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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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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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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*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

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.

7.2 Facilities, materials and equipment

The work area in the laboratory should be designed for preventing accidental DNA contamination originating from, for example, dust, human material and spreading aerosols, including consideration of:

— systematic containment of the methodological steps involved in the production of the results;

— a forward flow principle for sample handling.

For DNA-based methods, separation (temporal and/or physical) of work is required to prevent contamination. Designated contained/dedicated work areas with their own apparatus are recommended, as follows:

a) a work area for grinding and homogenization;

b) a work area for extraction of the nucleic acid from the test material;

c) a work area dedicated to the setup of PCR/amplification reactions;

d) a work area dedicated to subsequent processing, including analysis and characterization of the amplified DNA sequences, if applicable.

If human DNA is detected by the method, appropriate contamination prevention measures (e.g. use of masks, gloves and disposable coats) should be taken in order to prevent false-positive results by contamination with the operator's or other human DNA during analysis.

Physical separation through the use of different rooms is the most effective and preferable way of ensuring separate work areas, but other methods can be used as a protection against contamination, provided their effectiveness is comparable. The air flow system should be set up and directed in a way that prevents intrusion of dust/amplicons from work areas with higher contamination risk to work areas with lower contamination risk.

For the analysis, unless otherwise stated, only analytical grade reagents suitable for molecular biology, free from DNA and DNases should be used. 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, deionized or of comparable quality. Solutions should be prepared by dissolving the appropriate reagents in water and autoclaved, unless specified differently. Sterile filtration devices (possibly 0,22 µm pore size) may be used when autoclaving is not possible.

In order to avoid contamination, sterile technique should be adopted in the PCR set-up area, e.g. powder-free gloves, sterilized plastic ware, autoclaved reagents, disposable plasticware and aerosol-protected, DNA/RNA free and DNase/RNase free filtered pipette tips should be used.

Materials and all containers and disposables containing reagents shall be preserved from any contaminating agent (e.g. dust).

Manufacturers' recommendations for the use of reagents should be followed. Appropriate controls can be used to assess the integrity of reagents and the absence of DNase.

No unintended enzyme activities (e.g. exonuclease) that might interfere with PCR shall be present in the preparation. The reaction buffer shall be suitable for the polymerase used.

7.3 Sample preparation and DNA extraction

A representative sample should be tested. It shall be ensured that the test samples used for DNA extraction are representative of the laboratory sample, such as by homogenizing the sample or appropriate portions thereof. At least two aliquots should be taken from the homogenized laboratory sample as test portions for DNA extraction and subsequent analysis.

If possible, the sample material should not be taken from the surface of the laboratory sample in order to minimize the risk of the amplification of adhering contaminants.

If the analytical method to be used for the sample detects human DNA, special contamination prevention measures should be taken.

Concerning the preparation of DNA from the test portion, the general instructions and measures described in ISO 21571 should be followed. One of the DNA extraction methods described in ISO 21571:2005, Annex A, should be considered. Alternatively, commercial kits can be used for the extraction and purification of DNA.

7.4 Use of controls

Controls should be carried out according to Table 1 (see also ISO 24276).

Negative DNA target control should be prepared from DNA extracted from non-target species prevalent in the sample (e.g. for a horse assay in cattle meat, the non-target species is cattle).

Table 1 — Flow diagram showing intersection of successive steps and inclusion of controls

Control step	Environment control[b]	Extraction blank control[c]	Positive extraction control[d]	Positive DNA target control[e]	Negative DNA target control[f]	PCR reagent control[g]	PCR inhibition control[h]
Homogenization	Recommended	—	—	—	—	—	—
Nucleic acid extraction	↓[a]	One per series	Mandatory at regular intervals	—	—	—	—
Assessment of nucleic acid quality	↓	↓	↓	—	—	—	—
Nucleic acid amplification	↓	↓	↓	Mandatory	Recommended	Mandatory	Recommended, but mandatory in certain cases[i]
Assessment of results of nucleic acid amplification	↓	↓	↓	↓	↓	↓	↓
Interpretation	—	↓	↓	↓	↓	↓	↓
Test report	—	↓	↓	↓	↓	↓	↓

[a] The arrows indicate that this control should be applied in the subsequent analytical steps.

[b] The use of environment controls helps the laboratory to identify sources of contamination at an early stage and can be used to identify in which work area the contamination is present. This can be demonstrated in various ways, e.g. if negative samples included in the series of homogenized samples showed negative results, starting at the first step of the process (e.g. grinding step if relevant).

[c] At least one extraction blank control shall be included each time DNA is extracted from one or more samples. The tube shall always be the last in each series. It may be appropriate to put one extraction blank on, for example, a rack of eight tubes or a microplate of 96 wells for automated extraction.

[d] A positive extraction control shall be included regularly. This control reveals if something is wrong with the reagents or the performance of the extraction protocol.

[e] The positive DNA target control demonstrates the ability of the nucleic acid amplification procedure to detect the target DNA sequence at a low copy number in order to confirm the LOD.

[f] The negative DNA target control demonstrates the ability of the nucleic acid amplification procedure to avoid false positive amplification in the absence of the target DNA sequence.

[g] The PCR reagent control demonstrates the absence of contaminating nucleic acid in the PCR reagent batches used. The PCR reagent control can be omitted when the extraction blank control is used.

[h] The PCR inhibition control can be used to demonstrate the absence of soluble inhibitors. This can also be demonstrated by serial dilutions of the template nucleic acid. However, some type of assessment of the effect of soluble inhibitors on the results of the analysis of the sample shall be made.

[i] A PCR inhibition control is mandatory, if all PCR test on the sample give negative results.

7.5 Data analysis

7.5.1 Control

Each control shall have a valid value and, if the observed result for any control is different from the valid value, the analysis shall be repeated. Environmental controls with a positive result, shall always initiate measures to remove and prevent contamination of the laboratory environment. If a non-valid result for any of the other controls is obtained repeatedly, measures shall be taken to locate and remove/replace

the source(s) responsible for the error, and the analysis then repeated. Analytical results shall only be reported when all controls yield valid values and the valid values for the controls are as follows:

— extraction blank control shall always be negative;

— positive extraction control shall always be positive;

— negative results shall always be negative (negative sample results are valid, even if the negative DNA target control is not valid, if all other controls are valid);

— positive DNA target control shall always be positive;

— negative DNA target control should be negative;

— PCR reagent control shall always be negative;

— PCR-inhibition control shall not show significant inhibitory effects in case of samples with negative qualitative results and for samples with quantitative results.

The above controls shall be used for interpreting/reporting the test sample result.

7.5.2 Conventional PCR

The amplicons generated by conventional PCR shall have the expected length (e.g. gel visualization). To avoid false-positive results, verification of the obtained amplicon can be performed by hybridization, sequencing, restriction enzyme analysis or another suitable sequence specific method of verification in addition to the length confirmation.

NOTE Melting curve analysis is sometimes used for amplicon verification but is not sequence specific.

7.5.3 Real-time PCR amplification curves

Real-time PCR amplification curves should be visually checked for a sigmoid shape in order to exclude artefacts/false-positive results.

NOTE Melting curve analysis is sometimes used for amplicon verification but is not sequence specific.

7.6 Expression of results

7.6.1 Expression of positive results

Results shall be described to show detection of target derived DNA.

EXAMPLE For target analyte X, the presence of DNA derived from (state specific target sequence and animal species or taxonomic group) was detected.

NOTE The scope of this analysis is only to show the presence/absence of DNA of the named animal species, not to show the presence/absence of tissue of the animal species (e.g. DNA derived from egg, gelatine).

Results for all test portions of one sample in one analysis shall be consistent. When at least one test portion gives a positive result and at least one gives a negative result, the PCR analysis shall be repeated.

If the PCR results of the second analysis are not identical for all test portions, DNA extraction and PCR analysis shall be repeated.

If at least two repetitions of the procedure (beginning with the DNA extraction) give ambiguous results, such as a positive and a negative result, the report shall state that the sample is negative at the limit of detection.

The result shall provide the specificity (species or taxonomic group or groups) and target (nuclear, mitochondrial or other) of the method in order to allow an unambiguous interpretation and comparability of the results.

7.6.2 Expression of negative results

Negative results shall be described to show no detection of target derived DNA.

EXAMPLE For target analyte X, animal species-derived DNA was not detected.

7.6.3 Expression of quantitative results

The results of quantitative methods shall state the unit of measurement.

For an example, see Annex B.

The result shall provide the measurement uncertainty and also the calibrators and the calculation method used, where applicable.

The applicability of the measured result with regard to mass/mass percentage of the target species in the sample shall be explained.

8 Test report

The test report should refer to ISO/IEC 17025 and shall contain at least the following:

— all information needed to identify the sample;

— a reference to this document, i.e. ISO 20813, and the respective annex(es) followed;

— the date and type of sampling procedure(s) used (if known);

— the date of receipt;

— the analysis start/end date, if applicable;

— the organization responsible for the analysis;

— the unit of measurement and the measured method, if applicable;

— the test portion amount used for the analysis;

— the result according to the requirements of the specific method and as specified under Clause 6;

— the methods and systems used;

— LOD of the analytical method as LOD_{rel} and/or LOD_{abs} for qualitative results;

— practical LOQ for quantitative results;

— any particular observations made during testing;

— any deviations, additions to or exclusions from the test specification.

Information shall be given with regard to the units of measurement.

The measurement uncertainty and its level of confidence shall, on request, be made available to the interested party in the same units as the result (e.g. %).

Annex A
(informative)

List of typical species used for inclusivity and exclusivity testing

The typical species used for inclusivity and exclusivity testing are listed in Tables A.1 to A.8.

Table A.1 — Examples of relevant mammals

No	Name	Latin name
1	Springbok	*Antidorcas marsupialis*
2	Minke whale	*Balaenoptera acutorostrata*
3	Antarctic minke whale	*Balaenoptera bonaerensis*
4	Sei whale	*Balaenoptera borealis*
5	Bryde's whale	*Balaenoptera edeni*
6	Fin whale	*Balaenoptera physalus*
7	Arnoux's beaked whale	*Barardius arnuxii*
8	Bryde's beaked whale	*Barardius bairdii*
9	Bison	*Bison bison*
10	Yak	*Bos mutus*
11	Cattle	*Bos taurus*
12	Water buffalo	*Bubalus bubalis*
13	Camel	*Camelus bactrianus*
14	Dromedary	*Camelus dromedarus*
15	Dog	*Canis lupus familiaris*
16	Goat	*Capra aegagrus hircus*
17	Roe deer	*Capreolus capreolus*
18	Red deer	*Cervus elaphus*
19	Sika deer	*Cervus nippon*
20	Gnu	*Connochaetes spp.*
21	Fallow deer	*Dama dama*
22	Donkey	*Equus asinus*
23	Horse	*Equus caballus*
24	Cat	*Felis catus*
25	Short-finned pilot whale	*Globicephala macrorhynchus*
26	Human	*Homo sapiens*
27	Capybara	*Hydrochoerus hydrochaeris*
28	Hare	*Lepus europaeus*
29	Red kangaroo	*Macropus rufus*
30	Rabbit	*Oryctolagus cuniculus*
31	Gemsbok	*Oryx gazelle*
32	Sheep	*Ovis aries*
33	Sperm whale	*Physeter catodon*
34	Dall's porpoise	*Phocoenoides dalli*
35	Rat	*Rattus norvegicus*

Table A.1 *(continued)*

No	Name	Latin name
36	Chamoise	*Rupicapra rupicapra*
37	Striped dolphin	*Stenella coeruleoalba*
38	Pig	*Sus scrofa domesticus*
39	Wild boar	*Sus scrofa scrofa*
40	Kudu	*Tragelaphus strepsiceros*
41	Bottlenose dolphin	*Tursiops truncatus*

Table A.2 — Examples of relevant birds

No.	Name	Latin name
1	Mallard duck	*Anas platyrhynchos*
2	Green leg goose	*Anser anser*
3	Muscovy duck	*Cairina moschata*
4	Rock pigeon	*Columba livia*
5	Common quail	*Coturnix coturnix*
6	Chicken	*Gallus gallus*
7	Turkey	*Meleagris gallopavo*
8	Guinea fowl	*Numida meleagris*
9	Ostrich	*Struthio camelus*

Table A.3 — Examples of relevant reptiles

No.	Name	Latin name
1	Nile crocodile	*Crocodylus niloticus*
2	Salt-water crocodile	*Crocodylus porosus*

Table A.4 — Examples of relevant amphibians

No.	Name	Latin name
1	Smokey jungle frog	*Leptodactylus pentadactylus*
2	Bullfrog	*Rana catesbeiana*
3	Edible frog	*Pelophylax esculentus*
4	Indian valley bullfrog	*Hoplobatrachus tigerinus*

Table A.5 — Examples of relevant fishes

No.	Name	Latin name
1	European eel	*Anguilla anguilla*
2	Japanese eel	*Anguilla japonica*
3	Splendid alfonsino	*Beryx sp lendens*
4	Atlantic herring	*Clupea harengus*
5	Pacific herring	*Clupea pallasii*
6	Pacific saury	*Cololabis saira*
7	Argentine anchovy	*Engraulis anchoita*
8	European anchovy	*Engraulis encrasicolus*
9	Japanese anchovy	*Engraulis japonicus*
10	Californian anchovy	*Engraulis mordax*

Table A.5 *(continued)*

No.	Name	Latin name
11	Anchoveta	*Engraulis ringens*
12	Alaska pollock	*Gadus chalcogrammus*
13	Pacific cod	*Gadus macrocephalus*
14	Atlantic cod	*Gadus morhua*
15	Greenland cod	*Gadus ogac*
16	Atlantic halibut	*Hippoglossus hippoglossus*
17	Pacific halibut	*Hippoglossus stenolepis*
18	Striped marlin	*Kajikia audax*
19	Skipjack tuna	*Katsuwonus pelamis*
20	Japanese sea bass	*Lateolabrex japonicus*
21	Yellow goosefish	*Lophius litulon*
22	Humpback salmon/pink salmon	*Oncorhunchus gorbuscha*
23	Coho salmon	*Oncorhunchus kisutch*
24	Chinook salmon, king salmon, spring salmon	*Oncorhunchus tschawytscha*
25	Chum salmon	*Oncorhynchus keta*
26	Masu trout/masu salmon	*Oncorhynchus masou masou*
27	Rainbow trout/steelhead	*Oncorhynchus mykiss*
28	Sockeye salmon	*Oncorhynchus nerka*
29	Red seabream	*Pagrus major*
30	Olive flounder/bastard halibut	*Paralichthys olivaceus*
31	Yellow striped flounder	*Pseudopleuronectes herzensteini*
32	Greenland halibut	*Reinhardtius hippoglossoides*
33	Atlantic salmon	*Salmo salar*
34	Japanese pilchard	*Sardinops melanostictus*
35	South American pilchard	*Sardinops sagax*
36	Blue mackerel/spotted chub mackerel	*Scomber australasicus*
37	Chub mackerel	*Scomber japonicus*
38	Atlantic mackerel	*Scomber scombrus*
39	Marbled rockfish	*Sebastiscus marmoratus*
40	Greater amberjack	*Seriola dumerili*
41	Yellowtail amberjack	*Seriola lalandi*
42	Japanese amberjack	*Seriola quinqueradiata*
43	Shishamo smelt	*Spirinchus lanceolatus*
44	Tiger puffer/ocellate puffer	*Takifugu rubripes*
45	Albacore tuna	*Thunnus alalunga*
46	Yellowfin tuna	*Thunnus albacares*
47	Southern bluefin tuna	*Thunnus maccoyii*
48	Bigeye tuna	*Thunnus obesus*
49	Pacific bluefin tuna/northern bluefin tuna	*Thunnus orientalis*
50	Atlantic bluefin tuna	*Thunnus thynnus*
51	Japanese jack mackerel	*Trachurus japonicus*

Table A.5 *(continued)*

No.	Name	Latin name
52	Atlantic horse mackerel	*Trachurus trachurus*
53	Pouting	*Trisopterus luscus*
54	Swordfish	*Xiphias gladius*

Table A.6 — Examples of relevant molluscs

No.	Name	Latin name
1	Ocellated octopus	*Amphioctopus fangsiao*
2	North pacific giant octopus	*Enteroctopus dofleini*
3	Inshore squid	*Heterololigo bleekeri*
4	Common octopus	*Octopus vulgaris*
5	Bigfin reef squid	*Sepioteuthis lessoniana*
6	Japanese flying squid	*Todarodes pacificus*
7	Firefly squid	*Watasenia scintillans*

Table A.7 — Examples of relevant crustaceans

No.	Name	Latin name
1	Snow crab	*Chionoecetes bairdi*
2	Red snow crab	*Chionoecetes japonicus*
3	Snow crab	*Chionoecetes opilio*
4	Shiba shrimp	*Metapenaeus joyneri*
5	Alaskan pink shrimp	*Pandalus eous*
6	Japanese spiny lobster	*Panulirus japonicus*
7	Red king crab	*Paralithodes camtschatica*
8	Blue king crab	*Paralithodes platypus*
9	Black tiger prawn	*Penaeus mondon*

Table A.8 — Examples of relevant insects

No.	Name	Latin name
1	Pine and spruce aphids	*Adelgidae*
2	Aphids	*Aphididae*
3	Tiger moths	*Arctiidae*
4	Roaches	*Blattellinae*
5	American cockroach family	*Blattidae*
6	Blowflies	*Calliphoridae*
7	Ground beetles	*Carabidae*
8	Biting midges	*Ceratopogonidae*
9	Nonbiting midges	*Chironomidae*
10	Leaf beetles	*Chrysomelidae*
11	Green lacewings	*Chrysopidae*
12	Cicadas	*Cicadidae*
13	Flat bark beetles	*Cucujidae*
14	Mosquitos	*Culicidae*
15	Weevils	*Curculionidae*

Table A.8 *(continued)*

No.	Name	Latin name
16	Skin and larder beetles	*Dermestidae*
17	Pomace flies	*Drosophilidae*
18	Click beetles	*Elateridae*
19	Common earwigs	*Forficulidae*
20	Ants	*Formicidae*
21	Crickets	*Gryllidae*
22	Minute brown scavenger beetles	*Latridiidae*
23	Common skimmers/dragonflies	*Libellulidae*
24	Root-eating beetles	*Monotomidae*
25	House flies	*Muscidae*
26	Fungus gnats	*Mycetophilidae*
27	Sap-feeding beetles	*Nitidulidae*
28	Owlet moths	*Noctuidae*
29	Prominent moths	*Notodontidae*
30	Stink bugs	*Pentatomidae*
31	Humpbacked flies	*Phoridae*
32	Phylloxerans	*Phylloxeridae*
33	Skipper flies	*Piophilidae*
34	Booklice and barklice	*Psocoptera*
35	Sandflies and mothflies	*Psychodidae*
36	Snout moths	*Pyralidae*
37	Flesh flies	*Sarcophagidae*
38	Soldier flies	*Stratiomyidae*
39	Hover flies	*Syrphidae*
40	Darkling beetles	*Tenebrionidae*
41	Fruit flies	*Tephritidae*
42	Winter crane flies	*Trichoceridae*
43	Hornets/yellowjackets/wasp	*Vespinae*
44	Smoky moths	*Zygaenidae*

Annex B
(informative)

Examples of unit conversion methods from DNA copy numbers to the ratio of masses

B.1 General

This annex shows examples of unit conversion methods from DNA copy numbers to the ratio of masses.

The following examples are considered to be standardized methods, however the estimation is highly likely to contain an uncertainty due to different DNA content per weight equivalent in different tissue and degree of processing.

B.2 Example for converting measurement results expressed in the ratio of DNA copy numbers into the ratio of masses using reference materials

See Reference [6].

The ratio of DNA copy numbers obtained in quantitative testing can be converted into the ratio of mass by using reference materials (RMs).

When each specific value of conversion factors for each available RM and its lot is determined, a converted result expressed in the ratio of masses remains traceable and comparable to a result expressed in the ratio of copy numbers.

RM with no DNA copy number or DNA mass value can be used for specified meat as a matrix.

The mass/mass calculation of the conversion factor (CF) is shown by Formula (B.1):

$$F_c = \frac{C_{rt}}{M_{rt}} \tag{B.1}$$

where

F_c is the value of conversion factor;

C_{rt} is copy number of target animal species or taxonomic group in reference material;

M_{rt} is mass of reference material of target animal species or taxonomic group.

As the premise for measuring target copy number in the same matrix used for the CF calculation, an example formula for calculating target % (mass/mass) is shown by Formula (B.2):

$$T = \frac{C_s}{M_s} \times \frac{1}{F_c} \times 100 \quad \text{(B.2)}$$

where

T is the percentage of masses of target species in the matrix;

C_S is the copy number of target animal species measured according to the requirements, including those described in 4.8;

M_S is the mass of the whole sample for analysis.

The uncertainty of conversion factor is used combined with the measurement uncertainty. The amount of DNA varies depending on the types and parts of meat, and it is important to estimate the uncertainty by considering it.

B.3 Example of relative quantification of animal species content in meat products

B.3.1 Analytical results reported in mass fraction

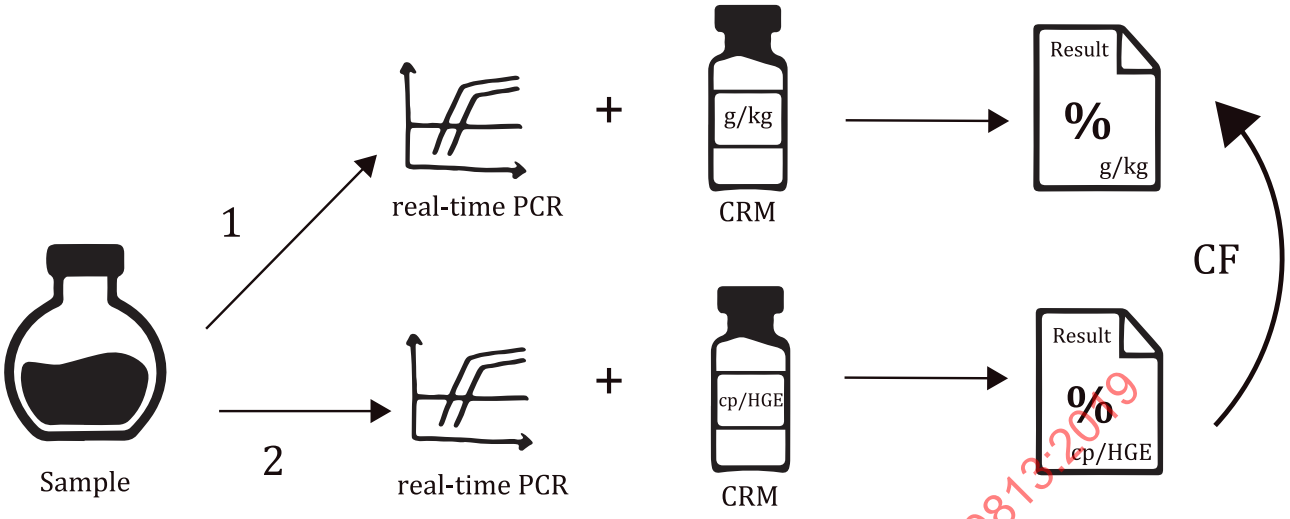
The expression of a quantitative result of analysis in mass fraction is a common approach in many areas (GMO, allergens, etc.) using DNA-based technologies, otherwise results would not be accepted/used by most food experts/assessors/regulators. Unfortunately, there is no consistent quantitative relationship between the DNA copy number and the meat content of a sample, therefore, an example of conversion is shown as published in EUR 28536 EN.[11] The document outlines how comparable and metrologically traceable results can be established from real-time polymerase chain reaction (real-time PCR) measurements, if the data are anchored to the certified value of a reference material.

Due to the lack of CRMs for testing animal species, an alternative strategy is routinely applied in official control laboratories using mass calibrated materials (e.g. meat mixture reference materials prepared in accordance with ISO 17034, or model food made under defined/controlled conditions, see also option 1 in Figure B.1). Different types of materials can be used to realize calibration curves for real-time PCR. The most common materials are solutions of genomic DNA (gDNA) or plasmid DNA (pDNA). However, an analyst can decide to extract gDNA from materials composed of different mixtures of animal materials that have been adjusted for their mass content.

B.3.2 Conversion of measurement results expressed in copy number per haploid genome equivalent (cp/HGE) into mass fraction

B.3.2.1 General

The conversion of a measurement result obtained by real-time PCR using a calibrant, expressed as copies per haploid genome equivalent (cp/HGE), into a mass fraction is needed. An overview of the various possibilities to measure the animal content present in a sample is presented in Figure B.1.



NOTE 1 In option 1, the animal content is determined by real-time PCR using a CRM or equivalent material (e.g. model samples) adjusted for its mass fraction as calibrant. The result is expressed in mass fraction (g/kg) or % mass.

NOTE 2 In option 2, the animal content is determined by real-time PCR using a calibration material for which the copy number is determined. The results obtained (in cp/HGE) are converted into a mass fraction (g/kg) using a CF.

NOTE 3 Adapted from Reference [11] with modification.

Figure B.1 — Overview of the various possibilities to measure the animal content present in a sample

By using a relative quantification approach, the DNA isolates are analysed using at least two real-time PCR systems targeting single copy genes: the species-specific real-time PCR assay to determine the content of the animal of interest and a reference real-time PCR assay to determine the total amount of animal DNA (e.g. myostatin gene, combination of singleplex systems to sum up the total amount of mammal and poultry DNA). Both real-time PCR assays are calibrated. Calibration curves are obtained by plotting the logarithm of the DNA concentration against the respective quantification cycle (Cq) value. After establishing the calibration curves for both PCR assays using linear regression, the total DNA amount of mammals and poultry in the sample is determined and the percentage of the specific animal calculated.

The DNA of all animal species, mammals and poultry is assumed to be extracted with approximately the same efficiency.

To evaluate the applicability of the quantification approach, analyse positive quantitative controls with known composition and calculate their recoveries. The matrix and composition of the positive quantitative controls are expected to be similar to the sample.

EXAMPLE The DNA in the PCR assay targeting the reference gene (e.g. myostatin) is diluted in buffer or nuclease free water (e.g. dilutions from 150 ng DNA/PCR to 1 ng DNA/PCR are used to establish a calibration curve for the reference gene). To generate a calibration curve for the animal-DNA target, 150 ng DNA/PCR extracted from a model material containing a decreasing amount of the target animal (e.g. from 50 g/kg to 1 g/kg) are used. The amount of target-DNA in the assay that has been extracted from a model material containing 50 g/kg target animal (corresponding to a mass fraction of 5 %) is also considered to be 5 % in terms of target DNA copies (i.e. 7,5 ng of target DNA per PCR well for a reaction containing 150 ng DNA). The same proportional approximation is made for the other standards that contain a smaller mass fraction of animal or reference gene targets. The amounts (or concentrations in %) of animal-target and reference gene DNA in a DNA solution extracted from an unknown sample are then calculated by converting the measured Cq values into mass values using the two calibration curves and dividing them. The animal mass fraction (animal target versus reference gene target) is finally multiplied by 100 to express it as a percentage.

Approaches already published for relative quantification of animal species content in meat products are listed in Table B.1.

Table B.1 — Real-time PCR based quantification strategy

Real-time PCR based quantification strategy	Calibration		Result expressed as	Literature	
	Calibrator	Unit			
Standard curve method using reference gene	DNA mixtures (copy number calculation)	cp/µl	% DNA (c/c)	Laube et al. (2007)[22] Iwobi et al. (2015)[18]	
	DNA mixtures	ng/µl	% DNA (w/w)	Druml et al. (2015)[15]	Kaltenbrunner et al. (2018)[19] Kaltenbrunner et al. (2018)[20]
	meat extract (lysate) mixtures	ng/µl	% DNA (w/w)		
	meat mixtures	ng/µl	% DNA (w/w)	Druml et al. (2016)[16]	
	model matrix	ng/µl	% DNA (w/w)		
Standard curve method using normalization	DNA mixtures	ng/µl	% DNA (w/w)	Köppel et al. (2012)[21] BVL L 08.00-61[13] BVL L 08.00-62[14]	
	model matrix	ng/µl	% DNA (w/w)	Eugster et al. (2009)[17]	

B.3.2.2 Example: Relative quantification based on reference gene (standard curve method)

B.3.2.2.1 Singleplex PCR amplification of species-specific target sequences and an endogenous universal animal target sequence (reference gene) based on copy number calculations.

Procedure:

— genomic DNA extracted from species muscle tissue samples (reference sample) and DNA concentration (in ng/µl) measurement (by Nanodrop, PicoGreen etc.) or plasmid DNA used as calibration standards;

— singleplex PCR run for the species target and the universal animal target with calibration DNAs (at least four standards with DNA dilutions and genome equivalents per reaction of defined numbers, e.g. 156 250, 31 250, 6 250, 1 250, 250, 50) and the sample DNA;

— Cq-values of standards are for the calibration curve, the Cq-values of the sample are used to calculate the species-specific concentration (copies/µl);

— the generated copy number for each species are extrapolated against the calculated copy numbers for the universal animal reference gene, to give the proportion of species x or species y, etc.

Formula (for each PCR targeted species):

$$P_x = \frac{C_{sx} \times 100}{C_r}$$

$$P_y = \frac{C_{sy} \times 100}{C_r}$$

where

P_x is the percentage of the target species X;

P_y is the percentage of the target species Y;

C_{sx} is the copy number of target species X (cp/µl);

C_{sy} is the copy number of target species Y (cp/µl);

C_r is the copy number of universal reference gene (cp/µl).

Genome sizes of some species are listed in Table B.2 as an example.

Table B.2 — Genome sizes to calculate the genome copies

Species	Base pair	Genome size (pg)
Cattle	$3{,}65 \times 10^9$	4,00
Pig	$3{,}11 \times 10^9$	3,41
Lamb	$3{,}25 \times 10^9$	3,56
Goat	$3{,}20 \times 10^9$	3,51
Chicken	$1{,}25 \times 10^9$	1,37
Turkey	$1{,}68 \times 10^9$	1,84
Duck	$1{,}54 \times 10^9$	1,69

For further information, see References [18] and [22].

B.3.2.2.2 Multiplex or singleplex PCR amplification of species-specific target sequences and an endogenous universal animal target sequence (reference gene, in singleplex PCR) based on amplified DNA (ng/µl).

Procedure:

— calibrator:

  — DNA mixtures: extraction of genomic DNA from species muscle tissue samples (reference sample) and DNA concentration (in ng/µl) measurement (by Nanodrop, PicoGreen, etc.) or use of plasmid DNAs with targets specific for different species; genomic DNAs or plasmid DNAs are diluted to the same concentration and mixed in appropriate ratios;

  — lysate mixtures (sample adapted standard calibration):

    — lysis (CTAB buffer + proteinase K) of muscle tissue samples (pure reference meat samples, e.g. 1 g);

    — after lysis, lysates are mixed in appropriate ratios (volume fraction), DNA is extracted (extraction protocol is the same for calibration material and samples); by imitating the composition of the tested sample species-specific PCR effects are eliminated;