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Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Polymerase chain reaction (PCR) based screening strategies

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National foreword

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Produits alimentaires - Méthodes d'analyse pour la détection des organismes génétiquement modifiés et des produits dérivés - Stratégies de criblage basées sur l'utilisation de la réaction de polymérisation en chaîne (PCR)

Lebensmittel - Verfahren zum Nachweis von gentechnisch veränderten Organismen und ihren Produkten - Strategien für das Screening mit Polymerase-Kettenreaktion (PCR)

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Foreword

This document (CEN/TS 16707:2014) has been prepared by Technical Committee CEN/TC 275 “Food analysis - Horizontal methods”, the secretariat of which is held by DIN.

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Introduction

Largely, detection of materials derived from genetically modified organisms (GMOs) in a given sample employs polymerase chain reaction (PCR) analysis, specifically real-time PCR.

A general strategy for GMO detection and identification by means of PCR analysis and a stepwise approach is described.

In initial screening analysis, DNA sequences of genetic elements common to many GMOs are targeted. According to its purpose, screening is a test to rapidly and reliably sort samples into groups. Once the samples are grouped, screening facilitates and potentially reduces subsequent analytical work and results interpretation. The screening strategy should be adjusted to the scope (food, feed or seed, crop-specific etc.) of the test(s).

This document takes the general principle of GMO detection strategies as a basis and describes the underlying analytical steps for complex screening (known as the matrix-approach [2]).

The document is written primarily for screening strategies applying real-time PCR methods. Other PCR methodologies may be applicable in the same way.

The terms “screening method” and “screening strategies” are not interchangeable and have different meanings in this document.

1 Scope

This Technical Specification describes screening strategies for the detection of genetically modified (GM) DNA in food products by means of PCR methods. The strategies have been established for food matrices, but it can also be applied to other matrices (e.g. feed, seed and samples from field grown plants).

Detection of GM DNA is based on PCR methods targeting segments of transgenic DNA sequences (genetic elements, genetic constructs or insertion sites of transgenes). Various combinations of these PCR methods are involved in screening strategies. The methods are applied simultaneously or hierarchically. The general strategy is based on the matrix approach. Examples for the implementation and application of this approach are described.

In order to ensure reliable analytical results, the document also provides guidelines for the validation of the performance of qualitative PCR methods applied in screening approaches.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN ISO 21569, *Foodstuffs - Methods of analysis for the detection of genetically modified organisms and derived products - Qualitative nucleic acid based methods (ISO 21569)*

EN ISO 21570, *Foodstuffs - Methods of analysis for the detection of genetically modified organisms and derived products - Quantitative nucleic acid based methods (ISO 21570)*

EN ISO 21571, *Foodstuffs - Methods of analysis for the detection of genetically modified organisms and derived products - Nucleic acid extraction (ISO 21571)*

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

3 Terms and definitions

For the purposes of this document, the terms and definitions given in EN ISO 24276 and the following apply:

3.1
GMO method matrix
relational presentation (e.g. a table) of symbols or numbers

Note 1 to entry: One dimension (e.g. columns) corresponds to genetic elements and genetic constructs detected by a defined PCR method and the other dimension (e.g. rows) corresponds to GM events. The entered symbols or numbers indicate the detectability or non-detectability of the target sequence for the GM event.

Note 2 to entry: The term matrix is commonly used for a defined composition of food, but this definition is not relevant here.

3.2
GMO target matrix
relational presentation (e.g. a table) of symbols or numbers

Note 1 to entry: One dimension corresponds to genetic elements or genetic constructs present in a GMO and the other dimension (e.g. rows) corresponds to GM events. The entered symbols or numbers indicate the presence or absence of the target for the GM event and copy number, if available.

Note 2 to entry: In contrast to GMO method matrix, the GMO target matrix is independent from a detection method.

3.3
screening method
method that rapidly and reliably eliminates (screens) a large number of negative (or positive) test samples and restricts the number of test samples requiring the application of a rigorous method

Note 1 to entry: In this document, a screening method refers to PCR methods detecting the presence of several GMOs in one test.

3.4
element-specific method
method that targets a single discrete DNA sequence of a specific genetic element

Note 1 to entry: A genetic element is a part of a gene, for example a promoter, terminator, intron or a coding sequence. Elements are often derived from naturally occurring viruses, bacteria, plants, etc.. However, elements in GMOs are commonly modified at the sequence level, relative to the original (natural) source, e.g. by altered codon-usage or specific single nucleotide polymorphisms (SNPs). This sequence modification may be based on adaptation of the nucleotide composition to the new host genome.

3.5
construct specific method
method that targets a combination of inserted DNA sequences that are only found in GMO-derived material composed of at least two elements that do not naturally co-exist in this conformation, and where the 5' and 3' end of the sequence are derived from separate genetic elements

3.6
event specific method
method that detects a specific sequence that is only present in that event

Note 1 to entry: The event may be the result of either a rearrangement or unique combination of insert and insertion locus during the transformation.

Note 2 to entry: This is commonly targeted at the integration-border region.

3.7
probability of detection (POD)
probability of a positive analytical outcome for a qualitative method for a given matrix at a given concentration

Note 1 to entry: It is estimated by the expected proportion of positive results for the given matrix at the given analyte concentration.

4 Principle

Total DNA is extracted from the sample by a suitable extraction method. The DNA quantity and quality shall be checked as specified in EN ISO 21571 to ensure the presence of sufficient analyte and to assess the presence of PCR inhibitors that could inhibit PCR amplification.

Subsequently, a set of validated PCR methods are selected and applied in a decision tree approach, for detection and identification of genetic modifications linked to the sample. This approach enables the user to choose between different courses of action. Commonly, screening starts with element-specific PCR tests prior to construct-specific and/or event-specific tests. Before, in parallel or after screening, taxon-specific tests are recommended.

Based on the combination of results at each level of the decision tree the total number of tests required for the analysis is reduced.

5 Reagents

5.1 General

The requirements and conditions for nucleic acid extraction and PCR analysis laid down in detail in EN ISO 24276, EN ISO 21569, EN ISO 21570 and EN ISO 21571 shall be followed. Only analytical grade reagents suitable for molecular biology shall be used.

5.2 PCR reagents

5.2.1 Concentrated PCR buffer solution[1)] (containing reaction buffer, dNTPs, $MgCl_2$ and Hotstart *Taq* polymerase) or equivalent.

5.2.2 Oligonucleotides at the concentrations specified in the method protocol.

6 Apparatus and equipment

See EN ISO 24276, EN ISO 21569, EN ISO 21570 and EN ISO 21571.

7 PCR analysis

7.1 General

A large number of validated PCR methods applicable for the detection of GM DNA in food samples are available in EN ISO 21569, EN ISO 21570 or are compiled in [3] and [4]. The complexity of these methods can be divided in four categories according to the targeted DNA sequence:

— taxon-specific (for the detection and identification of DNA of a species or a taxonomical group);

— element-specific (for the detection and identification of genetic elements present in more than one GMO);

— construct-specific (for the detection and identification of specific combinations of genetic elements present in a GMO as a result of genetic engineering);

— event-specific (for the detection and identification of the insertion site specific for a GM event).

Testing laboratories develop a strategy for selecting the methods according to their scope (food, feed or seed testing), and requirements of their clients (e.g. government authority or company). In addition, the incidence of products on the market containing or consisting of new GMOs may require the ad hoc implementation of new methods into an existing screening strategy in order to incorporate the detection and identification of the respective GM target DNA.

To confirm the identity of the PCR product generated by the PCR method, the method shall include a step for verification of the amplicon by an appropriate technique (e.g. probe hybridization, subsequent DNA sequence analysis or restriction enzyme digestion of the PCR product, respectively).

7.2 Screening

7.2.1 General

The first step of PCR based screening analysis is the selection of a defined set of methods targeting DNA-sequences present in several GM events. The set of methods selected depends on the availability of (validated) methods, the capability of the laboratory and the scope of the testing.

1) Ready-to-use reagent mixtures or single components may be used as PCR buffer solution. If other reagent mixtures are used than the ones stated in the method validation report, they should give comparable to or better results.

The selection of methods should allow a maximum of information obtained from a minimum number of the tests performed in the first step. The selection of methods should also allow a maximum coverage of the GM events under scrutiny, when applicable.

Selected methods should be evaluated for their sensitivity (8.2) and specificity (8.3).

7.2.2 Combination of targets

Different combinations of targets can be chosen depending on the purpose of the analysis.

If genetic elements or constructs commonly present in GMOs are targeted, a 'GMO target matrix' or a 'GMO method matrix' can be established for the interpretation of the combined outcome of these tests (Table 1). Practical examples of application of a GMO method matrix are described [5], [6], [7], [9] and [10], an example for an available GMO target matrix is given in Reference [7].

Table 1 is an example of a GMO method matrix used in screening for various GM events. The matrix is based on experimental or theoretical (in silico) verifications. The example tabulates five screening methods ("ScreenMeth" A to E) and seven GM events. Each GM event has a distinct profile of positive (denoted by a '+' representing 'target detected') and negative (denoted by a '-' representing 'target not detected') reactions with the set of screening methods [2]. The outcome of the PCR tests performed with the five methods allows considering the presence of the GM event(s) in the sample. For instance, if positive results are obtained with method A and B and negative results with methods C, D and E it can be assumed that "GM event 2" is present in the sample, and not the other GM events.

The use of a 'GMO method matrix' should indicate the copy number of the target sequence in the GM event and the absolute LOD (see 8.2) of each screening method, if available. Otherwise, it could lead to erroneous conclusions concerning the GMO(s) actually present in the sample. For example, a negative result by method A and a positive result by method B may be a consequence of (i) the disproportional copy number of the target sequence present in the GM event and/or of (ii) significantly different absolute LODs of both methods.

Table 1 — Example of a GMO method matrix

	Analytical method				
GM event	**ScreenMeth A**	**ScreenMeth B**	**ScreenMeth C**	**ScreenMeth D**	**ScreenMeth E**
GM event 1	+	+	+	-	-
GM event 2	+	+	-	-	-
GM event 3	+	+	-	+	-
GM event 4	+	-	-	+	-
GM event 5	-	+	+	-	-
GM event 6	-	-	-	-	+
GM event 7	+	+	-	-	+

The numbers of GMOs and PCR methods included in the GMO method matrix are flexible. Each variable can be increased or decreased according to the (1) available information, (2) needs for the specific analysis and (3) requested coverage. For the efficient use of GMO method matrices it is important to distinguish which species DNAs are present, in order to discriminate and reduce the number of required tests. For example, if the food sample consists of only a single species, the design and subsequent use of the GMO method matrix can be filtered for the GMOs belonging to this species group.

Alternatively, the coverage of GMOs that are potentially detected in the first screening step can be categorized based on the origin of the product (region, country, continent etc.) and available information on the use of GMOs to that geographic region.

Multiplex PCR methods (or other developed analysis platforms) are available for selected combinations of screening targets for example in [8], [11] and [15].

If methods have significantly different absolute LODs (e.g. > 10 copies), they should not be combined in the same GMO method matrix.

NOTE 1 According to the selected methods, the outcome may lead to a group of GM events instead of a single one.

NOTE 2 If different amounts of diverse target-species DNA in composite food products are determined, this may lead to disparate practical LODs for the applied tests. Assorted absolute LODs of the detection methods also need to be considered as this may also lead to dissimilar practical LODs.

7.2.3 Analysis of the output of the first screening

The GMO method matrix can be used to design decision trees. The pattern of the output (detection of specific screening targets) indicates the presence or absence of one or several GM events listed in a method matrix. The information obtained in a first round of screening can then be used to decide, which targets should be selected for a second round of screening.

The target taxon(s) of the DNA under study shall be identified. The determination of the species present in the sample could be done prior to, in parallel with, or after the first screening phase. If relevant, further quantification shall be undertaken in order to assess the practical LOD.

After the first round of screening, the next step in the decision tree process is to consider the following:

— If the sample is negative, no further analysis is required;

— If the sample is positive,

  — and the number of suspected events is low, event specific tests or tests for identification of the event(s) should be performed,

  — and the number of suspected events is high, a second round of screening may be required according to the species determined.

For output analysis, it shall be considered that more than one GMO or stacked GM events could be present, as well as a GMO(s) not listed in the GMO method matrix.

7.2.4 Additional screening tests

Further screening may be useful, where the output of the first screening round enables the sorting of GMOs into groups of events distinguishable by presence/absence of another target (e.g. a specific construct of genetic elements specific for a group of GM events). This additional screening test(s) could reduce the number of GM event-specific tests. For example, decision tree tools are described for GMO detection in plant products [4], [5], [6], [7], [8], [13] and [14].

7.3 GM event identification

7.3.1 Event specific tests

Neither interpretation nor conclusion about the presence of an event shall be made without stating the event-specific method used.

It may not be possible after the initial screening analysis to determine all the GM events potentially present in the sample by additional screening tests. If this is the case, the next step in the analysis is to set up tests for GM event identification.

Event-specific PCR methods are used to identify the GM event(s) unambiguously.

Different platforms can be used for GMO identification, either as individual event-specific tests or as groups of events in (i) prepared multi-well PCR strips or plates [8], [12], or (ii) multiplex PCR assays [11], [15]. The use of multi-well strips or plates may be also an applicable strategy for direct analysis of a sample for GMO identification. In some events, a genetic element or construct could be present in more than one copy per haploid genome. Therefore, if the GM DNA content is low, it is possible to obtain a positive result in the screening test, but a negative one in the event-specific test that is by definition a single copy target sequence.

NOTE Occasionally a unique genetic construct is present in a single GM event or a certain group of GM events. Assignment of a GMO to the corresponding group can then be achieved also by an appropriate construct-specific method, although the specific identity of the GMO cannot be determined unambiguously.

7.3.2 Additional tests

The output from the GMO identification step may not completely explain the results of the screening. Thus, additional tests can be performed to reduce the room for interpretation:

— It is recommended to first sum up the output of the taxon-specific tests. Then, consider the presence of other species than those indicated (or apparently present in the sample material). Traces of material from other species than those indicated could be caused by adventitious and technically unavoidable admixture during primary production (harvest, transport, storage). In addition, the contact to GMO-derived packaging materials (starch, cotton, flax, etc.), or the content of spices should be considered.

— The copy numbers of the targeted elements could be disproportionately present in some GMOs (see 7.2.2). This condition needs to be considered for the decision of further required testing.

— If a positive result is obtained with a method targeting a single genetic element and this signal cannot be related to the presence of a GMO, it is useful to test the sample for the presence of DNA derived from the donor organism (e.g. testing for CaMV in case of a positive P-35S signal not explained by the GMO identification step).

Some widely used GMOs occur frequently and their presence should be considered in some food products.

NOTE PCR methods are available to rule out the possibility that the detected signal is due to the presence of DNA derived from naturally occurring organisms [16], [17] and [18].

7.4 Interpretation of PCR results

7.4.1 General

Results from all test portions of a sample shall be consistent. When at least one test portion gives a positive result and at least one gives a negative result, the analysis shall be repeated [see EN ISO 24276].

NOTE 1 If possible, the quantity of template DNA in the reaction can be increased, in order to get consistent results for both test portions.

NOTE 2 Verification of a positive detection is obtained e.g. by sequence-specific hybridization of the real-time hydrolysis probe.

7.4.2 Interpretation of results at the limit of detection (LOD)

For the interpretation of results obtained with the matrix approach using screening tests it should be considered that the practical LOD depends on (i) the method performance, (ii) the quantity of the target taxon DNA and (iii) the number of target sequence copies per haploid genome in the GM event(s) present in the sample [EN ISO 24276]. The copy number of individual targets within the same GM event may vary. Thus, the observed output pattern (positive and negative test results) could also display negative test results for those targets present in lower quantity and below the LOD.

An acceptable signal threshold to ensure unambiguous results may be established: one possibility is to set it to 2 X the absolute LOD (as determined in the validation of the method) [2]. Alternatively a control DNA sample containing the target at a defined concentration (close to or at the absolute LOD) can be used [19].

For a better comparison of test results, laboratories should also report the practical limit of detection (LOD_{prac}) when appropriate. This requires testing for a species-specific target by a validated reference gene specific real-time PCR method.

The LOD_{prac} for genetically modified material can be determined in accordance with EN ISO 24276 and calculated for each sample using Formula (1):

$$LOD_{prac}\,(\%) = \frac{{}_{GM}LOD_{abs} \quad x100}{{}_{Ref}copies_{sample}} \tag{1}$$

with

${}_{GM}LOD_{abs}$ is the LOD of the real-time PCR method used for target detection (in copies per PCR); for calculations it is reasonable to set the absolute LOD at 10 copies, because experimental evidence from validations of GMO detection methods show that the LOD_{abs} is in the range of 5 to 10 copies.

${}_{Ref}copies_{sample}$ is the amount of amplifiable species-specific reference gene copies in the sample (copies per PCR), determined by means of real-time PCR method targeting a species-specific reference gene.

NOTE The overall interpretation of the results (combination of the different results from different PCR tools) is linked to the absolute LOD of the screening methods. For example, a negative result does not obligatory mean that the target DNA is absent from the tested sample, but the quantity might be below the LOD of the method used.

8 PCR method performance criteria and validation

8.1 General

General method performance criteria and guidelines for validation of qualitative methods are described in EN ISO 24276 and in Codex Alimentarius standard [20]. Method performance criteria particular for both event-specific PCR methods and DNA extraction methods are described in the ENGL document 'Definition of minimum performance requirements for analytical methods of GMO testing' [21].

A qualitative PCR should be validated as much as possible in the same way as it is intended to be used for routine analyses — that means the sensitivity of the method should be shown to be such that it can reliably detect a positive sample. By their very nature, qualitative test results refer to the detection above/below a detection limit. The absolute limit of detection (LOD_{abs}), specificity and robustness of a PCR method should be validated *in-house* (single laboratory), prior to use in a GMO method matrix. Furthermore performance data on the robustness, the false-positive rate and the false-negative rate of a screening method can be obtained in a collaborative validation study.

Additional performance characteristics, such as probability of detection (POD), may also be used to provide meaningful information about the performance of qualitative methods in collaborative studies. At present, POD is the only additional performance characteristic available. However, more may arise in the future.

Ideally screening methods should be validated by a collaborative trial (see EN ISO 24276), which is conducted to assess the performance of a method, according to the International Harmonised Protocol [22]. If the method has been already validated by collaborative trial, it should then be verified *in-house* prior to use in a GMO method matrix. This process is required to ensure that the chosen method shows performance characteristics similar to those assessed in the collaborative trial. Then for qualitative PCR methods, it is only necessary to verify the absolute LOD; it is not required to repeat the specificity or robustness experiments in a verification study [23].

For evaluation and comparison of different PCR methods (element-, construct- and/or event-specific methods) applied in screening strategies, it is recommended that DNA concentration is measured using the same technique (e.g. UV spectrophotometry, PicoGreen® etc.).

The DNA quality extracted from different matrices should be taken into consideration.

8.2 Absolute limit of detection (LOD_{abs})

The absolute limit of detection is the lowest amount of analyte in a sample, which can be reliably detected, but not necessarily quantified. Experimentally, methods should detect the presence of the analyte at least 95 % of the times at the LOD, ensuring ≤ 5 % false-negative results [19], [20], [21].

For each screening method, the absolute LOD needs to be assessed with at least two different GM events containing the target sequence.

Experimental evidence from validations of real-time PCR methods show that the LOD_{abs} is usually in the range of 5 to 10 copies [2].

For qualitative real-time PCR methods, it is recommended to estimate the LOD by testing at least 10 replicates of DNA samples at nominal concentrations of e.g. 10, 5, 2, 1 copies and a tenfold dilution of 1 copy of the target sequence. The estimated LOD_{abs} is then the lowest concentration where all replicates are positive. The probability distribution suggests that 1 copy should give approximately 30 % negative results. Therefore, as a requirement to verify that the copy numbers of the dilution series are approximately correct, at least one replicate shall be negative for the 1 copy dilution [24].

For the assessment of the absolute LOD alternative approaches may arise in the future and may be used if they improve the accuracy of the determination.

8.3 Specificity and reference materials

A PCR method should respond exclusively to the target sequence of interest [21].

The specificity of the PCR method shall be verified by *in silico* studies using publicly available sequence databases (e.g. EMBL, GenBank, etc.). The source of the DNA sequence information and the date of the *in silico* study shall be reported.

PCR methods shall be in-house validated, preferably by experimental verification using appropriate control samples. These can be Certified Reference Materials (CRMs), or — if suitable reference materials are not available — the verification shall be performed using a combination of *in silico* (bioinformatics) analysis and experimental analysis, by using other sufficiently defined materials, e.g. characterized material from interlaboratory validation studies, from certified seeds or synthetic positive controls.

The absence of amplification products with non-GM DNA of the closest related taxa and of the most important food crops shall be experimentally demonstrated for the screening methods.

A PCR screening method shall not produce amplification signals with target sequences different from the target sequence for which the method was developed. If element-/construct-specific methods show *in silico* sequence identities or empirical cross-reactivity to other sequences, this shall be only acceptable if the method remains ´fit for purpose‘. This information shall be given in the scope of the method.

Information on the specificity experiments performed should be documented, e.g. reference to the validated method, specification of the sequence data (GenBank accession number) and/or the reference material used.

When testing the specificity of a screening method with DNA of a target GM event, it is recommended to conduct the test with 100 copies of the target DNA. For tests with DNA of a non-target GM event, approximately 2.500 copies of the haploid genome are recommended. Unexpected positive results with CRMs

can occur in these tests if a trace of another GMO and of its genetic element(s) or construct(s) is present and is detected. CRMs are verified for the presence of a specific GM event but not for the absence of other possibly contaminating GM events.

8.4 Robustness

The robustness of a method is a measure of its capacity to remain unaffected by small, but deliberate deviations from the experimental conditions described in the procedure [20].

For a PCR method used in the GMO method matrix, the following factors should be tested: thermal cycler or real-time PCR instruments (preferably different brands), master mixes (different brands and volumes), primer concentration, probe concentration (when applicable), annealing temperature.

The method shall provide the expected results when small deviations are introduced from the experimental conditions described in the procedure.

8.5 False-positive rate and false-negative rate

The false positive rate is the probability that a known negative test sample has been classified as positive by the method [20]. For convenience this rate can be expressed as percentage:

% false-positive results = 100 × number of misclassified known negative samples / total number of known negative samples

The false negative rate is the probability that a known positive test sample has been classified as negative by the method. For convenience this rate can be expressed as percentage:

% false-negative results = 100 × number of misclassified known positive samples / total number of known positive samples

The collaborative validation study should demonstrate that a rate of false-negative results of 5 % or less is achieved. False-positive results above 5 % should be clarified and evaluated from case to case.

8.6 Probability of Detection (POD)

The POD assessment is an optional approach to obtain additional performance data for a qualitative PCR method at given target sequence concentrations close to the LOD_{abs} [25], [26], [27], [28]. It is done preferably in the collaborative validation study. To obtain data for the determination of the POD participating laboratories measure replicate DNA samples at increasing GM target concentrations in the range close to the LOD_{abs}. With an appropriate design of the study, POD response curves can be calculated and could give an overview of the performance of the PCR method. This response curve can be compared to the “ideal curve” calculated on basis of the underlying probability (Poisson) distribution of the GM target across the concentration series. The POD can be calculated for all laboratories or separately for each single laboratory to identify outliers.

The POD response curve can also be used to calculate the average GM target concentration at POD = 0,95 (95 % probability of detection).

POD can be assessed in addition to, but not replacing, false-positive rate and provides additional information on the performance of the qualitative PCR method.

NOTE The relations between the average POD across laboratories, the reproducibility standard deviation of the POD and the interval within which the laboratories PODs are expected to lie are not the same as the analogous relations for measurement results (RSD_R) by quantitative methods in collaborative validation studies.

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