SLOVENSKI STANDARD
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Analiza molekulskih biomarkerjev - Splošne smernice za validacijo kvalitativnih metod PCR v realnem času v posameznem laboratoriju (ISO/DIS 11781:2023)

Molecular biomarker analysis - General guidelines for single-laboratory validation of qualitative real-time PCR methods (ISO/DIS 11781:2023)

Molekulare Biomarker-Analyse - Allgemeine Anleitung für die Einzellaborvalidierung qualitativer Real-time-PCR Verfahren (ISO/DIS 11781:2023)

Analyse de biomarqueurs moléculaires - Lignes directrices générales pour la validation intralaboratoire des méthodes de PCR qualitative en temps réel (ISO/DIS 11781:2023)

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Molecular biomarker analysis — General guidelines for single-laboratory validation of qualitative real-time PCR methods

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Contents

Page

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

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.

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 statistical model described has been practically applied.[3] Other models can be applicable, see ISO/TS 16393.

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 — General guidelines for single-laboratory validation of qualitative real-time PCR methods

1 Scope

This document describes 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 can be applied 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 detection of genetically modified foodstuffs and for species determination, including species known to produce allergenic proteins.

The document does not cover 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 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 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 — The notes have been removed and a new note 1 to entry was added.]

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

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

[SOURCE: ISO 24276, 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 should be assessed to show that the method complies with the quality criteria stipulated in relevant documents[1],[2].

For a qualitative real-time PCR method, the main focus of the validation should be 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 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 (inter-laboratory 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 probability of detection (POD) across laboratories, can be evaluated by a collaborative study, if the design is appropriate[3].

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, etc., is described in other relevant documents[1],[2] or ISO 21569.

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

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

The $LOD_{95\ \%}$ is expressed as the number of copies of the target sequence and should 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 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 (λ x $LOD_{95\ \%}$ = 2,996)[3].

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

Practical guidance for determining the $LOD_{95\ \%}$ that has been verified 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[4]) and the genome mass.[5],[6],[7] 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[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 that is relevant for extracted DNA[18].

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

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 generalized linear mixed model (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[9] or by using the R package POD (see C.5[16]).

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[3].

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.