained from the ISTA Secretariat. Both types of ISTA Certificates are 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. …………….
1.4 Completing ISTA Certificates	**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: …………… **1.4.2 Orange International Seed Lot Certificate** It is stated on the ~~back of the~~ Orange International Seed Lot Certificate: …………… **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: …………… **1.6 Validity of certificates** …….. Previously issued certificates do not need to be returned to the issuing laboratory.	**1.4.1 General** ISTA Paper 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. ISTA eCertificates may be completed in any language. Blank ISTA eCertificates must not be digitally stored. A completed certificate must show the following information: …………… **1.4.2 Orange International Seed Lot Certificate** It is stated on the Orange International Seed Lot Certificate: …………… **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 Blue International Seed Sample Certificate: …………… **1.6 Validity of certificates** …….. Previously issued paper certificates do not need to be returned to the issuing laboratory.

VOTE TO ACCEPT ITEM	YES VOTES	NO VOTES	RESULT
C.1.1	**27**		**PASS**

C.1.2 Removing the restriction to only issue dust-like species test results on a BIC

This proposal removes the restriction that the other seed determination test for dust-like species (see ISTA Rules 4.5.3) can only be issued on a Blue International Seed Sample Certificate (BIC).

The restriction was based on previous discussions between BSC and PUR technical committees on whether a modified ISTA sampling methodology was needed when sampling for dust like species in seed lots. More recent discussions have concluded that the sampling methodology does not need to be modified therefore testing results for dust like species can be reported on either the Orange International Seed Lot Certificate or the Blue International Seed Sample Certificate.

The following proposal was developed and approved by the ECOM in consultation with the BSC and PUR TCOMs.

CURRENT VERSION	PROPOSED VERSION
1.5.2.4 Determination of other seeds by number ………… Upon request, the presence of one or more of the following genera: *Aeginetia*, *Alectra*, *Orobanche*, *Phelipanche* and *Striga*~~, can~~ be reported ~~only on a Blue International Seed Sample Certificate (see 1.2.2)~~ and must be reported as: 'Test for presence of ... species: … seeds of ... species were found in … g of seed examined.' **Concurrent change in 4.7 required** **4.7 Reporting results** ………….. Upon request, the presence of one or more of the following genera: *Aeginetia, Alectra, Orobanche, Phelipanche* and *Striga*, can be reported ~~only on a Blue International Seed Sample Certificate (see 1.2.2) and~~ must be reported as: 'Test for presence of ... species: … seeds of ... species were found in … g of seed examined.'	**1.5.2.4 Determination of other seeds by number** ………… Upon request, the presence of one or more of the following genera: *Aeginetia*, *Alectra*, *Orobanche*, *Phelipanche* and *Striga* can be reported. These genera must be reported as: 'Test for presence of ... species: … seeds of ... species were found in … g of seed examined.' **Concurrent change in 4.7 required** **4.7 Reporting results** ………….. Upon request, the presence of one or more of the following genera: *Aeginetia, Alectra, Orobanche, Phelipanche,* and *Striga*, can be reported. These genera must be reported as: 'Test for presence of ... species: … seeds of ... species were found in … g of seed examined.'

VOTE TO ACCEPT ITEM	YES VOTES	NO VOTES	RESULT
C.1.2			**WITHDRAWN**

Chapter 3: The purity analysis

C.3.1 Additional guidance on reporting results

The proposal is to indicate how other seeds and inert matter found in the second whole sample test shall be reported. This proposal is to provide clear guidance to the second whole sample test.

The following proposal was developed and approved by the PUR Committee.

CURRENT VERSION	PROPOSED VERSION
3.7 Reporting results The results of a purity test must be reported in the spaces provided as follows: • …… • 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. *Elymus 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 2C, column 4 (Purity analysis), no statement regarding the weight of the working sample is required on the ISTA Certificate.	**3.7 Reporting results** The results of a purity test must be reported in the spaces provided as follows: • …… • 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, (e.g. *Elymus repens*). • When testing the second whole sample (3.6.3), the name of every species of other seeds and the kind of inert matter found in the tests that are averaged according to 3.6.3.2 must be reported. • When the weight of the working sample tested for purity equals or is no more than 10 % higher than the weight specified in Table 2C, column 4 (Purity analysis), no statement regarding the weight of the working sample is required on the ISTA Certificate.

VOTE TO ACCEPT ITEM	YES VOTES	NO VOTES	RESULT
C.3.1	**27**		**PASS**

Chapter 5: The germination test

C.5.1 Revised germination method for *Anethum graveolens*

An ISTA Peer Method Validation Study was conducted to approve the addition of a new temperature regime (20°C) for the germination of *Anethum graveolens*. The temperatures 20°C, 20<=>30°C, and 10<=>30°C were compared, combined with Top of Paper (TP), and Between Paper (BP). Statistical analyses showed that the germination tests performed at 20°C gave results with good repeatability. In addition, reproducibility was equal to or better than existing methods for this species in the ISTA Rules.

This proposal is supported by a ISTA peer validation study.

The following proposal was developed and approved by the ISTA GER Committee.

CURRENT: Table 5A Parts 1 and 2. Detailed methods for germination tests

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
Anethum graveolens	TP; BP	20<=>30; 10<=>30	7	21	Prechill	-	-

PROPOSED: Table 5A Parts 1 and 2. Detailed methods for germination tests

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
Anethum graveolens	TP; BP	20<=>30; 10<=>30 20	7	21	Prechill	-	-

VOTE TO ACCEPT ITEM	YES VOTES	NO VOTES	RESULT
C.5.1	**25**	**2**	**PASS**

C.5.2. Additional guidance on conducting a tetrazolium test at the end of a germination test

This proposal gives clarification that species listed in Table 5A can have a tetrazolium test conducted to determine the viability of fresh ungerminated seeds, at the germination test. This is different to using Table 6A to carry out a Tetrazolium Test and report this on an Orange International Seed Lot Certificate.

The following proposal was developed by the ISTA GER Committee and approved by the Germination and TZ Committees.

CURRENT VERSION	PROPOSED VERSION
5.6.5.3 Ungerminated seeds ... **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...	**5.6.5.3 Ungerminated seeds** ... **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. Tetrazolium may be used to determine the germination potential of all species listed in Table 5A Parts 1, 2, and 3, regardless of whether these species are listed in Table 6A Part 1 or 2. Those determined to have the potential to germinate...

VOTE TO ACCEPT ITEM	YES VOTES	NO VOTES	RESULT
C.5.2	**27**		**PASS**

C.5.3. Addition of information on prechilling temperatures

This proposal gives clarification that during prechilling, the temperature prescribed for prechilling, should be measured from on or in the substrate.

The following proposal has been developed and approved by the ISTA GER Committee.

CURRENT VERSION	PROPOSED VERSION
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.	**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. The temperatures prescribed for prechilling are those to which the seed is exposed on or inside the substrate. They should be as uniform as possible throughout

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. **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.	the prechilling apparatus or room. 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. **5.6.2.3 Temperature** The temperatures prescribed in Table 5A for the germination, or for the prechilling period, 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.

VOTE TO ACCEPT ITEM	YES VOTES	NO VOTES	RESULT
C.5.3	**27**		**PASS**

C.5.4. Addition of Seedling Evaluation Group onto Table 5A

This proposal is to add the Seedling Evaluation Groups to Table 5A (Parts 1, 2 and 3). This should be beneficial to seed analysts and ensure that the correct seedling evaluation group is used.

This is helpful when a species has had a change in the rules (e.g. *Helianthus annuus*) but the ISTA Handbook on Seedling Evaluation has not been updated.

See Appendix for Changes to Tables 5A Parts 1, 2, and 3.

The following proposal was developed and approved by the ISTA GER Committee.

CURRENT VERSION	PROPOSED VERSION
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 2C may be considered to be covered…….	**5.10 Germination methods** Table 5A indicates the prescribed substrates, temperatures and test durations, recommended procedures for breaking dormancy, additional directions, additional advice, and Seedling Evaluation Group (further guidance is available in the ISTA Handbook on Seedling Evaluation). Where methods are prescribed for a group of

<table>
<tr>
<td>……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 H2SO4 is used in combination with any other method, they must be used prior to the other methods…………</td>
<td>species, only those species specifically listed in Table 2C may be considered to be covered.

………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 H2SO4 is used in combination with any other method, they must be used prior to the other methods…………

Seedling Evaluation Groups: The Seedling Evaluation Groups can be found in the ISTA Handbook on Seedling Evaluation. Genera have been classified into two categories:

• Agricultural and horticultural species, represented by the letter A; and
• Trees and shrubs, represented by the letter B

Categories A and B are then sub-divided into groups according to the following criteria:

<table>
<tr><td>• systemic class</td><td>1</td><td>monocotyledons</td></tr>
<tr><td></td><td>2</td><td>dicotyledons</td></tr>
<tr><td></td><td>3</td><td>conifers</td></tr>
<tr><td>• germination mode</td><td>1</td><td>epigeal germination</td></tr>
<tr><td></td><td>2</td><td>hypogeal germination</td></tr>
<tr><td>• shoot development</td><td>1</td><td>without epicotyl elongation</td></tr>
<tr><td></td><td>2</td><td>with epicotyl elongation</td></tr>
<tr><td></td><td>3</td><td>no shoot elongation; shoot apex enclosed within a sheath (coleoptile)</td></tr>
<tr><td></td><td>4</td><td>tuberous hypocotyl</td></tr>
<tr><td>• root system and its significance for evaluation</td><td>1</td><td>primary root essential</td></tr>
<tr><td></td><td>2</td><td>secondary roots may compensate for the primary root</td></tr>
<tr><td></td><td>3</td><td>several equal seminal roots</td></tr>
</table>
</td>
</tr>
</table>

	The letters and numbers are combined to form the group number. Thus, for example, Group A-2-1-2-2 comprises seedlings: • **A**-2-1-2-2 of agricultural or horticultural plants • A-**2**-1-2-2 belonging to the dicotyledons • A-2-**1**-2-2 with epigeal germination • A-2-1-**2**-2 with epicotyl elongation • A-2-1-2-**2** with secondary roots that are taken into account if the primary root is defective In this way any genus can be assigned to the group that covers its systematic and morphological characteristics.

CURRENT: Table 5A Parts 1. Detailed methods for germination tests

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	–	–

PROPOSED: Table 5A Part 1. Detailed methods for germination tests

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	Seedling Evaluation Group
1	2	3	4	5	6	7	8	9
Abelmoschus esculentus	TP; BP; S	20<=>30	4	21	–	–	–	A-2-1-1-2
Achillea millefolium	TP	20<=>30	5	14	–	–	–	A-2-1-1-1
Aeschynomene americana	TP	20<=>35; 20<=>30	4	14	–	–	–	A-2-1-1-1
Agropyron cristatum	TP	20<=>30; 15<=>25	5	14	KNO_3; prechill	–	–	A-1-2-3-1
Agropyron desertorum ……	TP	20<=>30; 15<=>25	5	14	KNO_3; prechill	–	–	A-1-2-3-1

CURRENT: Table 5A Part 2. Detailed methods for germination tests

Table 5A Part 2. Detailed methods for germination tests: tree and shrub seeds

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,	TP	20<=>30	7	28	–	Double test: no prechill and	–

Abies concolor, *Abies grandis,* *Abies nordmanniana,* *Abies pinsapo,* *Abies procera,* *Abies veitchii*						prechill 21 d	

PROPOSED: Table 5A Part 2. Detailed methods for germination tests

Table 5A Part 2. Detailed methods for germination tests: tree and shrub seeds

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendation for breaking dormancy	Additional directions	Additional advice	Seedling Evaluation Group
1	2	3	4	5	6	7	8	9
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	–	–	B-3-1-1-1
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	–	B-3-1-1-1
…….								

CURRENT: Table 5A Part 3. Detailed methods for germination tests

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

PROPOSED: Table 5A Part 3. Detailed methods for germination tests

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	Seedling Evaluation Group
1	2	3	4	5	6	7
Abutilon ×hybridum	TP; BP	20<=>30; 20	5–7	21	–	A-2-1-1-2
Achillea clavennae	TP; BP	20<=>30; 20	5	14	Light	A-2-1-1-1
Achillea filipendulina	TP; BP	20<=>30; 20	5	14	Light	A-2-1-1-1
Achillea ptarmica	TP; BP	20<=>30; 20	5	14	Light	A-2-1-1-1
Achillea umbellata	TP; BP	20<=>30; 20	5	14	Light	A-2-1-1-1
Adonis vernalis	TP; BP	15; 10	7–14	35	KNO_3; prechill	A-2-1-1-1
Ageratum houstonianum	TP	20<=>30; 20	3–5	14	–	A-2-1-1-1
Agrimonia eupatoria	TP	20<=>30	7–14	60	Soak in water for 24 h; chip or file off fragment of testa	A-2-1-1-1
Alcea rosea	TP; BP	20<=>30; 20	4–7	21	Pierce seed; or chip or file off fragment of testa at cotyledon end	A-2-1-1-2
………..						

VOTE TO ACCEPT ITEM	YES VOTES	NO VOTES	RESULT
C.5.4	27		PASS

Chapter 7: Seed health testing

C.7.1 Addition of an optional filtration method for detection of *Ditylenchus dipsaci* in *Medicago sativa*; *D. dipsaci* and *D. gigas* in *Vicia faba* seed

Proposal to give the option of a sieve with filter paper or an equivalent nematode-permeable container for methods in 7-031. Specifically, the proposal recommends the optional use of a non-woven plant growth bag for filtration to detect *Ditylenchus dipsaci* in *Medicago sativa* (alfalfa) and the detection of *D. dipsaci* and *D. gigas* in *Vicia faba* (faba bean) seed. A supplementary MVR study shows that this, single use, nematode permeable container is equivalent to the current method and allows for decreased risks of cross contamination.

This proposal is supported by a validation study.

The following proposal was developed and approved by the ISTA SHC Committee.

CURRENT VERSION	PROPOSED VERSION
Materials **….** Sieves: 250 µm sieve cover with soft filter paper and 20 µm mesh sieve **Methods** 1. Sieving method 1.1 Place a 250 µm mesh sieve in a plastic basin. ….. 1.6 Rinse the 250 µm sieve and the basin with tap water through the 20 µm mesh sieve in order to collect all nematodes. **Critical control points (CCP)** …... The 250 µm and 20 µm sieves used in the filtration protocol must be rinsed thoroughly to ensure all nematodes present are collected.	**Materials** **….** Sieves: 250 µm sieve cover with soft filter paper or equivalent nematode-permeable container (i.e. non-woven plant growth bag) and 20 µm mesh sieve **Methods** 1. Sieving method 1.1 Place a 250 µm mesh sieve or equivalent nematode-permeable container (i.e., non-woven plant growth bag) in a plastic basin. ….. 1.6 Rinse the 250 µm sieve or equivalent nematode-permeable container (i.e., non-woven plant growth bag) and the basin with tap water through the 20 µm mesh sieve in order to collect all nematodes. **Critical control points (CCP)** …... The 250 µm sieve or equivalent nematode-permeable container (i.e., non-woven plant growth bag) and 20 µm sieves used in the filtration protocol must be rinsed thoroughly to ensure all nematodes present are collected. ………

VOTE TO ACCEPT ITEM	YES VOTES	NO VOTES	RESULT
C.7.1	26		PASS

Chapter 8: Species and variety testing

C.8.1 New method on *Pisum*

Identification and verification of varieties or testing genetic purity by means of DNA-based techniques are being used extensively in many laboratories nowadays. In 2017 the first DNA-based method was included in the Rules for testing wheat. The interest for including such a technical procedure has increased over time. The Variety Committee propose the inclusion of a DNA-based test for testing *Pisum* varieties.

This proposal is supported by a validation study.

The following proposal was developed by a working group within the ISTA VAR Committee and approved by the Committee.

PROPOSED VERSION

8.10.4 *Pisum* (peas)

The standard reference DNA-based method for verifying varieties of *Pisum* is by analysis of a minimum set of eleven 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.

8.10.4.1 Microsatellite markers

Table 8I contains prescribed microsatellite markers required for reports and issuance of ISTA Certificates.

The 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. 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.

Notice that "Null" alleles (no PCR amplification) are known to occur in peas. “Null” is called when repeated attempts to amplify a specific marker fail to produce a detected allele for specimens of the same variety, while the other markers work well. It is advisable to confirm the absence of amplification for those particular markers to rule out chemical and enzyme malfunction. For markers that may display “Null” alleles, see references in Table 8.I.

Table 8.I. List of prescribed microsatellite markers and PCR primers for verification of pea varieties.

SSR	Forward 5' to 3'	Reverse 5' to 3'	Size range of PCR product in base pairs
A9	GTGCAGAAGCATTTGTTCAGAT	CCCACATATATTTGGTTGGTCA	340-420
AA67	CCCATGTGAAATTCTCTTGAAGA	GCATTTCACTTGATGAAATTTCG	370-425 *
AA135	CCGTTACACATCATTAAGATG	TCCATATCCAGATTAGTCAGA	360-370 *
AA205	TACGCAATCATAGAGTTTGGAA	AATCAAGTCAATGAAACAAGCA	220-250 *
AA285	TCGCCTAATCTAGATGAGAATA	CTTAACATTTTAGGTCTTGGAG	230-260
AA355	AGAAAAATTCTAGCATGATACTG	GGAAATATAACCTCAATAACACA	180-250
AB72	ATCTCATGTTCAACTTGCAACCTTTA	TTCAAAACACGCAAGTTTTCTGA	250-290
AD59	TTGGAGAATGTCTTCTCTTTAG	GTATATTTTCACTCAGAGGCAC	310-340 *
AD73	CAGCTGGATTCAATCATTGGTG	ATGAGTAATCCGACGATGCCTT	220-280
AD270	CTCATCTGATGCGTTGGATTAG	AGGTTGGATTTGTTGTTTGTTG	250-320 *
D23	ATGGTTGTCCCAGGATAGATAA	GAAAACATTGGAGAGTGGAGTA	170-200

* Marker that may display “Null” alleles.

8.10.4.2 Recommended DNA extraction protocol

DNA can be extracted using column-based kits; procedures such as those based on CTAB are also adequate for extraction of DNA from pea seeds. For any DNA extraction procedure chosen, it is recommended to use ½ seed as starting material. For each ½ seed to be extracted, it is recommended to use a single grinding bead (such as a Qiagen 5 mm stainless steel bead) placed in a 2 mL round-bottom microcentrifuge tube. ½ seeds can be crushed with needle-nose pliers as they are placed into the tubes. The seeds are then pulverized for 1 min at 28 Hz in a mixer mill (such as a Qiagen TissueLyser II). A rotation in the unit is performed and the seeds are pulverized a second time for 1 min at 28 Hz. Following DNA extraction, DNA quality and quantity can be verified by means of a 1 % agarose gel 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.

8.10.4.3 Recommended PCR procedures

The microsatellite markers set was selected based on performance in two comparative tests. Each marker can be run individually or multiplexed. If multiplexed, it is recommended to use PCR master mixes that were specially designed for multiplexing. However, it may be still required to optimize the primer concentration within a multiplex. Table 8.J. shows recommended primer concentrations for each marker.

Table 8.J. Recommended final primer concentration for pea markers.

SSR	Final primer concentration in PCR mix
A9	[3.75 µM]
AA67	[1.25 µM]
AA135	[3.0 µM]
AA205	[1.25 µM]
AA285	[2.5 µM]
AA355	[3.75 µM]
AB72	[2.0 µM]
AD59	[5.0 µM]
AD73	[2.5 µM]
AD270	[1.25 µM]
D23	[3.0 µM]

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 hybridizes 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.

8.10.4.3.1 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. Table 8.K. contains a list of reagents for a single 10 µL reaction with multiplexed primers.

Table 8.K. Recommended reaction composition for PCR amplification of microsatellite markers for verification of pea varieties.

Amount per reaction (µL)	**Component**	**Final concentration a**
5	2X PCR Master Mix (for multiplexing)	1X
1	10X Pea Primer Mix[a]	1X
3	Ultrapure H20	

[a] Suggested final concentrations for all markers are listed in Table 8.J. Concentrations in multiplexed reactions may require adjustment depending upon relative product intensities observed. 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 or two extra to accommodate for 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 (approximately 10 ng/µL) of template DNA is added to each reaction tube, resulting in a final reaction volume of 10 µL.

8.10.4.3.2 Thermal cycling profile

The thermal cycling profile presented in Table 8.L has been used successfully with the prescribed markers in comparative tests, thus it is recommended for verification of pea varieties. The total number of cycles may require alteration based on product intensities achieved.

Table 8.L. Recommended thermal cycling profile for PCR amplification of microsatellite markers for verification of pea varieties.

Number of cycles	Temperature	Duration
1 cycle	95°C	5' (Initial denaturation conditions according to manufacturer of Taq polymerase used)
28 cycles	95°C	30s
	60°C	90s
	72°C	30s
1 cycle	60°C	30min
1 cycle	4°C	forever

8.10.4.4 Evaluation of results

Alleles are called according to their sizes in base pairs. However, since every detection system and chemical used differ from one lab to another, this may alter the migration of the PCR products. To minimize this variation, it is recommended to use this method 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.

References

Loridon K., McPhee K., Morin J., Dubreuil P., Pilet-Nayel M. L., Aubert G., Rameau C., Baranger A., Coyne C., Lejene-Hénaut I. and Burstin J. (2005) Microsatellite marker polymorphism and mapping in pea (*Pisum sativum* L.). Theor Appl Genet 111: 1022–1031.

VOTE TO ACCEPT ITEM	YES VOTES	NO VOTES	RESULT
C.8.1	**26**		**PASS**

C.8.2 New method on *Avena sativa*

Identification and verification of varieties or testing genetic purity by means of DNA-based techniques are being used extensively in many laboratories nowadays. In 2017 the first DNA-based method was included in the Rules for testing wheat. The interest for including such a technical procedure has increased over time. The Variety Committee propose the inclusion of a DNA-based test for testing *Avena* varieties.

Method supported by a validation study.

The following proposal has been developed and approved by the ISTA VAR Committee.

PROPOSED VERSION

8.10.5 *Avena sativa* (oats)

The standard reference DNA-based method for verifying varieties of *Avena sativa* is by analysis of a minimum set of nine 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.

8.10.5.1 Microsatellite markers

Table 8.M. contains prescribed microsatellite markers required for reports and issuance of ISTA Certificates.

The 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. 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.

Occasionally NULL alleles have been observed for oats markers. An allele is called NULL when repeated attempts to amplify a specific marker fail to produce a detected allele for samples of the same variety, while the other markers work well. It is advisable to confirm the absence of amplification for those particular markers to rule out chemical and enzyme malfunction.

Table 8.M. List of prescribed microsatellite markers and PCR primers for verification of oats varieties.

SSR	Forward 5' to 3'	Reverse 5' to 3'	Size range of PCR product in base pairs
AM031	GCAAAGGCCATATGGTGAGAA	CATAGGTTTGCCATTCGTGGT	145-195
AM102	TGGTCAGCAAGCATCACAAT	TGTGCATGCATCTGTGCTTA	206-220
AM112	AGCGGTGTAGGGGAAAGAGT	TTCTTGGTTTAGATGGAGGA	233-266
MAMA-1	GTGCGCCTCTAACGAAAAAT	CATGCTGGCGAAATCTATCA	162-226
MAMA-3	ATGTTCTCCAATGGGACTGC	ATCGCGATGACTGTGTGA	364-402
MAMA-4	GGAGTGGGCGTTTGACATTA	CAGCTACCGGTTTTCATTCC	290-366
MAMA-5	GGATTGGGACTTCGCATCTA	AACCCTAATTACTGCTCCGTTTC	156-254
MAMA-6	GACTAAATCACACAACCCAACC	GCAGAATCGCGGGAAAGA	167-223
MAMA-7	ATAAATACGCGCCACCACTC	TCCGGTGTGAGTAGGGTAGG	324-362

8.10.5.2 Recommended DNA extraction protocol

DNA can be extracted using column-based kits; procedures such as those based on CTAB are also adequate for extraction of DNA from oat seeds. For any DNA extraction procedure chosen, it is recommended to use 1 seed as starting material. For each seed to be extracted, it is recommended to use a single grinding bead (such as a Qiagen 5 mm stainless steel bead) placed in a 2 mL round-bottom microcentrifuge tube. Seeds can be crushed with needle-nose pliers as they are placed into the tubes. The seeds are then pulverized for 1 min at 28 Hz in a mixer mill (such as a Qiagen TissueLyser II). A rotation in the unit is performed and the seeds are pulverized a second time for 1 min at 28 Hz. Following DNA extraction, DNA quality and quantity can be verified by means of a 1 % agarose gel 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.

8.10.5.3 Recommended PCR procedures

The microsatellite markers set was selected based on performance in two comparative tests. Each marker can be run individually or multiplexed. If multiplexed, it is recommended to use PCR master mixes that were specially designed for multiplexing. However, it may be still required to optimize the primer concentration within a multiplex. Table 8.N. shows recommended primer concentrations for each marker. Final concentration in PCR mix may vary depending on reagents.

Table 8.N. Recommended final primer concentration for oats markers.

SSR	Final concentration in PCR mix
AM031	[0.18 µM]

AM102	[0.85 µM]
AM112	[0.35 µM]
MAMA-1	[0.55 µM]
MAMA-3	[0.18 µM]
MAMA-4	[0.39 µM]
MAMA-5	[0.30 µM]
MAMA-6	[1.10 µM]
MAMA-7	[0.80 µM]

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.

8.10.5.3.1 Reaction components

For each marker or multiplex, 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.O. contains a list of reagents for a single 10 µL reaction with multiplexed primers.

Table 8.O. Recommended reaction composition for PCR amplification of microsatellite markers for verification of oat varieties.

Amount per reaction (µL)	**Component**	**Final concentration a**
5	2X PCR Master Mix (for multiplexing)	1X
1	10X AM or MAMA Primer Mix[a]	1X
3	Ultrapure H20	

[a] Suggested final concentrations for all markers are listed in Table 8.N. Concentrations in multiplexed reactions may require adjustment depending upon relative product intensities observed. 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 or two extra to accommodate for 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), then distributed

into reaction tubes or wells (9 µL each). Lastly, 1 µL (approximately 10 ng/µL) of template DNA is added to each reaction tube, resulting in a final reaction volume of 10 µL.

8.10.5.3.2 Thermal cycling profile

The thermal cycling profile presented in Tables 8.P and Q has been used successfully with the prescribed markers in comparative tests, thus it is recommended for verification of oat varieties. The total number of cycles may require alteration based on product intensities achieved.

Table 8.P. Recommended thermal cycling profile for PCR amplification of “AM” microsatellite markers for verification of oat varieties.

Number of cycles	Temperature	Duration
1 cycle	95°C	5’ (Initial denaturation conditions according to manufacturer of Taq polymerase used)
10 cycles	95°C	30s
	63°C	90s (touchdown: -0.5°C/cycle)
	72°C	30s
20 cycles	95°C	30s
	58°C	90s
	72°C	30s
1 cycle	60°C	30min
1 cycle	4°C	forever

Table 8.Q. Recommended thermal cycling profile for PCR amplification of “MAMA” microsatellite markers for verification of oat varieties.

Number of cycles	Temperature	Duration
1 cycle	95°C	5’ (Initial denaturation conditions according to manufacturer of Taq polymerase used)
1 cycle	58°C	1min
	72°C	1min
29 cycles	95°C	30s
	58°C	90s
	72°C	30s
1 cycle	60°C	30min
1 cycle	4°C	forever

8.10.5.4 Evaluation of results

Alleles are called according to their sizes in base pairs. However, since every detection system and chemical used differ from one lab to another, this may alter the migration of the PCR products. To minimize

this variation, it is recommended to use this method 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.

References

-C.D. Li, B.G. Rossnagel and G.J. Scoles (2000) The development of oat microsatellte markers and their use in identifying relationships among *Avena* species and oat cultivars. Theoretical and Applied Genetics 101:1259–1268.

-C. P. Wight, W, Yan, J. Mitchell Fetch, J. Deyl and N.A. Tinker (2010) A set of new simple sequence repeat and avenin DNA markers suitable for mapping and fingerprinting studies in oat (*Avena* spp.). Crop Science 50:1207–1218.

VOTE TO ACCEPT ITEM	YES VOTES	NO VOTES	RESULT
C.8.2	**26**		**PASS**

Chapter 9: Determination of moisture content

C.9.1 Addition of bullets in 9.2.7 and 9.3.2.7 to improve clarity

The current version of this section was proposed last year and was accepted. However, a missing bullet made the new addition seem as if it only referred to pelleted seed. This was not the intent of the proposal. This new proposal corrects the issue.

The following proposal was developed and approved by the ISTA MOI Committee.

CURRENT VERSION	PROPOSED VERSION
9.2.7 Reporting of results ……. • 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	**9.2.7 Reporting of results** ……. • 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'. ~~If the species being tested is not listed in Table 9A but is listed in Table 2C, the result must be reported according to 1.5.2.22. 'N/A' for 'not applicable' must be entered in the moisture test result space on the ISTA Certificate.~~ • 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)'	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)' • If the species being tested is not listed in Table 9A but is listed in Table 2C, the result must be reported according to 1.5.2.22. 'N/A' for 'not applicable' must be entered in the moisture test result space on the ISTA Certificate.
9.3.2.7 Reporting of moisture meter results … • 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.' If the species being tested is not listed in Table 9A but is listed in Table 2C, the result must be reported according to 1.5.2.22. "NA" must be entered in the moisture test result space on the ISTA Certificate.	**9.3.2.7 Reporting of moisture meter results** … • 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.' • If the species being tested is not listed in Table 9A but is listed in Table 2C, the result must be reported according to 1.5.2.22. "NA" must be entered in the moisture test result space on the ISTA Certificate.

VOTE TO ACCEPT ITEM	YES VOTES	NO VOTES	RESULT
C.9.1	**27**		**PASS**

Chapter 19: Testing for seeds of genetically modified organisms

C.19.1 New acronym in Object

The acronym TP for "trait purity" is introduced. It was pointed out during the discussion the TP is used in Chapter 5 to indicate "top of paper". However, the Committee agrees that the use of TP for trait purity in the context of Chapter 19 runs a very small risk of ambiguity and that the benefit in using it TP outweighs this risk.

In cases where ambiguity might exist, "trait purity" should be used in full.

The following proposal has been developed and approved by the ISTA GMO Committee.

CURRENT VERSION	PROPOSED VERSION
19.1 Object The object of this chapter is to give guidelines to detect, quantify or confirm the presence of genetically modified organism (GMO) seeds in seed lots. These guidelines can be applied to testing adventitious presence (AP) of GMOs and to GMO trait purity testing.	**19.1 Object** The object of this chapter is to give guidelines to detect, quantify or confirm the presence of genetically modified organism (GMO) seeds in seed lots. These guidelines can be applied to testing adventitious presence (AP) of GMOs and to GMO trait purity (TP) testing.

New definitions

Several new definitions have been added in 19.2. As a result, the numbering of the definitions throughout this section will be modified if this proposal is approved.

PROPOSED VERSION
19.2.6 Trait Purity Trait purity (TP) represents the percent level at which a GMO or a trait is present in a seed lot. **19.2.7 Detection** In this chapter detection indicates the assessment of the presence of a GMO or of a trait in a seed sample or in a seed lot. **19.2.8 Quantification**

In this chapter, quantification indicates the estimation of the level of a GMO or of a trait in a seed sample or in a seed lot. It can be obtained by testing multiple individuals (e.g. seeds or seedlings) or multiple groups with qualitative assays, or by testing one or more seed groups or bulks with quantitative assays.

19.2.9 Qualitative assay

An assay that allows the assessment of the presence of a GMO or of a trait in the unit of observation through the detection of the trait itself (e.g. herbicide tolerance) or of an associated analyte (e.g. the responsible enzyme or its DNA coding sequence). Possible qualitative assays: bioassay, Lateral Flow Stick, ELISA, or PCR.

19.2.11 Quantitative assay

An assay that, in addition to detection, allows an estimate of the level of GMO or of a trait in the unit of observation through the measurement of an associated target analyte (e.g. the responsible enzyme or its DNA coding sequence) to be obtained. Quantitative assays: ELISA, real time PCR, digital PCR. Often, in laboratory practice, a potentially quantitative assay can be used to obtain only qualitative information from the unit of observation.

19.2.13 Unit of observation

The whole or part of the working sample on which a single observation is made. It can be a single seed/seedling or a seed group/bulk.

19.2.14 Seed bulk

A seed bulk is a unit of observation defined only by its weight and for which the number of individuals (seeds or seedlings) composing it is not known. It can be the whole working sample or each of the subsamples that are prepared from it for subsequent grinding and testing. Testing on bulks does not allow ~~to make~~ statistical inferences to be made about the seed lot, just about the sample (when using quantitative assays).

19.2.15 Seed group

A seed group (or “pool” in SeedCalc) is a unit of observation for which the number of individuals (seeds or seedlings) composing it is known. A seed group can be the whole working sample or each of the subsamples that are prepared from it for subsequent grinding and testing. Testing on groups allows statistical inferences about the seed lot to be made, both with quantitative assays as well as with qualitative assays, provided that multiple groups are tested.

9.2.16 Testing approach

The testing approach is defined by the choice of the assay type to be applied and of the unit of observation to be submitted to the testing process (Table 19A).

Improved definitions

Several existing definitions have been improved for clarity in Chapter 19. If proposals C.19.2 and C.19.3 pass, the numbering of the definitions throughout this Chapter will be modified.

-- Improvement of the definition of the term "analyte" and relates it to the term "target" widely used in the Chapter.

-- Improved definition of performance-based approach. PBA is not an approach to testing, rather to ensure performance uniformity in testing.

-- The object of a proficiency test is to assess the ability of the subject (laboratory, operator) to carry out a test, not a specific method.

CURRENT VERSION	PROPOSED VERSION
~~19.2.2~~ **Analyte**	**19.2.17 Analyte**
An analyte is a substance or chemical constituent that is of interest in an analytical procedure.	An analyte is a substance or chemical constituent that is of interest in an analytical procedure (e.g., protein, DNA). In GMO testing the "target analyte" is the assay target of a method (e.g., a specified transgenic protein or its DNA sequence).
~~19.2.9~~ **Performance-based approach**	**19.2.20 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.	The performance-based approach (PBA) is an approach by which ISTA ensures the performance uniformity in GMO testing for its accredited labs. It relies on the evidence of reliability of each laboratory when testing for GMOs. This evidence must comply with defined criteria. Under the PBA individual laboratories can choose the test method, as long as it has been validated as fit for purpose and complies with given performance standards.
~~19.2.10~~ **Proficiency test**	**19.2.21 Proficiency test**
A proficiency test is a standardized test or series of tests that assess~~es~~ the ability of a laboratory	A proficiency test is a standardized test or series of tests that assess the ability of a

or an individual operator to carry out a particular ~~method~~.	laboratory or an individual operator to carry out a particular test.

New Table

The table summarises the types of analytical outputs that can be attained using the different approaches in GMO testing.

PROPOSED VERSION

Table 19A Outputs attainable with different approaches in GMO testing.

		Unit of observation			
		Individuals	**Group (known number of seeds)**		**Bulk (unknown number of seeds)**
			One Group	**Multiple Groups**	**One or more bulks**
Assay type	**Qualitative**	- Assessment of presence - Estimation of level - Statistical inference on seed lot	- Assessment of presence - Statistical inference on seed lot (only in case of negative result)	- Assessment of presence - Estimation of level - Statistical inference on seed lot	- Assessment of presence
	Quantitative	Not performed in routine testing	- Assessment of presence - Estimation of level - Statistical inference on seed lot		- Assessment of presence - Estimation of level - Statistical inference on sample, but not on seed lot

Revised text of General principles

More precise definition of the pieces of evidence a laboratory must provide to apply for accreditation under the PBA.

The requirement of method validation or verification is introduced. A more precise specification of the requirement of production of performance data on seed samples is given. The use by laboratories of results obtained in non-ISTA proficiency test is accepted as evidence (also considering the difficulties of covering all species/events in ISTA PTs).

CURRENT VERSION	PROPOSED VERSION
19.3 General principles …. The various methodologies and equipment available make the international standardization of GMO testing difficult. ~~The~~ ISTA ~~approach therefore has~~ targe~~ted~~ the uniformity in GMO testing results, not by the uniformity in testing methodology, but by ~~using the adoption of~~ 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. 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.	**19.3 General principles** …. The various methodologies and equipment available make the international standardization of GMO testing difficult. Therefore, ISTA is targeting the uniformity in GMO testing results by laboratory accreditation based on a performance-based approach (PBA), not by the uniformity in testing methodology. The PBA requires that laboratories demonstrate that the GMO detection, identification, or quantification methods that they are using for reporting results on ISTA Certificates meet acceptable standards set by ISTA. These standards include sampling, testing, and reporting. 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: • validation and/or verification data for the assay/method being used; • performance data based on seed samples for each species, objective and approach for which the laboratory is seeking ISTA accreditation, and • participation in all ISTA GMO proficiency tests dealing with the species, objectives, and approaches for which the laboratory is accredited. The results of other proficiency tests can also be considered in order to provide additional evidence of satisfactory performance using methods that the laboratory has in its scope of accreditation. Compliance with these requirements will ensure the reliability of the analysis and the final test result reported on the ISTA Certificate. The PBA gives seed laboratories the choice to use different testing technologies, e.g. bioassays, protein-based methods and DNA-based methods.

New title, revised text - Objectives and approaches

"Objectives and approaches" instead of "Procedure" consistently with revised terminology.

Testing objectives and approaches are defined in relation to each other, also with reference to Figure 1 renamed Fig 19.1.

Two paragraphs anticipating "technical aspects", which seemed misplaced, were moved at the end of the section.

The sentence indicating the need of reference material in quantitative PCR was deleted since digital PCR does not necessarily require it.

Several editorial changes are proposed.

CURRENT VERSION	PROPOSED VERSION
19.4 ~~Procedure~~	**19.4 Objectives and approaches**
Testing for ~~AP of~~ GMO and for GMO ~~trait purity~~ in seeds are "two sides of the same coin"; both ~~applications make~~ use of the same tests, and follow a very similar workflow (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 an estimate of a low 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 characterized 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 organ- ism 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~~ apply~~y~~- ~~ing a qualitative test~~ (see 19.5.1.2 and 19.5.2.2), ~~while~~ aquantitative ~~question~~, e.g. "How much GM seed is there in a seed lot?"~~can be answered~~	Testing for GMO AP and for GMO TP in seeds are "two sides of the same coin"; for both testing objectives use is made of the same tests, and follow a very similar workflow (Fig. 19.1). The expected results differ in the two applications. In GMO AP testing, most of the time the expected outcome is "not detected" or an estimate of a low proportion of GMO present. In GMO TP testing, the expected result is the quantification of a high percentage of presence of the specified trait. The second testing objective to be defined is either the assessment of presence or the estimation of level of GMO. A suitable approach to GMO testing is chosen according to the defined objectives and the available technologies, taking into account other factors, such as time and costs (see Fig. 1). If only detection is requested, e.g. "Is there any GM seed in the sample?", a qualitative test can be applied (see 19.5.1.2 and 19.5.2.2). On the other hand, if a quantitative determination is requested, e.g. "How much GM seed is there in a seed lot?", either a group testing approach (Remund *et al.,* 2001), also

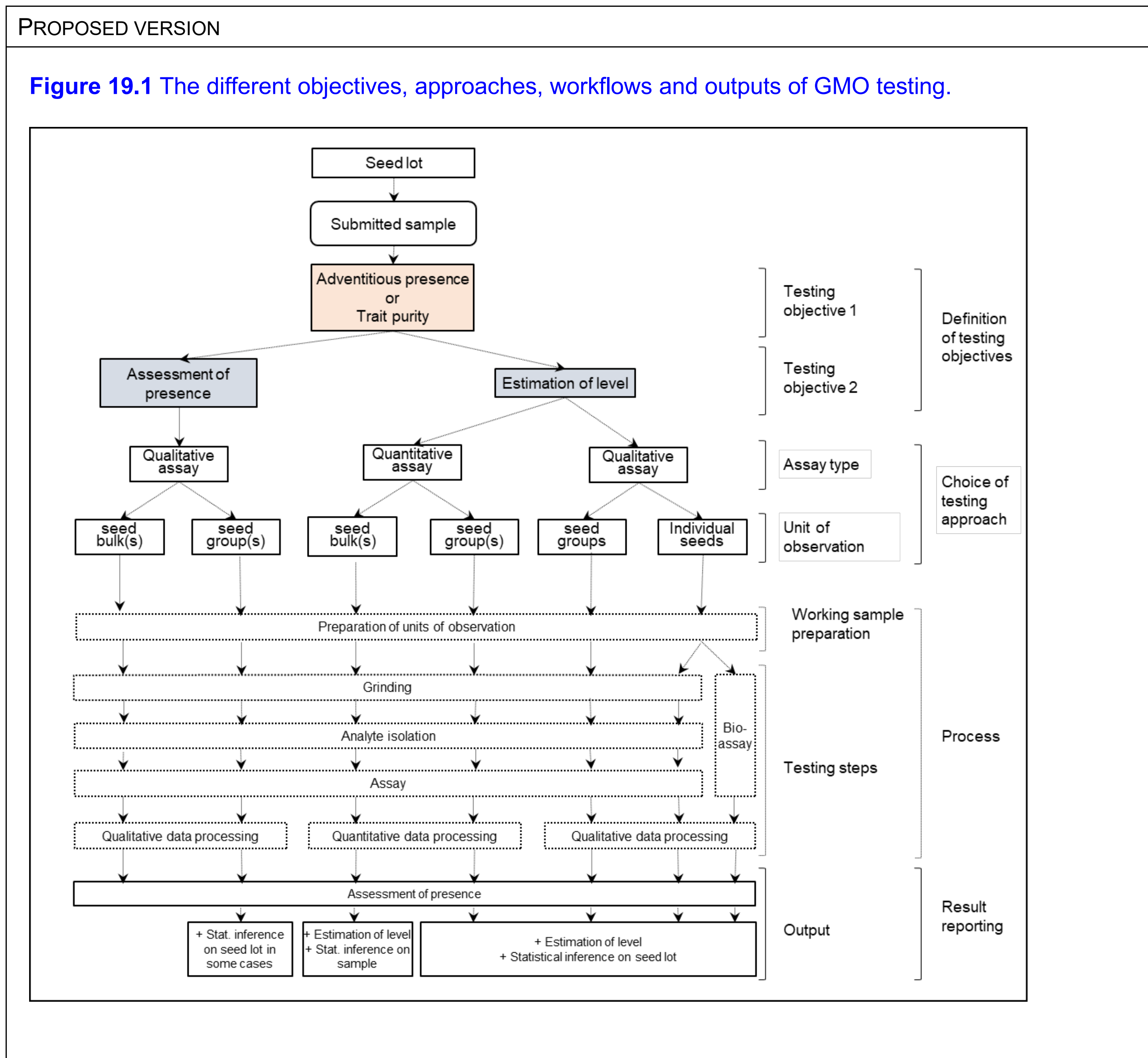
by using either a group-testing approach (Remund *et al.,* 2001), also known as the semi-quantitative method (which relies on qualitative tests of seed groups) or a quantitative test (Laffont et al., 2005) if appropriate certified reference material is available. Another classification is in relation to the specificity of the method, as described further in sections 19.5.1.1 and 19.5.2.1. 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, alternatively, the corresponding protein or coding DNA. However, a test can be performed on seed bulks at the DNA level aimed at detecting the absence of transgenic DNA, by targeting the wild type sequence at the insertion site (Battistini and Noli, 2009.	known as the semi-quantitative method (which relies on qualitative tests of multiple seed groups) or a quantitative test on one (usually) or more seed groups or bulks (Laffont *et al.,* 2005) can be used (19.5.1.3 and 19.5.4). Both AP GMO testing and GMO TP testing can be performed on seed bulks or groups, or on individual seeds. Seed bulk or group testing are more common in AP GMO testing, where the test target is a transgenic protein or a DNA segment. GMO TP tests are usually performed on a representative sample of individual seeds or seedlings, and target the GMO trait or, alternatively, the corresponding protein or DNA. However, a test can be performed on seed bulks or groups at the DNA level aimed at detecting the absence of transgenic DNA, by targeting the wild type sequence at the insertion site (Battistini and Noli, 2009). The methods used for these analyses can be classified and characterized 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). Another classification is in relation to the specificity of the method, as described further in sections 19.5.1.1 and 19.5.2.1.

Redrawn Figure 19.1

Figure 19.1 was almost completely redrawn according to revised terminology. It provides more information, including new concepts (unit of observation, testing approach) and relating the workflow to the different analytical outputs.

PROPOSED VERSION

Figure 19.1 The different objectives, approaches, workflows and outputs of GMO testing.



Revision of text - Sample size

Eliminated redundant sentence regarding the size of working sample compared to submitted sample.

Specified that the working sample can be analysed in a single or in multiple units of observation.

The concept of the limit of bulk/group size (and not of working sample size, since from the working sample multiple units of observation can be taken) in relation to the limit of quantification was clarified.

CURRENT VERSION	PROPOSED VERSION
19.4.1 Sample size	**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~~.	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. According to the different testing approaches, the working sample can be analysed as one or more units of observation (i.e., as multiple individual seeds, one or more groups or bulks). The sizes of seed groups and bulks 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 assays, the size of the bulk(s) or group(s) must be such that one single GM seed in a bulk/group would correspond to a level of presence above the limit of quantification.

Revised title - Testing technologies

Technologies instead of approaches consistently with revised terminology.

Other minor changes and specifications.

CURRENT VERSION	PROPOSED VERSION
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.	**19.5 Testing technologies** **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 essential steps are the following: • check of the seed sample; • grinding of the seed to produce a homogenous matrix; • matrix subsampling and DNA extraction; • DNA amplification; and • detection of the amplified DNA.

Addition of specification - Real time PCR

Specification regarding the need to verify the identity of PCR products in real time PCR when using intercalating dyes.

CURRENT VERSION	PROPOSED VERSION
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	**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 certified reference material.	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 addition, when using intercalating dyes, the identity of the amplification product must be verified by checking either Tm (melting temperature), or the restriction pattern, or by other suitable means. • In quantitative real-time PCR tests, the assay is designed to quantify the target against a standard curve produced from certified reference material.

Simplification of text - Lateral flow strip test

Simplification of text.

CURRENT VERSION	PROPOSED VERSION
19.5.2.2 Lateral flow strip test … An LFS may be used to test ~~bulk seed samples using seed groups, and also single seeds, or seedlings~~. Strips are available that can detect up to four proteins for efficiency; ~~these are commonly used for analysing single seeds, seedlings or plants in trait purity assays.~~	**19.5.2.2 Lateral flow strip (LFS) test** … An LFS may be used to test any type of unit of observation. Strips are available that can detect up to four proteins for efficiency.

Simplification of text - ELISA

Simplification of text.

CURRENT VERSION	PROPOSED VERSION
19.5.2.3 Enzyme-linked immunosorbent assay … ELISA assays can be used to test ~~bulk seed samples using seed groups, and also single~~	**19.5.2.3 Enzyme-linked immunosorbent assay** … ELISA assays can be used to test any type of unit of observation.

seeds, or seedlings.	

Replacement of term - Bioassays

Tolerance is more appropriate than resistance when referred to herbicides.

CURRENT VERSION	PROPOSED VERSION
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 the 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 concentration of herbicide must be appropriate for the crop, trait 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 multiple events may exist with the same herbicide-resistant phenotype. Therefore, in such cases herbicide bioassays can only be used to screen for the presence of GMO trait, but cannot indicate the presence of a particular event.	**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-tolerance traits. In this case the seeds or seedlings are exposed to the herbicide, and the expected effect on the plant is lack of normal development when the seeds do not contain the herbicide tolerance trait. All seeds or plants that continue to germinate or grow normally are scored as positive for the GMO trait. The concentration of herbicide must be appropriate for the crop, trait 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 multiple events may exist with the same herbicide-tolerant phenotype, similarly to the limitation for protein-based methods. Therefore, in such cases herbicide bioassays can only be used to screen for the presence of GMO trait but cannot indicate the presence of a particular event.

Revision of text - Consideration of testing objectives

Important specification that testing results for both assessment of presence or estimation of level can be referred either to the seed sample or the seed lot.

Deletion of a full sentence that has become unnecessary (since overcome) in the proposed version of the Chapter.

Other minor changes and specifications.

CURRENT VERSION	PROPOSED VERSION
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.	**19.6.1 Consideration of the testing objectives** 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 are: • reporting the presence or absence of a GMO or of a trait in the seed sample or in the seed lot; • estimating the proportion of the GMO or of a trait in the seed sample or in the seed lot with the associated measurement uncertainty. To assess the presence of two or more events in the same seed (stacked events), testing individual seeds is the appropriate approach. When seeds are tested in bulks or groups, 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.

Revision of text - Units of measurement

Modification of text to be consistent with revised terminology and to improve clarity.

CURRENT VERSION	PROPOSED VERSION
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~~ seeds ~~with GMO traits is required, and~~ in concordance with the PBA, ~~it is acceptable, for~~ accredited laboratories, ~~to~~ report ~~quantitative~~ test results on ISTA certificates using 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 reference material certified by % mass (g/kg) and under the ~~assumption~~ that the GMO present in the sample is genetically the same. % 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 The acceptance of more than one unit can avoid raising the difficult question of converting factors. ~~A simple mechanica~~l conversion between units is complex or even impossible. The unit adopted has to be clearly stated in the certificate. Whatever the unit used to express results, the resulting GM estimate should be methodologically sound, that is, a laboratory	**19.6.2 Units of measurement in estimation of the level of GMO** The calculation and expression of results depend on the testing objectives, approaches and assays, and on the associated units of measurement. The request of the applicant will need to be carefully considered. For the estimation of the level of GMO or trait presence, in concordance with the PBA, accredited laboratories can report test results on ISTA certificates using one of the following units: a) % in number of seeds: the estimate of the percentage of GM seeds in the seed lot. This is the unit to be used when individual testing or a group testing approach is chosen. b) % in mass of seeds: the estimate of the percentage of GMO content by mass. This unit should be used when the estimate is based on a calibration curve prepared using reference material certified by % mass (e.g. g/kg) and under the condition that the GMO present in the sample is genetically the same as the one in the reference material. c) % DNA copies: the estimate of the percentage of GMO content by a number of copies of the assay target. This unit should be used when the estimate is based on a calibration curve prepared using reference material certified by % DNA copies or when using digital PCR. The acceptance of more than one unit can avoid raising the difficult question of converting factors. In fact, the conversion between units is complex or even impossible. The unit adopted has to be clearly stated in the certificate. Whatever the unit used to express results,

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.	the resulting GM estimate should be methodologically sound, that is, a laboratory using quantitative real-time PCR should not report a value that is lower than its validated and verified limit of quantification. Moreover, when using quantitative assays (e.g. real-time PCR and digital PCR) results should be biologically meaningful. The lab should pay attention to results that are lower than 1 divided by the number of seeds in the working sample.

Revised text with additions - Reporting results

New specification of conditions for reporting results of GMO testing on OIC or on BIC.

Specifications of details to be provided when reporting results on an ISTA certificate.

CURRENT VERSION	PROPOSED VERSION
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) according to the value verified by the laboratory; • the limit of quantification of the method (when testing seed bulks with a quantitative method) according to the value verified by the laboratory.	**19.7 Reporting results** As for any other testing in the ISTA Rules, GMO testing results can also be reported on an Orange International Certificate under the condition that both sampling and testing have been carried out by accredited laboratories and that the total number of seeds in the working sample is known. In the case the size of the working sample is unknown (bulk sample) then only a Blue International Certificate can be issued. The result of a genetically modified organism test must be reported under 'Other determinations' as follows: • the request of the applicant (testing objectives); • the testing approach; • the testing technology; • the assay target; • 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 (in this case the results can be reported on an Orange International Certificate) or alternatively, the weight of the working sample (in this case the result can be

	reported only on a Blue International Certificate); • 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 bulks) according to the value verified by the laboratory; • the limit of quantification of the method (when testing seed groups or bulks with a quantitative method) according to the value verified by the laboratory. For the items indicated above, the terms to be used on an ISTA Certificate should be those reported in the List of Standardised Terms.

Revised text with addition - Assessment of presence

Title changed according to revised terminology.

Here it is defined how to express the compliance to a given standard when a working sample tests negative to a qualitative assays for a given target (in case multiple groups are tested, all are negatives).

Editorial changes.

CURRENT VERSION	PROPOSED VERSION
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.'~~	**19.7.1 Assessment of presence of GMO** Suggested phrases for reporting the detection of test targets depending upon the result are as follows: a) If the test target(s) was (were) detected: 'The test target(s) was (were) detected.' b) If the test target(s) was (were) not detected: 'The test target(s) was (were) not detected.' A negative result obtained with a qualitative assay even on a single group can allow the report to declare that the seed lot meets the specification of ...% (maximum or minimum) with …% confidence.

Revised title - Estimation of the level of GMO by multiple qualitative tests

Title changed according to revised terminology.

CURRENT VERSION	PROPOSED VERSION
19.7.2 ~~Quantitative results obtained~~ by multiple qualitative tests of individuals or groups ~~of seeds or seedlings~~	**19.7.2 Estimation of the level of GMO by multiple qualitative tests of individuals or groups**

Revised title and text with additions - Estimation of the level of GMO by quantitative measurements

Title changed according to revised terminology. Specification of conditions for reporting results of GMO testing on OIC or on BIC. Other changes to improve clarity. Editorial changes.

CURRENT VERSION	PROPOSED VERSION
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. ~~(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:	**19.7.3 Estimation of the level of GMO by quantitative measurements on groups or bulk*s*** Results should be reported relative to the percentage of the test target specified by the applicant by mass or number of DNA copies. If an Orange International Certificate is to be issued, the total number of seeds tested must be reported. If a Blue International Certificate is to be issued, either the number or the weight of the seeds tested must be reported. In any case, the number of groups or bulks, the number of replicate flour samples per group/bulk, and the number of replicate measurements per flour sample 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.'	a) If the test target(s) was (were) not detected (no signal or below the limit of detection): 'The test target(s) was (were) 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	b) If the test target(s) was (were) detected at a level above the limit of detection and below the limit of quantification: 'The test target(s) was (were) detected at a level below the limit

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 at the desired confidence, then the estimated percentage by mass or number of copies with the 95 % confidence interval will be reported.	of quantification of the method used.' c) If seeds showing the test target(s) were found at a level above the limit of quantification: 'The test target(s) percentage in the seed sample (in case of bulks)/in the seed lot (in case of groups) 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 sample (in case of bulks)/seed lot (in case of groups) 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 at the desired confidence, then the estimated percentage by mass or number of copies with the 95 % confidence interval will be reported.

VOTE TO ACCEPT ITEM	**YES VOTES**	**NO VOTES**	**RESULT**
C.19.1	**25**		**PASS**