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# Molecular biomarker analysis — Method for the statistical evaluation of analytical results obtained in testing sub-sampled groups of genetically modified seeds and grains — General requirements

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# National foreword

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BS ISO 22753:2021

# INTERNATIONAL STANDARD

# ISO 22753

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# Molecular biomarker analysis — Method for the statistical evaluation of analytical results obtained in testing sub-sampled groups of genetically modified seeds and grains — General requirements

*Analyse moléculaire de biomarqueurs — Méthode pour l'évaluation statistique des résultats d'analyse obtenus lors des essais de sous-échantillons multiples de semences et de graines génétiquement modifiées — Exigences générales*

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# Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 16, *Horizontal methods for molecular biomarker analysis*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

# Introduction

Seed and grain testing is used throughout the world to commercially define the purity of seed and grain lots.

Commercial requirements for labelling agricultural products with genetically modified organism (GMO) content at a specified threshold level both as a seed/grain contaminant and a food ingredient have become common to satisfy regulations and consumer demands. Conformance with these specifications is evaluated at various points of the supply chain, often starting with the harvested grain.

Quantitative real-time polymerase chain reaction (PCR) can be used to determine the GMO content by analysis of the ratio of GMO DNA copy numbers to plant-species specific DNA copy numbers followed by a conversion to genetically modified (GM) mass fraction.

Multiple events stacked in a crop, such as those generated by crossing two or more single events, are widely used in agricultural production. A stacked event seed or grain containing GMO DNA corresponding to two or more GM events commingled in lot cannot be differentiated by quantitative PCR alone from multiple seeds within the lot each containing a single GM event. Consequently, if the actual measured GMO arises only from GM stacked event seeds, GM content measured by quantitative real-time PCR of a single sample will lead to an overestimation of the actual number of GM seeds or grains present.

The group testing strategy described in this document provides a reliable alternative to estimate the GM content on the basis of the fact that whole seeds/grains are the sample material.

The process described in this document can provide a method to accurately estimate the percentages of GM seeds/grains in a lot irrespective of the presence of stacked event seeds/grains. GM content is determined for representative subsampled groups of seed/grain from a lot and statistically analysed.

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INTERNATIONAL STANDARD ISO 22753:2021

# Molecular biomarker analysis — Method for the statistical evaluation of analytical results obtained in testing sub-sampled groups of genetically modified seeds and grains — General requirements

## 1 Scope

This document describes general requirements, procedures and performance criteria for evaluating the content of genetically modified (GM) seeds/grains in a lot by a group testing strategy that includes qualitative analysis of sub-sampled groups followed by statistical evaluation of the results.

This document is applicable to group testing strategy estimating the GM content on a percentage seed/grain basis for purity estimation, testing towards a given reject/accept criterion and for cases where seed/grain lots are carrying stacked events.

This document is not applicable to processed products.

NOTE Description of the use of group testing strategy are available in References [1], [7], [8], [18], [19] and [20].

## 2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 16577, *Molecular biomarker analysis — Terms and definitions*

ISO 21572, *Foodstuffs — Molecular biomarker analysis — Immunochemical methods for the detection and quantification of proteins*

ISO 24276, *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — General requirements and definitions*

## 3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 16577 and the following apply.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

**3.1**
**absolute PCR limit of detection**
**absolute polymerase chain reaction limit of detection**
**absolute PCR LOD**
lowest nominal (average) number of target copies in the template volume distributed to individual PCRs that would allow for an acceptable probability of detecting the target

**3.10**
**LQL**
$L_{QL}$
**lower quality limit**
highest impurity that is acceptable to the consumer

Note 1 to entry: This can be equivalent to the *threshold* (3.22).

**3.11**
**mass fraction**
ratio of GM seeds/grains relative to the total seeds/grains corresponding to mass ratio

**3.12**
**number of deviant seed/grain groups**
number of *seed/grain groups* (3.17) including one or more *deviant seeds/grains* (3.4)

**3.13**
**operating characteristic curve**
**OC curve**
graph plotting the percentage of deviant seeds/grains and the probability of acceptance respectively on the horizontal and the vertical axes and used in quality control to determine the probability of accepting *seed/grain lots* (3.18) in a *testing plan* (3.21)

**3.14**
**producer risk**
**producer (alpha) risk**
probability of rejecting a lot at the *AQL* (3.2)

**3.15**
**representative sample**
sampling units (samples or groups) that have been extracted from a lot with the process ensuring all sampling units of the lots have an equal probability of being selected and not altered in any way that would change the analytical result

Note 1 to entry: The extraction process can be a multi-stage process.

**3.16**
**reject/accept criterion**
maximum *number of deviant seed/grain groups* (3.12) that can be detected in the *test sample* (3.20) of an acceptable *seed/grain lot* (3.18)

**3.17**
**seed/grain group**
**group**
determined number of seeds/grains prepared from a seed/grain *test sample* (3.20) by representative sampling

**3.18**
**seed/grain lot**
**lot**
population for which sampling is intended to estimate the measured parameter

**3.19**
**stacked event**
accumulation of two or more transformation events as a result of traditional breeding and/or successive transformation steps)

Note 1 to entry: In the context of this document a stacked event refers to a stack in which the two or more events are not genetically linked.

[SOURCE: ISO 16577:2016, 3.197, modified — Note 1 to entry has been added.]

**3.20**
**test sample**
sample prepared for testing or analysis, the whole quantity or part of it being used for testing or analysis at one time

Note 1 to entry: The test sample is prepared from the *laboratory sample* (3.9).

Note 2 to entry: The test sample is expected to represent the *laboratory sample* (3.9).

[SOURCE: ISO 16577:2016, 3.210, modified — Note 1 to entry and Note 2 to entry have been added.]

**3.21**
**testing plan**
plan specifying *group testing* (3.8) conditions including *group size* (3.7), the number of *seed/grain groups* (3.17) and the *number of deviant seed/grain groups* (3.12) in *test sample* (3.20) resulting in rejection of *seed/grain lot* (3.18)

**3.22**
**threshold**
maximum acceptable content of GMO presence in a lot

Note 1 to entry: This can be a prescribed value.

Note 2 to entry: Thresholds can be expressed in *mass fraction* (3.11) with the proviso that an uncertainty factor is involved in the conversion to a seed/grain percentage threshold.

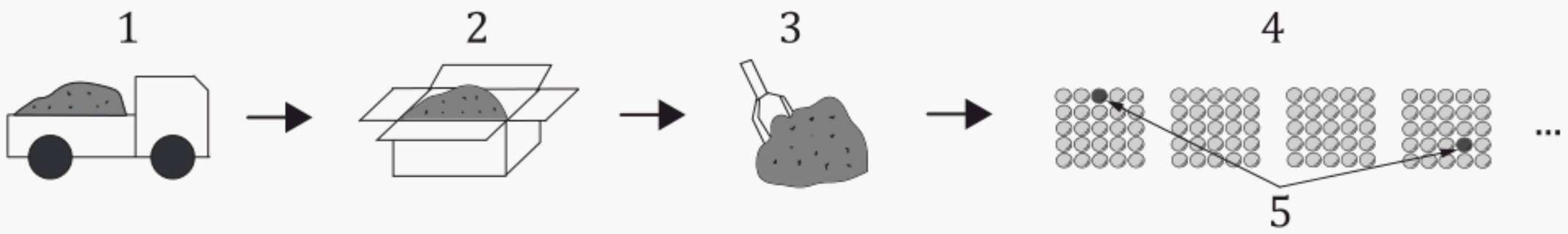
# 4 Principle

## 4.1 General

In this method, the test sample is divided into a predetermined number of groups. Each group consists of a determined number of seed/grain and is tested qualitatively for the presence or absence of a GM target. A statistical evaluation is performed on the number of GM positive groups relative to the total number of seed/grain groups to determine the GM content in mass fraction.

A statistical calculation determines the optimal testing conditions, namely, the number of seeds/grains per group (group size), the number of seed/grain groups, and the maximum number of GMO positive seed/grain groups for seed/grain lot acceptance. Alternatively, a statistical calculation provides an estimate of the percentage by number of the GM seeds/grains in a lot, according to a given testing plan.

## 4.2 Preparation of seed/grain groups




**Key**
1 bulk seed/grain lot
2 laboratory sample
3 test sample
4 seed/grain groups
5 deviant seed/grain

NOTE Each group is represented as an array on the right.

**Figure 1 — Sampling illustration of the obtention of seed/grain groups from a bulk seed/grain lot**

The process of forming seed/grain groups from a series of sampling steps starting with the bulk seed/grain lot is shown in Figure 1, (1).

Although the procedures for obtaining a laboratory sample from a seed/grain lot is not the subject of this document, a laboratory sample (2) from a seed/grain lot shall be obtained appropriately. The procedures can be designed according to the References [3], [6], [10], [11], [12], [15], [19] and [23].

The laboratory sample shall be thoroughly mixed and divided/reduced to create the test sample (3). Likewise, the test sample shall be thoroughly mixed (i.e. homogeneous) and divided into seed/grain groups (each group represented as an array in Figure 1, (4)) following simple random sampling principles. The seed/grain groups can vary in size from one single seed/grain up to the complete test sample (i.e. a single bulk). In most cases, multiple seed/grain groups are created from the test sample.

A determined number of seeds/grains can either be obtained by weighing or a volumetric measurement, where an approximation of number is made based on a determined conversion factor (e.g. thousand seeds/grains weight). For the case that weight is used to obtain the seed/grain groups, the operator shall have an estimate of the variability introduced by using weight rather than seed/grain count.

The group testing procedure described in Clause 7 is carried out on the collective qualitative (positive or negative) results for each seed/grain group.

### 4.3 Detection methods for the qualitative analysis of GM seed/grain in seed/grain groups

In general, GMO detection methods are categorized into two classes[ 21 ]. The first class of assays targets a nucleic acid sequence for detecting GMO presence. The second class includes methods for detecting a specified protein that confers a specific transgenic trait. Detection methods from either or both classes should be selected considering fitness-for-purpose. Guidance on the selection of qualitative methods is provided in Clause 8. Further details can be found in ISO 21569 [ 4 ] and ISO 21572.

### 4.4 Statistical evaluation

Sampling and measurement uncertainty shall be considered. Sampling uncertainty can be adequately considered using the binomial distribution[ 18 ][ 2 ]. The FPR and the FNR of the qualitative assay should be considered[ 2 ]. The LOD of the applied detection method should be considered.

The group testing described here can be used to set reject/accept criteria based on a given threshold by GMO content, as well as to estimate the GMO content and associated upper and lower confidence limits.

## 5 Reagents

All reagents used in the analysis should be those specified in the method.

Otherwise, all reagents should be of molecular biology grade.

These reagents shall be stored and used as recommended by the supplier or according to the laboratory quality assurance specifications. It can also be appropriate to aliquot the reaction solutions required for the analytical method in order to avoid subjecting them to repeated freeze–thaw cycles, or to reduce the chances of cross contamination or both. Further details shall refer to ISO 24276 and ISO 21572.

## 6 Apparatus and equipment

The laboratory should use properly maintained equipment suitable for the methods employed.

Further details shall refer to ISO 24276 and ISO 21572.

## 7 Design of testing plan

### 7.1 General

The number of seeds/grains tested, the reject/accept criteria, the sample preparation steps and the method used for testing shall be determined depending on the analytical purpose.

In seed/grain sample classification, it can be determined whether the number of deviant seeds/grains or seed/grain groups is above a given reject/accept criterion or not. Then, it can be decided to reject or accept the seed/grain lot based on the test results.

A basic testing plan for group testing consists of three fundamental parameters:

a) the number of seed/grain groups;

b) the size of the seed/grain groups;

c) the maximum number of deviant seed/grain groups for seed/grain lot acceptance (reject/accept criterion).

The risks associated with the AQL and the LQL are the producer (alpha) and consumer (beta) risks respectively, and together with the FPR and FNR allow the design of an appropriate testing plan.

The OC curve can be used to develop a testing plan. Explanations for the estimation of the LOD for a zero deviant testing plan, the effect of the genome size on the group size if methods targeting DNA are applied, and the effect of the individual seed size on the sample preparation are given in Annex C.

Annex D provides guidance on the determination of the maximum group size whatever analytical method is used in the laboratory.

NOTE Seedcalc[16] is a statistical program (Microsoft Excel spreadsheet application) that is freely available from the International Seed Testing Association and has procedures to facilitate the design. Seedcalc is located on the ISTA website.

### 7.2 Single-stage testing plan

A single-stage testing plan consists of one testing stage. Groups are taken from the test sample and evaluated once, and a decision is then made based on the results to accept or reject the seed/grain test sample. In a single-stage testing plan, a specified number of individual seeds/grains or seed/grain groups shall be selected randomly from the test sample and tested. Depending on the number of deviants detected and the maximum number of deviants specified in the plan, the seed/grain lot is either accepted or rejected.

The probability that an individual seed/grain or seed/grain group is deviant, $p_b$, can be calculated as given in Formula (1):

$$p_b = 1 - P = 1 - (1-p)^m \quad (1)$$

where

$P$ is the probability that there are no deviant seeds/grains in the group;
$p$ is the true unknown impurity in the seed/grain lot;
$m$ is the number of individual seeds/grains in a seed/grain group (if seeds/grains are tested individually, $m = 1$).

Then, the probability that a lot will be accepted, *P(a)* is calculated as given in Formula (2):

$$P(a) = \sum_{i=0}^{c} \binom{n}{i} {p_b}^i (1-p_b)^{n-i} \quad (2)$$

where

*P(a)* is the probability that a lot will be accepted;
$n$ is the number of individual seeds/grains or seed/grain groups tested;
$c$ is the maximum number of deviant seed/grain groups for acceptance.

By combining Formulae (1) and (2), *P(a)* is a function of $p$, $n$, $m$ and $c$.

After $n$, $m$ and $c$ are determined, an OC curve can be drawn by plotting $p$ and *P(a)* on the x-axis and y-axis, respectively.

## 7.3 Double-stage testing plan

A double-stage testing plan is generally set up so that additional seed/grain groups are tested in the second stage. Initial seed/grain groups are taken from the test sample and tested. Based on this test result, three different decisions can be made:

a) accept the seed/grain lot;

b) reject the seed/grain lot; or

c) draw a second set of seed/grain groups from the test sample and retest.

The test results from the first and second stages of testing are combined and used to determine whether the seed/grain lot is accepted or rejected (see Figure B.1). In Annex B examples for implementation of a double-stage testing plan to evaluate GMO content in seeds/grains are provided. Subclause B.1 can also be applied for cases where seed/grain lots are carrying stacked events.

Some additional terms are defined as follows:

— $n_1$ , the number of independent seed/grain groups to be tested in the first stage;

— $n_2$ , the number of independent seed/grain groups to be tested in the second stage;

— $c_1$ , the maximum number of allowable deviant seed/grain groups for acceptance in the first stage;

— $c_2$ , the minimum number of deviant seed/grain groups that will result in rejection at the first stage;

— $c_3$ , the maximum number of deviant seed/grain groups in the first and second stages combined allowed for acceptance;

— $d_1$ , the number of deviant seed/grain groups in the first stage;

— $d_2$ , the number of deviant seed/grain groups in the second stage.

*P(a)* is calculated as given in Formula (3):

$$P(a)=\sum_{i=0}^{c_1}\binom{n_1}{i}{p_b}^{i}(1-p_b)^{n_1-i}+\sum_{i=c_1+1}^{c_2-1}\left[\left\{\binom{n_1}{i}{p_b}^{i}(1-p_b)^{n_1-i}\right\}\times\sum_{j=0}^{c_3-i}\binom{n_2}{j}{p_b}^{j}(1-p_b)^{n_2-j}\right] \quad (3)$$

# 8 Selection of qualitative methods

## 8.1 General

An analytical method shall be chosen to meet the purpose of testing. The performance characteristics of the method should be determined before application in seed/grain testing.

Analytical methods have been developed to detect specific genes encoding transgenic traits or specific characteristics expressed by specific genes in seeds/grains. Nucleic-acid-based methods such as PCR are available that detect specific DNA sequences encoding elements, constructs or GMO events[ 4 ][ 5 ]. Protein-based methods such as ELISA and lateral flow immunoassays require a specific antibody for detecting a specific GM protein (see ISO 21572).

## 8.2 Performance criteria

The analytical methods applied for the test plan protocol shall detect at least one GM seed/grain in a group with high probability of detection. Refer to Reference [2].

In the case of PCR, detection methods shall be chosen to meet the purpose of group testing. General methods performance criteria are described in ISO 24276. General criteria for the design of the testing plan which should be considered include

a) physical and genome size of seed/grain species as it affects the number of seed/grain that can be easily ground per group and the number of genome equivalents that can be analysed in a standard PCR, respectively,

b) absolute PCR limit of detection of the qualitative method, and

c) false-negative rates associated with the method of detection or identification in addition for both nucleic acid- and protein-based methods should be considered[ 8 ][ 34 ].

Detection-method-specific performance criteria can refer to ISO 24276, ISO 21569 [ 4 ] and ISO 21572.

The seed/grain testing plans discussed in this document assume that the seeds/grains tested are a representative sample drawn from the seed/grain test sample. Simple representative sampling implies that each seed/grain in the test sample has both an equal and an independent chance of being included in the seed/grain group.

# 9 Interpretation

In determining whether to “accept” or “reject” a given seed/grain lot, the test results shall be compared with the predetermined reject/accept criterion, e.g. the maximum number of GM-positive groups allowable for acceptance.

Statistical calculation using the formulae shown below permit the evaluation of a GMO content with confidence intervals from the test results. Statistical calculation programs such as Seedcalc[1] facilitate the calculation. In this manner, one can obtain quantitative information on the GMO content of the seed/grain lot based on how many groups proved to be GM-positive in the qualitative analysis. Together, test results and their statistical evaluation reveal the level of impurity in the seed/grain lot. Ninety-five

1) Seedcalc is an example of a statistical tool for seed testing. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

percent upper and lower confidence limits for this impurity evaluation can then be calculated. The true impurity in the seed/grain test sample can be expected with 95 % confidence to fall within these limits.

The most likely value of GMO content, $p$, can be evaluated from the test results as given in Formula (4).

$$p = 1 - \left(1 - \frac{d}{n}\right)^{\frac{1}{m}} \tag{4}$$

where

$n$ is the number of individual seeds/grains or seed/grain groups tested;

$m$ is the number of individual seeds/grains in a seed/grain group (if the seeds/grains are tested individually, $m = 1$);

$d$ is the number of deviant seeds/grains or seed/grain groups.

The group testing approach, like quantitative methods, has limitations concerning the GM levels that can be estimated. Table 1 gives two examples of the highest computed GM estimate for test sample sizes of 200 seeds/grains and 3 000 seeds/grains. These highest estimates are obtained when all, but one group is positive. Associated 95 % confidence limits are given to the estimates to show the sampling uncertainty.

NOTE For seed/grain group sizes greater than one, when all groups are positive for GM presence, there is very limited utility in this approach.

**Table 1 — Examples of highest computed GM estimate of the content of the deviant seeds/grains for various seed/grain group sizes (when all but one group is positive) and the 95 % confidence limits (when all but one group is positive)**

| Seeds (total) | Groups | Seeds per groups | GM positive groups | Estimated percentage GM seed | Range of GMO content (%) (for 95 % confidence level) |
|---|---|---|---|---|---|
| 200 | 1 | 200 | 0 | 0,0 | 0,0 to 1,8 |
| | 5 | 40 | 4 | 3,9 | 0,8 to 12,4 |
| | 10 | 20 | 9 | 10,9 | 4,0 to 25,8 |
| | 20 | 10 | 19 | 25,9 | 13,0 to 48,7 |
| 3 000 | 1 | 3 000 | 0 | 0,0 | 0,0 to 0,1 |
| | 5 | 600 | 4 | 0,3 | 0,1 to 0,9 |
| | 10 | 300 | 9 | 0,8 | 0,3 to 2,0 |
| | 20 | 150 | 19 | 2,0 | 0,9 to 4,4 |
| | 30 | 100 | 29 | 3,3 | 1,7 to 6,8 |
| | 60 | 50 | 59 | 7,9 | 4,7 to 14,4 |

If the confidence level for evaluation is set at $x$ %, the upper confidence limit of GMO content in the evaluation can be calculated using the following Formulae (5) and (6):

$$\alpha = 1 - \frac{x}{100} \tag{5}$$

where $x$ is the confidence level in percentage terms.

$$P_{UL} = 1 - \left(1 - \frac{(d+1)F_{1-\alpha,2d+2,2n-2d}}{(n-d)+(d+1)F_{1-\alpha,2d+2,2n-2d}}\right)^{\frac{1}{m}} \tag{6}$$

where the quantity $F_{1-\alpha,2d+2,2n-2d}$ is the $1 - \alpha$ quantile from an $F$-distribution with $2d + 2$ and $2n - 2d$ degrees of freedom.

Also, the two-sided confidence interval (upper limit, $P_{UL}$ ; lower limit, $P_{LL}$ ) can be calculated using the following Formulae (7) and (8):

$$P_{UL} = 1 - \left( 1 - \frac{(d+1)F_{1-\alpha/2,2d+2,2n-2d}}{(n-d)+(d+1)F_{1-\alpha/2,2d+2,2n-2d}} \right)^{\frac{1}{m}} \quad (7)$$

$$P_{LL} = 1 - \left( 1 - \frac{d}{d+(n-d+1)/F_{\alpha/2,2d,2n-2d+2}} \right)^{\frac{1}{m}} \quad (8)$$

# 10 Expression of results

## 10.1 Classification of a seed/grain lot into "accept" or "reject" category

To classify a seed/grain lot into the "accept" or "reject" category, a statement can be made such that the seed/grain lot is acceptable or that the seed/grain lot should be rejected.

The upper 95 % confidence limit of the concentration based on the result can be included, or the number of groups tested, or the number of deviant pools or all of these.

The OC curve expressing the characteristic of sampling can be attached along with the alternative decision result in order to facilitate understanding.

## 10.2 Estimation of the level of molecular biomarker in the seed/grain lot

The GMO content in the seed/grain lot can be estimated as described in Clause 9. A statement can be made such that the most probable value of GMO content is $p$ %, and the $(1-\alpha)\times$ 100 % confidence interval ranges from $P_{LL}$ % to $P_{UL}$ %.

# 11 Test report

The test report shall be written in accordance with ISO 24276 and shall contain at least the following additional information:

a) the sample;

b) a reference to the method that was used for the extraction of nucleic acid or protein;

c) a reference to the methods used for the amplification of the nucleic acid target sequences or the methods used for the detection of the target protein or both;

d) the LOD of the method used to test the groups and the matrix used to identify the LOD;

e) the reference material used if applicable;

f) the results expressed according to Clause 10;

g) the International Standard used (i.e. ISO 22753:2021);

h) any deviations from the procedure;

i) any unusual features observed;

j) the date of the test.

# Annex A
(informative)

# Terms and definitions comparison table

## A.1 Comparison of terms defined other documents

Synonymous terms defined in other documents or organizations are shown in Table A.1.

**Table A.1 — Terms comparison table**

| This document | | ISTA[a,b] | | JRC[c] | |
|---|---|---|---|---|---|
| **Term** | **Definition** | **Term** | **Definition** | **Term** | **Definition** |
| seed/grain lot | population for which sampling is intended to estimate the measured parameter | seed lot | a seed lot is a specified quantity of seed that is physically and uniquely identifiable | lot | a lot is a distinct and specified quantity of material dispatched or received at one time and covered by a particular contract or shipping document |
| laboratory sample | sample or subsample(s) received by the laboratory<br>Note 1 to entry: The seed/grain sample received is expected to represent the seed/grain lot. | submitted sample | a submitted sample is a sample that is to be submitted to the testing laboratory and may comprise either the whole of the composite sample or a subsample thereof. The submitted sample may be divided into subsamples packed in different material meeting conditions for specific tests (e.g. moisture or health). | laboratory sample | sample as prepared (form the lot) for sending to the laboratory and intended for inspection or testing |

[a] INTERNATIONAL SEED TESTING ASSOCIATION, Chapter 2: Sampling. *International Rules for Seed Testing 2021*, 2021, Bassersdorf, Switzerland[15].

[b] INTERNATIONAL SEED TESTING ASSOCIATION, Chapter 19: Testing for seeds of genetically modified organisms. *International Rules for Seed Testing 2021*, 2021, Bassersdorf, Switzerland[17].

[c] EUROPEAN COMMISSION, JOINT RESEARCH CENTRE. (2014), JRC Technical Report: Guidelines for sample preparation procedures in GMO analysis[9].

| This document | | ISTA[a,b] | | JRC[c] | |
|---|---|---|---|---|---|
| **Term** | **Definition** | **Term** | **Definition** | **Term** | **Definition** |
| test sample | sample prepared for testing or analysis, the whole quantity or part of it being used for testing or analysis at one time<br>Note 1 to entry: The test sample is prepared from the laboratory sample.<br>Note 2 to entry: The test sample is expected to represent the laboratory sample. | working sample | the working sample is the whole of the submitted sample or a subsample thereof, on which one of the quality tests described in these ISTA Rules is made and must be at least the weight prescribed by the ISTA Rules for the particular test. | test sample | (sub-)sample prepared from the laboratory sample and from which test portions will be taken |
| seed/grain group | determined number of seeds/grains prepared from a seed/grain test sample by representative sampling | seed group | A seed group is one of the portions of the working sample that is separately prepared (e.g. grinding, DNA or protein extraction) and analysed (e.g. end-point PCR, ELISA, real-time PCR) when using the group testing approach | - | - |

[a] INTERNATIONAL SEED TESTING ASSOCIATION, Chapter 2: Sampling. *International Rules for Seed Testing 2021*, 2021, Bassersdorf, Switzerland[ 15 ].

[b] INTERNATIONAL SEED TESTING ASSOCIATION, Chapter 19: Testing for seeds of genetically modified organisms. *International Rules for Seed Testing 2021*, 2021, Bassersdorf, Switzerland[ 17 ].

[c] EUROPEAN COMMISSION, JOINT RESEARCH CENTRE. (2014), JRC Technical Report: Guidelines for sample preparation procedures in GMO analysis[ 9 ].

# Annex B
(informative)

# Implementation of the method to evaluate GMO content in seeds/ grains example

## B.1 Example 1: Group testing to evaluate GMO content in maize grains

### B.1.1 General

This annex provides an example to evaluate GMO content in grains using a double-stage testing plan.

The double stage testing plan is used for checking the appropriateness of food labelling in Japan.

### B.1.2 Analytical purpose

If there is commingling of stacked event(s) into seed/grain lots, GMO amounts measured by real-time PCR lead to an overestimation as compared to the actual proportional GM amount in a lot. The group testing strategy was introduced to estimate the GM content towards the given reject/accept criterion, irrespective of the presence of stacked event seeds/grains in the lot.

The specified sampling strategy was devised to determine if the GMO content in maize grain lots exceeds 5 % (mass/mass) or not.

### B.1.3 Properties of the analytical method

Properties for each item are shown in Table B.1. Flow chart for decision making is shown in Figure B.1.[19][22][13]

**Table B.1 — Analytical method example for double stage sampling plan**

| Item | Value |
|---|---|
| Type of analytical sample | Maize grain |
| Analyte | GMOs in maize grains |
| Qualitative molecular biomarker detection method | Real-time PCR method targeting either or both of Cauliflower mosaic virus derived 35S promoter and *Agrobacterium tumefaciens* derived NOS (nopaline synthase) terminator GM elements, which is validated to be able to detect at least 1 GM grain in 20 grains |
| Type of sampling plan | Double stage |
| Group size in the first stage | 20 grains |
| Number of groups in the first stage | 10 groups |
| Reject/accept criterion in the first stage | 6 groups<br>The second stage is carried out if 7 or more groups show positive. |
| Group size in the second stage | 20 grains |
| Number of groups in the second stage | 10 groups |
| Reject/accept criterion in the second stage | 12 groups after combining the numbers of positive group in the first and second stages.<br>Sample lot rejected if $\geq$ 13 groups test positive for either or both GM targets. |

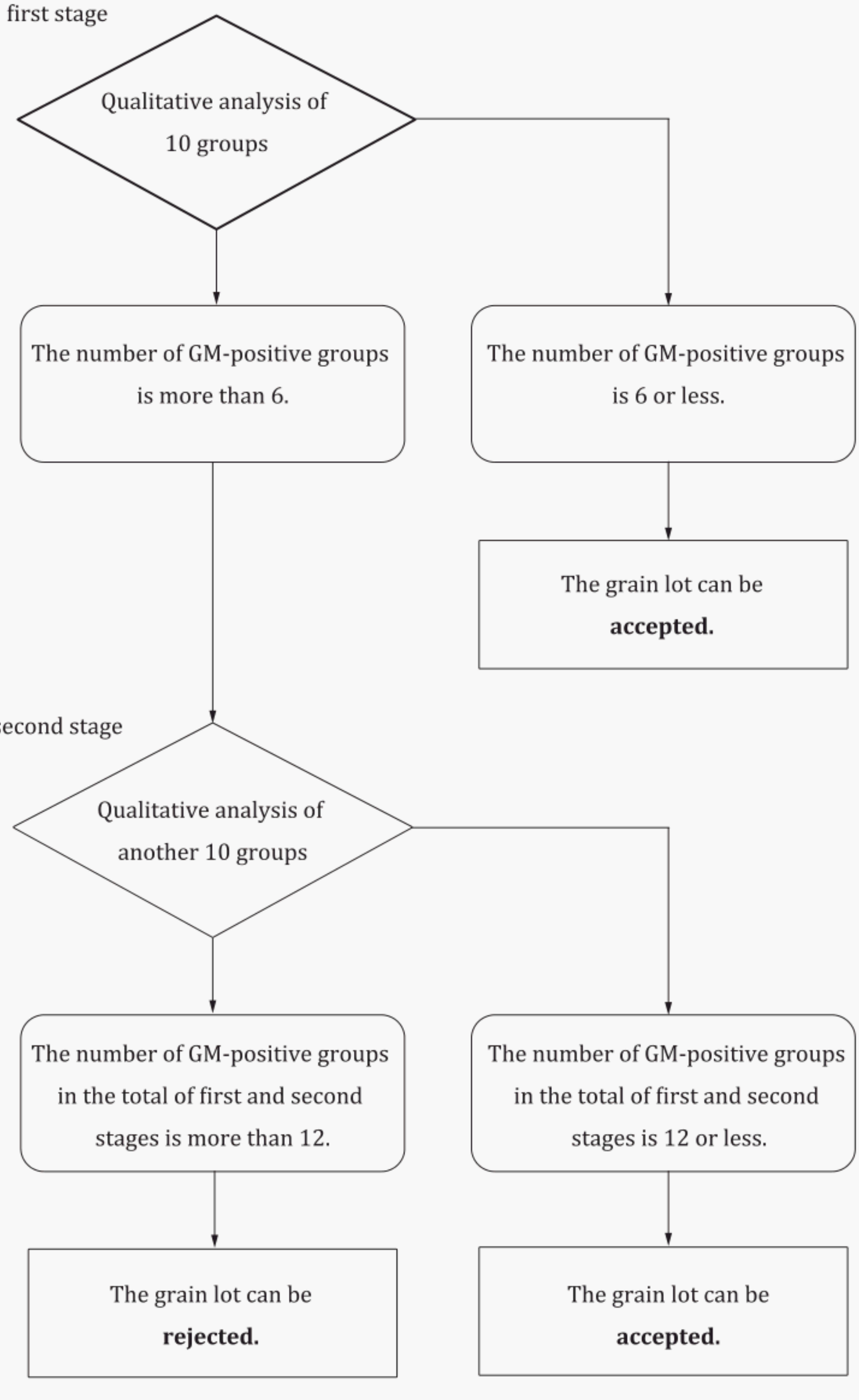



**Figure B.1 — Flow chart for decision making**

## B.2 Example 2: Group testing to evaluate GMO content in seeds

### B.2.1 Analytical purpose

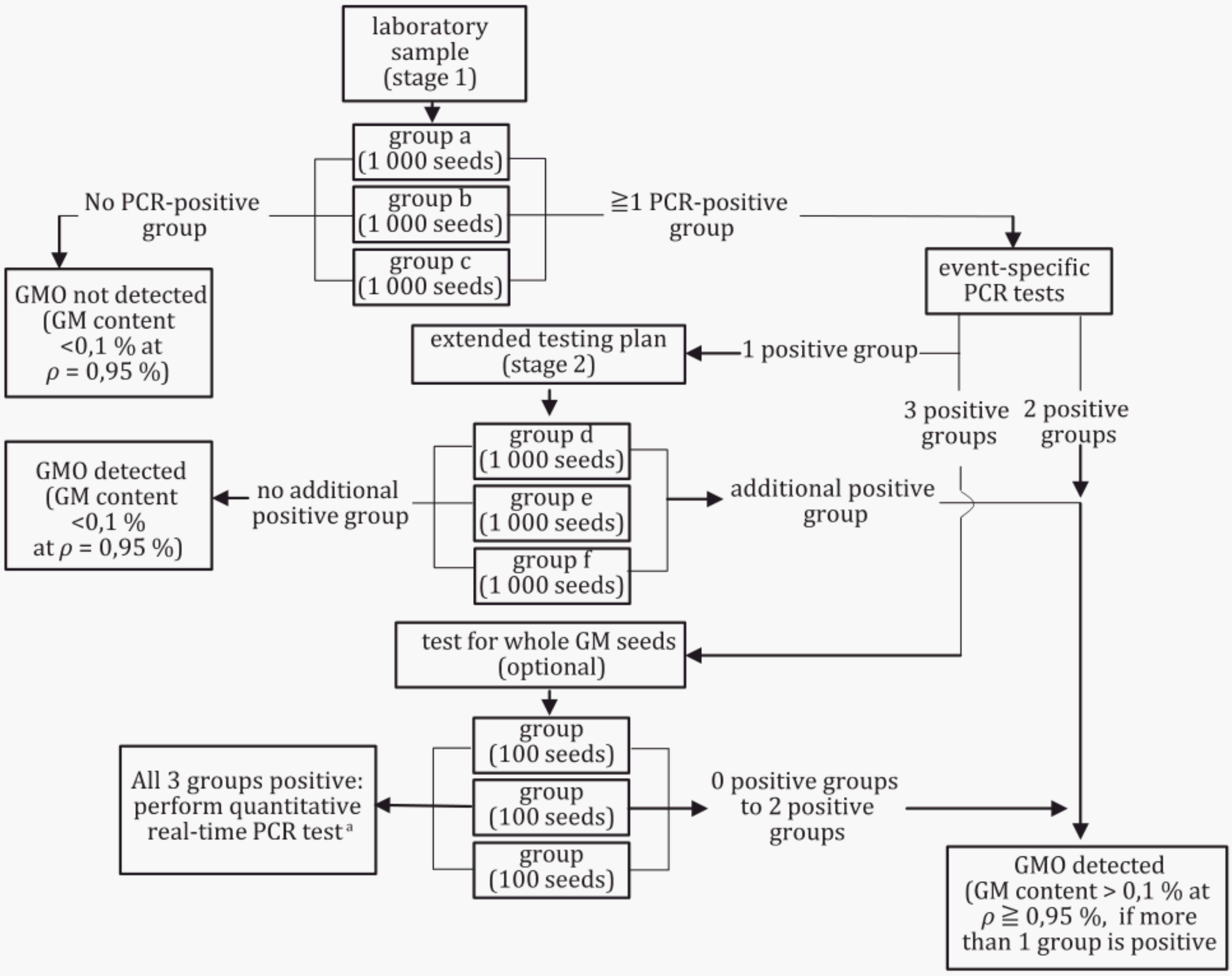
This example describes a validated German official approach for seed testing[20]. It can be applied to determine whether the GMO content exceeds 0,1 % or not.

### B.2.2 Properties of the analytical method

Properties for each item are shown in Table B.2. Flow chart for decision making is shown in Figure B.2. The Seedcalc based estimations of GM seed content based on the number of GM positive results obtained in the qualitative tests are given in Tables B.3 and B.4.[ 20 ][ 14 ]

**Table B.2 — Analytical method example for German official approach**

| Item | Value |
|---|---|
| Type of analytical sample | Seeds (maize; rapeseed) |
| Analyte | Genetically modified seed |
| Qualitative molecular biomarker detection method | Real-time PCR method validated to be able to detect at least 1 genetically modified seed in 3 000 seeds |
| Type of sampling plan | Double stage |
| Group size in the first stage | 1 000 seeds |
| Number of groups in the first stage | 3 groups |
| Reject/accept criterion in the first stage | When GMO is detected in more than 1 group: GM seed content exceeds 0,1 %;<br>second stage is carried out, if GMO is detected only in 1 group. |
| Group size in the second stage | 1 000 seeds |
| Number of groups in the second stage | 3 groups |
| Reject/accept criterion in the second stage | In additional group(s) GMO detected: GM seed content exceeds 0,1 % |




a Quantitative real time PCR tests or comparison of Cq values can help to distinguish whole GM seeds present in the sample from positive signals caused by traces of other homogenously present material (e.g. dust, seed coating).

**Figure B.2 — Flow chart for decision making**

**Table B.3 — Seedcalc estimations of GM seed content based on the number of GM positive results obtained in the qualitative tests of three groups of 1 000 seeds**

| Seeds (total) | Groups | Seeds per group | GM positive groups | Percentage GM seeds in laboratory sample | Range of GMO content (%) (for 95 % confidence level)[a] | Probability for a true GMO content of ≤ 0,1 % |
|---|---|---|---|---|---|---|
| 3 000 | 3 | 1 000 | 0 | 0,00 | 0,000 0 to 0,122 9 | 0,950 |
| 3 000 | 3 | 1 000 | 1 | 0,04 | 0,000 8 to 0,235 8 | 0,694 |
| 3 000 | 3 | 1 000 | 2 | 0,11 | 0,009 9 to 0,476 8 | 0,253 |

a The true GMO content of the seed lot (expressed as percentage GM seed) at a confidence level of 95 % is statistically significant in the given range.

**Table B.4 — Seedcalc estimations of GM seed content and corresponding probabilities based on the number of GM positive results obtained in the qualitative tests of six groups of 1 000 seeds**

| Seeds (total) | Groups | Seeds per groups | GM positive groups | Estimated percentage GM seed | Range of GMO content (%) (for 95 % confidence level)[a] | Probability for a true GMO content of ≤ 0,1 % |
|---|---|---|---|---|---|---|
| 6 000 | 6 | 1 000 | 0 | 0,00 | 0,000 0 to 0,061 5 | 0,998 |
| 6 000 | 6 | 1 000 | 1 | 0,02 | 0,000 4 to 0,102 5 | 0,972 |
| 6 000 | 6 | 1 000 | 2 | 0,04 | 0,004 4 to 0,150 0 | 0,862 |
| 6 000 | 6 | 1 000 | 3 | 0,07 | 0,012 6 to 0,213 4 | 0,611 |
| 6 000 | 6 | 1 000 | 4 | 0,11 | 0,025 2 to 0,313 5 | 0,287 |
| 6 000 | 6 | 1 000 | 5 | 0,18 | 0,044 4 to 0,545 5 | 0,064 |

[a] The true GMO content of the seed lot (expressed as percentage GM seed) at a confidence level of 95 % is statistically significant in the given range.

## B.3 Example 3: Implementation of the method to evaluate GMO purity in seeds

### B.3.1 General

Clause B.3 provides an example to evaluate the GMO purity in seeds for checking the percent herbicide tolerant seeds, or the percent seeds containing another desired characteristic. The purpose of the test is to determine whether a seed lot contains the desired characteristic at above a desired threshold.

A representative sample of seeds are, for example, sown in soil. At a specific leaf stage, the seedlings are sprayed with the herbicide at an appropriate rate. The proportion of herbicide tolerant seedlings is calculated as the share of the number of germinated seeds that show herbicide tolerance. Alternatively, seeds are placed on a towel or other medium containing the herbicide and the proportion of herbicide tolerant seeds is counted. Abnormal or non-germinating seeds are excluded from the count. This approach can be used on any seed. It can also be applied to seeds that are not GM seeds, that exhibit a phenotype or genotype that can be tested on individual seeds.[ 4 ][ 17 ][ 24 ][ 25 ]

### B.3.2 Analytical purpose

This is a testing method used to check if the percentage of seeds with a desired characteristic (e.g. herbicide tolerant seeds in herbicide tolerant GM) in a seed lot exceeds a specified threshold.

### B.3.3 Properties of the analytical approach

Properties of each item are shown in Table B.5. The analytical method is typically carried out on a sample of 400 seeds. Four replicates of 100 seeds is commonly used in testing seed lots for germination tests and for testing herbicide tolerance of a lot. Using 400 seeds gives a reasonable chance of a meaningful result where the threshold for acceptance is in the range of 97 % to 99 % of the seeds tested. If the seed lot is expected to be very pure, a smaller number of seeds (e.g. 200 seeds) can be tested, but this approach should only be used when the outcome is predictable.

**Table B.5 — Analytical method example for checking seed purity**

| Item | Value |
|---|---|
| Type of analytical sample | Soybean seeds |
| Analyte | Herbicide tolerance |
| Detection method | Tolerance (resistance) to an herbicide applied to the seeds or seedlings |
| Type of sampling plan | Double stage |
| Group size in the first stage | Single seed |

| Item | Value |
|---|---|
| Number of groups in the first stage | 400 groups (individual seeds) |
| Reject/accept criterion in the first stage | Dependent on the required threshold |
| Group size in the second stage | Single seed |
| Number of groups in the second stage | 400 groups (individual seeds) |
| Reject/accept criterion in the second stage | Dependent on the required threshold |

### B.3.4 Analytical method

The seeds can be assayed using a number of analytical methods. These depend on the tolerance to herbicide applied to the seeds, or to seedlings grown from the seeds, using PCR to determine the presence of the desired gene[4][5] or a protein assay (see ISO 21572) to determine expression of a protein. For the purpose of this annex, the example used is herbicide tolerance.

### B.3.5 Interpretation of results

A threshold is typically determined as the observed percentage purity assuming all the seeds tested have germinated. This threshold corresponds to a LQL with an associated consumer risk of 5 % equal to:

$$L_{QL} = \frac{y}{y+(n-y+1)q_{0,95}^{F_{\nu_1,\nu_2}}} \quad \text{(B.1)}$$

where $y$ is the number of tolerant seeds observed out of $n$ tested and $q_{0,95}^{F_{\nu_1,\nu_2}}$ is the 0,95 quantile of the $F$ distribution with $\nu_1 = 2(n - y + 1)$ and $\nu_2 = 2y$ degrees of freedom.

When all the seeds do not germinate, the threshold on the observed tolerant seedlings (or on the non-tolerant seedlings) needs to be adjusted by considering the LQL determined assuming all the seeds are germinating. Given that the distribution of the number of tolerant seedlings is binomial with parameters the number of seedlings (i.e. the number of germinated seeds) and the true percentage of purity in the lot, the threshold on the seedlings can be calculated for a true percentage of purity equal to the LQL.

For example, for a test where the threshold is 98 %, the LQL is 96,42 %. Suppose that 390 seeds germinate out of 400 and that after treatment with the herbicide at an appropriate rate, five die out of the 390 that were sprayed. Then the estimated trait purity is (390 – 5) / 390 = 98,7 % and the 95 % lower bound for the seed lot is 97,32 %. This lower bound is higher than the LQL of 96,42 %, and thus the seed lot is accepted.

A two-stage testing plan can be used to reduce producer risk. The decision to carry out a second stage test on a lot is conditioned on the result of the first stage of testing and therefore the statistics used for a single stage test cannot be used (e.g. by adding the results). The reject/accept criteria for two-stage testing can be determined using the “Qual Plan Design” tab of Seedcalc.

In order to simplify the performance and scoring of assays, a pass/fail table can be constructed showing the ranges of germination (seeds counted) and the number of deviant seeds allowed for a single stage test. Table B.6 is an example of such a table, using a 98 % count of 400 seeds as the baseline (LQL of 96,42 % for a consumer risk of 5 %). Calculations of low numbers of germinant seeds (<75 %) are not shown as such a situation would mean that the seed lot was not commercially saleable.

**Table B.6 — Example of an acceptance table for a test for 98 % tolerant seeds (by count)**

| Test 1 | | |
|---|---|---|
| **Nominal number seeds tested** | **Number germinating** | **Non-tolerant (deviant) seedlings** |
| 400 | 400 | ≤ 8 |
| 400 | 364 to 399 | ≤ 7 |
| 400 | 328 to 363 | ≤ 6 |
| 400 | 291 to 327 | ≤ 5 |

Table B.7 is an example of a table constructed for two-stage testing with an equal number of seeds at each stage. In this example, the required estimated trait purity by count of germinating seeds is 98 % or greater and the LQL is 96,4 % with a consumer risk of 5 % or less. Both criteria shall be met for an acceptable testing plan. Table B.7 gives only a few two stage testing plan examples and is not meant to be an exhaustive list of all testing plans meeting these criteria. Calculations of low numbers of germinant seeds (<75 %) are not shown because the seed would not normally be commercially saleable.

**Table B.7 — Example of an acceptance table for a two-stage test for 98 % tolerant seeds (by count)**

| Nominal number seeds tested in each stage | Number germinating in each stage | Non-tolerant seedlings accepted in first stage | Non-tolerant seedlings in first stage for retest | Non-tolerant total seedlings in first and/or second stage to reject lot |
|---|---|---|---|---|
| 400 | 400 | ≤ 8 | 9 to 16 | > 16 |
| 400 | 375 to 399 | ≤ 7 | 8 to 15 | > 15 |
| 400 | 350 to 374 | ≤ 6 | 7 to 14 | > 14 |
| 400 | 331 to 349 | ≤ 6 | 7 to 13 | > 13 |
| 400 | 325 to 330 | ≤ 5 | 6 to 13 | > 13 |
| 400 | 301 to 324 | ≤ 5 | 6 to 12 | > 12 |

# Annex C
(informative)

# Estimation of the limit of detection for a testing plan to detect GM seeds/grains in seed lots

## C.1 General

This annex provides a procedure to estimate the limit of detection (LOD) for a testing plan using qualitative PCR methods for detecting GMO presence. The procedure has been elaborated by the Working Group 'Seed Testing' of the European Network of GMO Laboratories (ENGL)[ 8 ].

A testing plan specifies the required group size and the number of seed/grain groups. The LOD of a testing plan can be estimated according to the described procedure to check if it addresses the goal of the test.

This annex is only applicable for PCR testing with the assumption that at least 10 copies of a target DNA need to be present in the PCR reaction.

## C.2 Calculations

Seed/grain groups which are comprised of $m$ seeds/grains are taken from a laboratory sample. The number of seeds/grain groups is $n$. The seed/grain groups are analysed independently using a detection method with a false negative rate (due to for example genome size and sample volume and random sporadic blunders) $f_N$ and a low false positive rate. If one or more seed/grain groups produce a positive result then the presence of GM seeds/grains in the lot is indicated.

For a group sample from a laboratory sample taken from a lot that contains a proportion $L$ GM seeds/grains the probability of at least one positive result $p_D$ is given in Formula (C.1):

$$p_D = 1-[1-(1-f_N)(1-(1-L) \tag{C.1}$$

The LOD of the testing plan $L_D$ is the value of $L$ for which $p_D$ = 0,95.

Then the group size needed to achieve a LOD $L_D$ (or LOD for a testing plan) can be directly estimated using Formulae (C.2) and (C.3):

$$m = \frac{\log\left(1-\frac{1-0{,}05^{1/n}}{1-f_N}\right)}{\log(1-L_D)} \tag{C.2}$$

$$L_D = 1-\left(1-\frac{1-0{,}05^{1/n}}{1-f_N}\right)^{1/m} \tag{C.3}$$

This approach is similar to that implemented on the "Qual Impurity Estimation" tab in Seedcalc[ 16 ].

## C.3 The effect of genome size on the number of analytical group size

PCR methods tend to reliably (at least 95 % of the time) give a positive response where a few copies of target DNA are present in a reaction. A fixed mass $M$ of DNA is delivered to each PCR. If we consider that we need to be confident of at least 10 copies[ 8 ] being present in each PCR from each single seed

in a seed/grain group for our PCR method to provide a probability of detection of at least 95 % for a GM seed, then, from the Poisson distribution, we require that the DNA contains an expected average of 16,7 genomes from each seed, i.e. that the group size *m* shall be at least that given in Formula (C.4):

$$m \leq \frac{g \times M}{16,7} \quad \text{(C.4)}$$

where *g* is the number of genome copies per ng of DNA and *M* is the mass of DNA delivered to each PCR. If 200 ng of DNA are delivered to each PCR reaction, *M* is 200 ng.

Examples for test plans to detect the presence of GMO seeds in a maize seed lot with a limit of detection of 0,1 % are as follows.

a) For a test plan with a $L_D$ of 0,1 % we require that the analytical method has a zero false negative rate if there is one GM seed in a 2 995 seed sample [according to Formula (C.2)]. If 200 ng of DNA is taken for the PCR reaction, then a total of 38 536 haploid maize genomes are analysed (see Table C.1). If at least one GM seed is present in the 2 995 seeds working sample, then 12 haploid GM genomes are expected to be present in the PCR (based on the simple calculation: number of haploid maize genomes divided by number of seeds). Hence, under this scenario of the analytical performance that we would require for the PCR method that $f_N$ = 0,1 haploid GM genomes are expected to be present in 200 ng of DNA.

b) If the detection method has a $f_N$ = 0,005 in a PCR test for 12 or more haploid GM genomes per 200 ng DNA, then the detection method can be applied in a test plan that provide a $L_D$ of 0,1 % by taking a working sample of 3 095 seeds [according to Formula (2)]. This working sample is divided into two equal group samples. Each group sample is grinded and each of the three flours is tested for GM presence can be tested with a method that has $f_N$ = 0,005 where 12 haploid GM genomes per 200 ng of DNA are present.

Table C.1 gives the expected number of copies of DNA in an extract containing 200 ng of DNA and the estimated volume per seed for a number of products.

**Table C.1 — Expected size of seeds/grains and number of DNA copies per extract**

| Common name | Scientific name | 1 000-seed mass g [a] | Bulk density kg·m$^{-3}$ | Average volume per seed ml | Genome copies in 200 ng DNA [b] |
|---|---|---|---|---|---|
| Papaya | *Carica papaya* | 23 | 440 [f] | 0,052 3 | 259 740 |
| Rice | *Oryza sativa* | 27,5 | 579 [d] | 0,047 5 | 227 272 |
| Sugar beet | *Beta vulgaris* | 16,5 | 447 [c] | 0,036 9 | 127 388 |

[a] http://data.kew.org/sid/ [26].

[b] Arumuganathan K., and Earle E.D. Nuclear DNA content of some important plant species. *Plant Mol. Biol. Rep.* 1991, **9**(3) pp. 208–218[27].

[c] Dursun I., Tugrul K.M, and Dursun E. Some physical properties of sugar beet seed. *J. Stored Products Res.* 2007, **43** pp. 149–155[28].

[d] Jayas D.S., and Cenkowski S. Grain Property Values and Their Measurement. *Handbook of Industrial Drying* (ed. Mujumdar A.S.), pp. 575–603, 2006, CRC Press, Boca Raton, FL[29].

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[f] Athmaselvi K.A., Jenney P., Pavithra C., and Roy I. Physical and biochemical properties of selected tropical fruits. *Int. Agrophys.* 2014, **28** pp. 383–388[31].

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[h] Yalcin I., Ozarslan C., and Akbas T. Physical properties of pea (*Pisum sativum*) seed. *J. Food Eng.* 2007, **79**(2) pp. 731–735[33].

[i] Median value of bulk density was used to calculate.

| Common name | Scientific name | 1 000-seed mass g [a] | Bulk density kg·m$^{-3}$ | Average volume per seed ml | Genome copies in 200 ng DNA [b] |
|---|---|---|---|---|---|
| Tomato | *Lycopersicon esculentum* | 1,97 | 300 [e] | 0,006 6 | 101 010 |
| Soybean | *Glycine max* | 185 | 772 [d] | 0,239 6 | 86 580 |
| Oilseed rape | *Brassica napus* | 3,3 | 669 [d] | 0,004 9 | 81 632 |
| Alfalfa | *Medicago sativa* | 2 | 772 [d] | 0,002 6 | 63 898 |
| Cotton | *Gossypium hirsutum* | 93,5 | 481 to 589 [g] | 0,174 8 [i] | 42 918 |
| Maize | *Zea mays* | 245,4 | 721 [d] | 0,340 4 | 38 536 |
| Sunflower | *Helianthus annuus* | 38,8 | 412 [d] | 0,094 2 | 31 847 |
| Pea | *Pisum sativum* | 139,9 | 712,1 [h] | 0,196 5 | 23 135 |
| Barley | *Hordeum vulgare* | 41,8 | 618 [d] | 0,067 6 | 19 802 |
| Wheat | *Triticum aestivum* | 37,7 | 772 [d] | 0,048 8 | 6 044 |

[a] http://data.kew.org/sid/ [ 26 ].

[b] Arumuganathan K., and Earle E.D. Nuclear DNA content of some important plant species. *Plant Mol. Biol. Rep.* 1991, **9**(3) pp. 208–218[ 27 ].

[c] Dursun I., Tugrul K.M, and Dursun E. Some physical properties of sugar beet seed. *J. Stored Products Res.* 2007, **43** pp. 149–155[ 28 ].

[d] Jayas D.S., and Cenkowski S. Grain Property Values and Their Measurement. *Handbook of Industrial Drying* (ed. Mujumdar A.S.), pp. 575–603, 2006, CRC Press, Boca Raton, FL[ 29 ].

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[f] Athmaselvi K.A., Jenney P., Pavithra C., and Roy I. Physical and biochemical properties of selected tropical fruits. *Int. Agrophys.* 2014, **28** pp. 383–388[ 31 ].

[g] Ramesh B., Sanjeeva R.B., Veerangoud M., Anantachar M., Sharanagouda H., and Shanwad UK. Properties of Cotton Seed in Relation to Design of a Pneumatic Seed Metering Device. *Indian J. Dryland Agr. Res. and Dev.* 2015, **30**(1) pp. 69–76[ 32 ].

[h] Yalcin I., Ozarslan C., and Akbas T. Physical properties of pea (*Pisum sativum*) seed. *J. Food Eng.* 2007, **79**(2) pp. 731–735[ 33 ].

[i] Median value of bulk density was used to calculate.

## C.4 The effect of seed size on sample preparation

In order to extract the DNA seeds/grains shall be finely ground. In addition to the effect of genome size, if the volume $v$ of the group is larger than the capacity of the grinder $V$ then each group sample shall be split into $h_{ss}$ grinding samples prior to being ground, where, according to Formula (C.5)

$$h_{ss} \geq \frac{m \times v}{V} \quad \text{(C.5)}$$

where

$m$ is the number of seeds/grains in a group;

$v$ is the volume of a seed in ml;

$V$ is the capacity of the grinder.

If for an example a grinder with a volume of 1 l is used, these provide a usable capacity of approximately 800 ml ($V$ = 800 ml).

# Annex D
(informative)

# Experimental determination of maximum group size

## D.1 General

This annex provides a procedure to determine the maximum group size through experiments conducted at the laboratory. This procedure is independent from the analytical method used in the laboratory, therefore it is suitable for DNA as well as for protein-based tests.

This annex has been elaborated by members from the ISTA GMO Committee and is one of the components of the ISTA approach for the design and the analysis of GMO testing plans.[ 34 ]

## D.2 Experimental procedure

In this approach, the maximum group size is defined as the group size with an acceptably low FNR. For example, a group size of 3 000 seeds might yield a FNR of 15 % which may be unacceptable, whereas a group size of 300 seeds could yield a lower FNR of 4 % which can be acceptable. There exist numerous testing plans which are robust for a FNR less than or equal to 5 %, i.e. for which the consumer and producer risks are maintained below a target value, whatever the FNR is in the 0 % to 5 % range.

An experiment is therefore set up by the laboratory for the assessment of the FNR where positive samples (seed/grain groups) are prepared by spiking a single GM seed in a group of conventional seeds of size ($m$ – 1). If the outcome from the experiment provides sufficient confidence that the true FNR is less than or equal to 5 %, then m is an acceptable group size. If not, the group size needs to be revised (i.e. group size reduction) and a new assessment of the FNR needs to be set up again.

One possible experiment design for the FNR assessment which minimizes the laboratory workload is the following:

— Thirty positive groups (one positive seed spiked into each group) are prepared and blindly tested; if all the tests are positive, then there is sufficient confidence (i.e. 79 %) that the true FNR is less than or equal to 5 %.

— If there is one negative group, then an additional 30 positive groups are prepared and tested. If all the additional tests are positive, then there is sufficient confidence (i.e. 71 %) that the true FNR is less than or equal to 5 %.

— If more than one negative group is found, then a revision of the group size is needed.

Increasing the number of groups to be tested allows higher confidence levels and/or the assessment of lower FNRs. For example, if 60 groups are tested, and no negative groups are found, it can be concluded that there is 95 % confidence that the true FNR is less than or equal to 5 %. On the other hand, if 300 groups are tested, and no negative groups are found, it can be concluded that there is 95 % confidence that the true FNR is less than or equal to 1 %.

Multiple combinations of FNRs and FPRs can be acceptable regarding consumer and producer risk targets for a given testing plan. The robust option in the Find Plan procedure of the Seedcalc Qual Plan Design worksheet allows to find testing plans which satisfy the consumer and producer requirements over a range of FNRs and FPRs. For example, consider a testing plan with 11 groups of 500 seeds and a maximum deviant groups equal to 7 as an acceptance criterion. The consumer and producer risks for a LQL and AQL equal to 0,5 % and 0,1 % respectively will then be less than or equal to 4,15 % and 4,23 % respectively whatever the FNRs and the FPRs are in the 0 % to 5 % range.

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