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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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Foreword

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

A list of all parts in the ISO 22942 series can be found on the ISO website.

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

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.

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.

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.

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.

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

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.

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:

— a matrix-specific extraction (where required);

— any further purification step(s);

— 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.

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].

Table 1 *(continued)*

IsoPCR strategy	Target nucleic acid	Enzymes involved	Initial heating	Time (h)	Amplification Power	Amplification Temperature (°C)	Measurement method(s)	LOD (copies)	Analyte	Detection method(s)	Equipment needed
NASBA	RNA, DNA	Reverse transcriptase, RNA polymerase, RNase H	No	1 to 3	Exponential	41	Qualitative, quantitative	1	DNA, RNA, miRNA, Protein	Gel electrophoresis; fluorescent probes; ELISA, fluorometry	Electrophoresis chamber, UV transilluminator; microplate reader, isothermal fluorometer
Cas9nAR	DNA	Cas9 polymerase	No		Exponential	37	Qualitative		DNA	Gel electrophoresis; fluorescent dyes	Electrophoresis chamber, UV transilluminator; isothermal fluorometer or visual

NOTE Adapted from Reference [19].

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

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.

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

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.

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.

5.6.6.4 Reproducibility standard deviation (s_R)

The reproducibility standard deviation shall be determined for a range of analyte concentrations for validation and collaborative trials.

5.6.7 Accuracy

The accuracy of an isoPCR method should be determined by comparison to the results from a different method for the same target or results from a calibrator. The accuracy is sometimes given based upon the extraction recovery, however, bias contributed by the amplification should also be included in this estimate.

5.6.8 Selectivity

Selectivity of the method should be tested against the most likely potential interference of target analytes, e.g. rRNA, similar DNA, polyglycosides, protein. An isoPCR method should be sufficiently selective for any interferences to be ignored.

5.6.9 Linearity

The calibration function should be linearizable, pass through the origin, and be unaffected by the cellular matrix of the test material. The linearizable component of the response from a dynamic range determination shall permit the concentration of the analyte in the test samples to be determined, if a quantification is performed. At least six calibration standards should be used to determine linearity. The POD should be known for a qualitative determination.

5.6.10 Limit of detection (LOD)

Method LOD is determined for both qualitative and quantitative analysis. The LOD is the true net concentration or amount of the analyte in the material to be analysed that will lead, with probability (1-β), to the conclusion that the concentration or amount of the analyte in the analysed material is larger than that in the blank material. It is defined as shown by Formula (1):

$$P_r\left(\hat{L} \le L_c \mid L = L_D\right) = \beta \tag{1}$$

where

L_D is the LOD;

$\hat{L}$ is the estimated value;

L is the expectation or true value;

L_c is the critical value.

NOTE 1 The limit of detection is estimated by:

$$L_D \approx 2t_{1-\alpha\nu}\sigma_o$$

where

L_D is the LOD;

$\alpha = \beta$;

$t_{1-\alpha\nu}$ is Student's t-distribution value, based on ν degrees of freedom for a one-sided confidence interval of 1-α;

σ_o is the standard deviation of the true value (expectation).

$L_D = 3{,}29\ \sigma_o$, when the uncertainty in the mean (expected) value of the blank is negligible, $\alpha = \beta = 0{,}05$ and L is normally distributed with known constant variance. However, L_D is not defined simply as a fixed coefficient (e.g. 3, 6) times the standard deviation of a pure solution background. To do so can be extremely misleading. The correct estimation of L_D can take into account degrees of freedom, α and β, and the distribution of L as influenced by factors such as analyte concentration, matrix effects and interference.

This definition provides a basis for taking into account exceptions to the simple case that is described, i.e. involving non-normal distributions and heteroscedasticity (e.g. "counting" (Poisson) processes as those used for real time PCR).

It is essential to specify the measurement process under consideration, since distributions, standard deviations and blanks can be dramatically different for different measurement processes.

At the L_D, a positive identification can be achieved with reasonable or previously determined confidence or both in a defined matrix using a specific analytical method.

NOTE 2 An empirically derived determination based on the results of a collaborative trial is called the "practical LOD". It is defined as the lowest relative quantity of the target DNA that can be detected, given a known (determined/estimated) number of target taxon copies. The practical LOD is related to the test portion, and the quality/quantity of the template DNA, and $L_D = 3{,}29\ \sigma_o$ which has also been called the absolute LOD of the method with 95 % confidence.

NOTE 3 In qualitative testing, an estimate of the L_D is measured at the chosen POD. The L_D can only be discretely determined for a quantitative method.

5.6.11 Limit of quantification (LOQ)

The LOQ is a method performance characteristic generally expressed in terms of the signal or measurement (true) value that will produce estimates having a specified reproducibility coefficient of variation ($C_{V,R}$), commonly less than 25 % for isoPCR. LOQ is estimated as shown by Formula (2):

$$L_Q = k_Q\ \sigma_Q,\ k_Q = 1/C_{V,R} \tag{2}$$

where

L_Q is the LOQ;

σ_Q is the standard deviation at that point;

k_Q is the multiplier whose reciprocal equals the selected $C_{V,R}$.

The approximate $C_{V,R}$ of an estimated σ, based on ν-degrees of freedom is $1/\sqrt{2\nu}$.

NOTE If σ is known and constant, then $\sigma_Q = \sigma_o$, since the standard deviation of the estimated quantity is independent of concentration. Substituting 25 % in for k_Q gives:

$$\text{LOQ} = (25 * \sigma_Q) = 25\ \sigma_o$$

In this case, the LOQ is just 7,60 times the limit of detection, given normality and $\alpha = \beta = 0{,}05$.

At the LOQ, a positive identification can be achieved with reasonable or previously determined confidence or both in a defined matrix using a specific analytical method.

This definition provides a basis for taking into account exceptions to the simple case that is described, i.e. involving non-normal distributions and heteroscedasticity (e.g. "counting" (Poisson) processes as those used for real time PCR).

5.6.12 Range

5.6.12.1 General

In general, the operative range of applicability for an isoPCR method should be established. The approach to establish the applicable range depends on whether the method will be used qualitatively or quantitatively or both.

5.6.12.2 Quantitative methods

For quantitative isoPCR methods, the dynamic range shall be established. The dynamic range shall satisfy conditions for repeatability and reproducibility coefficients of variation, $C_{V,r}$ and $C_{V,R}$.

NOTE 1 Mitochondrial and chloroplast isoPCR amplification targets cannot be used for reliable quantification of haploid genome copy number ratios of different species, because the number of mitochondrial DNA targets differs with tissue type.

NOTE 2 Different plant and animal tissue types can have variable DNA contents per mass equivalent.

5.6.12.3 Qualitative methods

5.6.12.3.1 General

Qualitative (binary) analytical isoPCR amplification methods for use in the analysis of food or food products (including seeds of food crops) with the purpose of demonstrating the presence or absence of a given biomarker in a sample shall include objective evidence that they are adequate for their intended purpose. The POD for the test method should be determined in accordance with ISO/TS 16393 or equivalent.

5.6.12.3.2 Probability of detection (POD)

The POD is the probability of a positive analytical outcome of a qualitative method for a given matrix at a given concentration in a single laboratory. The LPOD is the mean probability of detection across laboratories. The hybrid modified Wilson interval or other suitable model can be used to establish a validation experiment to determine the LPOD for isoPCR amplification methods (see ISO/TS 16393). The following criteria described in ISO/TS 16393 should be taken into consideration when validating a qualitative method of analysis:

— applicability;

— robustness;

— specificity;

— POD at specific measurand concentrations.

The number of replicate samples required to get a good estimation of the LPOD (at 95 % confidence) for a two-sided coverage is 12 per level for the range 25 % to 75 % LPOD for the case where 8 laboratories are included. If more participants are available, the number of replicate samples can be lowered in consultation with a statistician. Although the use of larger numbers of replicates is helpful to obtain ideal estimates of the LPOD at high and low measurand concentrations, a large number of replicates can be impracticable in a multi-laboratory trial. Two other models have also been evaluated (see ISO/TS 16393) for determining the POD, the maximum profile likelihood based on the probit model and the maximum likelihood estimate based on beta binomial distribution.

5.6.13 Robustness

Robustness, the capacity of the isoPCR method to resist small, but deliberate, deviations from the experimental conditions described in the procedure, shall be evaluated for the method with

a specific target sequence in a specific matrix. Some examples of parameters that can be purposely altered include, but are not limited to, the ability to evaluate targets from impurified nucleic acid, pH dependency, temperature and synchronization of polymerase, and accessory proteins. Robustness can also depend on a specific detection format. It is important to define the parametric limits of a chosen (or use the most reliably applicable) amplification and detection strategy for each specific target in each matrix tested.

Number of target copies accurately detected can be used as a measurement of individual assay performance. Copy number of different targets or between genotypes, and genome or cellular organization (i.e. variable ploidy and multinucleate cells) should be considered in comparing performance of different assays. Alternate measurements such as the number of positive samples within a counted group, or genome equivalents can be used if they provide additional context for understanding assay utility.

NOTE Robustness testing of the method is established at the single laboratory level when developing the method.

6 General laboratory and procedural requirements

6.1 Competence

The laboratory can follow the guidelines provided in ISO/IEC 17025[20] regarding the general requirements for competence, impartiality and consistent operation.

6.2 Sample preparation

6.2.1 General

Sampling and sample preparation guidelines applicable to biomarker analysis should be followed (e.g. for plant material, meat and plant pathogens).

6.2.2 Obtaining a representative sample

6.2.2.1 General

The laboratory sample shall be a representative sample. If the laboratory or field sample is subsampled, then care should be taken not to introduce heterogeneity with respect to both composition and biomarker distribution. Measurement uncertainty due to sampling error at this stage should be estimated.

6.2.2.2 Ensuring homogeneity of the test portion

If the laboratory or field sample must be reduced to a manageable size to produce a test portion, then care shall be taken not to introduce heterogeneity with respect to both composition and biomarker distribution. The sample shall be homogenized and divided so that the test portion(s) taken from this homogenized sample is representative of the laboratory or field sample.

NOTE Guidance on obtaining a representative sample of seeds for submission as a laboratory sample is provided by ISTA[21], and by national associations or regulations, and for grains by ISO 24333[22] and corresponding national standards.

6.2.3 Preparation of the test portion

Sampling plans and nucleic acid extraction can be different depending upon the analyte, the circumstances for the test and the isoPCR amplification strategy. Samples shall be reduced to the appropriate amount for analysis of the appropriate number of replicates. Where a portion is derived from a laboratory sample, the test portion shall be of sufficient size, and shall contain a sufficient

number of particles (if it is a ground or powdered material) to be representative of the laboratory sample and allow a statistically valid conclusion to be made. A sample can also consist of a single seed, leaf piece or piece of tissue as required for the intended analysis.

6.2.4 Nucleic acid extraction

The nucleic acid extraction method shall be validated for the isoPCR amplification procedure used. Many isothermal methods are designed to provide extraction and analysis in the same container for ease of use. The reagents are very robust, and purification of the nucleic acids is not required to be at the level required by thermal cycled PCR. When nucleic acid is not purified from the cell matrix prior to amplification, measurement of the nucleic acid concentration prior to assay is not required. In this case, an estimate of the amount of nucleic present can be determined during the experimental verification or validation. Where the nucleic acid extraction and isoPCR assay steps are discrete procedures, the method should define whether the nucleic acids are quantified and evaluated for quality prior to their use in the analytical test. In certain applications, especially in non-laboratory field settings, a single extraction and assay can be performed, as appropriate. In a laboratory setting, extraction and testing can be performed with appropriate controls (see Annex B) to produce a consistent result. Sufficient nucleic acid shall be present in the isoPCR to satisfy the minimum percentage dynamic range for the method.

NOTE The "quality" of nucleic acid depends on the average length of the extracted nucleic acid molecules, chemical purity and structural integrity of the nucleic acid sequence and of the double helix (e.g. intra-, inter-strand linking between nucleic acid bases, single-strand gaps, cross-linking with polyols, haemin. Moreover, such alterations are often sequence-specific and, consequently, not randomly distributed all over the genome.

6.3 Use of controls

6.3.1 General

Controls shall be used according to Annex B.

6.3.2 Environmental controls

Environmental controls will vary based on the isoPCR application. For example, in a closed amplification system, no external contamination will be introduced during amplification. However, amplicon contamination from the laboratory or field site can contaminate subsequent amplification assays during sample preparation. Environmental controls shall be established to disclose potential contamination of the isoPCR assay.

6.3.3 Positive controls

Positive controls can be any reliable source of well-characterized positive sample material, containing intact target nucleic acid sequences for isoPCR. This control can be purified nucleic acid or tissues. Tissues can also serve as the nucleic acid extraction control. When used for a quantitative assay, the material should adequately represent the range of the desired threshold.

6.3.4 Negative controls

The negative DNA target control shall be well-characterized DNA preparation material that does not contain target nucleic acid. A negative process control shall be a recognized reference sample lacking target analyte and that should be put through the same process steps as the test samples.

6.3.5 Extraction controls

6.3.5.1 Endogenous extraction controls

Internal controls extracted from host tissue can be used as both a positive control and extraction control.

6.3.5.2 Exogenous extraction controls

Extraction controls are used to ensure reliability of the extraction method. If appropriate, an extraction control can also be used as a positive control.

6.4 Workspace organization

6.4.1 General

IsoPCR amplification requires minimal instrumentation. Many isoPCR amplification methods can be used in non-laboratory field settings without an electrical power source.

Prevention of contamination from positive samples and previously undertaken amplifications is necessary whether the analysis is taking place in a laboratory or a non-laboratory field setting. Preparation of reagents shall be carried out in an environment which is protected from contamination by amplified products or positive controls. Accidental nucleic acid contamination can originate from dust and aerosols containing amplicons. One of the highest risks of nucleic acid contamination is from the amplified products of the previous assays. Consequently, the organization of the work area should provide systematic containment of the steps leading to the production of the results. Care shall also be taken when discarding amplified products.

Many isoPCR amplification systems are self-contained. These systems are not as susceptible to contamination as systems where nucleic acid extraction and amplification require separate steps that expose the analyses to aerosols.

Manufacturers' safety recommendations shall be followed.

6.4.2 Design of the workspace — Laboratory design

Physical, temporal or environmental separation between reagent preparation and performing the assay is essential for isoPCR. A laboratory should be configured to prevent secondary contamination from the products. The layout of a laboratory work area should provide for systematic containment and (if possible) a "forward flow" principle for sample handling. For isoPCR-based methods, the following laboratory design should be optimized:

— a separate room or laboratory section that is used exclusively for the mixing and preparation of non-nucleic acid reagents;

— a laboratory section for the isoPCR amplification reaction;

— a separate room or laboratory for the detection of the amplified product; this is only necessary if the amplification and detection are not completed in a closed chamber.

If grinding techniques producing dust or aerosolized particles are used, this work can preferably be carried out in an additional work area, but other measures can be used as a protection against contamination, provided their effectiveness is comparable.

6.4.3 Design of non-laboratory workspaces

Assays designed to be used in a non-laboratory setting are generally in a more contained or semi-contained system than those designed for laboratory use. This is often achieved by use of closed disposable containers that are discarded after use. Reagents are generally prepared and dispensed in a remote location before the assay is used on site. However, the non-laboratory work area should still be configured to prevent cross-contamination from handling and grinding of samples or between reagents used in reactions, should these be supplied in a bulk form.

6.4.4 Personnel

Staff shall wear appropriate personal protection equipment.

Powder-free gloves should be used. Where possible and at appropriate frequencies, gloves and laboratory coats should be changed and decontaminated.

All personnel who perform steps in the testing procedure should be trained and demonstrate competence in the performance of the method(s) and use of equipment.

6.4.5 Apparatus and equipment

The laboratory or non-laboratory location where the tests are performed should have available properly maintained equipment suitable for the methods employed.

7 Materials and reagents

For the analysis, unless otherwise stated, use only analytical grade reagents suitable for molecular biology, free from DNA, DNases, RNA and RNases. Water shall be double-distilled (≥18 MΩ) and nuclease-free or of comparable quality unless otherwise specified. Reagents and solutions should be stored at room temperature unless otherwise specified. Reagents should be stored in small aliquots for use to minimize the risk of contamination. Acceptable activity after storage or handling of reagents (such as enzymes) for use in the assay shall be verified.

Materials and all containers and disposables containing reagents shall be protected from contaminating agents (e.g. dust).

Procedures should be designed to minimize the opportunity for unintended enzyme activities (e.g. exonuclease) that can interfere with the reaction. Reagents specific to each type of isoPCR methodology are described in the appropriate annexes.

In many cases, isoPCR reagents and equipment are supplied as kits. Manufacturers' recommendations for the use of kits or reagents or both should be followed. Appropriate controls can be used to verify the integrity of reagents.

8 Interpretation of results

8.1 General

The analysis of nucleic acids in plants, animals or foods, or in determining the presence of a pathogen, aims to detect a very small amount of specific nucleic acid target in a background of large amounts of non-target nucleic acids. In most applications, the target nucleic acid sequence will be present at a low concentration compared with a taxon-specific nucleic acid sequence present at concentrations 10 to 1 000 times higher. Food matrices can contain significant amounts of nucleic acid from many sources.

8.2 Interpretation of controls

Each control is expected to have a valid value as described below and, if the observed result for any control is different from the valid value, the analysis shall be repeated. Environmental controls can be positive (amplification product of expected size detected) or negative (no amplification product detectable), but a positive result from a negative control shall always initiate measures to remove and prevent further contamination of the work environment. In certain applications, such as pre-assembled kits, it is not necessary or appropriate to include all of these controls. Operators should follow manufacturers' recommendations.

If a non-valid result for any controls is obtained, measures shall be taken to locate and remove the source(s) responsible for the error, and the analysis shall be repeated. Analytical results shall only be reported when all controls yield valid values. The valid values for the controls are as follows:

— positive extraction controls shall always be positive;

— extraction blank controls shall always be negative;

— positive nucleic acid target controls shall always be positive;

— negative nucleic acid target controls shall always be negative;

— amplification reagent controls shall always be negative.

IsoPCR amplification inhibition controls shall not show significant signs of inhibitory effects on the reaction.

Possible isoPCR amplification results of the controls are listed in Table C.1. These are used for interpreting/reporting the test sample result.

8.3 Expression of results

8.3.1 General

Qualitative determinations will return a binary result: positive or negative. The criteria used to choose a method for a particular application, i.e. in a specific matrix where the analyte falls within a specific range of concentration can be based on the POD for the minimum and maximum levels of target and the measurement uncertainty associated with the method (see ISO/TS 16393).

Quantitative determinations should be expressed as the ratio of a target-specific DNA sequence relative to a taxon-specific DNA sequence. This determination requires a minimum of two isoPCR-based measurements: quantitative isoPCR of 1) the target-specific DNA sequence and 2) the endogenous or taxon-specific sequence. Each of these determinations will have unique measurement uncertainties, and the two can have distinct measurement characteristics. The results can be reported in various units of measurement, e.g. mass per cent or copy number. ISO 20813[23] provides guidance for calculating mass per cent from a molecular result for meat products.

In certain applications such as pathogen detection and food adulteration, the target nucleic acid that is detected or quantified, or both, can be from a different taxon than the tissue that was sampled, e.g. horsemeat in beef. Quantitative results can be expressed:

a) in terms of signal strength per gram of tissue; or

b) against a predetermined scale based on the ratio of target copies to the sample taxon genome copies.

Quantitative results can also be based on the signal versus the amount of material present (e.g. virus titre per gram of leaf) or on a comparative scale. Such results should also provide the measurement uncertainty of the result reported.

8.3.2 Expression of a negative result

A negative result shall never be expressed as zero or “target not present”.

The following sentence shall appear in the test report:

> “For target analyte X, the target sequence Y was not detected. The LOD of the method is x % determined with ABC (identify the reference material).”

If it cannot be demonstrated that the amount of target DNA included in the PCR is sufficient for a positive identification to be obtained with reasonable or previously determined confidence or both in a defined matrix using a specific analytical method, then the following sentence shall be added:

> “However, the amount of the target DNA extracted from species X may not have been sufficient for a positive identification to be obtained for this sample.”

In addition, if applicable:

> “The limit of detection is x %.”

8.3.3 Expression of a positive result

In case of a qualitative analysis, the following text shall appear in the test report:

> "For sample X, target sequence Y was detected."

In addition, if applicable:

> "The probability of detection is x %."

The identity of the molecular biomarker can be included, if available.

8.3.4 Expression of quantitative results

If the target taxon-specific sequence and the GM target sequence are both detected but the quantity is below the LOQ of at least one of the target sequences, the following text shall appear in the test report for each molecular biomarker sequence:

> "DNA derived from a specific biomarker from organism X (specify the organism, species, variety, biomarker, etc.) as determined by detection of (specify target sequence) derived from (specify species) was detected, below the limit of quantification."

If it cannot be demonstrated that the amount of target DNA included in the PCR was sufficient for a positive identification to be achieved with reasonable or previously determined confidence or both in a defined matrix using a specific analytical method, then the following sentence shall be added:

> "However, the amount of the target DNA extracted from species X may not have been sufficient to produce a positive result for this sample."

In addition, if applicable:

> "The limit of detection is x (% or copies)."

If the target taxon-specific sequence and the biomarker target sequence are both detected and the quantity is above the LOQ for both target sequences, for each organism, state:

> "The content of organism X (specify the organism) derived DNA as determined by detection of (specify target sequence) derived from (specify species) is x ± umeas %" where umeas is the measurement uncertainty."

The results of quantitative methods shall state the unit of measurement.

8.3.5 Expression of ambiguous results

Results from all test portions shall be consistent. When at least one test portion gives a positive result and at least one gives a negative result, the analysis shall be repeated. If at least one repetition of the procedure, beginning with the nucleic acid extraction, gives ambiguous results such as a positive and a negative result, the report should state that the sample is negative at the limit of detection. Results within the same test portion shall be consistent. In case of +/− results for the two replicates, repeat the two isoPCR analyses for the respective test portion. If the two novel replicates are tested +/− or −/−, the test portion is considered as negative.

9 Test report

The test report shall contain at least the following information:

a) all information needed to identify the sample;

b) any particular information relating to the sample (e.g. insufficient size, degraded state);

c) reference to this document, i.e. ISO 22942-1;

d) statement about date and type of sampling procedure(s) used, if available;

e) date of sample receipt (if sample is sent to a laboratory for analysis);

f) person responsible for the analysis;

g) results according to the requirements of the specific method, the units used to report the results, the calibrators and the calculation method used;

h) any particular observations made during testing;

i) any deviations, additions to or exclusions from the method specification;

j) any statements required as specified in Clause 8.

The test report can also contain:

— storage conditions, if applicable;

— analysis start/end date;

— size of the sample and test portion.

The measurement uncertainty and its level of confidence, where applicable, should be made available to the user of the results.

Annex A
(informative)

Minimum information for an isoPCR experiment (MIIPCRE)

Table A.1 — Minimum information for an isoPCR experiment (MIIPCRE)

	Item to check	Importance[a]
Experimental design		
	Intended purpose, scope and limitations: scientific basis	E
	IsoPCR strategy: target genes, reference genes, controls, cellular location	E
	Single or multiplex analysis	E
	Will the method be carried out in a laboratory or a non-laboratory setting?	D
Sample/test portion		
	Sample preparation guidelines used	E
	How was the sample obtained?	D
	Sample representativeness and homogeneity, control samples	D
	Size and number of laboratories and test samples	D
	Description of test sample	D
	Processing procedures: grinding or homogenization	E
	Sample storage conditions and duration of storage	E
Nucleic acid extraction (if required)		
	Procedure or instrumentation or both	E
	Name of kit (if used) and details of any modifications	E
	Source of additional reagents used	D
	Details of DNase or RNase treatment	E
	Contamination assessment (DNA or RNA)	E
	Nucleic acid quantification	E
	Instrument and method	D
	Purity (A260/A280/A230) – if required	D
	Yield	D
	DNA/RNA integrity: method/instrument	D
	Extraction controls used for isoPCR strategy	E
	Gel electrophoresis of extracted nucleic acid	D
	Inhibition testing (spike or other)	D
Reverse transcription (if necessary)		
	Complete reaction conditions	E
	RNA amount and reaction volume	D
	Priming oligonucleotide(s)-gene-specific? and concentration	D
	Reverse transcriptase, other enzymes and concentration	E
	Temperature and time	E

[a] All essential information (E) should be submitted with the manuscript. Desirable information (D) can be submitted if available.

NOTE Adapted from Reference [24].

Table A.1 *(continued)*

	Item to check	Importance[a]
	Manufacturer of reagents and catalogue numbers	D
	Detection result with and without reverse transcription	D
IsoPCR target information		
	Target gene identification	E
	Sequence accession reference	E
	Location of amplicon	D
	Amplicon length	E
	In silico nucleotide sequence specificity screen (BLAST, etc.)	E
	Inclusivity/exclusivity	E
	Sequence alignments	E
	Secondary structure analysis of amplicon	D
	Location of each primer by exon or intron (if applicable)	E
	Are specific variants targeted?	E
IsoPCR oligonucleotides		
	Number of primers used	E
	Primer sequences	E
	Probe sequences (if used)	D
	Location and identity of any modifications	E
	Manufacturer of oligonucleotides	D
	Purification method	D
IsoPCR protocol		
	IsoPCR strategy used	E
	Complete reaction conditions	E
	Reaction volume and amount of RNA/DNA	
	Primers, (probe), Mg^{2+}, dNTP concentrations other components	E
	Polymerase(s) and enzyme(s) – identit(ies) and concentration(s)	E
	Buffer/kit identity and manufacturer	E
	Exact chemical composition of the buffer	D
	Manufacturer of plates/tubes and catalogue number	D
	Denaturation temperature and isothermal conditions, as required	E
	Reaction setup (manual/robotic)	D
	Manufacturer of instrument(s), if required	D
IsoPCR validation		
	Sensitivity-calibration curves with slope and y-axis intercept	D
	10× dilution gradient calibration series – know sample result	E
	Inclusivity, exclusivity	E
	Specificity (gel, sequence, melting curve or restriction digestion)	E
	IsoPCR efficiency calculated from slope – amplifiability	E
	Selectivity	D
	Linear dynamic range	E
	Repeatability, $C_{v,r}$	E

[a] All essential information (E) should be submitted with the manuscript. Desirable information (D) can be submitted if available.

NOTE Adapted from Reference [24].

Table A.1 *(continued)*

	Item to check	Importance[a]
	Reproducibility, $C_{v,R}$	E
	LOD	D
	LOQ	E
	POD	E
Data analysis		
	IsoPCR analysis program (source, version)	E
	Method of concentration determination	E
	Outlier identification and disposition	E
	Results for control samples	E
	Justification of number and choice of reference genes	E
	Description of quantification method	E
	Number and concordance of biological replicates	D
	Number and stage (reverse transcription or isoPCR) of technical replicates	E
	Repeatability (intralaboratory variation, $C_{v,r}$)	E
	Reproducibility (interlaboratory variation, $C_{V,R}$)	D
	Power analysis – replicate determination	D
	Statistical methods for results significance	E
	Software (source, version)	E
	Raw data submission with standardized PCR data format	D

[a] All essential information (E) should be submitted with the manuscript. Desirable information (D) can be submitted if available.

NOTE Adapted from Reference [24].

Annex B
(normative)

Use of controls

Table B.1 — Flow diagram showing intersection between successive steps and inclusion of controls

Control step	Environment control[a]	Extraction blank control[b]	Positive extraction control[c]	Positive nucleic acid target control[d]	Negative nucleic acid target control[e]	Amplification reagent control[f]	isoPCR amplification inhibition control[g]
Homogenization	mandatory						
Nucleic acid extraction	↓[h]	one per series	mandatory at regular intervals				
Assessment of nucleic acid quality	↓	↓	↓				
Nucleic acid amplification	↓	↓	↓	mandatory	recommended	mandatory	recommended, but mandatory in certain cases[i]
Assessment of results of nucleic acid amplification	↓	↓	↓	↓	↓	↓	↓
Interpretation		↓	↓	↓	↓	↓	↓
Test report		↓	↓	↓	↓	↓	↓

[a] The use of environmental controls will help the laboratory identify sources of contamination at an early stage and can be used to identify the work area where contamination is present.

[b] At least one extraction blank control shall be included each time nucleic acid is extracted from one or more samples. The tube shall always be the last in each series, e.g. it can be useful to put one extraction blank on a rack of tubes or a microplate for automated extraction.

[c] A positive extraction control should be included regularly, and always when a new batch of extraction reagents is used. This control will reveal if something is wrong with the reagents or the performance of the extraction protocol.

[d] The positive nucleic acid target control demonstrates the ability of the nucleic acid amplification procedure to detect the nucleic acid representative of the biomarker or target taxon. This condition can also be fulfilled by an appropriate positive extraction control.

[e] The negative nucleic acid target control demonstrates the presence or absence of false positive amplification.

[f] The amplification reagent control demonstrates the absence of contaminating nucleic acid in the isoPCR amplification reagent batches used. The amplification reagent control can be omitted when the extraction blank control is used.

[g] The isoPCR amplification inhibition control can be used to demonstrate the absence of soluble inhibitors. This can also be demonstrated by serial dilutions of the template nucleic acid. However, some type of assessment of the effect of soluble inhibitors on the results of the analysis of the sample shall be made.

[h] The arrows indicate that the control measure listed above it should be applied in subsequent analytical steps.

[i] An isoPCR amplification-inhibition control is mandatory, if all isoPCR amplification-tests on the sample are negative and for matrices where the yield of amplifiable nucleic acid is not known.

NOTE Adapted from ISO 24276[25].

Annex C
(informative)

Examples of isothermal nucleic acid isoPCR amplification results

Table C.1 — Examples of isothermal nucleic acid isoPCR amplification results

Test sample	Positive extraction control	Extraction blank control	Negative nucleic acid target control	Positive nucleic acid target control	Interpreted result
+[a]	+	–	–[b]	+	positive
–	+	–	–	+	negative
+	+	+	–	+	inconclusive[c]
–	–	+	–	–	inconclusive[c]
–	–	–	–	–	inconclusive[d]

[a] IsoPCR amplification product is detectable.

[b] No isoPCR amplification product is detectable.

[c] The procedure is repeated beginning with the extraction step (possible contamination).

[d] The procedure is repeated using another extraction method or a further purification step (possible inhibition).

NOTE Adapted from ISO 21569[26].

Annex D
(informative)

Loop mediated isothermal amplification (LAMP)

D.1 General

LAMP can amplify a few copies of DNA or RNA to 10^9 copies in as little as 1 h. This strategy uses four primers recognizing six distinct regions of the target DNA or RNA. A strand-displacing DNA polymerase initiates synthesis and two primers form loop structures to facilitate subsequent rounds of amplification forming bi-modal intermediate stem-loop structures. The amplification products are stem-loop DNA structures with inverted repeats of the target and cauliflower-like structures with multiple loops (see Figure D.1). An additional set of loop primers and loop priming sites can be added to improve the rate of amplification. When RNA is targeted, a reverse transcriptase is required. Amplification is extensive, and the synthesis of a visible by-product, magnesium pyrophosphate, can be sufficient for field diagnostics to correlate turbidity with target amplification. However, other detection systems are available when method sensitivity is important. For example, an intercalating dye such as Sybr green, Pico green, or propidium iodide can be used. Since there is a pH change and Mg^{2+} ions are depleted during nucleic acid synthesis, colorimetric substrates calcein or hydroxy napthol can be added to the reaction to track the pH and depletion of magnesium.

D.2 Amplification strategy

Specific primer sets are designed to bind to six priming sites, two of which are typically 23 nt to 24 nt in length (F3, F2) and two are typically 17 nt to 21 nt in length (R3, R2). Three priming sites occur on each side of the target about 40 nt apart to allow the dumbbell-loop formation. These are identified in Figure D.1 as forward priming sites F1, F2 and F3, and reverse priming sites R1, R2 and R3. The complementary sequences of the forward and reverse priming sites are F1c, F2c, F3c, R1c, R2c and R3c. The inner priming sites are F1 and R1 while the outer priming sites are F3 and R3. The four primers used for amplification are:

— a forward internal primer (FIP) containing priming site sequences F2 and F1c;

— a reverse internal primer (RIP) containing priming site sequences R2 and R1c separated by a spacer;

— a reverse external primer (R3);

— a forward internal primer (F3).

The outer primers R3 and F3 have the complementary sequences of R3c and F3c, respectively.

In the first step (A), primers, polymerase, buffer and DNA or RNA are added, and the reaction occurs efficiently at approximately 65 °C (can be adjusted to optimize reaction). The heat denaturation of the template can be performed prior to the LAMP reaction[27].

As shown in Figure D.1, the reaction occurs with primer RIP binding to the target primer site R2c and initiating complementary strand polymerization (A). Shortly thereafter, primer R3 binds to R3c and initiates strand displacement synthesis (B) resulting in the formation of a double-stranded product (C), a FIP complementary strand and the first form of looped-out structure (D). The looped-out structure can self-prime and synthesize a complementary strand. The same process occurs at the other end of the target with FIP and F3. The single-stranded looped-out product from the RIP primer binding also serves as a template for F3, producing a dumbbell structure (E). Subsequently, the dumbbell structure is repeatedly primed by FIP and RIP (F) producing looped structures (G). Continued elongation and

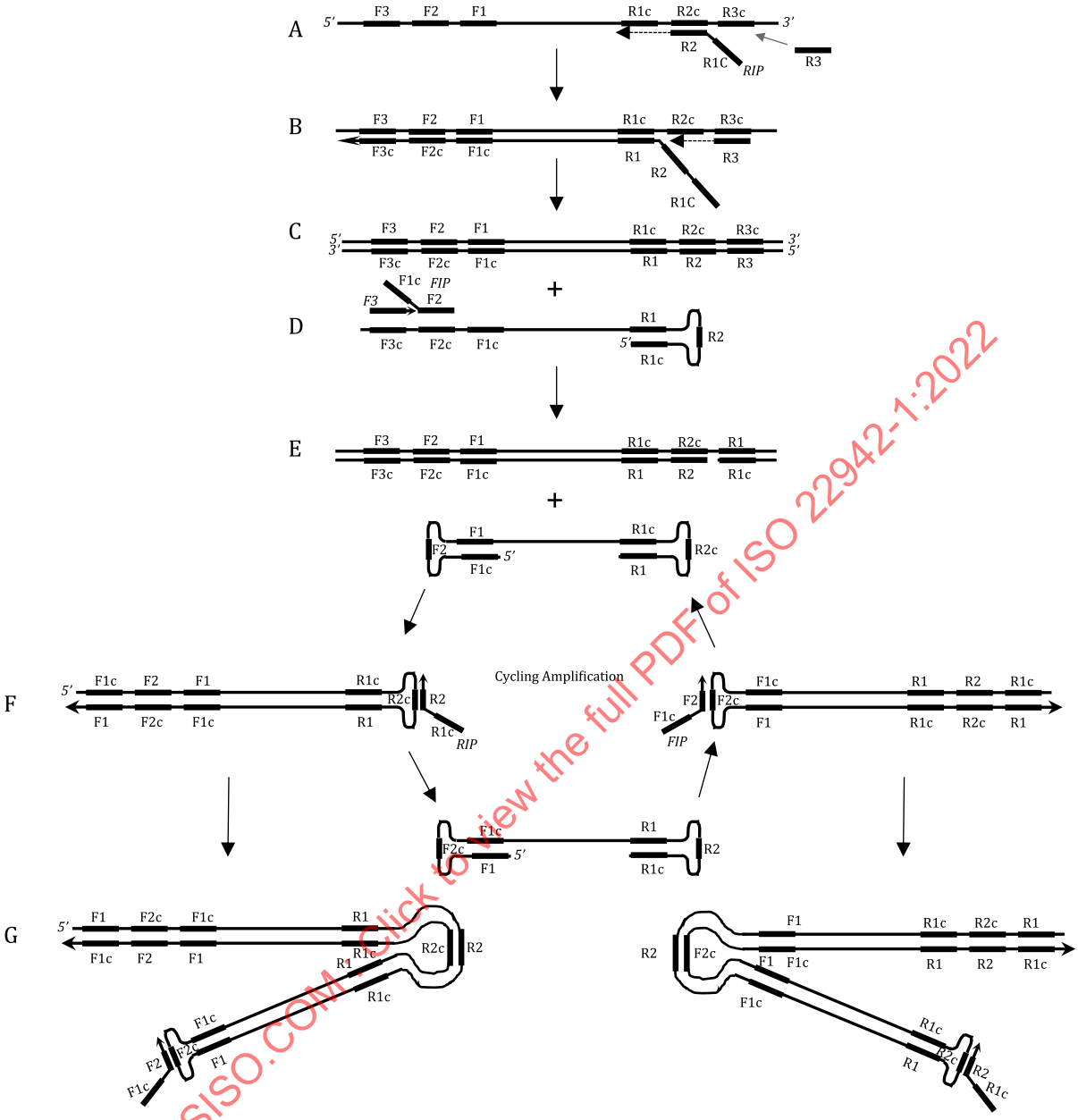
displacement steps result in large amounts of amplification product in cauliflower-like structures with multiple loops.

Additionally, loop priming sites can be added between F1-F2 and B1-B2 in the forward and reverse primer, respectively. Loop primer can accelerate reaction and reduce amplification time to half or one-third that of the original LAMP method[28].

D.3 Advantages and disadvantages

LAMP is a sensitive and rapid method. Reactions can be completed in less than an hour. LAMP has a wide applicability in diagnostic products. It is not sensitive to many common PCR contaminants. Crude DNA extracts can be used. There are a number of visualization and detection strategies. The LAMP reaction produces a complex amplification product, of which fragment size determination requires nuclease cleavage flanking the target nucleotide.

Lateral flow strip methods can also be applied for multiplex analysis to perform simultaneous detection. This system can hybridize immobilized unique single-strand nucleotides with their respective complementary strands attached to LAMP products[29].



Key

F1 internal forward primer site
F2 middle forward primer site
F3 external forward primer site
F1c complement to internal forward primer site
F2c complement to middle forward primer site
F3c complement to external forward primer site
R1 internal reverse primer site
R2 middle reverse primer site
R3 external reverse primer site
R1c complement to internal reverse primer site
R2c complement to middle reverse primer site
R3c complement to external reverse primer site
RIP reverse internal primer
FIP forward internal primer

Figure D.1 — Loop mediated amplification (LAMP)

Annex E
(informative)

Rolling circle amplification (RCA)

E.1 General

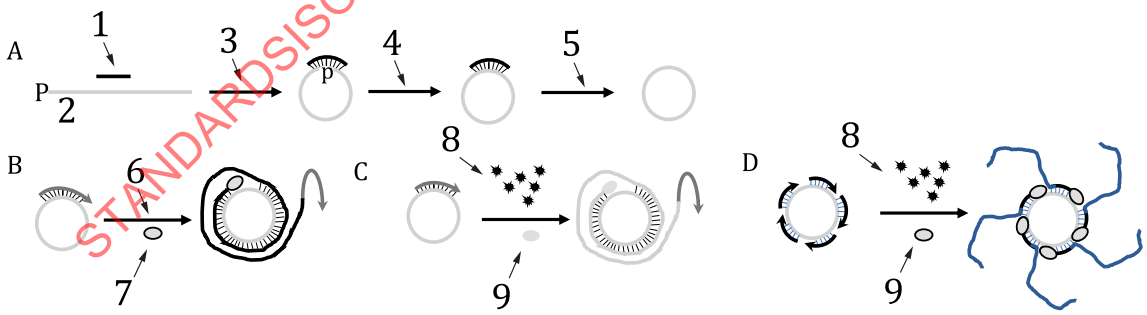
RCA is an isothermal enzymatic process where a short DNA or RNA primer is amplified to form a long single-stranded DNA or RNA using a circular DNA template and DNA or RNA polymerases. The RCA product is a concatemer containing tens to hundreds of tandem repeats that are complementary to the circular template[30][31].

E.2 Amplification strategy

RCA is depicted in Figure E.1[32]. Rolling circle consists of two steps A and B. In A, circularization of the template is facilitated by a ligation template (or padlock probe). These templates consist of two target-complementary segments connected by a linker sequence, typically carrying arbitrary sequences for detection and identification. The ligation template anneals to the template forming a circular hybrid in which the 5′ and 3′ ends are juxtapositioned. A ligation step circularizes the template. After ligation (step B) the ligation template acts as a primer for processive strand displacing DNA polymerase or reverse transcriptase (step C) that subsequently produces many linear copies of the template. A second primer is added to catalyse the production of the second strand. Additional primers that bind to other sites in the template can also be added to functionally increase amplification, e.g. hyperbranched RCA (step D)[31].

E.3 Advantages and disadvantages

RCA is simple, although in some cases it requires an initial denaturation step, such as when using a probe for diagnostic purposes[33][34][35].



Key

1 ligation template
2 circular template
3 annealing
4 ligation
5 purification
6 dNTPs
7 DNA polymerase
8 NTPs
9 RNA polymerase

Figure E.1 — Rolling circle amplification

Annex F
(informative)

Helicase dependent amplification (HDA)

F.1 General

HDA uses a DNA helicase to separate double helix strands into single helices. The unwinding of double-stranded DNA (dsDNA) generates single-stranded templates for primer annealing, hybridization and subsequent extension by a second DNA polymerase. Only two primers are required[9]. With the addition of reverse transcriptase, HDA can reverse transcribe and amplify RNA as well as the resulting DNA. HDA has been employed in several diagnostic devices and FDA-approved tests.

Since it was first described in 2004 by Vincent et al.[9] there have been numerous optimizations for HDA. The strategy is compatible with TaqMan assays and has been used in biothreat detection. In addition, there have been lateral flow, microfluidic chips and solid phase applications successfully developed for disease detection. The latter incorporate a microarray format with one of the primers immobilized. Several sample types have been tested and proven effective including crude bacterial preparation and soybean meal. Due to the similarities with PCR, the strategy is familiar and straightforward. Amplicons upwards of 2 300 base pairs can be generated. As with standard PCR, the time required is approximately 1 h to 2 h. Time is dependent upon the sample type and target concentration in the sample.

F.2 Amplification strategy

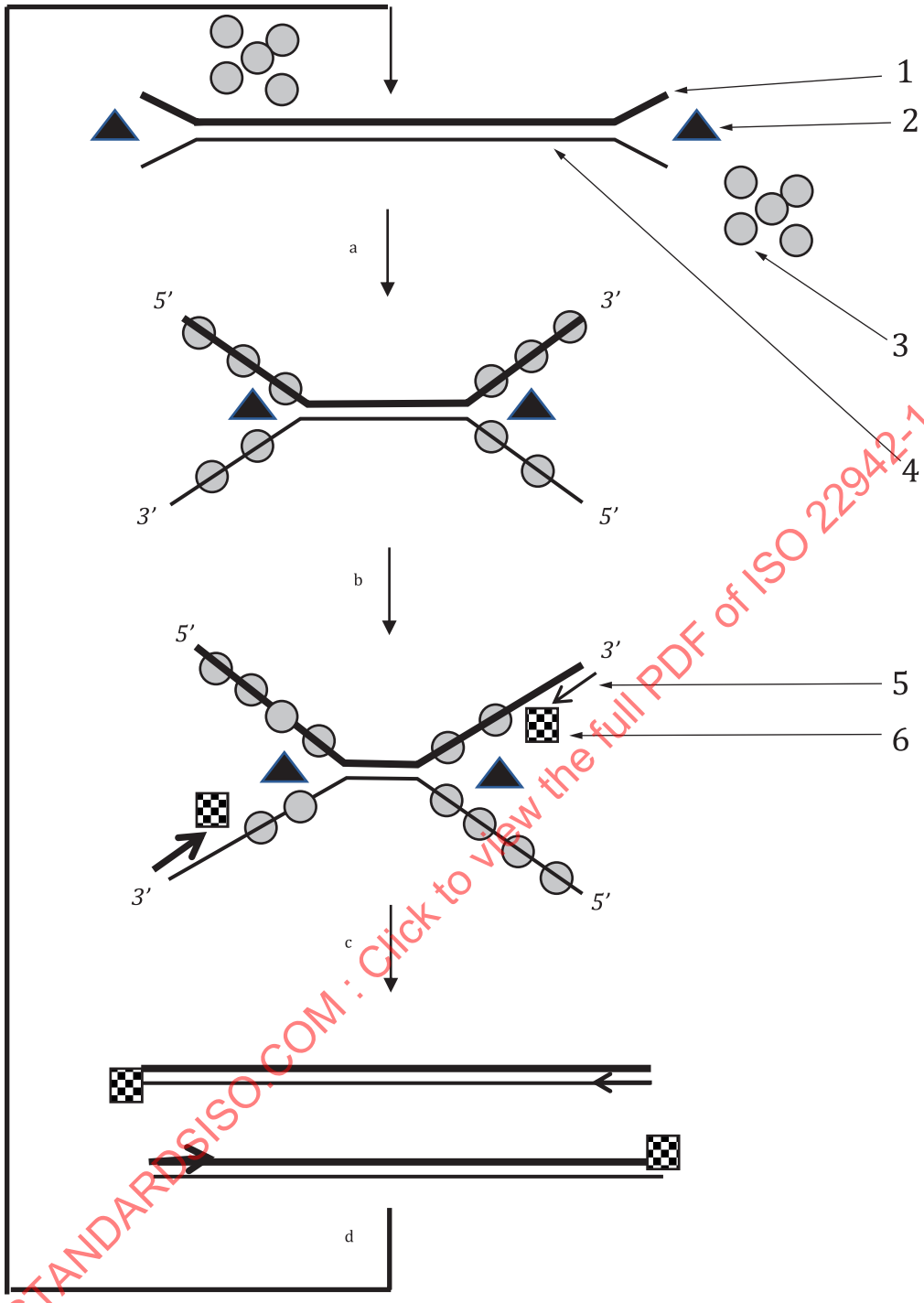
DNA helicase mimics the natural DNA replication fork mechanism. Double-stranded DNA is unwound by the enzyme at 60 °C and stabilizing proteins are added to the resultant single strands. The addition of restriction endonucleases acting upstream of the target region can enhance helicase activity. Target specific primers hybridize the template. The primers are designed to be complementary to the sense (e.g. forward) and antisense (e.g. reverse) strands. Primer melting temperatures should promote annealing in the temperature range of 60 °C to 65 °C. A DNA polymerase extends the primers producing two dsDNA target copies. The original strategy used a DNA polymerase deficient of 5′ to 3′ exonuclease activity. However, later applications were able to include a polymerase with exonuclease activity in a TaqMan platform. Following elongation, helicase acts on the newly formed dsDNA products. The products are continually copied resulting in exponential amplification of the target molecule.

In step A (see Figure F.1), duplex DNA is separated by helicase (triangle) and coated by single-stranded binding protein (ssb, circles). Primers anneal to their cognate sequence displacing ssb and initiating polymerase-mediated elongation (checkerboard box) of the 3′ ends of the primers producing two duplex DNA molecules. The process repeats with a twofold increase in DNA copies each cycle.

F.3 Advantages and disadvantages

The simplicity of the strategy enables rapid design and optimization compared with other isothermal methods. The downstream platforms for detection are as varied as those for standard PCR. That flexibility allows for a wide range of technical complexity and user enablement from gel electrophoresis to microfluidic chip. A 10-fold increase in sensitivity can be realized by utilizing a two-step process. The enzyme mix is separated from primer annealing. However, that has the potential to increase the risk for introduction of contaminants. A limitation is the speed of the strategy particularly in samples with < 100 copies of target.

As the DNA helicase unwinds dsDNA enzymatically, the initial heat denaturation and subsequent thermal cycling steps required by PCR can all be omitted. HDA provides a simple DNA amplification scheme: one temperature from the beginning to the end of the reaction.



Key

1 complimentary DNA strand (top)
2 helicase (black triangle)
3 ssb (grey circles)
4 complimentary DNA strand (bottom)
5 primers (lines with arrow heads)
6 DNA polymerase (squares with mosaic patterns)

a Helicases unwind DNA duplexes in the presence of ssb and accessory protein.
b Primers anneal to ssDNA.
c DNA polymerases extend the primers; one duplex is amplified to two duplexes.
d dsDNA is separated by helicases and this chain reaction repeats itself.

Figure F.1 — Helicase dependent amplification

Annex G
(informative)

Recombinase polymerase amplification (RPA)

G.1 General

RPA is an isothermal technology that utilizes the enzymatic activities of recombinases and polymerases to amplify either DNA or RNA templates. Two primers and a probe along with the recombinases and polymerases in a suitable reaction environment facilitate the creation of amplicons. The cyclic nature of RPA allows for exponential amplification of a very few copies of a target.

G.2 Amplification strategy

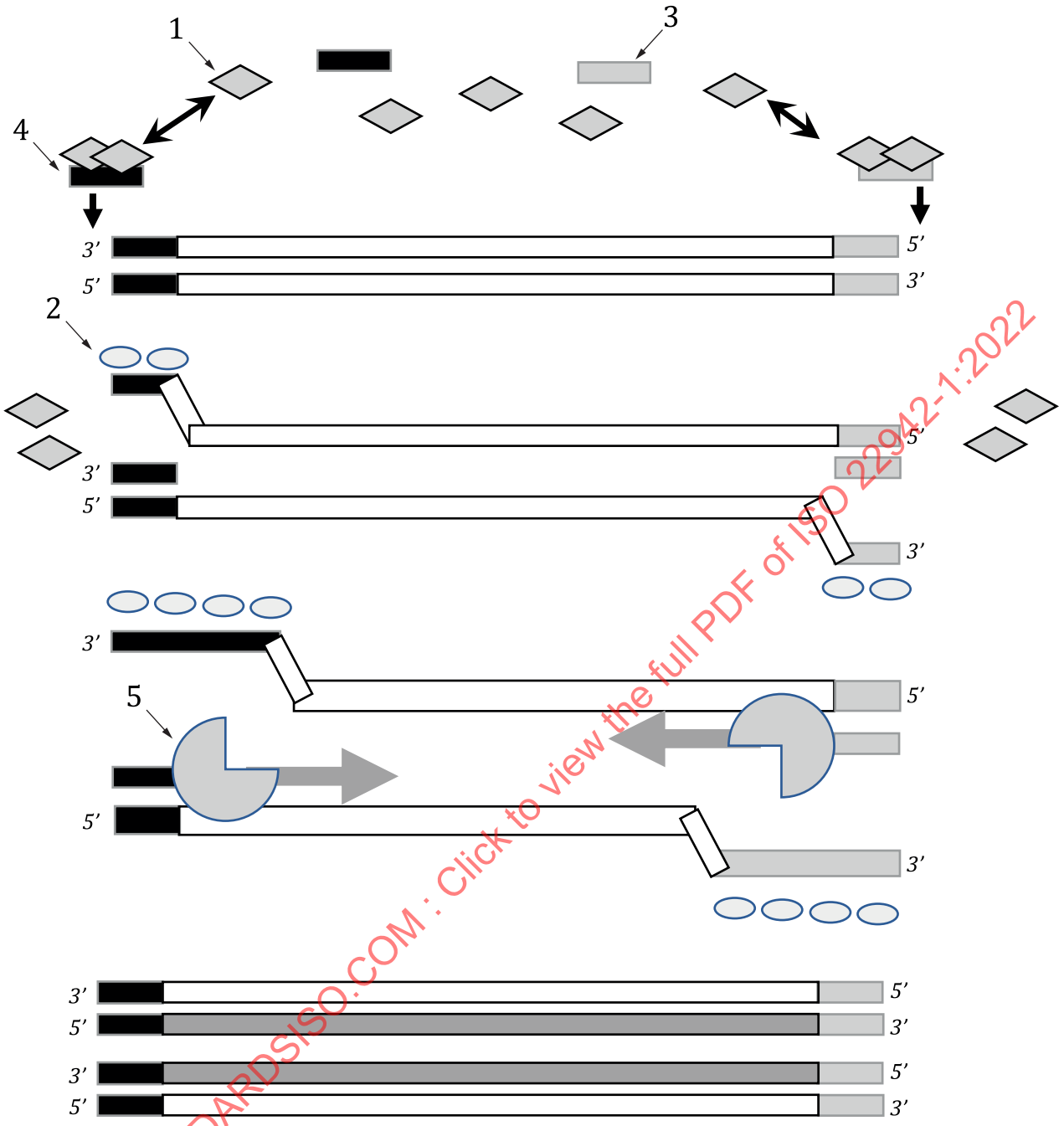
If the target template is RNA, a suitable reverse transcriptase is used to create DNA analogues. Primers, 30 nt to 35 nt long, bind to the DNA as part of recombinase enzyme (see 1 in Figure G.1) to primer complexes (see 3 and 4 in Figure G.1) that scan double-stranded DNA for complementary DNA sequences. Single-strand DNA binding proteins (see 2 in Figure G.1) stabilize the resulting DNA-primer-recombinase structure, preventing primer loss during the scanning process. Displacement of the recombinase by DNA polymerase (see 5 in Figure G.1) initiates extension of the primer. Cyclic repetition of this process enables exponential amplification of the target amplicon.

A labelled probe may be used to specifically identify the amplified product.

G.3 Advantages and disadvantages

The advantages of RPA include an extremely fast and sensitive reaction that does not require heat denaturation of the template. The assay is extremely portable and can be used in field or point of care diagnostics. Crude extraction of samples and the single transfer steps create a low maintenance operation with little change of amplicon contamination. Multiplex reactions can be designed to include one target and an internal control or multi-target reactions. Because of the DNA duplex formations, an additional specific-proofreading step is completed in the reaction. When compared side by side, the reverse transcriptase RPA isoPCR assay provides advantages over RT-PCR. Positive samples can be detected in less than 30 min and amplification only requires a single incubation temperature (optimum 37 °C)[36].

A disadvantage of RPA is the requirement for reverse transcriptase for RNA targets. Absence or low template concentration can eventually generate a non-specific reaction signal caused by primer-dependent artefacts. However, this can be overcome by utilizing a probe. The primer and probe design are more advanced, compared with PCR.



Key

1 recombinase enzyme
2 single-stranded binding protein
3 forward primer
4 backward primer
5 DNA polymerase

Figure G.1 — Recombinase polymerase amplification

Annex H
(informative)

Strand displacement amplification (SDA)

H.1 General

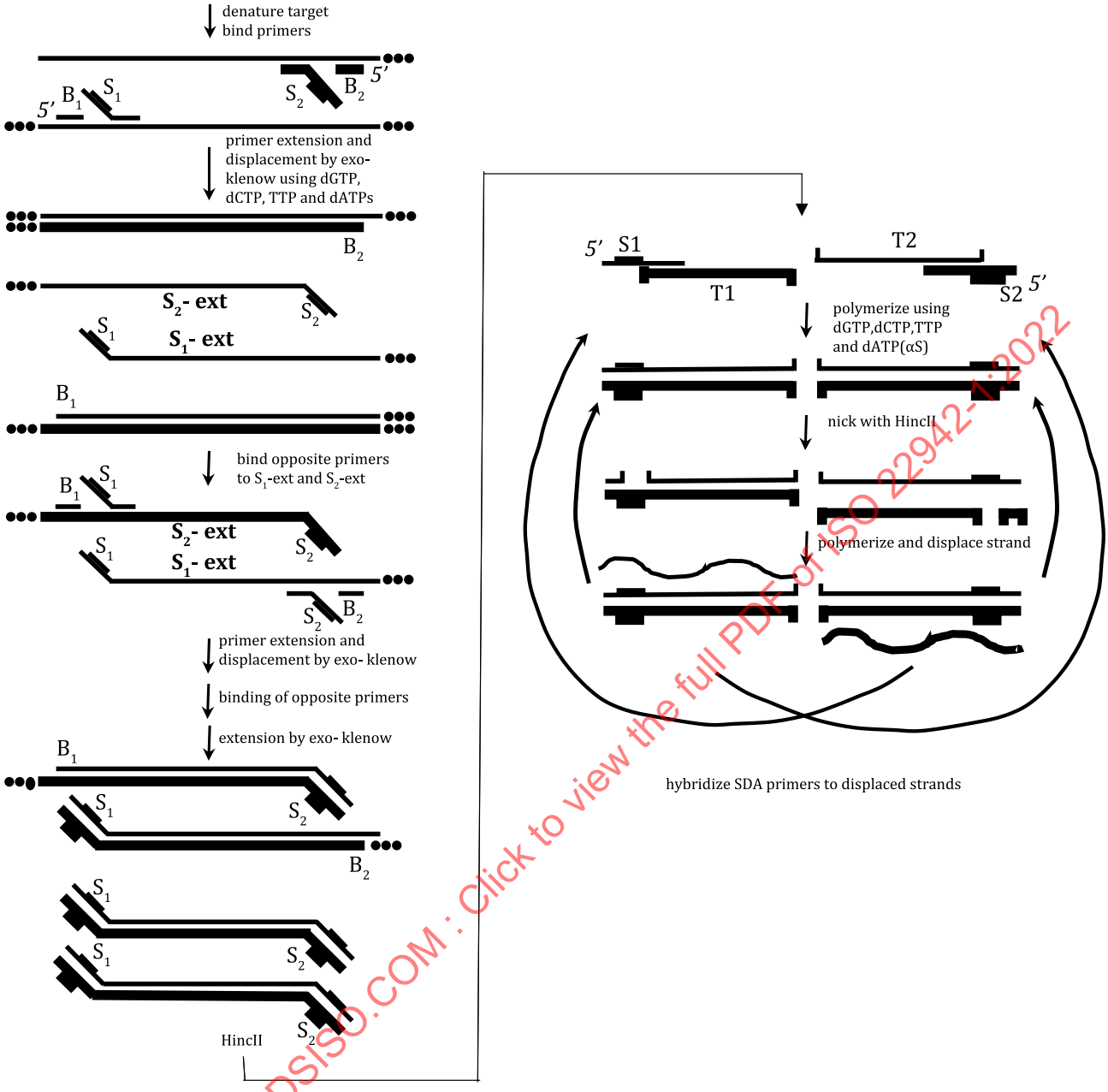
SDA relies on a strand-displacing DNA polymerase, typically Bst DNA or Klenow (large fragment, lacking 5′→3′ and 3′→5′ exonuclease activities) polymerase, to initiate extension at nicks created by a strand-limited restriction endonuclease or nicking enzyme at a site contained in a primer. The nicking site is regenerated with each polymerase displacement step, resulting in exponential amplification.

H.2 Amplification strategy

Amplification primer S1 and S2 followed by bumper primer B1 and B2 bind the target strands at positions flanking the sequence to be amplified (see Figure H.1). Amplification primer S1 and S2 contain hemiphosphorothioate HincII restriction enzyme cut sites specific for the target nucleic acids. The four primers are simultaneously extended by Klenow fragment. Extension of B1 displaces the S1 primer extension product, S1-ext. Likewise, extension of B2 displaces S2-ext. B2 and S2 bind to displace S1-ext. B1 and S1 bind to displace S2-ext. Extension and displacement reactions on templates S1-ext and S2-ext produce two fragments with restriction enzyme cut site at each end and two longer fragments with restriction enzyme cut site at just one end. Nicking, extension and displacement reactions by restriction enzyme and Klenow fragment are initiated at these four fragments and the cycle occurs continuously resulting in exponential amplification of the target nucleic acids.

H.3 Advantages and disadvantages

SDA has a wider range of application because it can amplify DNA and RNA. Primer design is complex in order to accommodate the restriction enzyme cut site. An initial denaturation step is required. SDA is useful for amplifying long templates[37]. Two or more enzymes are required for the reactions to occur.



Key

S1	amplification primer 1
S2	amplification primer 2
B1	bumper primer 1
B2	bumper primer 2
S1-ext	S1 primer extension product
S2-ext	S2 primer extension product

Figure H.1 — Strand displacement amplification