INTERNATIONAL STANDARD

ISO 22942-1

First edition
2022-03

Molecular biomarker analysis — Isothermal polymerase chain reaction (isoPCR) methods —

Part 1:
General requirements

Analyse de biomarqueurs moléculaires — Méthodes de réaction de polymérisation en chaîne isotherme (isoPCR) —

Partie 1: Exigences générales

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Reference number
ISO 22942-1:2022(E)

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Published in Switzerland

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Foreword

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This document was prepared by Technical Committee 34, *Food products*, Subcommittee SC 16, *Horizontal methods for molecular biomarker analysis*.

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Introduction

Isothermal nucleic acid amplification describes methods that use constant temperature polymerase-catalysed reactions to amplify a nucleic acid target sequence[1][2][3][4][5][6]. In contrast to thermal-cycler based polymerase chain reactions, isothermal nucleic acid amplification does not require variable temperature cycling for denaturation, annealing, and polymerization although, in some cases, primer binding requires a single high temperature denaturation and an annealing step. Isothermal amplification methods can be described by the term “isothermal PCR (isoPCR)”.

Naturally, living organisms isothermally replicate DNA during cell division and transcribe RNA to produce structural, and regulatory components. IsoPCR leverages both natural and synthetic isothermal enzymatic processes. The enzymes include DNA and RNA polymerase, helicase, recombinase, exonuclease and nickase. Because isoPCR does not require variable temperature cycling for denaturation, polymerization and annealing there is no need for precision thermal cycling instruments. Reactions are run at a single temperature, except in cases where a nickase or displacing enzyme is not present in the reaction and an initial denaturation is required. In addition, various non-enzymatic nucleic acid binding proteins can be necessary. IsoPCR amplification in many applications can be performed on cell lysates without nucleic acid extraction. Some examples of amplification strategies are loop-mediated isothermal amplification (LAMP)[7], rolling circle amplification (RCA)[8], helicase dependent amplification (HDA)[9], recombinase polymerase amplification (RPA)[10], strand displacement amplification (SDA)[11], nucleic acid sequence-based amplification (NASBA)[12] and Cas9 nickase-based amplification reaction (Cas9nAR)[13]. The LAMP, RCA, HDA, RPA, SDA and NASBA strategies can incorporate both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) into amplified nucleic acids. Cas9nAR can only use DNA as the starting template for amplification.

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IsoPCR methods can be used for amplification, detection, identification, quantification, and analysis of specific low concentration nucleic acids in food and food products. These methods can, in most cases, amplify nucleic acids from un-purified nucleotide extracts. Detection of the target sequence is achieved through real-time or end-point techniques using one of several different amplification strategies and detection chemistries. Detection chemistries include turbidimetry, chromatography, gel electrophoresis and fluorescence, and can, in some applications, be achieved in a closed lateral flow device system.

Key features of isoPCR methods are constant temperature nucleic acid amplification, use of crude extracts, simple detection methods, and short reaction times without the need for precision thermal cycling instruments.

Because isoPCR methods are gaining in popularity and applicability, standardization of the acceptance criteria for these methods in food products is important.

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Molecular biomarker analysis — Isothermal polymerase chain reaction (isoPCR) methods —

Part 1: General requirements

1 Scope

This document specifies general criteria for development, validation and use of nucleic acid analytical methods based on the isothermal polymerase chain reaction (isoPCR). It provides additional information and guidance for specific isoPCR technologies.

This document is applicable to food, feed, plant matrices and their propagules, plant pathogens, and animals in which amplification of a specific biomolecular target sequence is required.

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.

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ISO/TS 16393, *Molecular biomarker analysis — Determination of the performance characteristics of qualitative measurement methods and validation of methods*

ISO 16577, *Molecular biomarker analysis — Vocabulary for molecular biomarker analytical methods in agriculture and food production*

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
extraction blank control
negative control reaction generated by performing all required steps in an extraction procedure except for the addition of the test portion

EXAMPLE By substitution of water for the test portion.

Note 1 to entry: This control is used to demonstrate the absence of contamination during extraction.

3.2
extraction control
positive control reaction generated by performing all required steps in an extraction procedure except with a known test portion containing a known amount of target nucleic acid or tissue

Note 1 to entry: This control is used to demonstrate the performance of the extraction process.

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3.3
isothermal polymerase chain reaction
isoPCR
isothermal nucleic acid amplification
isothermal nucleic acid amplification technology
isothermal amplification
polymerase chain reaction that polymerizes nucleic acids without *thermal cycling* (3.10), e.g., at constant temperature

Note 1 to entry: In some isoPCR applications, nucleic acids are denatured at a higher temperature prior to the start of the amplification reaction.

Note 2 to entry: Seven isoPCR strategies are described in this document. These strategies can be applied to a number of different methods consisting of DNA extraction, amplification and detection chemistries.

3.4
isoPCR method
analytical method that applies an *isoPCR* (3.3) strategy

3.5
non-laboratory field setting
workspace lacking conditions controlled for environmental aerosol contamination and sophisticated nucleic acid purification apparatus

3.6
nucleotide sequence specificity
capacity to exclusively recognize a specific nucleic acid sequence target to be amplified, distinguishing it from other nucleic acids and contaminants

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3.7
percentage dynamic range
percentage applicability range
percentage range of quantification
ratio as a percentage of upper and lower limits of quantification as expressed by a set of reference materials (or dilutions) with a suitable level of precision and accuracy

3.8
representative sample
sampling units (samples or groups) that have been extracted from the lot with a process ensuring all sampling units of the lots have an equal probability of being selected and not altered in any way that would change the analytical result

Note 1 to entry: The extraction process can be a multi-stage process.

[SOURCE: ISO 22753:2021, 3.15]

3.9
selectivity
extent to which a method can determine particular analyte(s) in a mixture(s) or matrix(matrices) without interferences from other components of similar behaviour

Note 1 to entry: The selectivity of an *isoPCR method* (3.4) for RNA or DNA or both can be determined with respect to inhibitors such as polyamines, polysaccharides and polyphenols, since these interfere with the ability of the reaction to amplify and disclose a specific target sequence.

Note 2 to entry: Selectivity is differentiated from *nucleotide sequence specificity* (3.6) which measures the recognition of the target sequence by the assay at the molecular or taxonomic levels.

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3.10
thermal cycling
thermocycling
process including numerous heating and cooling steps of a pre-determined temperature regime used to denature, anneal, and elongate nucleic acids in a polymerase chain reaction

4 Principle

Detection of the target sequence is achieved through real-time or end-point techniques that apply a specific amplification strategy and leverage several different detection chemistries. Detection chemistries include turbidimetry, chromatography, gel electrophoresis and fluorescence. In some isoPCR applications, a product can be detected in a closed lateral flow device system or fluorescence detection instrument.

A general overview for seven examples of isoPCR amplification strategies is provided in Table 1. Descriptions of each isoPCR strategy, their applications, advantages and disadvantages can be found in Annexes D to J.

5 Development of an isoPCR method

5.1 General

A DNA or RNA isoPCR method can be used to detect, identify and, as required, quantify an intended specific nucleic acid target(s). A method consists of:

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— a matrix-specific extraction (where required);

— any further purification step(s);

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— the enzymatic components and reagents;

— a description of the oligonucleotide primers and probes (labelled and non-labelled) that will be used (including how the target and oligonucleotide sequences were chosen);

— a description of how the amplified products will be detected;

— a protocol describing the conditions under which the isoPCR method is used including the use of controls and example calculations.

Guidelines for the minimum information for publication of isoPCR experiments (MIIPCRE) are provided in Annex A. MIIPCRE are guidelines for the minimum information necessary for evaluating isoPCR experiments. Annex A is a checklist for laboratories.

5.2 Intended purpose

Information regarding the intended purpose and the limitations of a method shall be provided. Specifically, the method shall be evaluated for fitness for purpose based on the criteria and requirements described in this document.

5.3 Scientific basis

An overview of the principles and application of the method shall be provided. Appropriate references to relevant scientific publications should be included.

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Table 1 — General overview for seven isoPCR amplification strategies

IsoPCR strategy	Target nucleic acid	Enzymes involved	Initial heating	Time (h)	Amplification		Measurement method(s)	LOD (copies)	Analyte	Detection method(s)	Equipment needed
					Power	Temperature (°C)					
LAMP	DNA, RNA	Polymerase	Yes	< 1	Exponential	60 to 65	Qualitative, quantitative	~5	DNA, RNA, Small molecules	Turbidimetry of pyrophosphate, fluorescent dye, electrochemistry, single-stranded nucleotide tag hybridization	Visual detection, turbidimeter (real-time); isothermal fluorometer; electrochemical LAMP microfluidic chip, lateral flow detection strips or printed array strip
RCA	DNA, RNA	Polymerase	Yes	1 to 4	Linear	30 to 65	Qualitative, quantitative	10	DNA, RNA, Protein, Methylated DNA, Small molecules, Cells	Fluorescent tags, fluorometry	Spectrophotometer, isothermal fluorometer
HDA	DNA, RNA	Helicase, polymerase	No	0,5 to 2	Exponential	64	Qualitative, quantitative	1	DNA, Protein	Gel electrophoresis; immunohistochemistry, fluorescent dyes	Electrophoresis chamber, UV transilluminator; closed lateral flow device system, isothermal fluorometer
RPA	DNA, RNA	Recombinase, polymerase	No	< 1	Exponential	37 to 42	Qualitative, quantitative	1	DNA, RNA, Protein	Gel electrophoresis; immunohistochemistry, fluorometry	Electrophoresis chamber, UV Transilluminator; closed lateral flow device system, isothermal fluorometer
SDA	DNA, RNA	Polymerase, restriction enzyme	Yes	1 to 2	Exponential	30 to 55	Qualitative	10	DNA, RNA, small molecules	Gel electrophoresis; pH indicator dyes; fluorescence	Electrophoresis chamber, UV transilluminator; visual detection; spectrophotometer or isothermal fluorometer

NOTE Adapted from Reference [19].

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Table 1 *(continued)*

IsoPCR strategy	Target nucleic acid	Enzymes involved	Initial heating	Time (h)	Amplification		Meas-urement method(s)	LOD (copies)	Analyte	Detection method(s)	Equipment needed
					Power	Tempera-ture (°C)					
NASBA	RNA, DNA	Reverse tran-scriptase, RNA pol-ymerase, RNase H	No	1 to 3	Exponen-tial	41	Qualitative, quantitative	1	DNA, RNA, miRNA, Protein	Gel electrophoresis; fluorescent probes; ELISA, fluorometry	Electrophoresis chamber, UV transil-luminator; microplate reader, isothermal fluorometer
Cas9nAR	DNA	Cas9 polymerase	No		Exponen-tial	37	Qualitative		DNA	Gel electrophoresis; fluorescent dyes	Electrophoresis chamber, UV transil-luminator; isothermal fluorometer or visual

NOTE Adapted from Reference [19].

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5.4 Units of measurement

Qualitative (binary) measurement with isoPCR methods provides a binary result based on a predetermined probability of detection (POD). Qualitative measurements are used to determine the presence or absence of molecular biomarkers in food or food products (including seeds and propagules of food crops). The performance characterization of a qualitative method shall be carried out as described in ISO/TS 16393.

Quantitative methods determine the amount of the target analyte present in a sample. Quantitative units of measurement (e.g. target copy number), performance and data reporting criteria shall be specified. Quantitative results can be reported as:

— nucleic acid copy number (c);

— copy number ratio (c/c_r, where c_r is a known reference copy number);

— percentage of the analyte;

— other criteria as described in the method.

The principles of calculation of any ratio used shall be reported. For quantification methods, the quantification strategy will depend on the application. Application of a calibration curve or copy number determination method evaluation can be carried out as described in ISO 20395[15].

5.5 Method validation

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The isoPCR method shall be developed considering its fitness for purpose. Validation and verification shall include sufficient testing to provide adequate confidence that the procedure is selective, repeatable and can detect the target in a known applicability range. Although collaborative studies are preferable, single laboratory validations can be acceptable. Thompson et al.[16] provides criteria for the single laboratory validation of a method and ISO/TS 16393 gives further guidance for collaborative validation of qualitative methods.

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ISO 20395 provides generic requirements for evaluating the performance and ensuring the quality of methods used for the quantification of specific nucleic acid sequences (targets) including method validation (precision, linearity, limit of quantification, limit of detection, trueness and robustness).

Collaborative trials for isoPCR methods should be undertaken during the validation step. For quantitative methods, the ISO/AOAC/IUPAC Harmonized Protocol[17] describes a process for validating a method via collaborative trials. The results of all interlaboratory or single-laboratory collaborative trials, or both, and the resulting performance characteristics should be analysed, described and included with the published method[16].

The JRC technical report “Verification of analytical methods for GMO testing when implementing interlaboratory validated methods” provides guidance on how to carry out the method verification of interlaboratory validated methods for the qualitative and quantitative detection of GMOs[18].

5.6 Performance criteria

5.6.1 General

Performance criteria shall be determined and set for method validation. Performance criteria includes sensitivity, nucleic acid extract quality, applicability, nucleic acid sequence specificity, precision (repeatability, intermediate precision, reproducibility), accuracy, selectivity, linearity, limit of detection, limit of quantification, range, measurement uncertainty and robustness.

5.6.2 Sensitivity

The sensitivity of an isoPCR amplification method for biomarker analysis shall be established by determining the slope of a calibration curve. The calibration curve can be constructed by assaying

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sequential samples descending in target DNA concentration by a 10-fold serial dilution. A minimum of five sample concentrations run in triplicate is required.

5.6.3 Nucleic acid extract quality

Nucleic acids should be extracted from the most relevant types of matrices, including those types reflecting the method scope, containing a known mass/mass content of the target(s) to genomic nucleic acid of the species (evenly distributed over the percentage dynamic range of the method) and tissues relevant for the application.

The nucleic acid extraction procedure used for validation and verification of the isoPCR method of a specific target in a specific matrix shall be identified. The extraction method shall produce nucleic acids that are of sufficient length, chemical purity and structural integrity for subsequent amplification and analysis. For amplification directly from cell extracts, determination of the nucleic acid extract quality is dependent upon the particular matrix. Performance should be determined based on the results from each cell matrix tested. Nucleic acid extract quality is affected by nucleic acid concentration, structural integrity, purity, presence of inhibitors, etc.

5.6.4 Applicability

The applicability or fitness for purpose of the isoPCR methods shall include the intended purpose, a protocol, the target, the cellular location of the target (nuclear or mitochondrial), and the range of copy numbers or concentration range for which the target is detectable. The nature of the matrix (e.g. organism, tissues, processed food) should also be considered.

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5.6.5 Nucleic acid sequence specificity

The theoretical nucleic acid sequence specificities of the primers and probes shall be assessed through a search of the relevant databases.

Primers for amplification shall be designed to recognize and anneal to their complementary sequences and allow specific target amplification. This determination should be performed *in silico* potentially using a primer design application before primers are tested experimentally. The nucleic acid sequence specificity of detection methods for a particular target depends on the specific properties of the targeted DNA sequence and can vary considerably between isoPCR applications. It is, therefore, important to ensure that the chosen method(s) provides the desired nucleic acid sequence specificity and nucleic acid selectivity (DNA or RNA). When RNA is the target, sometimes additional considerations need to be addressed.

5.6.6 Precision

5.6.6.1 General

The precision of the isoPCR amplification method shall be determined. Single laboratory validation and collaborative trials should be applied to the entire range of matrices and target species.

5.6.6.2 Reference and certified reference material

Certified reference material should be analysed multiple times in the single-laboratory validation of an isoPCR method to assess laboratory and method bias. Other reference materials, i.e. those left over from proficiency tests, can also be used for this purpose if the associated uncertainty is known. Spiking and recovery information can also be used although the measurement uncertainty is not always known.

5.6.6.3 Repeatability standard deviation (s_r)

The repeatability standard deviation shall be determined for a range of analyte concentrations for laboratory verification and single laboratory validation.

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