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Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Qualitative nucleic acid based methods

Produits alimentaires — Méthodes d'analyse pour la détection des organismes génétiquement modifiés et des produits dérivés — Méthodes qualitatives basées sur l'utilisation des acides nucléiques

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

ISO 21569 was prepared by the European Committee for Standardization (CEN) Technical Committee CEN/TC 275, *Food analysis — Horizontal methods,* in collaboration with Technical Committee ISO/TC 34, *Food products*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

Introduction

The search for a genetically modified origin of ingredients is performed by means of the following successive (or simultaneous) steps. After sample collection, nucleic acids are extracted from the test portion. Extracted nucleic acids can be further purified, simultaneously or after the extraction process. Afterwards, they are quantified (if necessary), diluted (if necessary) and subjected to analytical procedures (such as PCR). These steps are detailed in this International Standard and in the following series of International Standards with the general title *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products*:

— *Sampling* (ISO 21568);

— *Quantitative nucleic acid based methods* (ISO 21570);

— *Nucleic acid extraction* (ISO 21571).

Further information about general requirements and definitions involving the steps cited above are collected in ISO 24276.

The qualitative detection of DNA target sequences is performed in order to obtain a yes or no answer to the question whether a certain target sequence is detected or not relative to appropriate controls and within the detection limits of the analytical method used and test portion analysed.

The specificity of the methods, as described in Annexes A to D, ranges from screening methods to detect common DNA sequences characteristic of GMOs, to specific identification of a genetic construct or a specific transformation event.

The International Organization for Standardization (ISO) draws attention to the fact that it is claimed that compliance with this document may involve the use of a patent concerning the PCR technology.

ISO takes no position concerning the evidence, validity and scope of this patent right.

ISO has been informed that Applied Biosystems, Roche Molecular Systems, Inc. and F. Hoffman La Roche Ltd. hold patent rights concerning the PCR technology. The companies have assured the ISO that they are willing to negotiate licences under reasonable and non-discriminatory terms and conditions with applicants throughout the world. In this respect, the statements of the holders of these patent rights are registered with ISO. Information may be obtained from:

Licensing Department

Applied Biosystems
850 Lincoln Centre Drive
Foster City, CA 94404
USA

and

Roche Molecular Systems, Inc.
Licensing Department
1145 Atlantic Avenue
Alameda, CA 94501
USA

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights other than those identified above. ISO shall not be held responsible for identifying any or all such patent rights.

Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Qualitative nucleic acid based methods

1 Scope

This International Standard describes the procedure to qualitatively detect genetically modified organisms (GMOs) and derived products by analysing the nucleic acids extracted from the sample under study. The main focus is on polymerase chain reaction (PCR) based amplification methods.

It gives general requirements for the specific detection and identification of target nucleic acid sequences (DNA) and for the confirmation of the identity of the amplified DNA sequence.

Guidelines, minimum requirements and performance criteria laid down in this International Standard are intended to ensure that comparable, accurate and reproducible results are obtained in different laboratories.

This International Standard has been established for food matrices, but could also be applied to other matrices (e.g. feed and plant samples from the environment).

Specific examples of methods are provided in Annexes A to D.

2 Normative references

The following referenced documents are indispensable for the application 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 21571:2005, *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Nucleic acid extraction*

ISO 24276:—[1)], *Foodstuffs — Nucleic acid based 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 24276 apply.

1) To be published.

4 Principle of the method

4.1 General

Qualitative analysis consists of specific detection of target nucleic acid sequences in the test samples. Each method shall specify the target sequence.

A qualitative result shall clearly demonstrate the presence or absence of the genetic element under study, relative to appropriate controls and within the detection limits of the analytical method used and test portion analysed.

4.2 PCR amplification

Amplification of the target sequence occurs *in vitro* through a reaction catalysed by a DNA polymerase in the presence of oligonucleotide primers and deoxyribonucleoside triphosphates in a defined reaction buffer[1], [2]. An important prerequisite for the amplification of the target sequence is that the reaction mixture contains no polymerase inhibitors. Amplification of the DNA is a cyclical process consisting of

— denaturation of the double-stranded DNA into single-stranded nucleic acid by means of heating,

— annealing of the primers to the target sequence at a suitable temperature, and

— extension of the primers, which are bound to both single strands, by a DNA polymerase suitable for PCR, at an appropriate temperature.

4.3 Detection and confirmation of PCR products

PCR products are detected by gel electrophoresis or an appropriate alternative, if necessary, after isolation by means of a suitable separation procedure.

The identity of any detected target sequence can be verified by an appropriate technique (e.g. by restriction enzyme analysis, by hybridization or by DNA sequence analysis).

In the case of real-time PCR analysis, amplification and detection occur simultaneously.

5 Reagents

It is generally advisable to store the reaction solutions required for the analytical method at approximately –20 °C if not specified otherwise.

It may also be appropriate to aliquot the reaction solutions required for the analytical method in order to avoid subjecting them to repeated freeze-thaw cycles, and/or to reduce chances of cross contamination.

5.1 Target DNA/control

5.2 Water

5.3 Deoxyribonucleoside triphosphate (dNTP) solution, containing dATP, dCTP, dGTP, and dTTP or dUTP.

NOTE The use of dUTP can interfere with restriction enzyme analyses of PCR products.

5.4 PCR buffer solution

The PCR buffer solution is usually delivered with the DNA polymerase, which may or may not include $MgCl_2$ in a concentration specified by the manufacturer. The final $MgCl_2$ concentrations are method specific and are therefore listed in each annex. Ready-to-use reagents may be commercially available. The manufacturer's instructions for use should be considered.

5.5 Thermostable DNA polymerase

5.6 Forward primer

5.7 Reverse primer

6 Apparatus and equipment

See ISO 24276 and Annexes A to D for details.

7 Procedure

7.1 Quality, integrity and amplifiability of nucleic acid extracts

The nucleic acid solution shall be pure enough for subsequent analysis[3]. The quality and amount of nucleic acid extracted using a given method on a given matrix shall be both repeatable and reproducible.

NOTE The quality, integrity and amount of the DNA template influences the outcome of the PCR, and hence the analytical results obtained. The limit of detection of a specific method may therefore depend on whether the material to be analysed has been processed or refined, and on the degree of degradation of the DNA therein.

Nucleic acids for use in PCR should be substantially free of PCR inhibitors [4]. PCR inhibition controls shall be included as described in ISO 24276.

7.2 Performance criteria

General performance criteria are described in ISO 24276.

The values for the performance characteristics are given for each method as outlined in Annexes A to D and should take into account the genome sizes; see Reference [5].

The reaction conditions, especially the $MgCl_2$ concentration and the thermocycling conditions should be optimized for every primer pair and/or system. When any primer system is used for the first time, it is necessary to demonstrate beforehand that the cycle conditions chosen for the particular matrix to be studied avoid undesirable competitive products that would otherwise reduce the sensitivity of the PCR detection.

In an optimal reaction, less than 40 cycles are required to amplify $\geqslant$ 10 target molecules to produce a product that is readily detectable by standard methods. As the cycle number increases, non-specific products could accumulate. The optimized PCR should be able to amplify in 40 PCR cycles from a pure reference sample of 100 copies of template DNA enough copies of the PCR product to be detectable. The characteristic temperature/time profile for each primer system and the reaction mixture appropriate for the apparatus used and the number of cycles shall be strictly adhered to.

In general, the specificity of the reaction should be enhanced as much as possible (e.g. by using hot-start PCR). Hot-start PCR is recommended as a means of reducing side reactions such as the amplification of non-target sequences in background DNA (mispriming) and primer-oligomerization (it thus increases specificity).

The values derived from the validation study may not be applicable to analyte concentration ranges and matrices other than given in the respective annexes.

7.3 Aspects of PCR design

7.3.1 General

Because the performance of each specific PCR should be comparable with other specific PCRs, the following aspects of PCR design shall be taken into account.

7.3.2 Size of PCR products

The size of the target sequence shall be selected to match the range of molecular mass available in the nucleic acid extract being analysed.

EXAMPLE For highly degraded DNA from processed foodstuffs, the size of the PCR product should ideally be in the range of 60 bp to 150 bp. For raw materials, a broader range of PCR products up to, for example, 250 bp is applicable.

However, if prior experimental studies are carried out to determine the validity of primer sets yielding different sized PCR products, these may be used on the matrix for which they have been validated.

7.3.3 Primers

7.3.3.1 General

Primer sequence information is included in Annexes A to D.

7.3.3.2 Primer design

The primer sequences should preferably have the following characteristics wherever practicable:

— length of each primer: 18 to 30 nucleotides;

— optimal annealing temperature ≈ 60 °C (should be established experimentally), i.e. estimated melting temperature ⩽ 65 °C;

— GC:AT ratio = 50:50 if possible, or else as close to this ratio as possible;

— high internal stability (avoid concentration of Gs and Cs in short segments of primers);

— minimal 3′ end complementarity to avoid primer-dimer formation;

— minimal secondary structure;

— minimal dimer formation with specific detection probe(s) designed for the PCR.

Software packages are available to help with primer design.

7.3.3.3 Validation of primers

7.3.3.3.1 General

The ability of the primers to detect the target sequence shall be validated.

Primer validation should be carried out in two steps: a first theoretical evaluation, and a second experimental evaluation.

7.3.3.3.2 Theoretical evaluation of the specificity

Theoretical evaluation shall as a minimum be carried out by performing a sequence similarity search (e.g. FastA, Blast®[2]) against one of the major nucleic acid sequence databases (e.g. EMBL, GenBank®[2]). Homologous gene sequences may be retrieved from the sequence databases and aligned to obtain an estimate of the chance of finding similar sequences in the target taxon or other organisms.

7.3.3.3.3 Experimental evaluation of the specificity

Irrespective of the design criteria used, the specificity of primers shall always be experimentally evaluated to confirm the primers' ability to discriminate between the target and closely related non-target sequences.

Primers designed to detect taxon-specific target sequences should be shown to detect these sequences reliably in a representative number of different members of the taxon.

7.4 PCR target descriptions

For the qualitative detection and identification of GMOs, various PCR tests may be performed, depending on the type of matrices under study and/or the requirements of the analysis. These analyses may be directed at sequences specific for target taxa, genetic constructs and transformation events, as well as elements suitable for screening purposes.

7.5 Controls

Because of the risk of obtaining false positive and/or false negative results, appropriate controls shall be included in each diagnostic PCR assay (see ISO 24276).

If available and appropriate, certified reference materials should be used as positive and negative controls.

7.6 PCR set-up, detection and confirmation of PCR products

Annexes A to D give details on the specific PCR procedure steps.

NOTE In the case of detection of the PCR products by gel electrophoresis, the size of the PCR products can be estimated using a suitable DNA size marker of known length to run in parallel with the PCR products under test.

It may be desirable in some cases to confirm a positive or negative result for a particular genetic modification. This may be achieved by employing primers to an alternative target sequence; this is particularly suitable for confirmation of screening test results.

A positive identification of the specific target DNA sequence may be confirmed by an appropriate method other than size determination of the PCR product, for example

— by hybridization of the PCR product with specific probes, or

— by carrying out restriction analyses of the PCR product; the length of the resulting fragments has to correspond to the expected length of the target DNA sequence after restriction, or

— by sequencing of the PCR product, or

— other equivalent confirmation.

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2) Blast and GenBank are examples of suitable products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of these products. Equivalent products may be used if they can be shown to give the same results.

If the primers used are designed to detect sequences derived from infectious organisms (a naturally occurring non-genetically modified organism such as a virus or a bacterium), then it is highly recommended that it be verified that the detected DNA is indeed derived from a GMO. This can be done by checking for the absence of other DNA derived from the infectious organism.

EXAMPLE The 35S promoter is derived from cauliflower mosaic virus (CaMV), and consequently detection of the CaMV 35S promoter could be due to the presence of either GMO-derived and/or CaMV-derived DNA [6]. By checking for presence of the other CaMV-derived DNA, it may be possible to confirm the GMO origin of the CaMV 35S promoter if no other CaMV-derived DNA is detected.

8 Interpretation

8.1 General

The PCR result will be either

a) positive if a specific PCR product has been detected, and all the controls give results as specified in ISO 24276:—, Table 2, or

b) negative if a specific PCR product has not been detected, and all the controls give results as specified in ISO 24276:—, Table 2.

NOTE Event-specific target sequences are sometimes present together with other event-specific sequences in a single GMO (e.g. due to gene stacking [7]).

If the results are ambiguous, the procedure shall be repeated; see ISO 24276.

8.2 Verification

Verification of positive or negative results for target sequences may be achieved as described in 7.6.

9 Expression of results and quality assurance

9.1 General

The results shall be expressed unambiguously, i.e. not as “±”.

A negative result shall never be expressed as “GMO not present”.

Ideally, the limit of detection (LOD) should be provided with reference to the test sample. However, this requires particular materials, DNA of exceptionally high quality, and/or use of sophisticated laboratory equipment that is not available to all laboratories. Consequently, the analysis can become very labour intensive and/or expensive, and therefore not applicable in practice for routine purposes.

As a minimum, the LOD shall be provided with reference to a reference material, and a relative value based on a specified matrix (preferably a given amount of genomic DNA solution, e.g. 100 ng of 0,01 % GTS 40-3-2 DNA).

9.2 Expression of a negative result

The following text shall appear in the test report:

“For sample X, target sequence Y was not detected.

The LOD of the method is x % determined with ABC (identify the reference material).”

If it cannot be demonstrated that the amount of target DNA included in the PCR is sufficient for the LOD to be applicable, then the following sentence shall be added:

> "However, the amount of the target DNA extracted from species X may be/was insufficient for the LOD to be applicable for this sample."

NOTE The LOD of the sample is determined by the quantity of DNA of the species included in the analytical reaction (copy number), and the ratio relative to the absolute LOD of the GM target (copy number) [7].

9.3 Expression of a positive result

The following text shall appear in the test report:

> "For sample X, target sequence Y was detected."

The identity of the GMO may be included, if available.

9.4 Quality assurance requirements

Results from both test portions shall be consistent. If one test portion gives a positive result and the other gives a negative result, then the analysis shall be repeated (see ISO 24276), if possible by increasing the quantity of template nucleic acid in the reaction so as to obtain consistent results for both test portions. Moreover, as a minimum, the purity of the template nucleic acid should be checked by including a PCR inhibition control. Other controls to check the length and integrity of the template nucleic acid may be useful.

10 Test report

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

— the limit of detection, and the matrix used to identify the limit of detection;

— description of the specificity of the analytical method;

— the result expressed according to Clause 9.

Annex A
(informative)

Target-taxon-specific methods

A.1 Target-taxon-specific method for the detection of components derived from soya beans

A.1.1 General

This is a routine procedure for the detection of a species-specific, single copy gene occurring in soya beans (*Glycine max*).

This method may be used to assess the amplifiability of DNA from products derived from soya beans.

A.1.2 Validation status and performance criteria

A.1.2.1 Collaborative study

This method has been validated in collaborative studies [8], [9] organized by the working group “Development of methods for identifying foodstuffs produced by using genetic engineering techniques” of the German Federal Institute for Health Protection of Consumers and Veterinary Medicine (BgVV) according to Article 35 of the German Federal Foodstuffs Act. For DNA extraction, the CTAB method as outlined in ISO 21571:2005, A.3, was used (but with a test portion of 100 mg).

The data from the collaborative studies are listed in Table A.1.

Table A.1 — Results of the collaborative studies

Year of collaborative study	1997 [8]	1998/1999 [9]
Number of laboratories	25	27
Number of laboratories submitting results	22	20
Number of samples per laboratory	10	3
Number of accepted results	220	60
Number of samples containing soya beans	220	50
False positive results	0	1 (2 %)
False negative results	0	1 (2 %)

A.1.2.2 Molecular specificity

A.1.2.2.1 General

This annex fulfils the requirements as outlined in Clause 7.

The method has been designed for a target sequence described in GenBank®[3] accession No. K00821 = M30884.

A.1.2.2.2 Theoretical

The soya bean lectin gene *Le*1 [10] obtained from gene databases was chosen as a target sequence.

No sequence similarity with DNA sequences of other crop plants (legumes, cereals, vegetables) has been found (NCBI BlastN®[2] search, European Molecular Biology Laboratory (EMBL) database, September 28th, 2001). However, GM03 matched 100 % the sequences in the following database accessions: AX033509 (sequence 17 from patent DE19906169), AX033507 (sequence 15 from patent DE19906169) and AX033501 (sequence 9 from patent DE19906169), while GM04 matched only accession No. AX033509 (sequence 17 from patent DE19906169). Note that the accession No. M30884 is the same as K00821, a GenBank® entry originally submitted in 1993.

The number of target sequence copies was not determined, but was presumed to be a single copy gene.

A.1.2.2.3 Experimental

No amplification has been observed using DNA from other crop plants (legumes, cereals, vegetables) or from beef and pork. The soya bean PCR assay appears to be highly specific for soya bean DNA [10], [11].

A.1.2.3 Limit of detection (LOD)

The absolute LOD has not been determined, but the method has been demonstrated to detect at least 0,1 ng of soya bean DNA, determined fluorometrically.

A.1.3 Adaptation

No specific information is available.

A.1.4 Principle

A 118 bp fragment from the soya bean lectin gene is amplified by PCR and separated by agarose gel electrophoresis.

A.1.5 Reagents

For the quality of the reagents used, see ISO 24276.

A.1.5.1 Water

A.1.5.2 PCR buffer, (without $MgCl_2$), 10× [4].

A.1.5.3 $MgCl_2$ solution, $c(MgCl_2)$ = 25 mmol/l.

A.1.5.4 dNTP solution, c(dNTP) = 2,5 mmol/l (each).

A.1.5.5 Oligonucleotides

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3) GenBank and BlastN are examples of suitable products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to give the same results.

4) 10× means 10-fold; i.e. a PCR buffer containing 1,5 mol/l Tris-HCl, pH 8,3.