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# Molecular biomarker analysis — Methods of analysis for the detection and identification of animal species in foods and food products (nucleic acid-based methods) — General requirements and definitions

*Analyse moléculaire de biomarqueurs — Méthodes d'analyse pour la détection et l'identification des espèces animales dans les aliments et les produits alimentaires (méthodes basées sur l'utilisation des acides nucléiques) — Exigences générales et définitions*



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# Foreword

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

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# Molecular biomarker analysis — Methods of analysis for the detection and identification of animal species in foods and food products (nucleic acid-based methods) — General requirements and definitions

## 1 Scope

This document specifies minimum requirements of performance characteristics for the detection of nucleic acid sequences (DNA) by molecular methods, such as the polymerase chain reaction (PCR), including different post-PCR detection methods, real-time PCR, single and/or multiple probe-based detection techniques as well as the combination of such methods.

The document is applicable to the detection, identification and quantification of DNA from animal species of higher and lower taxonomic groups in foodstuffs, and the validation of applicable methods.

It is applicable to mammals, birds, reptiles, amphibians, fishes, molluscs, crustaceans and insects. Typical examples for each are listed in Annex A.

## 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 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, ISO 24276 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 http://www.electropedia.org/

**3.1**
**basic local alignment search tool**
**BLAST**
sequence comparison algorithm optimized for speed that is used to search sequence databases for optimal local alignments to a query

Note 1 to entry: This algorithm directly approximates alignments that optimize a measure of local similarity, the maximum signal pair (MST) score or high-scoring segment pair (HSP) score.

Note 2 to entry: See Reference [2].

Note 3 to entry: BLASTn is applicable to nucleotide sequence comparison.

**3.2**
**conventional polymerase chain reaction**
**conventional PCR**
PCR method that requires a post-PCR step such as gel electrophoresis for detection or visualization of amplification products to provide a qualitative result

# 4 Performance characteristics of the methods

## 4.1 General

The methods to be used for animal species analysis shall meet the performance characteristics in accordance with this document. The results of all interlaboratory and/or single-laboratory validations and the performance characteristics shall be described.

NOTE Some guidelines are available for implementation of methods, see Reference [10].

## 4.2 Scope of the method

Information regarding the intended use and the limitations of the methods shall be provided. In particular, information shall indicate that the criteria set out in this document have been fulfilled.

## 4.3 Scientific basis

An overview of the principles and references to relevant scientific publications should be provided.



## 4.4 Units of measurement

Qualitative analyses indicate the presence or absence (lack of detection) of a certain target.

In quantitative analyses, the measured value is calculated as ratio of DNA copy numbers (c/c). The use of this ratio should examine possible influences, including the number of DNA copies with regard to the target in the genome. Other units (e.g. ratio of masses) can be employed. The principles of calculation of the ratio shall be reported.



If a quantitative method is intended to judge the mass/mass ratio of different animal species ingredients in a sample, it should be indicated that the values measured for the DNA copy number ratio cannot reflect in all cases the mass/mass ratio of animal constituents in the sample.

## 4.5 Applicability

When assessing if a method is fit for purpose, the following aspects regarding the nature of the target should be considered:

— the location of the target (nuclear or mitochondrial);

— the copy number per cell;

— the length of the target sequence.

For quantitative species-specific methods, a nuclear gene, excluding mitochondrial DNA shall be targeted. The target sequence shall be present as a single copy per haploid genome or the copy number shall be determined/known.

When assessing if a method is fit for purpose, the following aspects regarding the matrix should be considered:

— the nature of the potential sample matrices;

— the degree of processing of the sample constituents;

— the different species and animal tissue types involved;

— the preparation of the sample matrix.

The applicability of the method shall be tested by extracting DNA from test samples reflecting the matrices and analytical scope.

DNA should be extracted from a minimum of three matrices of the most relevant types, including those types reflecting the method scope, containing a known mass/mass content of the target(s) species materials (evenly distributed over the percentage dynamic range of the method) and tissues relevant for the application.

NOTE 1 Mitochondrial PCR targets cannot be used for reliable quantification of haploid genome copy number ratios of different species, because the number of mitochondrial targets differs with tissue type.

NOTE 2 Different animal tissue types can have variable DNA contents per mass equivalent.

NOTE 3 The practical limit of detection (LOD) (see ISO 21569) can differ significantly for different matrices. Furthermore, different processing grades of animal constituents in the same product will further contribute to DNA degradation and a possible asymmetric DNA distribution between ingredients. For example, a product can be composed of different types of animal tissue containing different amounts of DNA. This imbalance can be further intensified if some ingredients underwent pre-processing, like cooking or acid treatment, lowering DNA quality, whereas other ingredients were added for example raw or processed differently.

## 4.6 Specificity

### 4.6.1 General



The specificity should be assessed in a two-step procedure: theoretical and experimental evaluation of the inclusivity and exclusivity.

*In silico* testing of the specificity of primers and probes with available bioinformatics tools shall be performed.



NOTE 1 Examples are testing primer-dimer formation with primer3[1] and BLAST[2] searches in nucleic acid sequence databases.

If sequence data are used for verification of animal speciation results, they should be based on appropriate databases with due consideration of the timing of submission of individual entries and any subsequent changes in taxonomic classification or naming.

NOTE 2 In cases of unexpected results, further investigation can be carried out with appropriate techniques, such as sequencing, gel electrophoresis or hybridization techniques in order to confirm reference material identity.

### 4.6.2 Requirements for inclusivity testing

Experimental results from testing the method with the target animal species should be provided. This testing should include relevant breeds of the animal species according to the scope of the method (see 4.2).

Material for experimental inclusivity testing should contain approximately 100 target DNA copies[6]. Each sample material shall be at a minimum tested in duplicate. Sequence variants of the target animal species should be detected with comparable amplification efficiency, if they occur.

NOTE The target animal species for inclusivity testing are normally more than five breeds.

### 4.6.3 Requirements for exclusivity testing

Experimental results from testing the method with non-target animal species shall be provided. This testing should include both taxonomically close and not closely related animal species. Animal species

or taxonomic groups relevant with regard to the scope of the method shall be tested, e.g. species commonly used in food in general and particularly in matrices considered in the scope of the method. The method should clearly distinguish between target and non-target animal species.

Sufficient DNA should be used for experimental exclusivity testing. A number of 2 500 target copies[6] ensures that cross reactivity can be identified.

Select a minimum of 10 species that could cause interference with the target animal species present in the food test material. Examples of suitable organisms are listed in Annex A.

Other species should be included if relevant, e.g. if there are sequence homologies of oligonucleotides to nucleic acid sequences.

Cross-reactivity of matrix should be characterized.

The suitability of the DNA used for amplification should be confirmed by an amplification control, e.g. by a single copy (chromosomal) DNA consensus PCR system (e.g. myostatin or actin).

## 4.7 Sensitivity

### 4.7.1 General

Experimental results from testing the method at different concentrations in order to test the range of use of the method shall be available. They shall be described in the validation report.

If applicable, detailed information about how a cut-off value can be established and used in the laboratory should be provided.

Animal species that require qualitative testing should be detected at levels relevant for the interested party, e.g. the consumer.



### 4.7.2 Limit of detection (LOD)



#### 4.7.2.1 Absolute LOD

The absolute LOD ($LOD_{abs}$) shall be indicated in copy numbers of the target sequence per reaction with the confidence level (typically 95 %) specified.

NOTE 1 Twenty copies or less can be applied for single copy genes and an appropriate number of haploid genome equivalents for high copy number genes.

NOTE 2 If for the LOD determination a DNA with known copy number of target sequence is not available, plasmid DNA can be used.

The $LOD_{abs}$ of the method is determined experimentally by preparing a dilution series of target material with dilutions in the range of the expected/targeted limit of detection. Guidance for assessment of the $LOD_{abs}$ is described in Reference [6].

#### 4.7.2.2 Relative LOD

The relative LOD ($LOD_{rel}$) shall be determined in relevant non-target animal species DNA as background. Depending on test requirements, the $LOD_{rel}$ is adjusted to this value. The $LOD_{rel}$ expresses the relative c/c % of the target animal species DNA in other animal species DNA which is detected with 95 % confidence.

The $LOD_{rel}$ should be determined experimentally by preparing one or more defined reference samples with defined percentage content of the target DNA in the range of the limit of detection. Each reference sample is analysed in at least 10 replicates. The percentage of the reference sample where at least 95 % of the replicates give positive results is considered the $LOD_{rel}$.

 

#### 4.7.2.3 Asymmetric LOD (for multiplex methods only)

In the case of multiplex methods where the detection of different targets is restricted by competitive effects, as in the case of multiplex real-time PCR methods, the LOD for the single targets in an asymmetric target situation expressed as target ratio needs to be validated. Different contents of the specific animal target sequence are mixed to obtain defined copy ratios (i.e. ratios of 1:1 000 and 1 000:1; 1:100 and 100:1). The ratio where each target animal is detected with 95 % confidence is determined experimentally with an appropriate number of replicates for the defined reference sample.

## 4.8 Specific requirements for quantitative methods

### 4.8.1 General

The upper and lower limit of the linear range of the method shall be determined. The assessment of these limits and the linear range shall be carried out on samples containing animal non-target DNA relevant to the food item.

### 4.8.2 Limit of quantification (LOQ)

The absolute LOQ ($LOQ_{abs}$) shall be indicated as copy numbers of the target sequence. It shall be equal to the smallest amount included in the dynamic range.

The relative LOQ ($LOQ_{rel}$) shall be determined in DNA of other relevant animal species. Depending on the test requirements, the $LOQ_{rel}$ should be adjusted to this value. The $LOQ_{rel}$ expresses the ratio of the target animal species DNA copy number to other animal species DNA copies or to the DNA copies of a reference gene representative for the whole taxonomic rank. The $LOQ_{rel}$ should be equal to the smallest concentration included in the dynamic range.



If, for the LOQ determination, a DNA with known copy number of target sequence is not available, plasmid DNA should be used. This plasmid can also serve as a calibrator.

A minimum of 15 replicates with a target concentration of the expected LOQ shall be tested. The criteria for precision and trueness shall be fulfilled for the results.



NOTE The LOQ values reported from collaborative study data generally refer to the lowest level of analyte that was observed to have a relative reproducibility standard deviation of 25 % or less.

### 4.8.3 Dynamic range

The dynamic range should cover the percentage values as well as the copy numbers according to the expected use and scope of the method.

In order to define the relevant minimum copy number, the desired dynamic range in terms of target copy percentages shall be determined. It should be considered that the genome size of the species in the expected sample material restricts the maximum copy number that can be used for the analysis (e.g. 100 ng to 200 ng, depending on the method).

NOTE 1 For example, for cattle, a genome size of 4 pg can be assumed, which results in a maximum copy number of 25 000 in 100 ng of sample DNA material. See Table B.2[18][22].

The copy numbers of the dynamic range for both, target and reference sequence, shall be then determined as follows:

— for the reference sequence, the maximum number of copies can be calculated considering genome sizes and amount of sample DNA used for analysis as described above;

— for the target, the lowest copy number should be the absolute LOQ; as a prerequisite, the lowest possible value considering the ratio compared to the maximum number of copies of total/reference DNA should be taken into consideration;

— the minimum copy number of the reference sequence and the maximum copy number for the target sequence should be given by the ratio of the minimum and maximum, respectively, percentage value.

NOTE 2 The dynamic range is established on the basis of a standard curve with a minimum of four concentration levels evenly distributed at least in duplicate.

NOTE 3 For a desired upper limit of the percentage dynamic range of 100 %, the minimum copy number of the reference can be equal to the lower limit of the copy number range of the target sequence, and for a desired LOQ of 0,1 % at an absolute LOQ of 30 copies, the upper limit of the reference target is 30,000 copies.

#### 4.8.4 Determination of precision and trueness for quantitative methods

The precision should be determined and expressed as relative repeatability standard deviation ($S_r$).

A sufficient number of replicates (at least 15) for at least three DNA materials with different target percentages covering the whole dynamic range should be analysed.

NOTE Mitochondrial DNA cannot be used for the targets of quantitative methods.

The $S_r$ for all replicates shall be ≤ 25 % over the whole dynamic range of the method.

The trueness shall be within 25 % of the accepted reference value for all replicates over the whole dynamic range of the method.

### 4.9 Robustness



#### 4.9.1 General

Results from the empirical testing of the method against small but deliberate variations in method parameters (e.g. variation in concentration of kit components, variation in apparatus) should be provided, if available.

#### 4.9.2 Robustness determination by interlaboratory study



An interlaboratory study introduces a deliberate change in the laboratory performing the method and meets the criteria for an evaluation of robustness. Empirically, a robust method shall be selected by considering that the results from different laboratories do not vary significantly.

#### 4.9.3 Robustness determination by a multifactorial orthogonal test design

The test should be carried out in a multifactorial approach where several alterations, including, but not limited to, mastermix concentration, reaction volume, primer and probe concentration, annealing temperature and thermocycler platform are assessed.

NOTE 1 A detailed procedure is described in Reference [6].

For qualitative methods, at least three replicates should be tested. The target animal species copy number used in the test should be in a concentration threefold the $LOD_{abs}$ (95 % confidence) of the method.

For quantitative methods, three defined target concentrations over the whole dynamic range of the method should be tested in three replicates each.

NOTE 2 The method is considered to be robust if all reactions give the expected results.

## 5 Single-laboratory validation

An analytical method should have been sufficiently tested within a laboratory to disclose the required specification prior to the interlaboratory study, see ISO 13495.

 

Reference materials or certified reference materials (CRMs) should be considered for use in the validation of detection and quantification methods of nucleic acids.

# 6 Interlaboratory study (collaborative study)

## 6.1 General

Information about the collaborative study (organizer, protocol, number of participating laboratories, etc.) and the performance data obtained by the study shall be reported with appropriate references to the relevant documents. Collaborative studies for the validation of PCR methods for detection, identification and quantification of specific DNA sequences can be performed according to other relevant documents (e.g. Codex Alimentarius CAC/GL 74-2010[7]).

NOTE A small-scale collaborative study (pre-validation study involving, for example, two to four laboratories) can be performed to test the general transferability of the method before the expense of organizing a large-scale study is incurred.

For validation of the precision of detection and identification methods, data are collected from multiple laboratories having facilities and proficiency in molecular biology testing. In ISO 13495:2013, the required number of laboratories is eight and four for the international and national levels of validation, respectively. According to AOAC International (2002)[23], the required number is eight laboratories. Statistical analysis is calculated based on ISO 5725-1:1994, 6.3.

## 6.2 Qualitative methods

A collaborative validation study of a qualitative PCR method shall be designed by considering the probability of detection (POD) (see ISO/TS 16393) within the range of the method.

NOTE Traditional nonparametric 5 % false positive and 5 % false negative rates reflect PODs of 5 % and 95 %.

## 6.3 Quantitative methods

The relative reproducibility standard deviation ($S_R$) should be ≤ 25 % over the whole dynamic range of the method.

NOTE At levels of 0,1 % (copy/copy) an $S_R$ of 50 % can be acceptable.

# 7 General laboratory and procedural requirements

## 7.1 General

The procedure shall be documented to include the following steps:

— sample representability shall be addressed;

— preparation of the test sample (optional: if the test sample is not the whole laboratory sample, homogenize the laboratory sample and obtain test samples in accordance with the relevant International Standards);

— grinding and homogenization of the test sample;

— preparation of test portions;

— extraction of DNA;

— testing, interpretation and reporting of the results.

Manufacturers' safety recommendations shall be followed.