SLOVENSKI STANDARD
kSIST-TS FprCEN/TS 17329-2:2019

01-januar-2019

Živila - Splošne smernice za validacijo kvalitativnih metod PCR v realnem času - 2. del: Medlaboratorijska študija

Foodstuffs - General guidelines for the validation of qualitative real-time PCR methods - Part 2: Collaborative study

Lebensmittel - Allgemeine Anleitung für die Validierung qualitativer Realtime-PCR-Verfahren - Teil 2: Ringversuch

Ta slovenski standard je istoveten z: **FprCEN/TS 17329-2**

ICS:

67.050	Splošne preskusne in analizne metode za živilske proizvode	General methods of tests and analysis for food products

kSIST-TS FprCEN/TS 17329-2:2019 **en,fr,de**

TECHNICAL SPECIFICATION

SPÉCIFICATION TECHNIQUE

TECHNISCHE SPEZIFIKATION

FINAL DRAFT FprCEN/TS 17329-2

November 2018

ICS 67.050

English Version

Foodstuffs - General guidelines for the validation of qualitative real-time PCR methods - Part 2: Collaborative study

Lebensmittel - Allgemeine Anleitung für die Validierung qualitativer Realtime-PCR-Verfahren - Teil 2: Ringversuch

This draft Technical Specification is submitted to CEN members for Vote. It has been drawn up by the Technical Committee CEN/TC 275.

CEN members are the national standards bodies of Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, Former Yugoslav Republic of Macedonia, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and United Kingdom.

Recipients of this draft are invited to submit, with their comments, notification of any relevant patent rights of which they are aware and to provide supporting documentation.

Warning : This document is not a Technical Specification. It is distributed for review and comments. It is subject to change without notice and shall not be referred to as a Technical Specification.

EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

CEN-CENELEC Management Centre: Rue de la Science 23, B-1040 Brussels

Contents

Page

European foreword

This document (FprCEN/TS 17329-2:2018) has been prepared by Technical Committee CEN/TC 275 “Food analysis - Horizontal methods”, the secretariat of which is held by DIN.

This document is currently submitted to the Vote on TS.

This Technical Specification consists of two parts,

— Part 1: Single-laboratory validation

— Part 2: Collaborative study

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 results obtained from different laboratories by such food analytical methods satisfy certain performance characteristics and quality criteria. The performance of a method is validated in a step-wise process from in-house (single laboratory) validation to a pre-validation study by few laboratories followed by a full validation in a collaborative study to gain information and data on the reproducibility of the analysis results obtained by different laboratories.

The aim of this document is to provide practical guidance for a collaborative validation study 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 collaborative trial studies. 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 provides information on how the performance characteristics of qualitative (binary) real-time polymerase chain reaction (PCR) methods for detection of specific DNA sequences present in foods should be evaluated and validated by conducting a collaborative study.

The guidelines are applicable for validation of qualitative PCR methods used for detection of DNA sequences derived from genetically modified foodstuffs. They can be applicable also for PCR methods used for detection of other target sequences in foodstuffs, e.g. for species detection and identification.

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 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 http://www.electropedia.org/

— ISO Online browsing platform: available at http://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.

3.2
laboratory standard deviation σ_L
expression of the standard deviation between laboratories which describes the dispersion of the log-transformed laboratory-specific values for the LOD95%

3.3
mean amplification probability λ
probability that, for a randomly selected DNA copy of the target sequence, PCR amplification will occur

3.4
slope parameter *b*
slope of the POD curve (across laboratories) that indicates the deviation from the ideal POD curve (with b = 1)

Note 1 to entry: The ideal POD curve is based on the assumption that the mean amplification probability is independent of the number of DNA copies of the target sequence.

3.5
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 and 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.

3.6
limit of detection
$LOD_{95\%}$
mean number of copies of the target sequence yielding a probability of detection of 0,95

4 Principle

At the first step, a qualitative PCR method shall be single-laboratory validated and needs to show satisfactory performance characteristics, see CEN/TS (wi 335)xxxx-1.

As next step of the validation process, an inter-laboratory (or collaborative) validation study is undertaken to assess the methods performance.

According to appropriate guidelines [1], [2], the main criterion in the validation of a qualitative real-time PCR method by means of a collaborative study concerns the determination of the false-positive rate and false-negative rate. Due to the use of different real-time PCR equipment from one laboratory to the next, additional information on the robustness of the method can also be derived. Moreover, the probability of detection (POD) of qualitative PCR methods can be evaluated, if the design of the collaborative study is appropriate [3].

5 Validation of the performance characteristics by means of a collaborative study

5.1 General

Guidance for conducting a collaborative validation study of qualitative PCR methods (i.e. organization, protocol, number of participating laboratories etc.) and the description of all required components is provided in other relevant documents [1]. Participants should have the required laboratory equipment and proficiency in PCR testing.

The reagents essential for the PCR (oligonucleotides, PCR master mix) should be supplied to the participants in order to ensure that different PCR reagents, which have not been checked for suitability, do not influence the results.

Information about the results and data obtained by the study concerning the performance criteria shall be reported.

It is recommended that a small-scale collaborative study (pre-validation study involving 2 to 4 laboratories) is performed to test the general transferability of the method before the expense of organizing a large scale trial is incurred.

According to experiences and statistical considerations it is recommended that at least 12 laboratories participate in the validation study.

5.2 False-positive rate and false-negative rate

5.2.1 General

Prepare a series of replicates of known negative test samples and of known positive test samples from reference materials. If pure reference materials are not available, other sources as negative and positive materials may be used.

Each participant receives the same number of encoded positive and negative samples. The positive test samples contain defined quantities of the target DNA sequence of the positive material. The negative samples only contain non-target DNA or matrix-specific negative material.

Each participant receives at least 6 positive and 6 negative samples, which have been encoded beforehand. The participants perform the PCR measurements in single determination. Thus, for each laboratory, at least 6 results for positive and 6 results for negative DNA samples are available for the evaluation.

Requirements for preparation and evaluation of the homogeneity of replicate test samples are described e.g. in [4].

5.2.2 Procedure with DNA as collaborative study test samples

In general, the test material used in collaborative studies of qualitative PCR methods consists of DNA solutions.

The DNA is extracted from the sample material (in general from reference material) at a central facility involved in the conduct of the study. This central laboratory also performs pre-tests with respect to the quality of the extracted DNA (absence of PCR inhibition, amplificability, homogeneity). Guidance is given in other relevant documents [2] [5].

The positive DNA samples should contain at least twice the copy number corresponding to the limit of detection ($LOD_{95\%}$) as determined in the course of the single-laboratory validation. A copy number of less than 20 copies of the target sequence per PCR reaction should not be used as positive test sample. The test samples are to be prepared with up to 100 ng per 25 µl PCR reaction of suitable background DNA. In case of inclusion of DNA extraction it will be a combination collaborative trial of DNA extraction method and a real-time PCR method.

Negative DNA samples should contain only background DNA at the given concentration.

5.2.3 Procedure with food material as collaborative study test samples

If the target DNA sequence is to be detected mainly for one specific food matrix and if, for this matrix, no validated extraction procedure is available, the study uses known positive and known negative sample materials from which DNA shall be extracted by the participants.

For a pre-test, a central laboratory should extract DNA from the sample material using a defined DNA extraction procedure and should pre-test the quality of the extracted DNA concerning absence of PCR inhibition, amplificability and homogeneity [2] [5].

The participants shall check the performance of the DNA extraction method. For this purpose, an additional sample for positive extraction control (P) is provided. This sample allows each participant to test the extraction method and its own reagents. To this end, a PCR analysis is performed both for the DNA from P and for a positive control DNA provided by the organizer. The analysis is carried out in duplicate and the mean Cq values are then compared. The mean Cq value for the DNA from P should not exceed the mean Cq value for the positive control by more than 1 (for example: Cq value of positive control is 23, then the Cq value of DNA from P should not exceed the value of 24). In case of GMO testing methods this applies to the target-sequence PCR as well as to the taxon-specific PCR. Moreover, it is recommended to carry out an inhibition control test [2] [5].

For screening PCR methods, samples from more than one relevant species (e.g. corn and soya) may be included in the pool of test samples. The reagents for the PCR (oligonucleotides, PCR master mix etc.) are provided by the organizer of the collaborative study.

5.2.4 Evaluation and performance criteria

On the basis of the available results, the false-positive rates, *fp* in %, and false-negative rates, *fn* in %, are calculated with Formula (1) and (2):

$$fp = 100 \times \frac{mkn}{tkn} \quad (1)$$

where

mkn is the number of misclassified known negative samples;

tkn is the total number of known negative samples.

$$fn = 100 \times \frac{mkp}{tkp} \quad (2)$$

where

mkp is the number of misclassified known positive samples;

tkp is the total number of known positive samples.

The false-positive rate and false-negative rate assessed by the study should demonstrate that neither the false-negative rate nor the false-positive rate exceeds 5 %.

If a false-positive rate above 5 % was observed it should be investigated on a case-by-case basis. The method documentation should then provide relevant instructions. Apparently false positive results could occur, for example, in qualitative PCR screening tests for the detection of genetic elements which have a high inter-species sequence homology or occur naturally.

NOTE Annex A provides additional detailed information regarding the conduct of the collaborative study, the preparation of test samples and the evaluation of the results and pertaining documentation.

5.3 Robustness

In the collaborative study, the robustness of a qualitative real-time PCR method is evaluated concerning the different types of real-time PCR equipment that are used and the different laboratory conditions.

The method shall produce the expected results despite these changes. There shall not be any noticeable difference between the results obtained using different real-time PCR equipment.

Robustness is primarily tested in the framework of single-laboratory validation. Additional parameters including any pre-analytical influence by transport and time lag before starting the PCR tests are assessed in frame of the collaborative study.

5.4 Probability of detection (POD)

5.4.1 General

For the validation of the probability of detection (POD) of a qualitative real-time PCR method the different performance characteristics (laboratory standard deviation σ_L, mean amplification probability λ, slope parameter b, $LOD_{95\%}$) are calculated on basis of data from a sufficient number of laboratories that assessed several replicates across a number of coy number concentrations (in the range of the $LOD_{95\%}$).

5.4.2 Preparation of concentration levels

The participating laboratories receive a standard DNA with a calculated number of copies of the target sequence. On the basis of this standard DNA, a dilution series with different concentration levels for the target sequence is prepared.

Four concentration levels are chosen covering the dynamic range to obtain data for a standard curve. For the validation of the POD parameters the copy number concentrations should be chosen carefully at levels where the PCR probability of detection is < 1,0, e.g. at copy numbers equivalent to POD values of 0,05, 0,25, 0,5, 0,75, 0,95 and > 0,95. An example is given in Annex A, Table A.1.

Alternative procedures for preparation and providing the sample DNAs with different copy number concentrations can be applied.

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 [6]) and the genome weight [7] [8] [9]. 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 [10].

The dilutions are prepared in a buffer solution having a uniform non-target DNA concentration. For this purpose, the standard DNA is added to the corresponding amount of background DNA and thereby stabilized for the PCR.

5.4.3 Number of PCR replicates per laboratory

The specified replicate numbers for the given concentration levels represent the minimum number necessary to obtain a sufficient statistical reliability for the $LOD_{95\%}$ and for the corresponding precision data [3].

Each laboratory performs PCR measurements in triplicate determination for the standard curve levels. For the POD curve six replicate determinations are performed with each copy number level.

5.4.4 Evaluation of POD data

On the basis of the standard curve, the values for the slope and the coefficient of determination of the calibration function are calculated for each laboratory and are presented in a table (see Annex A, Table A.3).

A calibration function slope of approximately -3,1 to -3,6 is considered to be an indication of good PCR efficiency. The coefficient of determination should be at least 0,98.

The numbers of positive qualitative PCR results obtained for the six concentration levels in the low copy number range are tabulated (Annex A, Table A.2).

On the basis of the qualitative data, the laboratory-specific POD curves can be calculated (Annex A, Figure A.1).

Plausibility checks and outlier tests (e.g. according to Grubbs) are carried out for the laboratory-specific amplification probabilities λ and slope parameters *b* (B.4).

The value calculated for *b* should be in the range $0,65 < b \leq 2$ in order to ensure sensitive POD curves. This range is based on the condition that the $LOD_{95\%}$ value is not >20 copies and that the amplification probability in the range from 0,1 to 100 copies is not >1.

The mean amplification probability λ and the corresponding 95 % confidence interval are calculated and tabulated with the slope parameter *b* of the POD curve across laboratories, laboratory standard deviation (σ_L) as well as the $LOD_{95\%}$ (in copies of the target sequence for the theoretical median laboratory at POD = 0,95). If no statistically significant deviation for $b = 1$ is observed, the calculation of the other performance characteristics can be done using this value [3].