International Standard

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Molecular biomarker analysis — Requirements and guidance for single-laboratory validation of qualitative real-time polymerase chain reaction (PCR) methods

Analyse de biomarqueurs moléculaires — Exigences et recommandations pour la validation intralaboratoire des méthodes de PCR qualitative en temps réel

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Contents

Page

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,* in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 275, *Food analysis - Horizontal methods,* in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

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Introduction

Qualitative real-time polymerase chain reaction (PCR) methods currently find broad application for the detection of specific DNA sequences in food (e.g. for the detection and identification of genetically modified organisms and the products derived thereof, for food authentication and speciation and for other purposes). It is important that a newly developed food analytical method is fit for purpose and meets certain performance characteristics and quality criteria as demonstrated by a particular set of validation experiments.

The data determined by the single laboratory validation are the basis for the decision to apply a method in-house. Furthermore, it helps to decide whether the method in question should be fully validated in the framework of a collaborative study. The statistical model described has been practically applied, e.g. some parts of the ISO/TS 21569 series[1][2] and ISO/TS 20224 series[3]. Other models can be applicable, see ISO/TS 16393[4].

The aim of this document is to provide a protocol for single-laboratory validation of qualitative real-time PCR methods that are applied for food analysis. Procedures for DNA extraction from the food matrix are not included in this document. The procedure described is a recommendation which is underpinned by practical experience in several laboratories. Alternate approaches may be applied if they can be shown to meet the performance criteria set in this document.

Molecular biomarker analysis — Requirements and guidance for single-laboratory validation of qualitative real-time polymerase chain reaction (PCR) methods

1 Scope

This document specifies minimum requirements and minimum performance criteria for conducting a single-laboratory validation study for qualitative (binary) real-time polymerase chain reaction (PCR) methods applied to the detection of specific DNA sequences present in foods.

The document is applicable to any single-laboratory validation of a qualitative real-time PCR method used for the detection of specific DNA sequences in food and food products (e.g. for the detection of genetically modified foodstuffs and for species determination, including species known to produce allergenic proteins).

The document does not apply to single laboratory validation of qualitative microbiological real-time PCR methods.

The document does not apply to the evaluation of applicability and practicability with respect to the specific scope of the PCR method.

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 — Vocabulary for molecular biomarker analytical methods in agriculture and food production*

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

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 16577 and the following apply.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
probability of detection
POD
probability of a positive analytical outcome of a qualitative method for a given matrix at a given concentration in a single laboratory

Note 1 to entry: For a qualitative real-time PCR method, it describes the probability that, for a given number of DNA copies of the target sequence, PCR amplification will take place.

[SOURCE: ISO 16577:2022, 3.9.12, modified — Note 1 to entry replaced Notes 1 and 2 to entry.]

3.2
polymerase chain reaction efficiency
PCR efficiency
measured amplification rate for a DNA copy of the target sequence per PCR cycle in relation to the theoretically achievable value of 1

Note 1 to entry: The PCR efficiency is calculated from the slope of a standard curve resulting from the decadic semi-logarithmic plot of quantification cycle (Cq) values over the DNA concentration. The slope from the calculated regression line can be used. The PCR efficiency can either be expressed as absolute number or as percentage.

3.3
limit of detection
$LOD_{95\ \%}$
mean number of DNA copies of the target sequence yielding a *probability of detection* (3.1) of 0,95

3.4
specificity
property of a method to respond exclusively to the characteristic or analyte under investigation

[SOURCE: ISO 24276:2006, 3.1.4]

4 Principle

Specific primers and also probes, depending on the detection system applied, are designed for specific amplification and detection of a DNA target sequence by a qualitative real-time PCR method. In the next step for single laboratory validation, the method's performance characteristics shall be assessed to show that the method complies with the quality criteria stipulated in relevant documents.[5][6]

For a qualitative real-time PCR method, the main focus of the validation shall be the limit of detection ($LOD_{95\ \%}$) (at which the probability of detection (POD) is ≥ 95 %), the specificity for the DNA target sequence and the robustness to small but deliberate variations in the method parameters.

On the basis of single laboratory validation data, fulfilment of the minimum required performance criteria for a qualitative real-time PCR method can be verified and should comprise the basis for applicability of the method by a single laboratory. A further decision whether to conduct a validation of the method in the framework of a collaborative study can then be taken.

Determination of the reproducibility (interlaboratory transferability) and how the method performs in different laboratories, in particular the false-positive/false-negative rate obtained with negative/positive test samples, and the POD across laboratories, can be evaluated by a collaborative study, if the design is appropriate.[6]

5 Single-laboratory validation of the performance characteristics

5.1 General

Guidance for compiling the information required for a complete and detailed description of all components that should be provided with the protocol of qualitative PCR methods (e.g. oligonucleotide sequences, amplicon length, instrument or chemistry specifications, PCR conditions, analytical controls) is described in other relevant documents, see References [5] and [6] and ISO 21569[8].

DNA extraction shall be in accordance with the requirements specified in ISO 21571.

5.2 Limit of detection

The $LOD_{95\ \%}$ is expressed as the number of copies of the target sequence and shall be determined by means of a dilution series of the target DNA, where, in addition to the target DNA, each dilution contains a uniform concentration of non-target DNA (background DNA).

A minimum of six target DNA concentration levels with 12 replicates per level are required.

The lowest dilution level, i.e. the lowest number of copies for which all 12 replicates are positive, is considered to be an approximate value for the $LOD_{95\,\%}$ (see Clause B.2).

The $LOD_{95\,\%}$ of qualitative real-time PCR methods should not exceed 20 copies of the target sequence.

NOTE 1 This document is applicable to the validation of new methods. However, for method verification, 10 replicates can be sufficient.

NOTE 2 If the $LOD_{95\,\%}$ equals 20 copies of the target sequence, the amplification probability (λ) of the entire PCR is approximately 15 % based on the parameter of the Poisson distribution ($\lambda \cdot LOD_{95\,\%} = 2{,}996$).[7]

Annex A provides additional detailed information regarding copy number estimation of target DNA.

Practical guidance for determining the $LOD_{95\,\%}$ experimentally is given in Annex B.

Annex C provides the basics of the specific statistical model adapted for PCR methods.

5.3 Determining copies of DNA target sequences in DNA test materials

A determination of copies of DNA target sequences is required for the validation.

The number of copies of the target sequence for a specified mass of nucleic acid (DNA) can be calculated on basis of haploid genome equivalents using the measured DNA concentration (see ISO 21571:2005, Annex B) and the genome mass.[9][10][11] The use of digital PCR equipment (e.g. droplet digital PCR) is an alternative approach that allows an accurate determination of the number of copies of a DNA target sequence or the concentration of a DNA solution.[12]

The quality and the concentration (very high or very low) of the background DNA used for the dilution can influence the validation experiment. It is therefore highly recommended to use DNA tested for the absence of PCR inhibitors (e.g. commercial molecular biology grade DNA preparations) and a concentration that is relevant for extracted DNA.[13]

5.4 Evaluation of data for the limit of detection

The $LOD_{95\,\%}$, the mean POD curve and the 95 % confidence interval should be determined by means of a statistical model.

The experimental work that also includes a Poisson component is based on the generalized linear mixed model (GLMM) with a complementary log-log link function. This model has been found to be most effective when the DNA copy number is low and follows a Poisson distribution.

Details for the GLMM with complementary log-log link function are given in Annex C. To perform this calculation, the nominal copies that are added to the PCR reaction, the number of replicates performed and the number of positive results obtained are required.

Using the results data of the dilution series, the $LOD_{95\,\%}$, the 95 % confidence interval and the mean POD curve, along with the corresponding 95 % confidence range, can be calculated via a web service[14] or by using the R package POD (see Clause C.5[15]).

Check the $LOD_{95\,\%}$ for plausibility. A value smaller than 2,996 suggests that the number of copies of the target sequence that were actually added to the PCR reaction did not correspond to the (nominal) numbers of copies estimated for the DNA solutions.[7]

If more than two results are positive at the level with 0,1 copies of the target sequence per PCR, the DNA dilutions cannot be considered as verified and the number of copies shall be re-examined.

NOTE 1 The calculation of $LOD_{95\,\%}$ is only valid if false-positive results are negligible, i.e. if the specificity testing was successful and PCR carry-over contamination can be excluded.

NOTE 2 The level that will be the result of a tenfold dilution of 1 nominal copy is designated “level with 0,1 copies per PCR” for the sake of better readability throughout this document.

5.5 PCR efficiency and variability of the measured copy number around the limit of detection

For an optional determination of the copy number variability around the $LOD_{95\,\%}$, assign the copy numbers to the respective Cq values on the basis of an additional calibration series of target DNA (for preparation of a calibration series, see Clause B.2).

The variability of the measured number of copies around $LOD_{95\,\%}$ should be assessed by comparing the repeatability standard deviations with the theoretical values of the Poisson model. An adjusted standard deviation of > 30 % indicates that the $LOD_{95\,\%}$ that can be achieved in routine analysis can be subject to high variability (see Clause B.5).

The experimental data collected will also permit determination of the PCR efficiency (see Clause B.6), the slope of the amplification curve and the coefficient of determination (R^2). A PCR without inhibitory influences will amplify as an exponential doubling with a slope (Cq/number of initial copies) of −3,321 9. An acceptable PCR efficiency should not deviate by more than ±10 % from the theoretical value of 100 % (corresponding to a slope in the interval −3,1 to −3,6). Where the PCR efficiency is found to be greater than the theoretical maximum, errors due to reaction setup and PCR inhibition should be considered. The coefficient of determination should be at least 0,98 (or 98 %).

5.6 Specificity

5.6.1 General

In silico analysis and experimental results from testing the method with genomic sequence databases and material containing the target sequence shall be provided. If available, testing should be inclusive and exclusive with relevant and representative data and materials according to the scope of the method.

5.6.2 Bioinformatic (in silico) test for specificity

Bioinformatic specificity tests shall be carried out, examining the oligonucleotide and the amplicon sequences with available bioinformatics tools (e.g. primer-dimer formation with primer 3).[16] Homology to other sequences shall be tested by searches in nucleic acid sequence databases (e.g. BLAST in GenBank®1)[17]).

The in silico analysis should not reveal any sequence similarities between the target and the sequences that can be present in the sample capable of influencing the analytical result. The oligonucleotide sequence(s) should be adapted accordingly, if appropriate. Additional guidance on in silico analysis can be found in Reference [18].

5.6.3 Practical test for specificity

Perform tests for unexpected cross-reactions with non-target DNA. Check the PCR detection system for cross-reactivity with DNA from organisms that have similar (homologous) genetic elements, genes or genetic constructs. Also check for species that are often present in food as ingredients (e.g. corn, soya, rape seed, rice, potato, wheat, cattle, chicken, pig, sheep, turkey, horse).

If non-target DNA is tested and a negative result is expected, at least 2 500 copies should be added to the PCR reaction. If no reference material with sufficiently high concentrations of the non-target DNA is available, lower concentrations can be used and the number of copies added should be indicated. Verify the amplifiability of the non-target DNA by means of an independent test.

Perform tests with target DNA. Add target DNA for which a positive result is expected in copy numbers in the range of the limit of quantification (LOQ) (empirically the copy number for $LOD_{95\,\%}$ can be multiplied by a factor of 3, i.e. in general 20 to 60 copies per PCR). Add non-target DNA at a concentration of 100 ng/25 µl to 200 ng/25 µl of PCR mix to the target DNA in order to simulate conditions that are relevant in practice and could influence the outcome.

1) BLAST in GenBank® is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

It is sufficient to carry out each of the PCR tests for inclusivity (using target DNA) and exclusivity (using non-target DNA) in duplicate determination.

PCR results for in silico and the experimental analyses should meet or exceed the requirements and criteria of the method.

If there is cross-reactivity that is considered acceptable, it shall be indicated and taken into account in the scope of the method.

5.6.4 Robustness

The robustness of a qualitative real-time PCR method shall be evaluated. The evaluation of robustness shall include small but deliberate variations, e.g. in the following method parameters:

— different types of real-time PCR equipment, if available;

— PCR reagent kits;

— annealing temperature applied in the thermal cycling program;

— master mix volume;

— primer and probe concentrations.

A practicable example of factors and modifications in the procedure conditions controlling all relevant aspects of qualitative real-time PCR is given in Table 1. Other factors or modifications may be applied if the gain of sufficient informative value is indicated by the addition.

Testing should be carried out in a multifactorial experimental design.[19] The PCR reactions with the different combinations of factors should be done with target DNA at a concentration around the number of copies corresponding to the $LOD_{95\ \%}$ multiplied by a factor of 3 (corresponding to approximately 20 to 60 copies per PCR). Dilute the target DNA in non-target DNA (background DNA), e.g. 20 ng/µl. For each factor-level combination, PCR tests should at least be performed in triplicate. An example of the procedure is given in Table D.1.

The method shall yield positive results for all combinations despite the modified conditions.

In the case of negative results, the PCR test for the corresponding combinations shall be repeated. In the case of repeated negative results, the method is not sufficiently robust and shall be optimized.

Considerable deviations between Cq values can be an indication that the robustness of the method is insufficient.

Tables 1 and 2 describe statistically proven examples for robustness testing. Other options can be applied when they produce comparable statistically proven outcomes. If the single laboratory is the basis for a collaborative study, the tests of several variables is strongly recommended, see Annex D.

Table 1 — Example of a robustness test of factors and modifications in the procedure and conditions of qualitative real-time PCR methods

Factor	1	0
PCR equipment	A	B
PCR master mix	X	Y
Primer concentration	unchanged	−30 %
Probe concentration	unchanged	−30 %
Volume of PCR reagent mix (if total volume is 25 µl)	Specified volume of PCR reagent mix − 5 % (19 µl) + 5 µl of DNA	Specified volume of PCR reagent mix + 5 % (21 µl) + 5 µl of DNA
Annealing temperature	+1 °C	−1 °C