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Foodstuffs - General guidelines for the validation of qualitative real-time PCR methods - Part 1: Single-laboratory validation

Lebensmittel - Allgemeine Anleitung für die Validierung qualitativer Realtime-PCR-Verfahren - Teil 1: Einzellaborvalidierung

Denrées alimentaires - Lignes directrices générales pour la validation des méthodes de PCR qualitative en temps réel - Partie 1 : Validation intralaboratoire

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Foodstuffs - General guidelines for the validation of qualitative real-time PCR methods - Part 1: Single-laboratory validation

Denrées alimentaires - Lignes directrices générales pour la validation des méthodes de PCR qualitative en temps réel - Partie 1 : Validation intralaboratoire

Lebensmittel - Allgemeine Anleitung für die Validierung qualitativer Realtime-PCR-Verfahren - Teil 1: Einzellaborvalidierung

This Technical Specification (CEN/TS) was approved by CEN on 16 May 2021 for provisional application.

The period of validity of this CEN/TS is limited initially to three years. After two years the members of CEN will be requested to submit their comments, particularly on the question whether the CEN/TS can be converted into a European Standard.

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European foreword

This document (CEN/TS 17329-1:2021) has been prepared by Technical Committee CEN/TC 275 “Food analysis - Horizontal methods”, the secretariat of which is held by DIN.

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

This document supersedes CEN/TS 17329-1:2019.

This new version was updated by inclusion of an option (in Annex C, C.4) to calculate the results of a single-laboratory validation by using the R-package POD [15]. This calculation tool can make the user of this document independent from consultation of a statistician or a professional statistical service provider, when evaluating the results of a single-laboratory validation. The R-package can be downloaded without being charged.

This series consists of two parts:

— *Part 1: Single-laboratory validation*;

— *Part 2: Collaborative study*.

According to the CEN/CENELEC Internal Regulations, the national standards organisations of the following countries are bound to announce this Technical Specification: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Republic of North Macedonia, Romania, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and the United Kingdom.

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 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 aim of this document is to provide a protocol for single-laboratory validation of qualitative real-time PCR methods which are applied for food analysis. The procedure described is a recommendation that is underpinned by practical experience in several laboratories. It is possible to apply alternative approaches for which it can be shown that the performance criteria mentioned in the present document are achieved.

1 Scope

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

The protocol was developed for qualitative real-time PCR methods for the detection of DNA sequences derived from genetically modified foodstuffs. It is applicable also for single-laboratory validation of qualitative PCR methods used for analysis of other food materials, e.g. for species detection and identification.

The document does not cover the evaluation of the applicability and the 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.

EN ISO 21571:2005,[1] *Foodstuffs - Methods of analysis for the detection of genetically modified organisms and derived products - Nucleic acid extraction (ISO 21571:2005)*

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

ISO 16577, *Molecular biomarker analysis - Terms and definitions*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 16577 and EN ISO 24276 and the following apply.

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

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

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

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

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.

[1] As impacted by EN ISO 21571:2005/A1:2013.

3.2
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 copies of the target sequence yielding a probability of detection of 0,95

4 Principle

Specific primers and also probes, depending on the detection system applied, have been designed for specific amplification of a DNA target sequence by a qualitative real-time PCR method. As next step the methods performance characteristics needs to be assessed to show that the method complies with the quality criteria stipulated in relevant documents [1], [2].

According to the published guidelines the main criteria in the single-laboratory validation of a qualitative real-time PCR method mainly concerns the limit of detection (at which the probability of detection 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 the validation data, it can be verified whether the minimum required performance criteria are fulfilled. This will be the basis for the 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 (inter-laboratory transferability) and how the method performs at different laboratories, in particular the false-positive/false-negative rate obtained with negative/positive test samples, and the probability of detection (POD) across laboratories are evaluated by a collaborative study, if the design is appropriate [3].

General guidelines for conducting a collaborative validation study are provided in Part 2 of this series.

5 Single-laboratory validation of the performance characteristics

5.1 General

Guidance for compiling the information required for complete and detailed description of all components that should be provided with the protocol of the qualitative PCR methods (i.e. oligonucleotide sequences, amplicon length, instrument or chemistry specifications, PCR conditions, analytical controls, etc.) is described in other relevant documents [1], [2].

DNA extraction shall be according to the requirements and procedures specified in EN ISO 21571.

5.2 Limit of detection ($LOD_{95\ \%}$)

In qualitative PCR analysis (especially for the detection of genetically modified foodstuffs), the limit of detection is usually defined as the amount of the target DNA at which an amplification product is detected with a probability of at least 0,95 ($LOD_{95\ \%}$). It is expressed in the number of copies of the target sequence.

The $LOD_{95\ \%}$ should be determined by means of a dilution series of the target DNA, using a uniform concentration of non-target DNA (background DNA) for each dilution level.

Annex A provides additional detailed information regarding the copy number estimation of the target DNA. Annex C provides the basics of the specific statistical model adapted for PCR methods.

For each dilution level, perform 12 PCR replicate measurements.[2] 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 $LOD_{95\ \%}$ (see B.2).[3] The $LOD_{95\ \%}$ of the qualitative real-time PCR method should not exceed 20 copies of the target sequence.

The number of copies of the target sequence can be calculated on basis of haploid genome equivalents using the measured DNA concentration (see EN ISO 21571:2005, Annex B[1]; [4]) and the genome weight [5], [6], [7]. The use of digital PCR equipment (e.g. digital droplet PCR) is an alternative approach which allows an accurate determination of the number of copies of a target sequence or the concentration of a DNA solution [8].

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 which is relevant for DNA extracted from food.

Practical guidance which has been experimentally proven is given in Annex B.

5.3 Evaluation of data for the limit of detection ($LOD_{95\ \%}$)

Determine the $LOD_{95\ \%}$, the mean POD curve, and the 95 % confidence interval by means of a statistical model, e.g. the complementary log-log model and the likelihood ratio test [3]. Details on the statistical model are given in Annex C. For the calculation, the nominal copies added to the PCR reaction, the number of replicates and the number of positive results are required.

The complementary log-log model corresponds to applications where we observe either zero events (e.g. defects) or one or more events where the number of events is assumed to follow the Poisson distribution.

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 [9] or by using the R package POD [15].

Check the $LOD_{95\ \%}$ for plausibility. A value significantly 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 [3].

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

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2 The statistical approach is described in [4].

3 This document is relevant for the validation of new methods. However, for method verification, 10 replicates can be sufficient.

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 which 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.

5.4 PCR efficiency and variability of the measured copy number around the $LOD_{95\ \%}$

For the optional determination of copy numbers around the limit of detection, assign the copy numbers to the respective Cq values on the basis of an additional calibration series of target DNA (preparation, see Annex B, Table B.1).

In addition, the variability of the measured number of copies around $LOD_{95\ \%}$ can be assessed (see B.5).

To this end, compare the repeatability standard deviations to the theoretical values resulting from the Poisson model.

The experimental data also allow the calculation of the PCR efficiency (see B.6), the slope and the coefficient of determination.

5.5 Specificity

5.5.1 General

Theoretical and experimental results from testing the method with the sequence databases and material containing the target sequence should be provided. If available, this testing should include all relevant and representative materials according to the scope of the method.

5.5.2 Theoretical test for specificity

Computer-aided (*in-silico*) 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 [10]). The homology to other sequences shall be tested by searches in nucleic acid sequence databases (e.g. BLAST in GenBank [11]).

The *in-silico* analysis should not show any unwanted similarities between sequences which could influence the analytical result. The oligonucleotide sequence(s) should be adapted accordingly, if appropriate.

5.5.3 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 which are often present in food, e.g. as ingredient (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 2500 copies should be added to the PCR reaction, if possible. 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 LOQ (here: the copy number for $LOD_{95\ \%}$ multiplied by a factor of 3, i.e. in general 20 to 60 copies per PCR). Add non-target DNA in a concentration of 100 ng/25 µl to 200 ng/25 µl of PCR mix to the target DNA, in order to simulate conditions which are relevant in practice and could influence the outcome.

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.

In the experimental test, all the PCR results should fulfil the theoretical expectations.

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

5.5.4 Robustness

In the single-laboratory validation, evaluate the robustness of a qualitative real-time PCR method concerning different types of real-time PCR equipment, PCR reagent kits, annealing temperature applied in the thermal cycling programme, the master mix volume and the primer and probe concentrations (Table 1).

Implement a multifactorial experimental design [12]. The PCR reactions with the different combinations of factors are 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 Annex D, 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 should be repeated. In the case of repeated negative results, the method is not sufficiently robust and needs to be optimized.

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

Table 1 — Robustness test of factors and modifications in the procedure 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)	19 µl of PCR reagent mix + 5 µl of DNA	21 µl of PCR reagent mix + 5 µl of DNA
Annealing temperature	+1 °C	−1 °C