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Foodstuffs — Detection of food allergens by molecular biological methods

Part 1: General considerations

ICS 07.100.30; 67.050

National foreword

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A list of organizations represented on this committee can be obtained on request to its secretary.

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Foreword

This document (EN 15634-1:2009) has been prepared by Technical Committee CEN/TC 275 “Food analysis - Horitontal methods”, the secretariat of which is held by DIN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by July 2009, and conflicting national standards shall be withdrawn at the latest by July 2009.

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Introduction

This European Standard describes the procedure to qualitatively detect and/or quantitate DNA as markers for potentially allergenic ingredients or constituents by analysing the nucleic acids extracted from the sample under study.

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

The quantitative detection of DNA targets is performed to express the quantity of DNA targets, relative to the quantity of a specific reference, appropriate calibrants and controls and within the dynamic range of the analytical method used and the test portion analysed. Appropriate procedures for extraction of nucleic acids are included in each method.

The main focus of this European Standard will be on PCR based amplification methods. However, because of the rapid rate of technological change in this area, other amplification technologies and detection methods may be considered.

1 Scope

This European Standard provides the overall framework for detection of sequences corresponding to species containing allergens using the polymerase chain reaction (PCR). It relates to the requirements for the specific amplification of target nucleic acid sequences (DNA) and for the confirmation of the identity of the amplified nucleic acid sequence.

Guidelines, minimum requirements and performance criteria laid down in the European Standard are intended to ensure that comparable and reproducible results are obtained in different laboratories. This European Standard has been established for food matrices.

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.

prEN 15842:2008, *Foodstuffs – Detection of food allergens – General considerations and validation of method*s

3 Terms and definitions

For the purposes of this document, the terms and definitions given in prEN 15842:2008 and the following apply.

3.1 Terms relative to extraction and purification of DNA

3.1.1
DNA extraction
separation of DNA from the other components in a test sample

[EN ISO 24276:2006] [1]

NOTE The factors of major importance for the isolated DNA are: a) purity, b) amount or concentration and c) quality (integrity).

3.1.2
DNA purification
method resulting in a higher purity of the extracted DNA

NOTE In this context, purity refers to the reduction of observable measurable effects of PCR inhibitors.

3.1.3
PCR quality
DNA template of sufficient quality to be amplified by PCR

3.2 Terms relative to amplification of DNA

3.2.1
species (class/order/family/genus) specific target sequence
sequence known to be specific for the species

3.2.2
identification of nucleic acid sequences
identification by comparison with a reference nucleic acid fragment/sequence

NOTE Identification is possible by e.g. positive hybridisation with probe, matching restriction digest profiles or matching nucleic acid sequences.

3.3 Definitions referring to controls

3.3.1
positive DNA target control
reference DNA or DNA extracted from a certified reference material or known positive samples representative of the sequence or target under study

NOTE The control is intended to demonstrate what the result of analyses of test sample containing the sequence under study will be.

3.3.2
negative DNA target control
reference DNA or DNA extracted from a certified negative (blank matrix) reference material or known negative sample not containing the sequence under study

NOTE The control is intended to demonstrate what the result of analyses of test samples not containing the sequence under study will be.

3.3.3
PCR inhibition control
control containing known amounts of positive template DNA added in the same amount as analyte DNA to the reaction

NOTE This control allows the determination of the presence of soluble PCR inhibitors, particularly necessary in case of negative amplification and of quantitative PCR.

3.3.4
amplification reagent control
control containing all the reagents, except extracted test sample template DNA

NOTE 1 Instead of the template DNA, a corresponding volume of nucleic acid free water is added to the reaction.

NOTE 2 The water used should be double distilled or equivalent, free from DNA and nucleases (molecular biology grade).

3.3.5
extraction blank control
control performing all steps of the extraction procedure, except addition of the test portion, e.g. by substitution of water for the test portion

NOTE 1 It is used to demonstrate the absence of contaminating nucleic acid during extraction.

NOTE 2 The water used should be double distilled or equivalent, free from DNA and nucleases (molecular biology grade).

3.3.6
positive extraction control
control sample meant to demonstrate that the nucleic acid extraction procedure has been performed in a way that will allow for extraction and subsequent amplification of the target nucleic acid, i.e. by using a sample material known to contain the target nucleic acid

NOTE Information about controls can be found in EN ISO 24276.

4 General laboratory requirements

4.1 General

A draft European Standard dealing with General considerations and validation criteria of methods was adopted as prEN 15842:2008.

4.2 Laboratory organisation

Compliance with applicable requirements with respect to safety regulations and manufacturer's safety recommendation shall be followed.

Accidental contamination of DNA can originate from dust or spreading aerosols. As a consequence, the organisation of the work area in the laboratory is logically based on:

— the systemic containment of the methodological steps involved in the production of the results, and

— a forward flow principle for sample handling.

A minimum of three separately designated work areas with their own apparatus is required:

a) a work area for extraction of the nucleic acid from the test portion (sample);

b) a work area dedicated to the set up of PCR/amplification reactions; and

c) a work area dedicated for subsequent processing including analysis and characterisation of the amplified DNA segments.

If dust particle producing grinding techniques are used, this has to be carried out in a separate work area.

Physical separation through the use of different rooms is the most effective and preferable way of ensuring separated work areas, but other physical or biochemical methods may be used as a protection against contamination provided their effectiveness is comparable.

Staff shall wear different sets of lab coats at each dedicated work area. They shall also wear disposable gloves. Gloves and lab coats should be changed at appropriated frequencies.

4.3 Apparatus and equipment

The laboratory should use properly maintained equipment suitable for the method employed, e.g. according to the requirements outlined by EN ISO/IEC 17025 [2]. In addition to standard laboratory equipment, additional apparatus are described in the specific methods.

Apparatus and equipment shall be maintained according to manufacturer's instructions. Calibration systems shall be available and calibration routinely performed for equipment which may impact the data produced, according to laboratory quality assurance programs.

4.4 Material and reagents

For the analysis, unless otherwise stated, use only analytically pure reagents suitable for molecular biology, free from DNA and DNAses. Reagents and solutions should be stored at room temperature, unless otherwise specified. PCR reagents should be stored in small aliquots to minimize the risk of contamination. The water used shall be double distilled or equivalent, free from DNA and nucleases (molecular biology grade). Solutions are prepared by dissolving the appropriate reagents in water and autoclaved unless specified differently. Ultra-filtration devices can be used, when autoclaving is not possible.

In order to avoid contamination, all the appliances and the parts of equipment in contact with the samples, as well as work surfaces should be easy to clean and decontaminate. Work surfaces cleansing and decontamination is recommended before use and additionally when/where there is a suspicion for contamination. Sterile techniques could be adopted in the PCR set up area – powder-free gloves, sterilised plastic-ware, sterilised glassware**,** autoclaved reagents, disposable plastic-wares, aerosol-protected pipette tips could be used.

5 Procedure

5.1 Principle

Qualitative analysis consists of specific extraction and detection of target nucleic acid sequences in the test portion. A qualitative result shall clearly demonstrate the presence or absence of the target (class, order, family, genus or species) under study, relative to appropriate controls and within the detection limits of the analytical method used and test portion analysed.

Quantitative analysis consists, in addition, of the quantitation of target DNA sequences in the test portion.

5.2 General

The analysis of food allergens by molecular biology methodology usually involves the following stages:

— isolation (/extraction) of nucleic acid;

— amplification and detection of specific target sequences;

— confirmation of the specificity of the amplified fragment; and

— quantitation of the amplified fragments relative to calibrants.

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

6 Isolation / Extraction of nucleic acid

6.1 Preparation of sample

Further information can be found in prEN 15842:2008 and in the respective method standard protocols. It is recommended that from each laboratory sample two test portions should be prepared.

6.2 DNA extraction

The basic principle of DNA extraction consists of releasing the DNA present in the matrix and further, concurrently or subsequently, purifying the DNA from polymerase chain reaction inhibitors. The DNA extraction /purification is described in each method, taking into account examples of matrices given in each method.

6.3 DNA quantitation

It may be performed by either physical (e.g. measure of absorbance at a specific wavelength), chemical-physical (e.g. use of intercalating or binding agents able to emit fluorescence), enzymatic (e.g. bioluminescence detection) methods or by quantitative PCR. The latter method is especially suitable for composite matrices or for samples with a low DNA content or whose DNA is degraded.

6.4 Quality of DNA

The resulting DNA solution shall be pure enough for subsequent amplification and quantitation of PCR products, and substantially free of PCR inhibitors. A PCR inhibition control shall therefore always be included. DNA extraction and purification quality shall be controlled in case of negative PCR amplifications on a sample.

The quality and presence of DNA extracted using a given method on a given matrix shall be both repeatable and reproducible in terms of amplification by PCR. In particular, the method used shall allow for recovery of DNA fragments larger than the amplicons.

Each method should include the most appropriate manner to isolate DNA depending on the amount and quality of DNA and on the matrix from which DNA has been extracted. An estimation of the quality of extracted DNA may be performed by e.g. gel electrophoresis. The quality of the extracted DNA may be demonstrated by use of a PCR system amplifying a universal target (e.g. 18S rDNA).

The quality, integrity and amount of the DNA template influence the outcome of the PCR, and hence the analytical results obtained. The applicability of a specific method may therefore depend on whether the material to be analysed is processed or refined or not, and on the degree of degradation of the DNA.

NOTE Gel electrophoresis only provides information about degradation of DNA.

6.5 Storage conditions of extracted DNA

The DNA extracted shall be stored under such conditions that its stability is ensured to perform the subsequent analysis. Repeated freezing and thawing of DNA solutions should be avoided.

7 Amplification of specific target sequences

7.1 Reagents

It is generally advisable to take aliquots of the reaction solutions required for the PCR and to store them at -20 °C.

7.1.1 Target DNA

The target DNA for the method should be specified and the sequences of primers and probes provided.

7.1.2 Water

The water used shall be double distilled or equivalent, free from DNA and nucleases (molecular biology grade).

7.1.3 Deoxynucleoside triphosphate (dNTPs) solution containing dATP, dCTP, dGTP, dTTP and/or dUTP

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

7.1.4 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 method. Ready-to-use reagents are commercially available. The manufacturers instruction for use should be considered.

7.1.5 Thermostable DNA Polymerase

Specified in each part of this European Standard.

7.1.6 Optional additional reagents

Specified in each part of this European Standard.

7.2 Primers

7.2.1 Primer design

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

— length of each primer should be 18 to 30 nucleotides;

— optimal annealing temperature shall be established experimentally;

— GC:AT ratio should be 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 internal secondary structure;

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

Software packages are available to help with primer design.

7.2.2 Forward and reverse primer

Specified in each part of this European Standard.

7.2.3 Validation of primers

7.2.3.1 General

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

7.2.3.2 Theoretical evaluation on the specificity

Theoretical evaluation shall as a minimum be done by performing a sequence similarity search[1)] against one of the major nucleic acid sequence databases[2)]. Homologous gene sequences may be retrieved from the sequence databases and aligned to evaluate the potential cross-reactivity in other classes, orders, families, genus or species.

1) FastA or Blast® are examples of suitable products available commercially. This information is given for the convenience of users of this standard and does not constitute an endorsement by CEN of these products.

2) EMBL or GeneBank® are examples of suitable products available commercially. This information is given for the convenience of users of this standard and does not constitute an endorsement by CEN of these products.

7.2.3.3 Experimental evaluation on the specificity

Irrespective of the design criteria used, the specificity of primers shall always be experimentally validated to confirm the primers ability to discriminate between the target and related classes, orders, families, genus or species (closely or distant). The targets used for cross-reactivity testing need to be stated.

7.3 PCR procedure

7.3.1 PCR profile and performance criteria

In an optimal reaction, less than 40 cycles are required to amplify ≥ 10 target molecules to produce a product that is readily detectable by standard methods. As cycle number increases, non specific products will accumulate. The optimised 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 amplicon to be detectable.

NOTE 1 PCR methods are generally able to repeatable detect around 20 to 50 target copies with ≤ 5% false negative results.

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 real time PCR applications, a higher number of cycles may be used.

NOTE 2 Due to different performances of PCR thermocyclers the validity of the method for each instrument, different from the one cited in the method, needs to be validated individually.

In general, 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 method to reduce side reactions such as the amplification of non-target sequences in background DNA (mispriming) and primer-oligomerization.

7.3.2 PCR design

The size of amplicons should be selected in order to match with the range of molecular mass available in the sample under study, e.g. for denatured DNA from processed foodstuffs, the size of the amplicon should ideally be in the range of 60 bp to 150 bp. For non-processed foods, a broader range of amplicons, up to e.g. 250 bp is applicable. However, if prior experimental studies are carried out to determine the validity of primer sets yielding different size amplicons, these may be used on the matrix for which they have been validated.

7.3.3 PCR target descriptions

For the qualitative detection and identification of DNA as markers for allergens, different PCR tests will be performed, dependent on the type of matrices under study and/or the requirements of the analysis. In general, class, order, family, genus or species specific PCR test will be performed. Since the allergenic proteins are not detected directly, the target sequences have not necessarily to be derived from DNA coding for the allergen sequences, but have to be specific for the target in question.

7.3.4 Controls

Because of the risk of obtaining false positive and/or false negative results, appropriate controls shall be included in each PCR assay. These shall as a minimum include a positive DNA target control, a negative DNA target control or/and an extraction blank and an amplification reagent control. Certified reference materials should be used, if available and appropriate as positive and negative controls. Confirmation of a positive or negative result for a particular allergen may be achieved by employing primers to an alternative target sequence; this is recommended particularly for confirmation of screening test results.

7.3.5 PCR amplification

Amplification of the target sequence occurs *in vitro* through a reaction catalyzed by a DNA polymerase in the

presence of primers and deoxynucleoside triphosphates in a defined reaction buffer. 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:

— 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;

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

8 Detection and confirmation of PCR products

8.1 General

The methods describe details on the extraction and the specific PCR procedure steps.

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

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

— by carrying out restriction fragment 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 methods.

8.2 Calibration

Calibrants (calibration materials) used for quantitation should be traceable to certified reference materials (CRMs), if available. If not available, other suitable reference materials should be used.

An appropriated number of calibration points and replicates covering the range of quantitation shall be applied, e.g. four calibration points in duplicate (altogether 4×2 values) or six calibration points with one measurement at each point (altogether 6 values). The quality of the calibration influences the measurement uncertainty.

8.3 Quantitation of PCR products

Quantitation can be carried out by determining the absolute amount of target DNA in a given amount of food matrix.

NOTE Prior to application for routine purposes, quantitative PCR methods should be validated for each representative matrix to be investigated, and quantitative test results can be generated for the representative matrix only. This is critical to avoid erroneous quantification results due to matrix effects, varying DNA integrity and DNA extraction yields in different products having undergone different processes.

9 Interpretation

The results obtained including the controls should be unambiguous and yield expected results, otherwise the whole procedure, starting from the extraction of DNA, should be repeated.

The PCR result will be either:

— positive if a specific PCR product has been detected and all the controls give expected results, or

— negative if a specific PCR product has not been detected and all the controls give expected results.

The PCR result will be fit for quantitation of the target sequence provided that:

a) the result is positive according to the above;

b) the target sequence is in the dynamic range of the method;

c) the analysis is calibrated in accordance with the criteria described in prEN 15842:2008; and

d) a validation has been performed on the matrix of interest.

The PCR result will not be fit for quantitation of the target sequence if any of the conditions listed above [a) to d)] are not fulfilled.

10 Expression of the results

10.1 General

The statements in 10.2 to 10.4 are given as examples.

10.2 Expression of a positive result

10.2.1 Qualitative PCR

The following text could appear in the test report:

'For laboratory sample X, target sequence Y was detected.'

'The LOD of the method is x copy number determined with ABC (identify the reference material). The copy number x corresponds to z mg allergenic constituents/kg food.'

10.2.2 Quantitative PCR

In case of quantitative results the following text could appear in the test report:

'For laboratory sample X, the copy number of DNA as a marker for potentially allergenic material derived from class, order, family, genus or species was quantified with reference to a specific reference material.'

The LOQ of the method is x copy number determined with ABC (identify the reference material).

'The copy number x is equivalent/equal to z mg allergenic constituents/kg food and to a limit of quantitation (LOQ) of y mg allergenic food in that commodity.'

The basis for conversion of DNA copy number to mg allergenic constituents/kg food should be provided as well as the limit of detection in mg allergenic constituents per kg food.

10.3 Expression of a negative result

The following text could appear in the test report:

'For laboratory sample X, target sequence Y was not detected.'

'The LOD of the method is x copy number determined with ABC (identify the reference material).'

'The copy number x corresponds to z mg allergenic constituents/kg food.'

A negative result shall never be expressed as "specific target sequence y is not present".

10.4 Ambiguous results

The results shall be expressed unambiguously, i.e. not as "+/-".

10.5 Quality assurance requirements

Results from two 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, if possible, by increasing the quantity of template nucleic acid in the reaction, so as to get consistent results for both test portions. In case ambiguous results persist, repeat the whole procedure from extraction of the DNA.

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.

11 Test report

The test report should contain at least the following information:

a) the result expressed according to Clause 9;

b) description of the specificity of the analytical method;

c) the reference material used;

d) reference to this European Standard, i.e. EN 15634-1.

Bibliography

[1] EN ISO 24276:2006, *Foodstuffs - Methods of analysis for the detection of genetically modified organisms and derived products - General requirements and definitions (ISO 24276:2006)*

[2] EN ISO/IEC 17025, *General requirements for the competence of testing and calibration laboratories (ISO/IEC 17025:2005)*

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