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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 and definitions

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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).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

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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.

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 and definitions

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 terminological 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

3.2
AQL
A_{QL}
acceptable quality limit
level of impurity that is acceptable to the producer and that production practices can support

3.3
consumer risk
consumer (beta) risk
probability of accepting a lot at the *lower quality limit* (3.10)

3.4
deviant seed/grain
considered non-conforming based on the presence or absence of a specific trait or characteristic

Note 1 to entry: For the purpose of this document, a deviant seed is considered to possess a GM characteristic that is not expected or is unintended based on the expected or known GM characteristics of the seed/grain.

3.5
false negative rate
FNR
probability that a known positive (seed/grain group) *test sample* (3.20) has been classified as negative by the method

Note 1 to entry: The false negative rate is the number of misclassified known positives divided by the total number of positive *test samples* (3.20).

[SOURCE: ISO 16577:2016, 3.63, modified — the abbreviation has been added, "positive test sample" has been changed to "positive (seed/grain group) test sample", and the formula has been deleted.]

3.6
false positive rate
FPR

probability that a known negative (seed/grain group) *test sample* (3.20) has been classified as positive by the method

Note 1 to entry: The false positive rate is the number of misclassified known negatives divided by the total number of negative *test samples* (3.20).

[SOURCE: ISO 16577:2016, 3.65, modified — the abbreviation has been added, "negative test sample" has been changed to "negative (seed/grain group) test sample", and the formula has been deleted.]

3.7
group size
number of seeds/grains comprising a group

3.8
group testing
statistical evaluation of analyte contents based on qualitative analysis results (i.e. positive or negative) from each seed/grain group in the *test sample* (3.20)

3.9
laboratory sample
sample or subsample(s) received by the laboratory

Note 1 to entry: The seed/grain sample received is expected to represent the *seed/grain lot* (3.18).

[SOURCE: ISO 16577:2016, 3.89, modified — Note 1 to entry has been added.]

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.

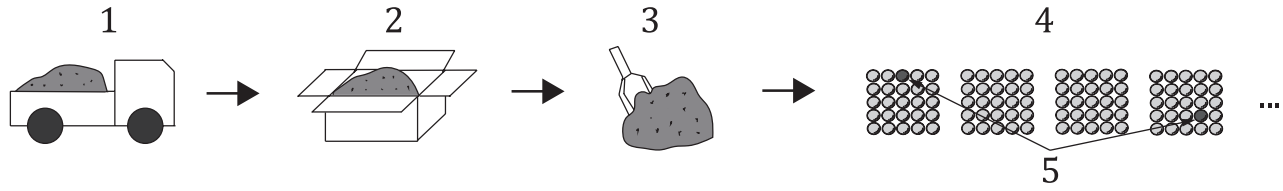
4 Principle

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 (e) 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/

