

Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Quantitative nucleic acid based methods

ICS 67.050

National foreword

This British Standard is the UK implementation of EN ISO 21570:2005+A1:2013, incorporating corrigendum February 2007. It is identical to ISO 21570:2005, incorporating amendment 1 and corrigendum December 2006. It supersedes BS EN ISO 21570:2005, which is withdrawn.

The start and finish of text introduced or altered by amendment is indicated in the text by tags. Tags indicating changes to ISO text carry the number of the ISO amendment. For example, text altered by ISO amendment 1 is indicated by **A1** **A1**.

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English Version

Foodstuffs - Methods of analysis for the detection of genetically modified organisms and derived products - Quantitative nucleic acid based methods (ISO 21570:2005)

Produits alimentaires - Méthodes d'analyse pour la détection des organismes génétiquement modifiés et des produits dérivés - Méthodes quantitatives basées sur l'utilisation des acides nucléiques (ISO 21570:2005)

Lebensmittel - Verfahren zum Nachweis von gentechnisch modifizierten Organismen und ihren Produkten - Quantitative auf Nukleinsäuren basierende Verfahren (ISO 21570:2005)

This European Standard was approved by CEN on 26 October 2005.

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
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Foreword

This document (EN ISO 21570:2005) has been prepared by Technical Committee CEN/TC 275 "Food analysis - Horizontal methods", the secretariat of which is held by DIN, in collaboration with Technical Committee ISO/TC 34 "Agricultural food products".

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by May 2006, and conflicting national standards shall be withdrawn at the latest by May 2006.

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Foreword to amendment 1

This document (EN ISO 21570:2005/A1:2013) has been prepared by Technical Committee ISO/TC 34 "Food products" in collaboration with Technical Committee CEN/TC 275 "Food analysis - Horizontal methods" the secretariat of which is held by DIN.

This Amendment to the European Standard EN ISO 21570:2005 shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by October 2013, and conflicting national standards shall be withdrawn at the latest by October 2013.

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Endorsement notice

The text of ISO 21570:2005/Amd 1:2013 has been approved by CEN as EN ISO 21570:2005/A1:2013 without any modification.

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Foreword

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

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

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ISO 21570 was prepared by the European Committee for Standardization (CEN) Technical Committee CEN/TC 275, *Food Analysis — Horizontal methods*, in collaboration with Technical Committee ISO/TC 34, *Food products*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

Introduction

The search for ingredients of genetically modified origin is performed by means of the following successive (or simultaneous) steps. After sample collection, nucleic acids are extracted from the test portion. Extracted nucleic acids can be further purified, simultaneously or after the extraction process. Afterwards, they are quantified (if necessary), diluted (if necessary) and subjected to analytical procedures (such as PCR). These steps are detailed in the present and in the following International Standards:

ISO 21569, *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Qualitative nucleic acid based methods*

ISO 21570, *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Quantitative nucleic acid based methods*

Further information about definitions and general items involving the steps cited above are collected in:

ISO 24276, *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — General requirements and definitions*.

The International Organization for Standardization (ISO) draws attention to the fact that it is claimed that compliance with this document may involve the use of a patent concerning the PCR technology.

ISO takes no position concerning the evidence, validity and scope of these patent rights.

ISO has been informed that Applied Biosystems, Roche Molecular Systems, Inc. and Hoffman-La Roche hold patent rights concerning PCR technology. The companies have assured the ISO that they are willing to negotiate licences under reasonable and non-discriminatory terms and conditions with applicants throughout the world. In this respect, the statements of the holders of these patent rights are registered with ISO. Information may be obtained from:

Licensing Department
Applied Biosystems
850 Lincoln Centre Drive
Foster City, CA 94404,
USA

and

Roche Molecular Systems, Inc.
Licensing Department
1145 Atlantic Avenue
Alameda, CA 94501,
USA

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Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Quantitative nucleic acid based methods

1 Scope

This International Standard provides the overall framework of quantitative methods for the detection of genetically modified organisms (GMOs) in foodstuffs, using the polymerase chain reaction (PCR).

It defines general requirements for the specific amplification of DNA target sequences, in order to quantify the relative GMO-derived DNA content and to confirm the identity of the amplified DNA sequence.

Guidelines, minimum requirements and performance criteria laid down in this International Standard are intended to ensure that comparable, accurate and reproducible results are obtained in different laboratories.

This International Standard has been established for food matrices, but is also applicable to other matrices, e.g. feed and plant samples from the environment.

Specific examples of methods are provided in Annexes A to D.

2 Normative references

The following referenced documents are indispensable for the application 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.

[A1] ISO 21569:2005 + AM1:2013, *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Qualitative nucleic acid based methods*

ISO 21571:2005 + AM1:2013, *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Nucleic acid extraction*

ISO 24276:2006 + AM1:2013, *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — General requirements and definitions* **[A1]**

ISO Guide 32, *Calibration in analytical chemistry and use of certified reference materials*

3 Terms and definitions

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

4 Principle

4.1 General

Quantitative analysis consists of the quantitation of target DNA sequences in the test samples. Each method specifies the target sequences(s).

Quantitation may be performed using competitive ^{[1],[2]} or real-time PCR ^{[3],[4]}.

A quantitative analysis should clearly express the quantity of the target genetic element, relative to the quantity of a specific reference, appropriate calibrants and controls, and be within the dynamic range of the analytical method used and the test portion analysed.

The analysis generally consists of

- amplification of one or more specific target sequences,
- detection and confirmation of the specificity of the PCR product(s), and
- quantitation of the amplified fragments relative to calibrants.

NOTE In the case of real-time PCR analysis, amplification, detection and confirmation occur simultaneously.

4.2 Amplification, detection and confirmation of PCR products

See ISO 21569 for the principles of amplification, detection and confirmation of the DNA sequences.

4.3 Quantitation of PCR products

The principle of quantitation is usually to determine the ratio (expressed as a percent) of two DNA target sequences; i.e. a sequence representing the genetically modified organism of interest and an (endogenous) target taxon-specific sequence. However, in some cases, quantitation can also be carried out relative to a specified amount of food matrix (e.g. when detecting GM microorganisms in foods).

Calibrants (calibration materials) used for quantitation should be traceable to certified reference materials (CRMs), if available. If not available, other suitable reference material should be used. Example guidance is provided in Reference [5]. Information on validation studies and measurement uncertainty has been gathered from international studies [6],[7],[8],[9].

5 Reagents

All reagents and materials used in the analysis should be identical, or equivalent, to those specified in the method. Otherwise, all reagents and materials should be of molecular biology grade. These reagents shall be stored and used as recommended by the supplier or according to the laboratory quality assurance specifications. For a list of reagents, see the specific annex.

6 Apparatus and equipment

See Annexes A to D and ISO 24276.

7 Guidelines concerning the procedure

7.1 General

General considerations relevant to PCR amplification for the detection of GMOs are described in ISO 21569.

Annexes A to D specify PCR detection methods together with details of their scope of application. The demonstrated performance characteristics for each method are detailed.

The concentration of the DNA sequence of interest should be within the dynamic range of the method.

NOTE A target taxon specific monitor run can be undertaken to determine whether the template DNA is of sufficient quality (length and structural integrity), purity and quantity to allow the detection and quantitation of a GMO belonging to the target taxon. This may be of particular relevance when DNA is extracted from composite or highly processed matrices.

The DNA extracted from each test portion should be analysed at least in duplicate.

Appropriate controls shall be included (see [\[A1\]](#) ISO 24276:2006 [\[A1\]](#), Table 1).

7.2 Target sequence stability

The allelic and copy number stability of the target sequence should be considered for cultivars of different geographic and phylogenetic origins.

7.3 Calibration of the analysis

An appropriate number of calibration points and replicates covering the range of quantitation shall be applied [e.g. four calibration points with two replicates (altogether 4×2 values) or six calibration points with one measurement at each point (altogether 6 values)]. The quality of the calibration influences the measurement uncertainty ^[9].

As an alternative to genomic DNA calibration reference materials, for example, a dilution series of a plasmid or synthetic dsDNA containing the target sequence may be used, provided that it is demonstrated to perform in an equivalent way to the genomic DNA reference material and the genomic DNA extracted from the sample.

7.4 Quantitation considerations

PCR methods should be appropriately designed to minimize the variability.

NOTE Depending on the method used and/or the material analysed, the presence of stacked genes can lead to overestimation of the true GMO content.

For the determination of the limit of quantitation (LOQ), see ISO 24276.

Calculation of the GMO content based on copy numbers of target sequences per haploid genome is influenced by the homo- and heterozygosity of the species under investigation. For details, see Annexes A to D.

Use of the $\Delta\Delta C_t$ (cycle of threshold) method is only valid if the amplification efficiencies of the target taxon-specific assay and the GMO-specific assay are very similar.

7.5 Quality assurance requirements

Consistency between measurements is desirable to obtain reliable estimates of target sequence quantities. However, knowledge of the relative standard deviation of repeatability of the method is required to establish whether the measurements are consistent (see the ISO 5725 series for details). To calculate the relative standard deviation of repeatability, the number of separate measurements per laboratory sample may exceed what is feasible in practice in terms of acceptable costs. Consequently, if a specified GMO-derived DNA is to be reported (in percent), a feasible solution should require the following as a minimum:

- a) within test portion consistency:
 - through rejection of measurements <LOQ, and
 - through maximum deviation observed between dilutions and individual measurements equals the value expected from the corresponding dilution factor ± 33 %;
- b) between test portion consistency:
 - estimated relative GMO-derived DNA concentrations obtained under a) for each test portion should not differ by values greater than -50 % to $+100$ % of the estimated quantity value (equal to a ΔC_t of 1 in real-time PCR) (i.e. for two test portions, measurements of $1,0$ % and $2,0$ % are acceptable, measurements of $0,9$ % and $2,1$ % are not).

In order to guarantee accuracy of the measurements, a reference material (RM), preferably certified (CRM), for the quantity of the event concerned, with an appropriate level of metrological reliability and with reasonable similarity of matrix shall be selected and analysed. In the absence of a CRM, in-house RM may be prepared by a procedure demonstrating stability, homogeneity and traceability, and ensuring the absence of bias. The quantified uncertainty shall fulfil the required uncertainty for the calibration (see ISO Guide 32).

A1 8 Interpretation

The PCR result will be either a) or b).

- a) Fit for quantification of the target sequence provided:
 - the result is positive according to ISO 21569:2005, 8.1;
 - the observable inhibition of the reaction is negligible;
 - the analysis produces an unambiguous measurement value;
 - the target sequence content is within the dynamic range of the method;
 - the analysis is calibrated in an acceptable way (see 7.3).
- b) Unfit for quantification of the target sequence if any of the conditions listed in a) are not fulfilled.

Interpretation of ambiguous results within the same test portion: in case of +/- results for the two replicates, repeat the two PCR for the relevant test portion. If the two novel replicates are tested +/- or -/-, the test portion is considered as negative.

Interpretation of ambiguous results between two test portions: in case of $\pm$ results for the two test portions of a sample, the extractions and analysis of two new test portions shall be performed. If again the results are +/-, the sample is considered as negative according to ISO 24276:2006, 6.3.

The measurement uncertainty shall be sufficiently small to enable the laboratory to draw the relevant conclusions.

Annexes A to D describe the measurement of the target DNA quantities. These quantities can be used to calculate the GMO content. These calculations usually take into consideration relevant biological factors, e.g. the homo- or heterozygosity of the target sequences.

If the GM target sequence content or the taxon-specific target sequence content is below the limit of quantification, the result shall only be expressed qualitatively.

NOTE Stating that the GMO-derived DNA content is below the practical LOQ accompanied by a specification of that LOQ is considered to be a qualitative expression of the result.

9 Expression of results

The results shall clearly state the quantity of the GM target sequence relative to the target taxon-specific sequence. The results should also provide values for the measurement uncertainty, such as the standard deviation or coefficient of variation. Furthermore, the LOD and LOQ of the method and the practical LOD and LOQ should be reported. The indication that the result refers only to GMO targets should be reported. In the case of quantitative screening analysis on complex matrices, it is recommended to specify that the GMO signal can come from non target taxa.

The target sequences can or cannot be detected, or the quantity of at least one of them can be below the limit of quantification. Table 1 describes the four alternative cases and the corresponding expression of the result to be included into the test report.

The GMO-derived DNA content can also be reported as being above or below a specific value, taking into account the measurement uncertainty. **A1**

A1 10 Test report

The test report shall be written in accordance with ISO 24276 and ISO 21569 and shall contain at least the following additional information:

- a) the LOQ of the method and the matrix used to establish it;
- b) the practical LOQ;
- c) a reference to the method which has been used for the extraction of DNA;
- d) a reference to the methods used for the amplification of the DNA target sequences;
- e) the reference material used;
- f) the results expressed according to Clause 9;
- g) the PCR target and whether considered “event specific” or “construct specific” or “screening”;
- h) the definition of the measurement uncertainty used.

NOTE For g) and h), information can figure in different documents (e.g. contract review, technical data sheets).

Table 1 — Expression of results

Result	Expression of the result
Target taxon-specific sequence is not detected.	“For species X, DNA was not detected.”
Target taxon-specific sequence is detected but GM target sequence is not detected.	According to ISO 21569. “For sample X, GM 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 test sample size and the amount of target DNA included in the PCR is sufficient for the LOD to be applicable, then the following sentence shall be added: “However, the amount of the target DNA extracted from species X can be/was insufficient for the LOD to be applicable for this sample. The LOD of sample is x %.” (Specify the unit used.) NOTE The LOD of the sample is determined by the quantity of DNA of the species included in the analytical reaction (copy number), and the ratio relative to the absolute LOD of the GM target (copy number), and in the case of grain and seeds, the number of grain or seeds in the portion that is ground.
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.	For each GMO, state: “GMO (specify the GMO) derived DNA as determined by detection of (specify target sequence) derived from (specify species) was detected, below the practical limit of quantification” In addition, if applicable: “The practical limit of quantification is x %.” (Specify the unit used.)
The target taxon-specific sequence and the GM target sequence are both detected and the quantity is above the LOQ for both target sequences.	For each GMO, state: “The content of GMO (specify the GMO) derived DNA as determined by detection of (specify target sequence) derived from (specify species) is $x \pm u_{\text{meas}}$ %” where u_{meas} is the measurement uncertainty. (Specify the unit used.)

A1

Annex A (informative)

Target taxon-specific methods

A.1 Target taxon-specific method for the absolute quantitation of the *adh1* gene DNA of maize using real-time PCR

A.1.1 Introduction

This annex describes a method for the specific amplification and quantitation of the taxon-specific (housekeeping) maize (*Zea mays*) *adh1* gene (coding for alcohol dehydrogenase 1) for determination of the content of maize DNA, or for testing for the presence/absence of detectable PCR inhibitors in DNA solutions extracted from products containing maize-derived DNA, e.g. foods.

For limitations, see A.1.8.

A.1.2 Validation status and performance characteristics

A.1.2.1 General

The method has been optimized for DNA extracted from pure ground maize kernels, maize leaves and certified reference materials (IRMM-411, IRMM-412, IRMM-413 series ^[10]) ^[11].

The reproducibility of the described method has been tested in a collaborative trial using unknown samples (U1 to U6) consisting of wild type maize DNA at different corresponding copy number of the target sequence (see A.1.2.2), and in other collaborative trials in combination with methods specific for GM maize events, e.g. Bt11 (see D.1).

The copy number of the target sequence per haploid genome is estimated to be 1 ^[11].

The allelic stability of the target sequence has been established ^[11].

A.1.2.2 Collaborative trial

The method has been validated in a collaborative trial organized by the European Commission's Joint Research Centre (EC-JRC), Institute for Health and Consumer Protection (IHCP), in agreement with the international harmonized protocol ^[12].

Six samples (S1-S6) of wild type maize DNA (extracted from leaf material ^[13]) containing known absolute copy numbers (183 486, 61 162, 20 387, 6 796, 2 265, 755) of haploid maize genomes were used to establish a calibration curve for absolute quantitation of haploid maize genomes in unknown samples. The absolute copy numbers in the known samples were determined by dividing the sample DNA mass (determined by fluorometric quantitation of dsDNA with PicoGreen, Molecular Probes, Cat. Number P-7589) by the published average 1C value for maize genomes (2,725 pg) ^[14].

Six samples (U1-U6) of wild type maize DNA (extracted from leaf material ^[13]) were used as unknown samples. The expected copy numbers in the unknown samples were determined in the same way as those of the known samples.

The results of the collaborative trial validation are summarized in Table A.1

The method has also been validated in combination with event-specific methods for several maize GMO, e.g. for Bt11 sweet maize. See References [15] and [16] and D.1 for details on the combined (relative quantitation) trial.

Table A.1 — Validation data

	Sample					
	U1	U2	U3	U4	U5	U6
Number of participating laboratories	12	12	12	12	12	12
Number of laboratories having returned results	10	10	10	10	10	10
Number of invalid laboratories	1	1	1	1	1	1
Number of retained laboratories	9	9	9	9	9	9
Number of samples per laboratory	4	4	4	4	4	4
Number of Cochran outliers	1	1	1	1	—	—
Number of Grubbs outliers	—	1	1	1	1	1
Number of accepted samples	35	34	34	34	35	35
Expected copy number value	7 339	18 349	36 697	55 046	91 743	146 788
Mean copy number value	9 985	23 885	46 918	75 161	100 541	122 080
Bias of true value (%)	36,1	30,2	27,9	36,5	9,6	–16,8
Repeatability standard deviation s_r^a	1 318,59	1 463,60	5 796,58	4 539,57	11 306,89	14 843,41
Repeatability relative standard deviation (%) ^b	13,21	6,13	12,35	6,04	11,25	12,16
Reproducibility standard deviation s_R^a	2 013,12	2 083,57	6 145,39	6 806,85	14 592,04	17 777,70
Reproducibility relative standard deviation (%) ^b	20,16	8,72	13,10	9,06	14,51	14,56
^a Expressed as copy number value.						
^b Expressed as percentage of the mean value.						

A.1.2.3 Molecular specificity

A.1.2.3.1 General

The method has been designed to target a part of the sequence described in EMBL/GenBank/DDBJ²⁾ accession number X04050. This sequence is unique to *Zea mays* (maize/corn) and *Zea mays* subsp. *diploperennis* (teosinte) [11].

A.1.2.3.2 Theoretical specificities

The theoretical specificities of the primers and probes were assessed through a search of the GenBank/EMBL/DDBJ databases ²⁾ using the nucleotide sequences as query sequences with the BLASTN programme ²⁾ at <http://www.ncbi.nlm.nih.gov/blast/> [October 9, 2003]. The result of the search confirmed a complete identity only with the expected target sequences.

2) These are examples of suitable products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

A.1.2.3.3 Experimental determination of specificity

The specificity of the method was tested against a wide range of non-target taxa and 20 different maize lines representing geographically and phylogenetically diverse samples [11]. No cross-reactivity was observed with the non-target taxa (except with teosinte *Zea mays* subsp. *diploperennis*, the wild ancestor of cultivated maize) [11], [17]. The copy number and allelic stability of the target sequence across different maize lines has been established [11].

A.1.2.4 Optimization

This was carried out for the ABI PRISM 7700[®] sequence detection system (SDS) ³⁾ and TaqMan[®] chemistry ³⁾. Primer and probe design were done with Primer Express[®] software (Applied Biosystems) ³⁾.

A.1.2.5 Limit of detection (LOD)

According to method developer, the absolute LOD is 10 copies of the target sequence [11].

The lowest number of copies of the target sequence included in the collaborative trial was 7 399 copies of the target sequence.

A.1.2.6 Limit of quantitation (LOQ)

According to the method developer, the absolute LOQ is 100 copies of the target sequence [11].

The lowest number of copies of the target sequence included in the collaborative trial was 7 399.

A.1.3 Adaptation

No specific information is available.

A.1.4 Principle

A 134 bp fragment of the *adh1* gene is amplified using two maize *adh1*-specific primers (see Table A.2). Accumulation of PCR products is measured at the end of each PCR cycle (real-time) by means of a maize *adh1*-specific oligonucleotide probe (ADH1-MDO, see Table A.2) labelled with two fluorescent dyes: FAM as reporter dye and TAMRA as quencher. For that purpose, TaqMan[®] ³⁾ chemistry was employed.

The measured fluorescence signal crosses a user-defined threshold value after a certain number of cycles. This number is called the C_t -value. For quantitation of the amount of maize *adh1*-DNA in an unknown sample, the C_t -value is converted into a corresponding copy number value by comparison with a calibration curve whose C_t -values are directly linked with known copy numbers (regression analysis).

A.1.5 Reagents

A.1.5.1 General

For quality of reagents to be used, see [A1](#) ISO 24276:2006 [A1](#), 6.6.

A.1.5.2 Water.

A.1.5.3 PCR buffer (without MgCl₂), 10-fold.

3) These are examples of suitable products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

A.1.5.4 MgCl₂ solution, $c(\text{MgCl}_2) = 25 \text{ mmol/l}$.

A.1.5.5 dNTP solution, $c(\text{dNTP}) = 2,5 \text{ mmol/l}$ (each).

A.1.5.6 Oligonucleotides

Details of the oligonucleotides are listed in Table A.2.

Table A.2 — Oligonucleotides

Name	Oligonucleotide DNA sequence	Final concentration in PCR
ADH-FF3	5'-CgT CgT TTC CCA TCT CTT CCT CC-3'	300 nmol/l
ADH-RR4	5'-CCA CTC CgA gAC CCT CAg TC-3'	300 nmol/l
ADH1-MDO	5'-FAM-AAT CAg ggC TCA TTT TCT CgC TCC TCA-TAMRA-3' ^a	200 nmol/l
^a FAM: 6-carboxyfluorescein; TAMRA: 6-carboxytetramethylrhodamine.		

The length of the *adh1* amplicon is 134 bp.

A.1.5.7 Thermostable DNA polymerase

AmpliTaq Gold® DNA polymerase⁴⁾.

A.1.5.8 Uracil *N*-glycosylase (optional).

A.1.5.9 Amplification reaction mixture

Details of the amplification reaction mixture are listed in Table A.3.

Table A.3 — Amplification reaction mixture in the final volume/concentration per reaction vial

Total reaction volume		25 µl
Template DNA (maximum 250 ng)		5 µl
Taq-DNA-polymerase	TaqMan® Universal Master Mix 2X	12,5 µl (1 X)
Decontamination system (dUTP included uracil <i>N</i> -glycosylase)		
Reaction buffer (containing passive reference ROX) ^a		
dNTP mix		
Primers	see Table A.2	see A.1.5.6
Probe	see Table A.2	see A.1.5.6
^a ROX = carboxy-X-rhodamine.		

4) These are examples of suitable products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

A.1.6 Apparatus

A.1.6.1 General

Standard laboratory apparatus should be used throughout unless otherwise specified.

A.1.6.2 Thermal cycler

The indicated temperature-time profile was originally tested with the ABI PRISM® 7700 SDS (Applied Biosystems) ⁵⁾. Other real-time PCR detection systems may be used after adaptation of the reaction conditions.

A.1.6.3 Reaction vials

The reaction vials shall be suitable for PCR amplification on a real-time thermal cycler, e.g. ABI PRISM® 96-Well Optical Reaction Plate, or MicroAmp® Optical Caps (8 caps/strip, flat) (Applied Biosystems) ⁵⁾.

A.1.7 Procedure: PCR set-up

A.1.7.1 General

The PCR set-up for the reference gene target sequence and for the GMO target sequence should be carried out in separate vials unless otherwise stated in the relevant GM target-specific annex.

The method is described for a total PCR volume of 25 µl per reaction mixture with the reagents as listed in Table A.3.

A.1.7.2 PCR controls

If the controls do not yield the expected results, the test results shall be rejected and the analysis shall be repeated.

As a positive control/calibration reference material, high-quality, pure genomic DNA extracted from maize (e.g. a CRM from the JRC, IRMM ⁵⁾) may be used ^[13]. Any other appropriate controls should be included as described in ISO 24276.

A.1.7.3 Temperature-time programme

The temperature-time programme as outlined in Table A.4 was optimized for the ABI PRISM® 7700 sequence detection system (SDS) (Applied Biosystems) ⁵⁾. In the validation study, it was used in combination with the AmpliTaq Gold® DNA polymerase ⁵⁾. The use of other thermal cyclers may require specific adaptation. The temperature and time required for enzyme activation depend on the particular polymerase used. Table A.4 describes the reaction conditions.

⁵⁾ These are examples of suitable products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

Table A.4 — Procedure: Reaction conditions

		Time s	Temperature °C
Pre-PCR: decontamination		120	50
Pre-PCR: activation of DNA polymerase and denaturation of template DNA		600	95
PCR (50 cycles)			
Step 1	Denaturation	15	95
Step 2	Annealing elongation	60	60

A.1.8 Limitations and interpretation of the results

The presence of PCR inhibitors may have a strong impact on the accuracy of the estimated copy number of the *adh1* in the analysed samples. Therefore the absence of detectable PCR inhibitors should be verified (see also ISO 21571:2005, Annex A), for example by setting up serial dilutions of the template DNA and examining the correspondence between dilutions and differences in C_t (cycle of threshold) values (i.e. one C_t corresponds to a doubling of template concentration).

For the use of this method in combination with a method for the quantitation of GMO-derived DNA, it is important that the absolute amount of template DNA (ng) be the same for both the *adh1* PCR and the GMO-specific PCR. If not, the absolute copy numbers of the reactions cannot be compared directly, and an adjustment of the corresponding copy numbers is required. Otherwise a relative GMO concentration cannot be calculated.

A.1.9 Calibration and calculation of results

Calibration points are produced with DNA containing defined amounts in absolute copy numbers of haploid genomic maize DNA containing the target sequence.

A calibration curve is produced by plotting C_t -values against the logarithm of the target copy number for the calibration points. This can be carried out, for example, by use of spread-sheet software such as Microsoft Excel⁶⁾, or directly by options available with the sequence detection system software.

The calibration curve is used to determine the absolute haploid genomic maize DNA copy numbers of the unknown samples. Although the sample DNA can be degraded due to food processing or may contain ingredients other than maize, this does not influence the calculated number of haploid genome copies of unknown samples.

6) These are examples of suitable products available commercially. This information is only given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

A.2 Target-taxon-specific method for the detection of DNA derived from rice

A.2.1 Principle

Rice *SPS* gene has been described as being suitable for use as an endogenous reference gene in GM rice identification and quantification (Reference [59]). The GMO Detection Laboratory of Shanghai Jiao Tong University (GMDL-SJTU) organized a collaborative trial for validation of the applicability of the rice sucrose phosphate synthase (*SPS*) gene as an endogenous gene for quantitative analysis of genetically modified (GM) or non-GM rice. The study involved 12 laboratories from Spain, Korea, Lithuania, Slovenia, Japan, Italy, and China.

The operational procedure of the collaborative study comprised the following modules.

Quantitative real-time PCR for quantification of blind rice DNA samples used to construct standard curves.

Quantitative real-time PCR for the quantification of blind rice DNA samples using the constructed standard curves.

The interlaboratory test was carried out in accordance with the following internationally accepted guidelines:

- ISO 5725; [51]–[56]
- the IUPAC protocol for the design, conduct and interpretation of method-performance studies (Reference [12]).

The results of the collaborative study as well as the related protocol are given in A.2.3.3.

A.2.2 Scope

The method has been optimized for rice grain and its processed products containing mixtures of rice and other matrices, e.g. maize and soybean. The applicability of the *SPS* gene was tested through collaborative trials using DNA samples extracted from rice grains.

A.2.3 Validation status and performance criteria

A.2.3.1 Robustness of the method

The robustness of the *SPS* gene quantitative real-time PCR system was tested by the method developer on different temperature–time programmes (i.e. two-step and three step) and on three different DNA samples containing known amounts of rice DNA (10 ng, 1 ng, 0,1 ng rice genome DNA samples). There were three repetitions per sample. The quantitative real-time PCR systems had the expected ruggedness and worked well at different temperature–time programmes and three concentrations of the rice DNA samples.

The quantitative PCR system for the *SPS* gene was also tested on different real-time PCR instruments (Rotor gene 3000A,¹⁾ Corbett Research and ABI7700,¹⁾ Applied Biosystems), with three different reaction volumes (20 µl, 25 µl, and 30 µl; three repetitions per volume). The quantitative real-time PCR system demonstrated appropriate ruggedness, working well on the different real-time PCR instruments and with the different reaction volumes. 

1) Product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

A.2.3.2 Intralaboratory trial

For sample preparation, all the DNA samples were extracted using the cetyl(trimethyl)ammonium bromide (CTAB) method adopted from ISO 21571. Spectrometric quantification of the amount of total DNA extracted was performed using a method adopted from ISO 21571:2005, B.1. After the DNA quantification, a quantitative real-time PCR run was carried out to provide data about possible PCR inhibition.

The *SPS* gene PCR system was tested by three researchers using the rice genome DNA, and gave satisfactory results; in particular, in quantitative PCR, the bias was below 25 % over the dynamic range (i.e. 0,05 ng to 1,00 ng).

A.2.3.3 Collaborative trial

Standard curves were constructed using serially diluted DNA samples extracted by the GMDL-SJTU from four rice cultivars by means of quantitative PCR. The PCR efficiency, calculated from the slope of the standard curve as $(10^{-1/a} - 1) \times 100$, where a is the slope, of the *SPS* gene PCR system ranging from 0,846 3 to 1,223 3, and the linearity (regression coefficient, R_c^2) was on average equal to 0,997.

The results of the eight blind DNA samples are reported in Table A.5. These are evaluated with respect to the method acceptance criteria and to the method performance requirements, as established by the European Network of GMO laboratories (ENGL) and adopted by the European Reference Laboratory for GM Food and Feed (EU-RL GMFF) (Reference [60]). In Table A.5, estimations of both repeatability and reproducibility for each rice concentration level are reported, after identification and removal of outliers according to Cochran's test.

Table A.5 — Results of quantitative real-time PCR

Parameter	Blind samples							
	0,5 ng	0,5 ng	1ng	1 ng	2 ng	5 ng	5 ng	10 ng
Laboratories returning results	12	12	12	12	12	12	12	12
Samples per laboratory	1	1	1	1	1	1	1	1
Total data no.	108	108	108	108	108	108	108	108
Data excluded	4	2	0	8	0	0	0	0
Reason for exclusion	Cochran's test	Cochran's test	—	Cochran's test	—	—	—	—
Mean value	0,407 8	0,412 1	0,814 6	0,734 4	1,857 5	5,261 0	5,445 7	10,889 6
Repeatability standard deviation	0,058 3	0,071 9	0,143 5	0,111	0,336 2	0,936 4	1,142 7	1,867 7
Repeatability coefficient of variation, %	14,29	17,44	17,62	15,11	18,10	17,80	20,98	17,15
Reproducibility standard deviation	0,130 2	0,061 8	0,249 6	0,092 7	0,201 3	0,810 9	0,989 6	1,617 5
Reproducibility coefficient of variation, %	31,92	14,99	30,65	12,63	10,84	15,41	18,17	14,85
Bias, absolute value	0,092 2	0,087 8	0,185 3	0,265 5	0,142 4	-0,261 0	-0,445 8	-0,889 6
Bias, %	-18,44	-17,57	-18,54	-26,53	-7,12	5,22	8,92	8,90

A1

A.2.3.4 Molecular selectivity

A.2.3.4.1 General

The primers and probe targeting the 81 bp *SPS* gene DNA fragment are listed in Table A.6.

Table A.6 — Oligonucleotide primers and probe sequences

Name	Oligonucleotide DNA sequence (5' to 3')
Quantitative-real time PCR primer and probe sequence	
<i>SPS</i> primer F	TTgCgCCTgAACggATAT
<i>SPS</i> primer R	CggTTgATCTTTTCgggATg
<i>SPS</i> probe	HEX- TCCgAgCCgTCCgTgCgTC -TAMRA

A.2.3.4.2 Experimental

DNA samples extracted from 11 different plant materials (including rice) were analysed using the *SPS* gene PCR method. Among the 11 samples, only rice DNA gave positive results. The 10 other samples (i.e. bamboo, green bristlegrass, barley, wheat, foxtail millet, rapeseed, tomato, potato, soybean and *Arabidopsis*) gave negative results.

DNA samples extracted from 12 different rice cultivars were analysed by the specific PCR method developed for the detection of the *SPS* gene. All 12 samples gave positive results.

A.2.3.4.3 Theoretical

The theoretical specificity of the *SPS* gene primers and probe was assessed through a similarity search using the BLASTN 2.0MP-WashU program (Reference [64], search date: 2010-01-09). The 81 bp sequence used as query is part of the NCBI accession number U33175 (nucleotides 1055–1135). The results of the blast search confirmed the complete identity of the query sequence with rice *SPS* gene sequence, and no similarity with other genes and species.

A.2.4 Principle and summary

An 81 bp fragment of the *SPS* gene is amplified using two rice *sps*-specific primers. Accumulation of PCR products is measured at the end of each PCR cycle (real-time) by means of a rice *sps*-specific oligonucleotide probe labelled with two fluorescent dyes: FAM as reporter dye and TAMRA as quencher (see Table A.6). For that purpose, TaqMan®¹ chemistry is employed. The fluorescence signal measured crosses a user-defined threshold value after a certain number of cycles. This number is called the C_t value. For quantification of the amount of rice *sps*-DNA in an unknown sample, the C_t value is converted into a corresponding copy number value by comparison with a calibration curve whose C_t values are directly linked with known copy numbers (regression analysis).

A.2.5 Terms and definitions

For the purposes of this clause, the terms and definitions of ISO 5725-1^[51] and ISO 24276 apply.

A.2.6 Sample type and amounts

DNA samples extracted from the grains of four rice cultivars, were used to construct the standard curves in this collaborative study. Then, eight blind samples were analysed using the four standard curves constructed. 

 The participants received the following samples.

- Four DNA samples from different rice varieties (3M, Indica variety from US; Balilla, Japonica variety from Italy; Guangluai, Indica variety from Southern China, and Shennong265, Chinese Japonica variety), 50 ng/μl, 30 μl each. Each rice cultivar DNA was diluted and used to generate the corresponding standard curve.
- Eight blind rice DNA samples from four different rice varieties with different concentrations (0 ng/μl to ~50 ng/μl), 50 μl each.
- Negative DNA target control (labelled N): salmon sperm DNA (20 ng/μl).
- Positive DNA target control (labelled P): (Guangluai4) genomic DNA (20 ng/μl). All the DNA samples were purified using the CTAB method by-GMDL-SJTU. The negative and positive DNA target controls were used for each PCR plate.
- Primers and probes for the SPS gene PCR system (see Table A.6) and further reaction reagents as follows:
 - real-time PCR master mixture (1 ml × 6);
 - DNA dilute solution (0,1× TE, 1,2 ml).

A.2.7 Limit of quantification (LOQ), range of use

According to the developed method, the absolute LOQ of the method is 0,01 ng/μl. The relative LOQ of quantitative PCR has not been assessed in a collaborative trial.

A.2.8 Estimation of measurement uncertainty

The global uncertainty of the method is given by the results of the collaborative trial (see Table A.5).

A.2.9 Interferences

The amount and the ability for amplification of the nucleic acid used as template for the real-time PCR is of major importance for the sensitivity of the method. In addition to this general point, no specific interferences are known for this method.

A.2.10 Physical and environmental conditions

The procedures require experience of working under sterile conditions.

Maintain strictly separated working areas for DNA extraction, PCR set-up and amplification.

Any residual DNA should be removed from equipment prior to its use.

In order to avoid contamination, filter pipette tips (A.2.11.6) protected against aerosol should be used.

Use only powder-free gloves (A.2.11.8) and change them frequently.

Clean laboratory benches and equipment periodically with sodium hypochlorite (10 % active chloride) solution (bleach).

Pipettes should be calibrated regularly, if necessary. 

A.2.11 Apparatus and equipment

Usual laboratory equipment and in particular the following.

- A.2.11.1 **Microcentrifuge.**
- A.2.11.2 **Freezer** maintained at $-20\text{ }^{\circ}\text{C}$ and **refrigerator** maintained at $4\text{ }^{\circ}\text{C}$.
- A.2.11.3 **Micropipettes.**
- A.2.11.4 **Vortex mixer.**
- A.2.11.5 **Tubes**, of capacities 0,2 ml, 1,5 ml, 2,0 ml.
- A.2.11.6 **Tips** and **filter tips** for micropipettes.
- A.2.11.7 **Rack** for reaction tubes.
- A.2.11.8 **Vinyl** or **latex gloves.**
- A.2.11.9 **Vacuum dryer** suitable for drying DNA pellets, optional.
- A.2.11.10 **Real-time PCR system** with plastic reaction vials suitable for fluorescence measurement.
- A.2.11.11 **Software:** Sequence Detection System¹⁾ version 1.7 (Applied Biosystems Part No 4311876¹⁾) or equivalent versions.
- A.2.11.12 **Optical 96 well reaction plates**, MicroAmp^{®1)} (Applied Biosystems Part No N801-0560¹⁾).
- A.2.11.13 **Optical adhesive covers**, MicroAmp^{®1)} (Applied Biosystems Part No 4311971¹⁾).
- A.2.11.14 **Optical caps**, MicroAmp^{®1)} (Applied Biosystems Part No. No 801-0935¹⁾).

A.2.12 Reagents and materials

A.2.12.1 General

Unless otherwise stated, use only reagents that conform to the specifications of ISO 24276 and only sterile distilled or demineralized water or water of equivalent purity.

A.2.12.2 DNA extraction

- A.2.12.2.1 **Cetyltrimethylammonium bromide** (CTAB), ultrapure grade.
- A.2.12.2.2 **Tris-(hydroxymethyl)aminomethane hydrochloride** (tris), molecular biology grade.
- A.2.12.2.3 **Ethylenediaminetetraacetic acid disodium salt** (EDTA), titration 99,9 % mass fraction.
- A.2.12.2.4 **Ethanol**, $\varphi[\text{CH}_3\text{CH}_2\text{OH}]$ at least 96 % volume fraction.
- A.2.12.2.5 **Isopropanol**, $\varphi[\text{CH}_3\text{CH}(\text{OH})\text{CH}_3]$ at least 99,7 % volume fraction.
- A.2.12.2.6 **Chloroform**, $\varphi[\text{CHCl}_3]$ at least 99 % volume fraction.
- A.2.12.2.7 **Sodium chloride**, $w(\text{NaCl})$ at least 99 % mass fraction.
- A.2.12.2.8 **Sodium hydroxide**, anhydrous, $w(\text{NaOH})$ at least 98 % mass fraction.
- A.2.12.2.9 **RNase A solution**, 10 mg/ml.
- A.2.12.2.10 **Proteinase K solution**, 20 mg/ml. 

A.2.12.2.10 Proteinase K solution, 20 mg/ml.

A.2.12.2.11 Dilution buffer: tris (A.2.12.2.2), 10 mmol/l, pH 9,0.

A.2.12.2.12 Hydrochloric acid, $\rho(\text{HCl}) = 370 \text{ g/l}$.

A.2.12.2.13 Herring testes DNA, calf thymus DNA, or Lambda DNA.

A.2.12.3 Quantitative real-time PCR

TaqMan®¹⁾ universal PCR master mix (1×) or suitable equivalent.

A.2.13 Sample collection, transport, preservation and storage

DNA solutions may be stored at 4 °C for a maximum of 1 week, or at -20 °C for long-term storage (A.2.11.2). For quantitative real-time PCR set up, PCR reagents shall be kept thawed at 1 °C to 4 °C on ice.

A.2.14 Preparation of test sample

Ensure that the test sample is representative of the laboratory sample, e.g. by grinding or homogenization. Measures and operational steps to be taken into consideration are described in detail in ISO 21571. For the collaborative study, a total amount of 1 g ground rice noodles was used.

A.2.15 Instrument calibration

Instruments, e.g. thermocyclers and pipettes shall be calibrated, e.g. according to ISO/IEC 17025.^[61]

A.2.16 Analysis steps

A.2.16.1 General

A.2.16.1.1 Preparation of the DNA for standard curve construction

Each of the 50 ng/μl rice DNA samples was diluted to 10 ng/μl, 1 ng/μl, 0,1 ng/μl, 0,01 ng/μl, 0,002 ng/μl using the DNA dilute solution provided by the method developer, and 5 μl of each of the diluted DNA samples was used for real-time PCR for the standard curve construction.

For specific requirements, see ISO 21571.

A.2.16.1.2 PCR reagents

A.2.16.1.3 PCR master mix

See A.2.12.3.

A.2.16.1.4 Primers and probe

See Table A.6.

A.2.16.2 Procedure

A.2.16.2.1 General

The quantitative PCR for rice *SPS* gene is developed for a total volume of 25 μl per reaction mixture.

Thaw, mix gently and centrifuge the quantitative real-time PCR master mix and the DNA samples needed for the run. Keep thawed reagents at 1 °C to 4 °C on ice. ^[A1]

- A1** Distribute 20 µl/tube of the master mixture to 200 µl PCR reaction tubes (A.2.11.5). Add 5 µl of DNA solution samples, rice positive control, negative control, and blank control (H₂O) to the tubes, respectively.

Mix the PCR tubes gently and centrifuge in the microcentrifuge (A.2.11.1) at $1\,000 \times g$ for 10 s.

Place the plate (A.2.11.12) into the instrument.

Run the PCR with quantitative real-time PCR cycling conditions.

A.2.16.2.2 PCR controls

See A.2.6.

A.2.16.2.3 Preparation of standards

Calibration curves are produced by plotting C_t values against the logarithm of the quantity of target DNA, in nanograms. This can be done by use of spreadsheet software [e.g. Microsoft Excel¹⁾], or directly by options available with the sequence detection system software (A.2.11.11).

The mass, in nanograms, measured for the unknown sample DNA is obtained by interpolation from the standard curve.

A.2.16.2.4 Temperature–time programme (PCR)

The PCR assay has been optimized for use in an ABI Prism^{®1)} 7700 sequence detection system and a Rotor Gene 3000A¹⁾ real-time PCR system (A.2.11.10). Other systems may be used. In these cases, thermal cycling conditions may have to be adjusted. The quantitative real-time PCR temperature–time programme is outlined in Table A.7.

Table A.7 — Quantitative real-time PCR temperature–time programme

Step	Stage		T °C	Time s	Acquisition	Cycles
1	Activation of DNA polymerase and denaturation		95	900	No	1×
2	Amplification	Denaturation	95	15	No	40×
3		Annealing and extension	60	45	Measure	

A.2.16.2.5 Accept or reject criteria

A.2.16.2.5.1 Acceptance and performance criteria are described in Reference [60].

Method performance requirements used to evaluate the results from the collaborative study are given in A.2.16.2.5.2 to A.2.16.2.5.4.

A.2.16.2.5.2 The dynamic range of the method should include 1/10 and at least five times the target concentration. Target concentration should be intended as the threshold relevant for legislative requirements.

A.2.16.2.5.3 The coefficient of variation of reproducibility should be below 35 % at the target concentration and over the entire dynamic range. A $C_{V,R} < 50$ % is acceptable for concentrations below 0,2 %.

A.2.16.2.5.4 The trueness should be within ± 25 % of the accepted reference value over the whole dynamic range.

A.2.16.2.6 Identification

The use of a TaqMan^{®1)} probe allows the unambiguous identification of PCR products.

A.2.17 Sample identification

See A.2.6.

A.2.18 Interpretation and calculations of the results

After the quantitative real-time PCR is completed, locate the threshold line in the area where the amplification profiles are parallel (exponential phase of PCR — logarithmic mode) and where there is no cross-effect between repetitions of the same sample. Determine the cycle number at which the threshold line crosses the first amplification curve and set the baseline three cycles before that value. Export all the data for further calculations.

After having defined a threshold value within the logarithmic phase of amplification as described in the previous paragraph, C_t values for each reaction are calculated using the instrument's software. The standard curves are constructed according to the C_t values at the threshold and the decimal logarithms of the original template DNA quantities.

Thereafter, the standard curves are used to estimate the DNA content in the blind sample DNA by interpolation from the standard curves.

A.2.19 Record keeping

Record keeping shall conform to ISO/IEC 17025^[61] requirements.

A.2.20 Reporting

Reporting shall conform to ISO 24276 and ISO/IEC 17025^[61] requirements.

A.2.21 Safety measures

No specific requirements. See ISO 24276.

A.2.22 Pollution prevention and waste disposal

No specific requirements. See ISO 24276.

A.3 Target-taxon-specific method for the detection of components derived from tomato (*Lycopersicon esculentum*)

A.3.1 Principle

The *LAT52* gene encodes a heat-stable, glycosylated, cysteine-rich protein that is necessary for tomato pollen development. The *LAT52* detection system has been demonstrated to be suitable for use as an endogenous reference gene in GM tomato identification and quantification (Reference [62]). The GMO Detection Laboratory of Shanghai Jiao Tong University (GMDL-SJTU) organized the collaborative trial for validation of the method of detection of the tomato *LAT52* gene as tomato endogenous gene for quantitative analysis of genetically modified (GM) or non-GM tomato. The study involved 13 laboratories from the USA, Singapore, Korea, Lithuania, Slovenia, Norway, Italy, and China. The results appear in Reference [63], appendices 1 and 2.

The operational procedure of the collaborative study comprised the following modules:

- Quantitative real-time PCR for the quantitative standard curve construction using tomato DNA samples from four different tomato cultivars.
- Quantitative real-time PCR for the quantification of blind tomato DNA samples using the constructed standard curves. 

A1 The ring trial was carried out in accordance with the following internationally accepted guidelines:

- ISO 5725,[51]–[56] especially considered in relation to the measure of precision (i.e. repeatability and reproducibility) and trueness;
- the IUPAC protocol for the design, conduct and interpretation of method-performance studies (Reference [12]).

A.3.2 Scope

This method specifies the detection of tomato DNA using quantitative PCR.

The method has been optimized for tomato seeds, tomato fruits, tomato ketchup, and tomato juice, containing mixtures of tomato and other matrices, such as maize and soybean. The applicability of the *LAT52* gene was tested through collaborative trial using DNA samples extracted from tomato seeds.

A.3.3 Validation status and performance criteria

A.3.3.1 Robustness of the method

The robustness of the *LAT52* quantitative real-time PCR system was tested by the method developer on three different temperatures-time programmes (i.e. two-step and three-step; three different annealing temperatures, i.e. 58 °C, 59 °C, and 60 °C), on three different DNA samples containing known amounts of tomato DNAs (10 ng, 1 ng, 0,1 ng tomato genome DNA samples, and three repetitions per samples). The quantitative real-time PCR systems showed the expected ruggedness at temperature–time programmes and three concentrations of the tomato DNA samples.

The *LAT52* gene quantitative PCR system has also been tested on different real-time PCR cyclers [Rotor gene 3000A,¹⁾ Corbett Research and ABI7700¹⁾], with three different reaction volumes (20 µl, 25 µl, and 30 µl, and three repetitions per volume). The quantitative real-time PCR systems showed the expected appropriate ruggedness when used with different real-time PCR cyclers and different reaction volumes.

A.3.3.2 Intralaboratory trial

The tomato *LAT52* gene has been validated for use as endogenous reference gene in GM tomato identification and quantification (Reference [62]). The *LAT52* PCR system was also tested by three operators in GMDL-SJTU using the tomato genomic DNA, providing satisfactory results; in particular, in quantitative PCR, the bias was below 25 % over the dynamic range (i.e. 0,05 ng to 1,00 ng).

A.3.3.3 Collaborative trial

The collaborative trial results are summarized and reported in Reference [63]. Briefly, for sample preparation for the collaborative study, all the DNA samples were extracted by the GMDL-SJTU using the CTAB method adopted from ISO 21571:2005, A.3. Spectrophotometric quantification of the amount of total DNA extracted was performed using a method adopted from ISO 21571:2005, B.1. After the DNA quantification, a quantitative real-time PCR run was carried out to provide data about possible PCR inhibition. Then the prepared DNA samples were sent to each participant laboratory.

Standard curves were constructed using the diluted DNA samples from four varieties of tomato seeds by means of quantitative PCR: the PCR efficiency, calculated from the slope of the standard curve as $(10^{-1/a} - 1) \times 100$, where a is the slope, of the *LAT52* gene PCR system was on average 96,6 %, and the linearity (regression coefficient, R_c^2) was on average equal to 0,997.

The collaborative trial results on the eight blind DNA samples are summarized in Table A.8. The method performance requirements, as established by European Network of GMO Laboratories (ENGL) and adopted by European Union Reference Laboratories (EURL) (Reference [60]) are used to evaluate these data. After removal of outliers through the 95 % confidence interval according to ISO 5725-2,[52] the deduced repeatability and reproducibility for each tomato concentration level are reported in Table A.8. **A1**

**Table A.8 — Validation data of the *LAT52* real-time PCR system**

Parameter	Unknown amount of tomato target DNA							
	0,5 ng				0,05 ng			
	Zhongsu5	R144	Zaofeng	Lichun	Zhongsu5	R144	Zaofeng	Lichun
Laboratories having returned results	13	13	13	13	13	13	13	13
Samples per laboratory	4	4	4	4	4	4	4	4
Data excluded	2	5	4	4	5	8	7	10
Reason for exclusion	Data not in the 95 % confidence interval							
Mean value	0,446 8	0,480 6	0,487 5	0,488 6	0,045 9	0,049 0	0,049 8	0,045 7
Repeatability standard deviation	0,120	0,122	0,109	0,133	0,015	0,015	0,014	0,011
Repeatability coefficient of variation, %	26,76	25,41	22,46	27,12	32,06	30,41	29,00	24,73
Reproducibility standard deviation	0,084	0,077	0,073	0,091	0,007	0,009	0,007	0,004
Reproducibility coefficient of variation, %	18,751	15,99	15,04	18,68	15,00	18,61	14,21	8,65
Bias, absolute value	0,053	0,019	0,012	0,011	0,004	0,001	0,000	0,004
Bias, %	10,64	3,88	2,50	2,29	8,26	1,92	0,43	8,68

A.3.3.4 Molecular selectivity

A.3.3.4.1 General

The method targets the *LAT52* gene which is stably present with a single copy per haploid genome of different tomato cultivars. The primers and probe targeting the 92 bp *LAT52* DNA fragment are listed in Table A.9.

Table A.9 — Oligonucleotide primers and probe sequences,

Name	Oligonucleotide DNA Sequence (5' to 3')
Quantitative real time PCR primer and probe sequence	
<i>LAT52</i> primer F	AgACCACgAGAACgATATTTgC
<i>LAT52</i> primer R	TTCTTgCCTTTTCATATCCAgACA
<i>LAT52</i> probe	HEX- CTCTTTgCAGTCCTCCCTTgggCT-BHQ

A.3.3.4.2 Experimental

DNA samples extracted from 11 different plant materials (including tomato) were analysed with the *LAT52* PCR method (Reference [62]). Out of the 11 samples, only tomato DNA gave a positive result. The remaining 10 samples, such as eggplant, potato, petunia, capsicum, maize, soybean, rapeseed, rice, tobacco and *Arabidopsis* gave negative results.

DNA samples extracted from 12 different tomato cultivars with different geographic and phylogenetic origin, e.g. Shengnong2, Jifan4, Zhongsu5, Yashu6, Jiafen1, Shenfeng2, Hongza9, R144, Nongyou30, Dongnong70, Lichun and Zaokui, were analysed by the *LAT52* gene PCR by the developed method. All 12 samples gave positive results.

A.3.3.4.3 Theoretical

The theoretical specificity of the *LAT52* primers and probe was analysed by a similarity search using the BLASTN 2.0MP-WashU program (Reference [64], search date: 2010-01-20). The 92 bp *LAT52* sequence used as query is part of the NCBI accession number X15855 (nucleotides 1385–1476), and the blast analysis result confirmed the complete identity of the query sequence with tomato anther specific *LAT52* gene sequences.

A.3.4 Principle and summary

This methodology is a real-time quantitative TaqMan®¹⁾ PCR procedure for the quantitative detection of the tomato *LAT52* target sequence. PCR products are quantified by the fluorescent signal during each PCR cycle by using a *LAT52* specific oligonucleotide probe labelled with two fluorescent dyes: HEX as a reporter dye at its 5'-end and TAMRA as a quencher dye at its 3'-end. The C_t value is used to calculate the tomato DNA contents, and the C_t value is the threshold cycle with observed fluorescent signal above a threshold value after a certain number of cycles.

For quantification of the copy number of the tomato *LAT52* gene in an unknown sample, the C_t value of the sample is determined. Four calibration standards are then employed to generate a standard curve and to calculate the corresponding copy numbers of unknown DNA samples.

A.3.5 Terms and definitions

For the purposes of this clause, the terms and definitions of ISO 5725-1^[51] and ISO 24276 apply.

A.3.6 Sample type and amounts

Four DNA samples from different tomato varieties, such as R144, Zhongsu5, Zaofeng and Lichun, 50 ng/ μ l, 30 μ l each were diluted to generate the standard curves.

Eight unknown tomato DNA samples from four different tomato varieties with different concentrations (0 ng/ μ l to 50 ng/ μ l) and different GM concentrations were used, 50 μ l each.

A.3.7 Limit of detection (LOD), limit of quantification (LOQ), range of use

The absolute LOQ of the quantitative PCR method is 0,01 ng per reaction, corresponding to about 11 copies of the haploid tomato genomic DNA. The LOD of the quantitative PCR method was not assessed in the collaborative trial.

A.3.8 Estimation of measurement uncertainty

The global uncertainty of the method is given by the results of the collaborative trial (refer to A.3.3.3).

A.3.9 Interferences

The prepared DNA samples should be checked for PCR inhibitors before the PCR analysis to avoid false-negative results.

A.3.10 Physical and environmental conditions

The procedures should be performed under sterile working conditions.

Maintain strictly separated working spaces for DNA extraction, PCR preparation, and amplification.

Any residual DNA should be removed from equipment prior to its use.

To avoid contamination, filter pipette tips (A.3.11.6) should be protected against aerosol.

Only powder-free gloves (A.3.11.8) should be used and changed frequently.

Laboratory benches and equipment should be cleaned periodically with sodium hypochlorite (10 % active chloride) solution (bleach).

Pipettes should be checked for precision and calibrated frequently.

A.3.11 Apparatus and equipment

Usual laboratory equipment and in particular the following. 

A.3.11.1 Microcentrifuge.

A.3.11.2 Freezer maintained at $-20\text{ }^{\circ}\text{C}$ and **refrigerator** maintained at $4\text{ }^{\circ}\text{C}$.

A.3.11.3 Micropipettes.

A.3.11.4 Vortex mixer.

A.3.11.5 Tubes, of capacities 0,2 ml, 1,5 ml, 2,0 ml.

A.3.11.6 Tips and **filter tips** for micropipettes.

A.3.11.7 Rack for reaction tubes.

A.3.11.8 Vinyl or **latex gloves**.

A.3.11.9 Vacuum dryer suitable for drying DNA pellets, optional.

A.3.11.10 Real-time PCR instrument.

A.3.11.11 Real-time PCR reaction tubes or **wells** and **optical caps** or **optical adhesive covers**, as appropriate.

A.3.11.12 Software: Sequence Detection System¹⁾ version 1.7 (Applied Biosystems Part No 4311876¹⁾) or equivalent versions.

A.3.12 Reagents and materials

Unless otherwise stated, use only reagents that conform to the specifications of ISO 24276 and only sterile distilled or demineralized water or water of equivalent purity.

A.3.12.1 Real-time PCR master mixture.

A.3.12.2 Oligonucleotides (primers and probe).

A.3.13 Sample collection, transport, preservation and storage

DNA solutions should be stored at $4\text{ }^{\circ}\text{C}$ for a maximum of one week, or at $-20\text{ }^{\circ}\text{C}$ for long-term storage (A.3.11.2). For quantitative real-time PCR set up, PCR reagents shall be kept thawed at $1\text{ }^{\circ}\text{C}$ to $4\text{ }^{\circ}\text{C}$ on ice.

A.3.14 Test sample preparation

The test sample should be representative of the laboratory sample, e.g. by grinding or homogenizing of the samples. Measures and operational steps to be taken into consideration are described in detail in ISO 21571.

A.3.15 Instrument calibration

Instruments, e.g. thermocyclers and pipettes shall be calibrated as per ISO/IEC 17025.^[61]

A.3.16 Analysis steps

A.3.16.1 General

A.3.16.1.1 Preparation of the DNA for standard curves construction

DNA was extracted, quantified and sent to all participating laboratories as outlined in A.3.3.3.

Each of the 50 ng/ μl tomato DNA sample was diluted to 10 ng/ μl , 1 ng/ μl , 0,1 ng/ μl , 0,01 ng/ μl , 0,002 ng/ μl using the $0,1 \times \text{TE}$, respectively. 

A.3.16.1.2 PCR reagents

A.3.16.1.3 Real-time PCR master mixture

See A.3.12.

A.3.16.1.4 Primers

See Table A.9.

A.3.16.2 Procedure

A.3.16.2.1 General

The PCR set-up for quantitative PCR targeting the tomato *LAT52* gene is developed for a total volume of 25 µl per reaction mixture.

Thaw, mix gently and centrifuge the quantitative real-time PCR master mix and the DNA samples needed for the run. Keep thawed reagents at 1 °C to 4 °C on ice.

Distribute 20 µl per tube of the master mixture to 200 µl PCR reaction tubes (A.3.11.7). Add 5 µl of DNA solution samples, tomato positive control, negative control, and blank control (H₂O) to the tubes, respectively.

Mix the solution in the PCR tubes gently and centrifuge briefly in a microcentrifuge (A.3.11.1).

Place the plate into the instrument.

Run the PCR with quantitative real-time PCR cycling conditions described in A.3.16.2.4.

A.3.16.2.2 PCR controls

Positive and negative target controls should be performed according to ISO 24276.

A.3.16.2.3 Preparation of standards

Calibration curves are generated by plotting C_t values against the logarithm of the target DNA amount, in nanograms. This can be done by use of spreadsheet software [e.g. Microsoft Excel¹⁾], or directly by options available with the sequence detection system software (A.3.11.11).

The mass, in nanograms, measured for the unknown sample DNA is obtained by interpolation from the standard curves.

A.3.16.2.4 Temperature–time programme (PCR)

The PCR assay has been optimized for use in an ABI Prism^{®1)} 7700 sequence detection system and a Rotor Gene 3000A¹⁾ real-time PCR system (A.3.11.10). Other systems may be used. In these cases, thermal cycling conditions may have to be adjusted. For quantitative real-time PCR, the temperature–time programme is given in Table A.10.

Table A.10 — Quantitative real-time PCR temperature–time programme

Step	Stage		T °C	Time s	Acquisition	Cycles
1	Activation of DNA polymerase and denaturation		95	900	No	1×
2	Amplification	Denaturation	95	15	No	40×
3		Annealing and extension	60	45	Measure	

A₁ A.3.16.2.5 Accept or reject criteria

A.3.16.2.5.1 Method performance requirements used to evaluate the results from the collaborative study are given in A.3.16.2.5.2 to A.3.16.2.5.4.

A.3.16.2.5.2 The dynamic range of the method should include 1/10 and at least five times the target concentration. Target concentration should be intended as the threshold relevant for legislative requirements.

A.3.16.2.5.3 The coefficient of variation of reproducibility should be below 35 % at the target concentration and over the entire dynamic range. A $C_{VR} < 50$ % is acceptable for concentrations below 0,2 %.

A.3.16.2.5.4 The trueness should be within ± 25 % of the accepted reference value over the whole dynamic range.

A.3.16.2.6 Identification

The use of TaqMan[®]¹⁾ probe allows the unambiguous identification of PCR products.

A.3.17 Sample identification

See A.3.6.

A.3.18 Interpretation and calculations of the results

After the quantitative real-time PCR run is completed, the threshold line is set in the area where the amplification profiles are parallel (exponential phase of PCR — logarithmic mode) and where there is no “fork effect” between repetitions of the same sample. Determine the cycle number at which the threshold line crosses the first amplification curve and set the baseline three cycles before that value. Export all the data for further calculations.

After having defined a threshold value within the logarithmic phase of amplification as described in the previous paragraph, the instrument software calculates the C_t values for each reaction.

The standard curves are constructed according to the C_t values at the threshold and the decimal logarithms of the original template DNA quantities.

Thereafter, the standard curves are used to calculate the DNA content in the unknown sample DNA by interpolation from the standard curves.

A.3.19 Record keeping

Record keeping shall conform to ISO/IEC 17025^[61] requirements.

A.3.20 Reporting

Reporting shall conform to ISO 24276 and ISO/IEC 17025^[61] requirements.

A.3.21 Safety measures

No specific requirements. Refer to ISO 24276.

A.3.22 Pollution prevention and waste disposal

No specific requirements. Refer to ISO 24276. **A₁**

Annex B (informative)

Screening methods

B.1 Screening method for the relative quantitation of the 35S-promoter DNA of soya bean line GTS 40-3-2 using real-time PCR

B.1.1 Introduction

This annex describes a method for the specific amplification and detection of a taxon-specific soya bean gene (lectin gene, *le1*) and of the 35S-promoter DNA originating from the cauliflower mosaic virus, and for quantitative determination of the amount of 35S-promoter DNA in soya bean ingredients containing genetically modified soya bean line GTS 40-3-2 (Roundup Ready®).

For limitations, see B.1.8.

B.1.2 Validation status and performance characteristics

B.1.2.1 General

The method has been optimized for certified reference materials (CRM IRMM-410)⁷⁾ [18] consisting of dried soya bean powder containing mixtures of GTS 40-3-2 and conventional soya bean.

The reproducibility of the described methods has been tested in collaborative trials using unknown samples (samples labelled as SA to SE, see Table B.1) consisting of mixtures of reference materials of the type mentioned above [19]. In addition, commercially available foodstuffs were tested [20].

The copy numbers of each of the target sequences per genome have not been assessed in detail [21], [22].

The method has been published in Reference [23].

B.1.2.2 Collaborative trial

Five unknown samples containing between 0,7 % and 3 % (mass fraction) of dried soya bean powder derived from the line GTS 40-3-2 were analysed by eleven participants.

The 35S-promoter-specific detection method resulted in a reproducibility relative standard deviation in the range of 17 % to 34 % (see Table B.1). During the initial experiments of the collaborative trial, it was determined that the 1 % soya bean CRM was inconsistent with its designated value. Investigations revealed that the diverging reference materials were manufactured using different procedures at different time points, leading to different degrees of DNA degradation. The collaborative trial participants were advised during the course of the collaborative trial to use the 2 % CRM and to dilute the DNA to get a 1 % reference DNA solution for use in quantitation.

For DNA extraction, the procedure described in Reference [23] was used. In brief, 200 mg of the sample material was lysed in 1 ml of guanidium hydrochloride/proteinase K buffer (0,5 mol/l//0,8 mg/ml) at 56 °C for

7) These are examples of suitable products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

3 h. After a RNase digestion step, 500 µl of the cleared extract was mixed with 1 ml of Wizard® silica resin ⁸⁾ and the resin with bound DNA was recovered by aspiration through a Wizard® filter column ⁸⁾. After isopropanol washing, the DNA was eluted from the silica resin in 10 mmol/l Tris buffer pH 9,0 at 70 °C. The DNA concentration was estimated by OD measurement at 260 nm and adjusted to 20 µg/ml. For subsequent PCR analysis, 200 ng of DNA from each sample was analysed in two independent reactions.

Samples were analysed in duplicate.

Since the samples labelled SA to SE were mixtures of these divergent standards, the results obtained with these samples could not be used for the evaluation of the trueness of the applied real-time PCR method. However the results could be used for assessing the precision of the applied method, whereby the described circumstances reflect a worst-case scenario leading to an underestimation of the precision of the applied real-time PCR method ^[19].

The exclusion of laboratories is based on the real-time PCR apparatus used and on the statistical calculation of outliers. The method was developed for block thermal cyclers. Therefore, two laboratories using a LightCycler® system ⁸⁾ were excluded before the calculation of outliers. The reason for the exclusion of a specific type of PCR apparatus was the observation that the applied method needs careful adaptation and optimization if run on real-time PCR apparatus other than those specified in the method. The retained laboratories were, in addition, examined for outliers according to Grubbs ^[24]. However, no outliers were identified. Details of the collaborative trial are listed in Table B.1.

Table B.1 — Validation data for 35S-Promotor-specific GMO detection

	Sample				
	SA	SB	SC	SD	SE
Year of interlaboratory test	1999	1999	1999	1999	1999
Number of laboratories having returned results	11	11	11	11	11
Number of samples per laboratory	1	1	1	1	1
Number of excluded laboratories	2	2	2	2	2
Number of laboratories retained after exclusion	9	9	9	9	9
Number of accepted samples	9	9	9	9	9
Expected value (% GMO)	1,4	1,8	3	0,7	1
Mean value (% GMO)	1,63	1,76	4,04	0,88	1,73
Median value (% GMO)	1,62	1,70	3,46	1,00	1,60
Reproducibility standard deviation s_R (% GMO)	0,28	0,39	1,36	0,21	0,35
Reproducibility relative standard deviation (%)	17	22	34	24	20
Reproducibility limit R ($R = 2,8 s_R$)	0,80	1,08	3,82	0,58	0,97

In addition, four unknown commercially available food samples containing between 0,3 % and 36 % (mass fraction) (mean values) of genetically modified soya bean line GTS 40-3-2 were analysed by four participants. The 35S-promoter-specific detection method resulted in a reproducibility relative standard deviation in the range of 17 % to 28 % ^[20].

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B.1.2.3 Molecular specificity

B.1.2.3.1 General

The method has been designed to target a sequence described in for example GenBank®⁹⁾ database accession number V00141.

A list of genetically modified plants containing the CaMV 35S promoter is provided in Reference [25].

A false positive result can occur since the amplified sequence is derived from cauliflower mosaic virus infecting cauliflower and other members of the family *Brassicaceae* (*Cruciferae*) as well as *Resedaceae* and *Solanaceae* [26], [27]. Positive results dealing with *Brassicaceae*, *Resedaceae* and *Solanaceae* should therefore be treated carefully. Positive results may indicate the presence of a GM-plant-derived product but should not be interpreted as proof of the presence of GM-plant-derived products without additional confirmation.

In order to distinguish between a viral infection and GM material, methods for the detection of cauliflower mosaic virus may be used [28].

B.1.2.3.2 Theoretical specificities

The theoretical specificities of the primers and probes were assessed through a search of the GenBank/EMBL/DDBJ databases⁹⁾ using the nucleotide sequences as query sequences with the BLASTN programme 2.2.3⁹⁾ [24th April 2002]. The result of the search confirmed a complete identity only with the expected target sequences.

B.1.2.3.3 Experimental determination of specificity

The experimental specificity of the method was assessed by analysis of CRM IRMM-410 from IRMM dried soya bean powder containing 0 % to 5 % of GTS 40-3-2 soya bean line and of reference material from Leatherhead Food Research Association International⁹⁾ full fat soya bean flour (Lot No. 2/99-01) containing 0 %, 0,3 %, 1,25 % and 2 % soya bean GTS 40-3-2 soya bean, respectively.

The commercial food matrices tested were soya bean flour, soya bean protein isolates, composite foodstuff containing soya bean flour and soya bean protein.

B.1.2.4 Optimization

Optimization was carried out on the ABI PRISM® 7700 sequence detection system⁹⁾ (SDS) using TaqMan® chemistry⁹⁾.

Primer and probe design were carried out applying the Primer Express® software (Applied Biosystems)⁹⁾.

B.1.2.5 Limit of detection (LOD)

Since the method is quantitative, the LOD was not assessed specifically. The LOD was expected to be better than or equal to the limit of quantitation; i.e. 50 genome copies of soya bean line GTS 40-3-2 in 82 000 genome copies of conventional soya bean flour.

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B.1.2.6 Limit of quantitation (LOQ)

The LOQ was assessed by measuring serial target DNA dilutions according to Reference [20].

According to the method developer, the LOQ is 50 genome copies of soya bean line GTS 40-3-2 in 82 000 genome copies of conventional soya bean flour. For 1 C value, see Reference [20]. This yields an estimated relative LOQ of 0,06 (= 50 copies/82 000 copies × 100 %).

The concentrations tested in the collaborative trial are listed in Table B.1

NOTE The number of copies was not determined in the collaborative trial.

B.1.3 Adaptation

No specific information is available.

B.1.4 Principle

An 82 bp fragment of the CaMV 35S promoter sequence is amplified by PCR using two 35S-promoter-specific primers. PCR products are measured during each PCR cycle (real-time) by means of a 35S-promoter-specific oligonucleotide probe labelled with two fluorescent dyes: FAM as a reporter dye and TAMRA as a quencher. For that purpose, TaqMan[®] chemistry ¹⁰⁾ is employed.

An 81 bp fragment of the soya bean lectin gene sequence is amplified by PCR in a separate real-time PCR reaction using two soya bean lectin gene specific primers, and the PCR products are measured over each PCR cycle by means of a soya bean lectin gene specific TaqMan[®] probe ¹⁰⁾.

Quantitation is performed using either the $\Delta\Delta C_t$ method or the double calibration curve method (see B.1.9).

B.1.5 Reagents

B.1.5.1 General

For the quality of reagents used see  ISO 24276:2006 , 6.6.

B.1.5.2 Water.

B.1.5.3 PCR buffer (without MgCl₂), 10-fold.

B.1.5.4 MgCl₂ solution, $c(\text{MgCl}_2) = 25 \text{ mmol/l}$.

B.1.5.5 dNTP solution, $c(\text{dNTP}) = 2,5 \text{ mmol/l}$ (each).

B.1.5.6 Oligonucleotides

Details of the oligonucleotides are listed in Table B.2.

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Table B.2 — Oligonucleotides

Name	Oligonucleotide DNA sequence	Final concentration in PCR
Reference gene target sequence		
Lectin-F	5'-TCC ACC CCC ATC CAC ATT T-3'	900 nmol/l
Lectin-R	5'-ggC ATA gAA ggT gAA gTT gAA ggA-3'	900 nmol/l
Lectin-TMP	5'-FAM-AAC Cgg TAg CgT TgC CAg CTT Cg-TAMRA-3' ^a	100 nmol/l
GMO target sequence		
35S-F	5'-gCC TCT gCC gAC AgT ggT-3'	300 nmol/l
35S-R	5'-AAg ACg Tgg TTg gAA CgT CTT C-3'	900 nmol/l
35S-TMP	5'-FAM-CAA AgA Tgg ACC CCC ACC CAC g-TAMRA-3' ^a	100 nmol/l
^a FAM: 6-carboxyfluorescein; TAMRA: 6-carboxytetramethylrhodamine.		

The length of the lectin PCR product is 81 bp; the length of the 35S PCR product is 82 bp.

B.1.5.7 Thermostable DNA polymerase

AmpliTaq Gold[®] DNA polymerase ¹¹⁾.

B.1.5.8 Uracil *N*-glycosylase (optional).

B.1.6 Apparatus

B.1.6.1 General

Standard laboratory apparatus should be used throughout unless otherwise specified.

B.1.6.2 Thermal cycler

The indicated temperature-time profile was originally tested with the ABI PRISM[®] 7700 SDS and GeneAmp[®] 5700 SDS (Applied Biosystems) ¹¹⁾. Other real-time PCR detection systems may be used after adaption of the reaction conditions.

B.1.6.3 Reaction vials

The reaction vials shall be suitable for PCR amplification on a thermal cycler, e.g. ABI PRISM[®] 96-Well Optical Reaction Plate, or MicroAmp[®] Optical Caps (8 caps/strip, flat) (Applied Biosystems) ¹¹⁾.

¹¹⁾ These are examples of suitable products available commercially. This information is only given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.