

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

ICS 67.050

National foreword

This British Standard is the UK implementation of EN ISO 21569:2005+A1:2013. It is identical to ISO 21569:2005, incorporating amendment 1:2013. It supersedes BS EN ISO 21569:2005, which is withdrawn.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.

The UK participation in its preparation was entrusted to Technical Committee AW/275, Food analysis — Horizontal methods.

A list of organizations represented on this committee can be obtained on request to its secretary.

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Foodstuffs - Methods of analysis for the detection of genetically modified organisms and derived products - Qualitative nucleic acid based methods (ISO 21569:2005)

Produits alimentaires - Méthodes d'analyse pour la détection des organismes génétiquement modifiés et des produits dérivés - Méthodes qualitatives basées sur l'utilisation des acides nucléiques (ISO 21569:2005)

Lebensmittel - Verfahren zum Nachweis von gentechnisch modifizierten Organismen und ihren Produkten - Qualitative auf Nukleinsäuren basierende Verfahren (ISO 21569:2005)

This European Standard was approved by CEN on 6 June 2005.

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Foreword

This document (EN ISO 21569:2005) 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of an identical text or by endorsement, at the latest by December 2005, and conflicting national standards shall be withdrawn at the latest by December 2005.

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Foreword to amendment A1

This document (EN ISO 21569:2005/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 21569:2005 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.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. CEN [and/or CENELEC] shall not be held responsible for identifying any or all such patent rights.

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Endorsement notice

The text of ISO 21569:2005/Amd 1:2013 has been approved by CEN as EN ISO 21569:2005/A1:2013 without any modification.

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detection of genetically modified
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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.

ISO 21569 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 a genetically modified origin of ingredients 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 this International Standard and in the following series of International Standards with the general title *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products*:

A1 Text deleted **A1**

- *Quantitative nucleic acid based methods* (ISO 21570);
- *Nucleic acid extraction* (ISO 21571).

Further information about general requirements and definitions involving the steps cited above are collected in ISO 24276.

The qualitative detection of DNA target sequences is performed in order to obtain a yes or no answer to the question whether a certain target sequence is detected or not relative to appropriate controls and within the detection limits of the analytical method used and test portion analysed.

The specificity of the methods, as described in Annexes A to D, ranges from screening methods to detect common DNA sequences characteristic of GMOs, to specific identification of a genetic construct or a specific transformation event.

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 this patent right.

ISO has been informed that Applied Biosystems, Roche Molecular Systems, Inc. and F. Hoffman La Roche Ltd. hold patent rights concerning the 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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ISO 21569:2005+A1:2013 (E)

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights other than those identified above. ISO shall not be held responsible for identifying any or all such patent rights.

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

1 Scope

This International Standard describes the procedure to qualitatively detect genetically modified organisms (GMOs) and derived products by analysing the nucleic acids extracted from the sample under study. The main focus is on polymerase chain reaction (PCR) based amplification methods.

It gives general requirements for the specific detection and identification of target nucleic acid sequences (DNA) and for the confirmation of 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 could also be applied 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.

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

ISO 24276:2006, *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — General requirements and definitions* ^{A1}

3 Terms and definitions

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

4 Principle of the method

4.1 General

Qualitative analysis consists of specific detection of target nucleic acid sequences in the test samples. Each method shall specify the target sequence.

A1 A qualitative result shall clearly demonstrate the presence or absence of the genetic element under study, relative to appropriate controls.

NOTE Detection limits and size of the test portion are critical aspects of a method. **A1**

4.2 PCR amplification

Amplification of the target sequence occurs *in vitro* through a reaction catalysed by a DNA polymerase in the presence of oligonucleotide primers and deoxyribonucleoside triphosphates in a defined reaction buffer^{[1], [2]}. An important prerequisite for the amplification of the target sequence is that the reaction mixture contains no polymerase inhibitors. Amplification of the DNA is a cyclical process consisting of

- denaturation of the double-stranded DNA into single-stranded nucleic acid by means of heating,
- annealing of the primers to the target sequence at a suitable temperature, and
- extension of the primers, which are bound to both single strands, by a DNA polymerase suitable for PCR, at an appropriate temperature.

4.3 Detection and confirmation of PCR products

PCR products are detected by gel electrophoresis or an appropriate alternative, if necessary, after isolation by means of a suitable separation procedure.

The identity of any detected target sequence can be verified by an appropriate technique (e.g. by restriction enzyme analysis, by hybridization or by DNA sequence analysis).

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

5 Reagents

It is generally advisable to store the reaction solutions required for the analytical method at approximately –20 °C if not specified otherwise.

It may also be appropriate to aliquot the reaction solutions required for the analytical method in order to avoid subjecting them to repeated freeze-thaw cycles, and/or to reduce chances of cross contamination.

5.1 Target DNA/control

5.2 Water

5.3 Deoxyribonucleoside triphosphate (dNTP) solution, containing dATP, dCTP, dGTP, and dTTP or dUTP.

NOTE The use of dUTP can interfere with restriction enzyme analyses of PCR products.

5.4 PCR buffer solution

The PCR buffer solution is usually delivered with the DNA polymerase, which may or may not include MgCl_2 in a concentration specified by the manufacturer. The final MgCl_2 concentrations are method specific and are therefore listed in each annex. Ready-to-use reagents may be commercially available. The manufacturer's instructions for use should be considered.

5.5 Thermostable DNA polymerase

5.6 Forward primer

5.7 Reverse primer

6 Apparatus and equipment

See ISO 24276 and Annexes A to D for details.

7 Procedure

7.1 Quality, integrity and amplifiability of nucleic acid extracts

The nucleic acid solution shall be pure enough for subsequent analysis^[3]. The quality and amount of nucleic acid extracted using a given method on a given matrix shall be both repeatable and reproducible.

NOTE The quality, integrity and amount of the DNA template influences the outcome of the PCR, and hence the analytical results obtained. The limit of detection of a specific method may therefore depend on whether the material to be analysed has been processed or refined, and on the degree of degradation of the DNA therein.

Nucleic acids for use in PCR should be substantially free of PCR inhibitors^[4]. PCR inhibition controls shall be included as described in ISO 24276.

7.2 Performance criteria

General performance criteria are described in ISO 24276.

The values for the performance characteristics are given for each method as outlined in Annexes A to D and should take into account the genome sizes; see Reference [5].

The reaction conditions, especially the MgCl_2 concentration and the thermocycling conditions should be optimized for every primer pair and/or system. When any primer system is used for the first time, it is necessary to demonstrate beforehand that the cycle conditions chosen for the particular matrix to be studied avoid undesirable competitive products that would otherwise reduce the sensitivity of the PCR detection.

In an optimal reaction, less than 40 cycles are required to amplify ≥ 10 target molecules to produce a product that is readily detectable by standard methods. As the cycle number increases, non-specific products could accumulate. The optimized PCR should be able to amplify in 40 PCR cycles from a pure reference sample of 100 copies of template DNA enough copies of the PCR product to be detectable. The characteristic temperature/time profile for each primer system and the reaction mixture appropriate for the apparatus used and the number of cycles shall be strictly adhered to.

In general, the specificity of the reaction should be enhanced as much as possible (e.g. by using hot-start PCR). Hot-start PCR is recommended as a means of reducing side reactions such as the amplification of non-target sequences in background DNA (mispriming) and primer-oligomerization (it thus increases specificity).

The values derived from the validation study may not be applicable to analyte concentration ranges and matrices other than given in the respective annexes.

7.3 Aspects of PCR design

7.3.1 General

Because the performance of each specific PCR should be comparable with other specific PCRs, the following aspects of PCR design shall be taken into account.

7.3.2 Size of PCR products

The size of the target sequence shall be selected to match the range of molecular mass available in the nucleic acid extract being analysed.

EXAMPLE For highly degraded DNA from processed foodstuffs, the size of the PCR product should ideally be in the range of 60 bp to 150 bp. For raw materials, a broader range of PCR products up to, for example, 250 bp is applicable.

However, if prior experimental studies are carried out to determine the validity of primer sets yielding different sized PCR products, these may be used on the matrix for which they have been validated.

7.3.3 Primers

7.3.3.1 General

Primer sequence information is included in Annexes A to D.

7.3.3.2 Primer design

The primer sequences should preferably have the following characteristics wherever practicable:

- length of each primer: 18 to 30 nucleotides;
- optimal annealing temperature $\approx 60\text{ }^{\circ}\text{C}$ (should be established experimentally), i.e. estimated melting temperature $\leq 65\text{ }^{\circ}\text{C}$;
- GC:AT ratio = 50:50 if possible, or else as close to this ratio as possible;
- high internal stability (avoid concentration of Gs and Cs in short segments of primers);
- minimal 3' end complementarity to avoid primer-dimer formation;
- minimal secondary structure;
- minimal dimer formation with specific detection probe(s) designed for the PCR.

Software packages are available to help with primer design.

7.3.3.3 Validation of primers

7.3.3.3.1 General

The ability of the primers to detect the target sequence shall be validated.

Primer validation should be carried out in two steps: a first theoretical evaluation, and a second experimental evaluation.

7.3.3.3.2 Theoretical evaluation of the specificity

Theoretical evaluation shall as a minimum be carried out by performing a sequence similarity search (e.g. FastA, Blast^{®2)}) against one of the major nucleic acid sequence databases (e.g. EMBL, GenBank^{®2)}). Homologous gene sequences may be retrieved from the sequence databases and aligned to obtain an estimate of the chance of finding similar sequences in the target taxon or other organisms.

7.3.3.3.3 Experimental evaluation of the specificity

Irrespective of the design criteria used, the specificity of primers shall always be experimentally evaluated to confirm the primers' ability to discriminate between the target and closely related non-target sequences.

Primers designed to detect taxon-specific target sequences should be shown to detect these sequences reliably in $\langle A_1 \rangle$ an appropriate $\langle A_1 \rangle$ number of different members of the taxon.

7.4 PCR target descriptions

For the qualitative detection and identification of GMOs, various PCR tests may be performed, depending on the type of matrices under study and/or the requirements of the analysis. These analyses may be directed at sequences specific for target taxa, genetic constructs and transformation events, as well as elements suitable for screening purposes.

7.5 Controls

Because of the risk of obtaining false positive and/or false negative results, appropriate controls shall be included in each diagnostic PCR assay (see ISO 24276).

If available and appropriate, certified reference materials should be used as positive and negative controls.

7.6 PCR set-up, detection and confirmation of PCR products

Annexes A to D give details on the specific PCR procedure steps.

NOTE In the case of detection of the PCR products by gel electrophoresis, the size of the PCR products can be estimated using a suitable DNA size marker of known length to run in parallel with the PCR products under test.

It may be desirable in some cases to confirm a positive or negative result for a particular genetic modification. This may be achieved by employing primers to an alternative target sequence; this is particularly suitable for confirmation of screening test results.

A positive identification of the specific target DNA sequence may be confirmed by an appropriate method other than size determination of the PCR product, for example

- by hybridization of the PCR product with specific probes, or
- by carrying out restriction analyses of the PCR product; the length of the resulting fragments has to correspond to the expected length of the target DNA sequence after restriction, or
- by sequencing of the PCR product, or
- other equivalent confirmation.

2) Blast and GenBank 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 these products. Equivalent products may be used if they can be shown to give the same results.

If the primers used are designed to detect sequences derived from infectious organisms (a naturally occurring non-genetically modified organism such as a virus or a bacterium), then it is highly recommended that it be verified that the detected DNA is indeed derived from a GMO. This can be done by checking for the absence of other DNA derived from the infectious organism.

EXAMPLE The 35S promoter is derived from cauliflower mosaic virus (CaMV), and consequently detection of the CaMV 35S promoter could be due to the presence of either GMO-derived and/or CaMV-derived DNA [6]. By checking for presence of the other CaMV-derived DNA, it may be possible to confirm the GMO origin of the CaMV 35S promoter if no other CaMV-derived DNA is detected.

8 Interpretation

8.1 General

The PCR result will be either

- a) positive if a specific PCR product has been detected, and all the controls give results as specified in  ISO 24276:2006,  Table 2, or
- b) negative if a specific PCR product has not been detected and all controls give results as specified in  ISO 24276:2006,  Table 2.

NOTE Event-specific target sequences are sometimes present together with other event-specific sequences in a single GMO (e.g. due to gene stacking [7]).

If the results are ambiguous, the procedure shall be repeated; see ISO 24276.

8.2 Verification

Verification of positive or negative results for target sequences may be achieved as described in 7.6.

9 Expression of results and quality assurance

9.1 General

The results shall be expressed unambiguously, i.e. not as “±”.

A negative result shall never be expressed as “GMO not present”.

Ideally, the limit of detection (LOD) should be provided with reference to the test sample. However, this requires particular materials, DNA of exceptionally high quality, and/or use of sophisticated laboratory equipment that is not available to all laboratories. Consequently, the analysis can become very labour intensive and/or expensive, and therefore not applicable in practice for routine purposes.

As a minimum, the LOD shall be provided with reference to a reference material, and a relative value based on a specified matrix (preferably a given amount of genomic DNA solution, e.g. 100 ng of 0,01 % GTS 40-3-2 DNA).

9.2 Expression of a negative result

The following text shall appear in the test report:

“For sample X, 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 the LOD to be applicable, then the following sentence shall be added:

“However, the amount of the target DNA extracted from species X may be/was insufficient for the LOD to be applicable for this sample.”

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) [7].

9.3 Expression of a positive result

The following text shall appear in the test report:

“For sample X, target sequence Y was detected.”

The identity of the GMO may be included, if available.

9.4 Quality assurance requirements

A1 Results within the same test portion shall be consistent. In case of +/- results for the two replicates, repeat the two PCR for the respective test portion. If the two novel replicates are tested +/- or -/-, the test portion is considered as negative.

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 (LOD). **A1**

10 Test report

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

- the limit of detection, and the matrix used to identify the limit of detection;
- **A1** the specificity of the analytical method (event specific, construct specific, or screening method); **A1**
- the result expressed according to Clause 9.

Annex A (informative)

Target-taxon-specific methods

A.1 Target-taxon-specific method for the detection of components derived from soya beans

A.1.1 General

This is a routine procedure for the detection of a species-specific, single copy gene occurring in soya beans (*Glycine max*).

This method may be used to assess the amplifiability of DNA from products derived from soya beans.

A.1.2 Validation status and performance criteria

A.1.2.1 Collaborative study

This method has been validated in collaborative studies [8], [9] organized by the working group “Development of methods for identifying foodstuffs produced by using genetic engineering techniques” of the German Federal Institute for Health Protection of Consumers and Veterinary Medicine (BgVV) according to Article 35 of the German Federal Foodstuffs Act. For DNA extraction, the CTAB method as outlined in ISO 21571:2005, A.3, was used (but with a test portion of 100 mg).

The data from the collaborative studies are listed in Table A.1.

Table A.1 — Results of the collaborative studies

Year of collaborative study	1997 [8]	1998/1999 [9]
Number of laboratories	25	27
Number of laboratories submitting results	22	20
Number of samples per laboratory	10	3
Number of accepted results	220	60
Number of samples containing soya beans	220	50
False positive results	0	1 (2 %)
False negative results	0	1 (2 %)

A.1.2.2 Molecular specificity

A.1.2.2.1 General

This annex fulfils the requirements as outlined in Clause 7.

The method has been designed for a target sequence described in GenBank[®]³⁾ accession No. K00821 = M30884.

A.1.2.2.2 Theoretical

The soya bean lectin gene *Le1* ^[10] obtained from gene databases was chosen as a target sequence.

No sequence similarity with DNA sequences of other crop plants (legumes, cereals, vegetables) has been found (NCBI BlastN[®]²⁾ search, European Molecular Biology Laboratory (EMBL) database, September 28th, 2001). However, GM03 matched 100 % the sequences in the following database accessions: AX033509 (sequence 17 from patent DE19906169), AX033507 (sequence 15 from patent DE19906169) and AX033501 (sequence 9 from patent DE19906169), while GM04 matched only accession No. AX033509 (sequence 17 from patent DE19906169). Note that the accession No. M30884 is the same as K00821, a GenBank[®] entry originally submitted in 1993.

The number of target sequence copies was not determined, but was presumed to be a single copy gene.

A.1.2.2.3 Experimental

No amplification has been observed using DNA from other crop plants (legumes, cereals, vegetables) or from beef and pork. The soya bean PCR assay appears to be highly specific for soya bean DNA ^[10], ^[11].

A.1.2.3 Limit of detection (LOD)

The absolute LOD has not been determined, but the method has been demonstrated to detect at least 0,1 ng of soya bean DNA, determined fluorometrically.

A.1.3 Adaptation

No specific information is available.

A.1.4 Principle

A 118 bp fragment from the soya bean lectin gene is amplified by PCR and separated by agarose gel electrophoresis.

A.1.5 Reagents

For the quality of the reagents used, see ISO 24276.

A.1.5.1 Water

A.1.5.2 PCR buffer, (without MgCl₂), 10× ⁴⁾.

A.1.5.3 MgCl₂ solution, *c*(MgCl₂) = 25 mmol/l.

A.1.5.4 dNTP solution, *c*(dNTP) = 2,5 mmol/l (each).

A.1.5.5 Oligonucleotides

3) GenBank and BlastN 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 this product. Equivalent products may be used if they can be shown to give the same results.

4) 10× means 10-fold; i.e. a PCR buffer containing 1,5 mol/l Tris-HCl, pH 8,3.

A.1.5.5.1 Forward primer

Soya bean lectin gene (GenBank® accession No. K00821).

Primer GM03: 5'-gCC CTC TAC TCC ACC CCC ATC C-3'.

A.1.5.5.2 Reverse primer

Soya bean lectin gene (GenBank® accession No. K00821).

Primer GM04: 5'-gCC CAT CTg CAA gCC TTT TTg Tg-3'.

A.1.5.6 Thermostable DNA polymerase (for hot-start PCR), 5 IU/μl.

A.1.5.7 Hybridization probe (GM)

5'-ggT AgC gTT gCC AgC TTC g-3'.

A.1.5.8 Saline sodium citrate buffer (SSC) 5×, pH 7,0.

A five-fold SSC is a solution containing 0,75 mol/l of NaCl and 0,075 mol/l of sodium citrate.

A.1.5.9 Prehybridization solution

Containing 5× SSC, 0,1 % (mass concentration) of *N*-lauroylsarcosine, 0,02 % (mass concentration) of sodium dodecyl sulfate (SDS) and 1 % Blocking Reagent⁵⁾ or 5 % (mass concentration) non-fat dried milk powder [12].

A.1.5.10 Hybridization solution

Containing 10 pmol of hybridization probe in 2,5 ml of prehybridization solution (A.1.5.9). The hybridization temperature is 50 °C. Further information on conditions for hybridization is given in Reference [12].

A.1.6 Apparatus

A.1.6.1 Thermal cycler

A.1.6.2 Electrophoresis chamber, with power supply.

A.1.7 Procedure

A.1.7.1 PCR set-up

The method is described for a total PCR volume of 25 μl per reaction with the reagents as listed in Table A.2. The PCR can also be carried out in a larger volume if the solutions are adjusted appropriately. The final concentrations of reagents as outlined in Table A.2 have proven to be suitable.

5) Blocking Reagent is an example of a suitable product available commercially from Boehringer, Mannheim. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to give the same results.

Table A.2 — Addition of reagents

Reagent	Final concentration	Volume per sample (µl)
Sample DNA	10 ng to 50 ng	1
Water		15,9
10 × PCR buffer (without MgCl ₂)	1 ×	2,5
MgCl ₂ solution ^a , 25 mmol/l	1,5 mmol/l	1,5
dNTP solution, 10 mmol/l	0,8 mmol/l	2
Primer GM03, 5 µmol/l	0,2 µmol/l	1
Primer GM04, 5 µmol/l	0,2 µmol/l	1
Taq DNA polymerase, 5 IU/µl	0,5 IU	0,1
^a If the PCR buffer solution already contains MgCl ₂ , the final concentration of MgCl ₂ in the reaction mixture is adjusted to 1,5 mmol/l.		

A.1.7.2 PCR controls

As a positive control, certified reference materials of GTS 40-3-2 produced by the Institute for Reference Materials and Measurements (IRMM) Geel, Belgium (IRMM-410) may be used.

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.3 has been used for the validation study using thermal cyclers GeneAmp® 2400 or GeneAmp® 9600 and AmpliTaq Gold® DNA polymerase⁶⁾. The use of other thermal cyclers might make an adaptation necessary. The time for activation/initial denaturation depends on the polymerase used. If using a hot-start polymerase, the recommendation of the manufacturer should be adhered to unless the protocol states otherwise.

Table A.3 — Temperature-time programme

Activation/initial denaturation	10 min/95 °C
Amplification	30 s/95 °C
	30 s/60 °C
	60 s/72 °C
Number of cycles	35
Final extension	3 min/72 °C

A.1.8 Identification

Because this method is only to be assessed as a control method for the determination of the quality of extracted DNA, the identification is based only on PCR product size.

6) GeneAmp® 2400 and 9600 and AmpliTaq Gold® DNA polymerase are examples of suitable products available commercially from Applied Biosystems, previously Perkin Elmer/Applied Biosystems. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of these products. Equivalent products may be used if they can be shown to give the same results.

If using the method for other purposes than mentioned above, the identity of the amplified product may be determined by Southern hybridization using a digoxigenine-labelled oligonucleotide probe GM (A.1.5.7 to A.1.5.10) or by sequencing of the PCR product and comparison with the GenBank® entries given in A.1.5.5.

A.1.9 General quality assurance and interpretation of the results

The target sequence is presumed to have been detected if the size of the PCR product corresponds to the expected length of the target DNA sequence, determined by comparison with products derived from certified reference materials prepared from GTS 40-3-2 (e.g. IRMM-410 series from IRMM, Geel, Belgium).

For identification purposes see A.1.8.

The detection of fragments with a size of 118 bp indicates that the sample DNA solution contains amplifiable DNA of soya bean origin within the assessed limitations of specificity described in A.1.2.2.

For details on electrophoretic stages, see ISO 21571:2005, B.2.

A.2 Target-taxon-specific method for the detection of multicopy DNA sequences generally present in plant chloroplasts

A.2.1 General

This is a routine procedure for the detection of multicopy DNA sequences generally present in plant chloroplasts (the chloroplast trnL intron).

This method is suitable to check if the DNA extraction of a food sample was successful and to check if the sample contains amplifiable plant DNA. In the case of processed material, the applicability of the method depends on the degree of degradation of the DNA.

A plant cell normally contains multiple copies of this DNA sequence and the size of the target DNA sequence is substantially larger than DNA sequences used for detection of specific genetic modifications. Therefore this method may not be used as a control for quantification purposes.

The number of copies per cell may vary between plant species and tissues.

A.2.2 Validation status and performance criteria

A.2.2.1 Collaborative study

The method was validated in a collaborative study ^[13] organized by the working group “Development of methods for identifying foodstuffs produced by means of genetic engineering techniques” of the German Federal Institute for Health Protection of Consumers and Veterinary Medicine (BgVV) according to Article 35 of the German Federal Foodstuffs Act. For DNA extraction, the CTAB method as outlined in ISO 21571:2005, A.3, was used (but with a test portion of 100 mg).

The data of the collaborative study are listed in Table A.4.

Table A.4 — Results of the collaborative study

Year	1995
Number of laboratories	18
Number of laboratories submitting results	18
Number of samples per laboratory	10
Number of total samples	180
Number of accepted results	180
Number of samples containing B33-INV-potato	71
Number of samples containing non-GMO potato	109
False positive results	0 (0 %)
False negative results	0 (0 %)

A.2.2.2 Molecular specificity

A.2.2.2.1 General

This annex fulfils the requirements as outlined in Clause 7.

The method has been designed to target sequences described in Reference [14], e.g. GenBank® accession No. Z00044, S54304, X15901.

A.2.2.2.2 Theoretical

No significant sequence similarity with DNA sequences of non-plant organisms has been found in databank searches (NCBI BlastN® search, EMBL database, September 28th, 2001).

The primers were designed to amplify a sequence unique to chloroplast DNA (the intron disrupting the *trnL* gene), which shows no known similarity with non-target sequences.

A.2.2.2.3 Experimental

No amplification has been observed using DNA from animals, fungi or bacteria [14].

Amplification has been demonstrated using DNA from algae, cyanobacteria, bryophytes, pteridophytes, gymnosperms and angiosperms [14].

The number of target sequence copies is multiple, depending on the plant species and tissue type.

A.2.2.3 Limit of detection (LOD)

The absolute LOD has not been determined, but the method has been demonstrated to detect at least 0,1 ng of soya bean DNA, determined fluorometrically.

A.2.3 Adaptation

No specific information is available.

A.2.4 Principle

A 500 bp to 600 bp DNA fragment, occurring in the tRNA gene of chloroplasts ^[14], is amplified by PCR and separated by agarose gel electrophoresis.

A.2.5 Reagents

For the quality of the reagents used, see ISO 24276.

A.2.5.1 Water

A.2.5.2 PCR buffer (without MgCl₂), 10×

A.2.5.3 MgCl₂ solution, *c*(MgCl₂) = 25 mmol/l.

A.2.5.4 dNTP solution, *c*(dNTP) = 2,5 mmol/l (each).

A.2.5.5 Oligonucleotides

A.2.5.5.1 Forward primer

Chloroplast tRNA gene (GenBank[®] accession No. Z00044, X15901).

Primer c ^[14]: 5'–CgA AAT Cgg TAg ACg CTA Cg-3'.

A.2.5.5.2 Reverse primer

Chloroplast tRNA gene (GenBank[®] accession No. Z00044, X15901).

Primer d ^[14]: 5'–ggg gAT AgA ggg ACT TgA AC-3'.

A.2.5.6 Thermostable DNA polymerase, 5 IU/μl.

A.2.6 Apparatus and equipment

As specified in A.1.6.

A.2.7 Procedure

A.2.7.1 PCR set-up

The method is described for a total PCR volume of 25 μl per reaction mixture with the reagents as listed in Table A.5. The PCR can also be carried out in a larger volume if the solutions are adjusted appropriately. The final concentrations of reagents as outlined in Table A.5 have proven to be suitable.

Table A.5 — Addition of reagents

Reagent	Final concentration	Volume per sample (µl)
Sample-DNA	10 ng to 50 ng	1
Water		13,9
10 × PCR buffer (without MgCl ₂)	1 ×	2,5
MgCl ₂ solution ^a , 25 mmol/l	1,5 mmol/l	1,5
dNTP solution, 10 mmol/l	0,8 mmol/l	2
Primer c, 10 µmol/l	0,8 µmol/l	2
Primer d, 10 µmol/l	0,8 µmol/l	2
Taq DNA polymerase, 5 IU/µl	0,5 IU	0,1
^a If the PCR buffer solution already contains MgCl ₂ , the final concentration of MgCl ₂ in the reaction mixture is adjusted to 1,5 mmol/l.		

A.2.7.2 PCR controls

As a positive control, certified reference materials of GTS 40-3-2 produced by the Institute for Reference Materials and Measurements (IRMM) Geel, Belgium (IRMM-410) may be used.

Any other appropriate controls should be included as described in ISO 24276.

A.2.7.3 Temperature-time programme

The temperature-time programme as outlined in Table A.6 has been used for the validation study using thermal cyclers GeneAmp® 2400 or GeneAmp® 9600 and AmpliTaq® DNA polymerase⁷⁾. The use of other thermal cyclers might make an adaptation necessary. The time for activation/initial denaturation depends upon the polymerase used. If using a hot-start polymerase, the recommendation of the manufacturer should be adhered to unless the protocol states otherwise.

Table A.6 — Temperature-time programme

Activation/initial denaturation	4 min/94 °C
Amplification	30 s/95 °C 30 s/55 °C 120 s/72 °C
Number of cycles	35
Final extension	5 min/72 °C

A.2.8 Identification

Because this method is only to be assessed as a control method for the determination of the quality of extracted DNA, the exact fragment size is not relevant for this method. As yet, identification is based only on PCR product size within the expected range of 500 bp to 600 bp.

7) GeneAmp® 2400 and 9600 and AmpliTaq® DNA polymerase are examples of suitable products available commercially from Applied Biosystems, previously Perkin Elmer/Applied Biosystems. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of these products. Equivalent products may be used if they can be shown to give the same results.

A.2.9 General quality assurance and interpretation of the results

The target sequence is presumed to have been detected if the size of the PCR product corresponds to the expected length of the target DNA sequence.

For identification purposes see A.2.8.

The detection of fragments with a size of 500 bp to 600 bp indicates that the sample DNA solution contains amplifiable DNA of plant origin within the assessed limitations of specificity described in A.2.2.2.

For details on electrophoretic stages, see ISO 21571:2005, B.2.

A.3 Target-taxon-specific and GMO screening method for the detection of DNA derived from tomato and/or Zeneca® genetically modified tomato

A.3.1 General

This is a routine procedure for the detection of a species-specific single copy DNA sequence occurring in tomato (*Lycopersicon esculentum* Mill).

The method may also be used as a screening method for the detection of ripening-delayed genetically modified tomatoes (Zeneca; *Lycopersicon esculentum* Mill cultivar Ailsa Craig strain Nema 282F).

No tool to verify the identity of the PCR product has been described. Therefore this method cannot be considered as an identification method. It may be used to assess the amplifiability of DNA extracted from tomato.

A.3.2 Validation status and performance criteria

A.3.2.1 Collaborative study

This method was validated in a collaborative study ^[15] organized by the working group “Development of methods for identifying foodstuffs produced by using genetic engineering techniques” of the German Federal Institute for Health Protection of Consumers and Veterinary Medicine (BgVV) according to Article 35 of the German Federal Foodstuffs Act. For DNA extraction, the CTAB method as outlined in ISO 21571:2005, A.3, was used.

The data of the collaborative study are listed in Table A.7.

Table A.7 — Results of first collaborative study

Year	1998
Number of laboratories	19
Number of laboratories submitting results	18
Number of samples per laboratory	5
Number of accepted results	90
Number of samples containing <i>Lycopersicon esculentum</i> Mill cv. Ailsa Craig strain Nema 282F (Zeneca)	43
Number of samples containing <i>Lycopersicon esculentum</i> Mill cv. Ailsa Craig strain Nema 282C (non-genetically modified)	47
False positive results	0 (0 %)
False negative results	0 (0 %)

Another collaborative study conforming to the criteria specified in ISO 5725-2 was carried out by the German Federal Institute for Health Protection of Consumers and Veterinary Medicine (BgVV) within a European Project SMT4-CT96-2072. For DNA extraction, the CTAB method as outlined in ISO 21571:2005, A.3, was used (but with a test portion of 100 mg).

Table A.8 — Results of second collaborative study

Year	1998
Number of laboratories	21
Number of laboratories submitting results	19
Number of samples per laboratory	10
Number of accepted results	190
Number of samples containing <i>Lycopersicon esculentum</i> Mill cv. Ailsa Craig strain Nema 282F (Zeneca)	88
Number of samples containing <i>Lycopersicon esculentum</i> Mill cv. Ailsa Craig strain Nema 282C (non-genetically modified)	102
False positive results	0 (0 %)
False negative results	0 (0 %)

A.3.2.2 Molecular specificity

A.3.2.2.1 General

This annex fulfils the requirements as outlined in Clause 7.

The method has been designed to target sequences described in, for example, GenBank® accession No. X04583.

A.3.2.2.2 Theoretical

No sequence homology with DNA sequences other than plant species has been observed in database searches (NCBI BlastN® search, EMBL database, September 28th, 2001). Both primers match 100 % the following database accessions: X14074 (Tomato gene for cell wall degrading polygalacturonase), X05656 (Tomato mRNA for polygalacturonase), M37304 (Tomato polygalacturonase (PG) gene), X04583 (Tomato mRNA for polygalacturonase-2a), A24194 (*L. esculentum* polygalacturonase clone), A15981 (*L. esculentum* mRNA for polygalacturonase-2a), I01809 (Nucleotide sequence 1 from patent US4801540), and AX062336 (sequence 1 from patent WO0078982).

A.3.2.2.3 Experimental

No amplification has been observed using DNA from other crop plants [16].

The number of target sequence copies was not determined, but was presumed to be a single copy gene.

A.3.2.3 Limit of detection (LOD)

The absolute LOD has not been determined, but the method has been demonstrated to amplify a DNA fragment from at least 0,1 ng DNA (determined fluorometrically), extracted from fresh tomato.

A.3.3 Adaptation

Using highly processed samples could give a negative result due to the potential absence of target fragments as large as 383 bp in length.

The detection of fragments with a size of 383 bp indicates that the sample DNA solution contains amplifiable DNA of tomato origin while fragments of 180 bp indicate that the sample DNA solution contains amplifiable DNA of genetically modified tomato origin (Zeneca; *Lycopersicon esculentum* Mill cv. Ailsa Craig strain Nema 282F).

A.3.4 Principle

The polygalacturonase gene (PG gene) codes for a PG-enzyme that is associated with ripening. This method amplifies the endogenous PG gene ^[17] with a fragment size of 383 bp. In Zeneca genetically modified tomato, a second fragment of 180 bp will be amplified resulting from the transferred truncated cDNA of the PG gene ^{[18], [19]}.

A.3.5 Reagents

For the quality of the reagents used, see ISO 24276.

A.3.5.1 Water

A.3.5.2 PCR buffer (without MgCl₂), 10×

A.3.5.3 MgCl₂ solution, *c*(MgCl₂) = 25 mmol/l.

A.3.5.4 dNTP solution, *c*(dNTP) = 2,5 mmol/l (each).

A.3.5.5 Oligonucleotides

A.3.5.5.1 Forward primer

PG gene (GenBank® accession No. X04583).

Primer PG34L: 5'-ggA TCC TTA gAA gCA TCT AgT-3'.

A.3.5.5.2 Reverse primer

PG gene (GenBank® accession No. X04583).

Primer PG34R: 5'-CgT Tgg TgC ATC CCT gCA Tgg-3'.

A.3.5.6 Thermostable DNA polymerase (for hot-start PCR), 5 IU/μl.

A.3.6 Apparatus

As specified in A.1.6.

A.3.7 Procedure

A.3.7.1 PCR set-up

The method is described for a total PCR volume of 25 μl per reaction with the reagents as listed in Table A.9. The PCR may also be carried out in a larger volume if the solutions are adjusted appropriately. The final concentrations of reagents as outlined in Table A.9 have proven to be suitable.

Table A.9 — Addition of reagents

Reagent	Final concentration	Volume per sample (µl)
Sample-DNA	10 ng to 50 ng	1
Water		16,8
10 × PCR buffer (without MgCl ₂)	1 ×	2,5
MgCl ₂ solution ^a , 25 mmol/l	1,5 mmol/l	1,5
dNTP solution, 10 mmol/l	0,4 mmol/l	1
Primer PG34L 10 µmol/l	0,4 µmol/l	1
Primer PG34R 10 µmol/l	0,4 µmol/l	1
Taq DNA polymerase, 5 IU/µl	1 IU	0,2
^a If the PCR buffer solution already contains MgCl ₂ , the final concentration of MgCl ₂ in the reaction mixture is adjusted to 1,5 mmol/l.		

A.3.7.2 PCR controls

As a positive control for the target-taxon-specific use of the method, DNA from fresh tomato may be used. However, no positive control for the truncated gene version present in the genetically modified tomato is commercially available⁸⁾.

Any other appropriate controls should be included as described in ISO 24276.

A.3.7.3 Temperature-time programme

The temperature-time programme as outlined in Table A.10 has been used for the validation in a collaborative study using thermal cyclers GeneAmp® 2400 or GeneAmp® 9600 and AmpliTaq Gold® DNA polymerase⁹⁾. The use of other thermal cyclers might make an adaptation necessary. The time for activation/initial denaturation depends on the polymerase used. If using a hot-start polymerase, the recommendation of the manufacturer should be adhered to unless the protocol states otherwise.

Table A.10 — Temperature-time programme

Activation/initial denaturation	10 min/95 °C
Amplification	30 s/94 °C 60 s/60 °C 60 s/72 °C
Number of cycles	35
Final extension	6 min/72 °C

A.3.8 Identification

As yet, identification is based only on PCR product size.

8) For the availability of appropriate control material, contact your national standards institute.

9) GeneAmp® 2400 and 9600 and AmpliTaq Gold® polymerase are examples of suitable products available commercially from Applied Biosystems, previously Perkin Elmer/Applied Biosystems. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of these products. Equivalent products may be used if they can be shown to give the same results.

A.3.9 General quality assurance and interpretation of the results

The target sequence is presumed to have been detected if the size of the PCR product corresponds to the expected length of the target DNA sequence, determined by comparison with products derived from reference material prepared from tomato.

For identification purposes, see A.3.8.

The detection of fragments with a size of 383 bp and 180 bp indicates that the sample DNA solution contains amplifiable DNA of tomato and of Zeneca genetically modified tomato, respectively, within the assessed limitations of specificity described in A.3.2.2.

For details on electrophoretic stages, see ISO 21571:2005, B.2.

A.4 Target-taxon-specific method for the detection of maize-derived components

A.4.1 General

This is a routine procedure for the detection of a single copy species-specific invertase gene sequence in maize (*Zea mays*).

No tool to verify the identity of the PCR product has been described. Therefore this method cannot be considered as an identification method. It may be used to assess the amplifiability of DNA extracted from maize.

A.4.2 Validation status and performance criteria

A.4.2.1 Collaborative study

This method was validated by the working group “Development of methods for identifying foodstuffs produced by using genetic engineering techniques” of the German Federal Institute of Consumer Protection and Veterinary Medicine (BgVV) for implementing Article 35 of the German Federal Foodstuffs Act in several collaborative studies. For DNA extraction, half of the participants used the CTAB method as outlined in ISO 21571:2005, A.3, and half of the participants used the Wizard[®] DNA-Clean-Up-System¹⁰⁾.

The data of the collaborative study are listed in Table A.11.

Table A.11 — Results of the collaborative study ^[20]

Year	1999
Number of laboratories	18
Number of laboratories submitting results	16
Number of samples per laboratory	6
Number of accepted results	96
Number of samples containing Bt-176	32
Number of samples containing Bt-11	32
Number of samples containing non-GM maize	32
False positive results	0 (0 %)
False negative results	0 (0 %)

10) Wizard[®] DNA-Clean-Up-System is an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

A.4.2.2 Molecular specificity

A.4.2.2.1 General

This annex fulfils the requirements as outlined in Clause 7.

The method has been designed to target the sequence described in GenBank® accession No. U16123.

A.4.2.2.2 Theoretical

The maize invertase gene obtained from a DNA sequence database was chosen as a target sequence.

Some sequence homology with DNA sequences of other agriculturally relevant plants (legumes, cereals, vegetables) and human and insect DNA has been found in database searches (NCBI BlastN® search, EMBL database, September 28th, 2001).

Primer IVR1-F retrieved:

- AF171874 *Zea mays* soluble acid invertase IVR1 (100 % match);
- AX033517 Sequence 25 from Patent DE19906169 (21 nucleotide contiguous match);
- AX033514 Sequence 22 from Patent DE19906169 (21 nucleotide contiguous match).

Primer IVR1-R retrieved:

- AF171874 *Zea mays* soluble acid invertase IVR1 (100 % match);
- AX150234 Sequence 30 from Patent WO0132919 (100 % match);
- AJ224681 *Triticum aestivum* mRNA for beta-fructosidase (20 nucleotide contiguous match);
- AF062735 *Saccharum officinarum* soluble acid invertase (19 nucleotide contiguous match);
- AF062734 *Saccharum robustum* soluble acid invertase. (19 nucleotide contiguous match).

The number of target sequence copies was not determined, but was presumed to be a single copy gene.

A.4.2.2.3 Experimental

The maize-PCR assay appeared to be highly specific for maize DNA [21].

A.4.2.3 Limit of detection (LOD)

The absolute LOD has not been determined, but the method has been demonstrated to amplify $\leq 0,1$ ng maize-DNA (determined fluorometrically), extracted from maize kernels [20].

A.4.3 Adaptation

No specific information is available.

A.4.4 Principle

The maize invertase gene codes for a carbohydrate metabolism enzyme.

A 226 bp fragment from the maize invertase gene is amplified by PCR and separated by agarose gel electrophoresis.

A.4.5 Reagents

For the quality of the reagents used, see ISO 24276.

A.4.5.1 Water

A.4.5.2 PCR buffer (without MgCl_2), 10×

A.4.5.3 MgCl_2 solution, $c(\text{MgCl}_2) = 25 \text{ mmol/l}$.

A.4.5.4 dNTP solution, $c(\text{dNTP}) = 2,5 \text{ mmol/l}$ (each).

A.4.5.5 Oligonucleotides

A.4.5.5.1 Forward primer

Maize invertase gene (GenBank® accession No. U16123).

Primer IVR1-F: 5'-CCg CTg TAT CAC AAg ggC Tgg TAC C-3'.

A.4.5.5.2 Reverse primer

Maize invertase gene (GenBank® accession No. U16123).

Primer IVR1-R: 5'-ggA gCC CgT gTA gAg CAT gAC gAT C-3'.

A.4.5.6 Thermostable DNA polymerase (for hot-start PCR), 5 IU/ μl .

A.4.6 Apparatus

As specified in A.1.6.

A.4.7 Procedure

A.4.7.1 PCR set-up

The method is described for a total PCR volume of 25 μl per reaction with the reagents as listed in Table A.12. The PCR may also be carried out in a larger volume if the solutions are adjusted appropriately. The final concentrations of reagents as outlined in Table A.12 have proven to be suitable.

Table A.12 — Addition of reagents

Reagent	Final concentration	Volume per sample (μl)
Sample-DNA	10 ng to 50 ng	2
Water		15,3
10 × PCR buffer (without MgCl_2)	1 ×	2,5
MgCl_2 solution ^a , 25 mmol/l	1,5 mmol/l	1,5
dNTP solution, 10 mmol/l	0,4 mmol/l	1
Primer IVR1-F, 10 $\mu\text{mol/l}$	0,5 $\mu\text{mol/l}$	1,25
Primer IVR1-R, 10 $\mu\text{mol/l}$	0,5 $\mu\text{mol/l}$	1,25
Taq DNA polymerase, 5 IU/ μl	1 IU	0,2
^a If the PCR buffer solution already contains MgCl_2 , the final concentration of MgCl_2 in the reaction mixture is adjusted to 1,5 mmol/l.		

A.4.7.2 PCR controls

As a positive control, certified reference materials from IRMM of, for example, maize Bt 11 (IRMM-412) or Event 176 maize (Bt 176) (IRMM-411) may be used.

Any other appropriate controls should be included as described in ISO 24276.

A.4.7.3 Temperature-time programme

The temperature-time programme as outlined in Table A.13 has been used for the validation study using thermal cyclers GeneAmp® 2400 or GeneAmp® 9600 and AmpliTaq Gold® DNA polymerase¹¹⁾. The use of other thermal cyclers might make an adaptation necessary. The time for activation/initial denaturation depends on the polymerase used. If using a hot-start polymerase, the recommendation of the manufacturer should be adhered to unless the protocol states otherwise.

Table A.13 — Temperature-time programme

Activation/initial denaturation	12 min/95 °C
Amplification	30 s/95 °C 30 s/64 °C 60 s/72 °C
Number of cycles	35
Final extension	10 min/72 °C

A.4.8 Identification

As yet, identification is based only on PCR product size.

A.4.9 General quality assurance and interpretation of the results

The target sequence is presumed to have been detected if the size of the PCR product corresponds to the expected length of the target DNA sequence, determined by comparison with products derived from certified reference material prepared from maize (e.g. IRMM-412 [Bt11 maize] or IRMM-411 [Event 176 maize] series from IRMM, Geel, Belgium).

For identification purposes, see A.4.8.

The detection of fragments with a size of 226 bp indicates that the sample DNA solution contains amplifiable DNA of maize origin within the assessed limitations of specificity described in A.4.2.2.

For details on electrophoretic stages, see ISO 21571:2005, B.2.

11) GeneAmp® 2400 and 9600 and AmpliTaq Gold® polymerase are examples of suitable products available commercially from Applied Biosystems, previously Perkin Elmer/Applied Biosystems. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of these products. Equivalent products may be used if they can be shown to give the same results.

A.5 Target taxon-specific method for the detection of DNAs derived from rice

A.5.1 Purpose, relevance and scientific basis

The GMO Detection Laboratory of Shanghai Jiao Tong University (GMDL-SJTU) organized a collaborative study for validation of the applicability of a target taxon-specific method using the rice sucrose phosphate synthase (*SPS*) gene as an endogenous gene for qualitative analysis of genetically modified (GM) or non-GM rice. This study involved 12 laboratories from Spain, Korea, Lithuania, Slovenia, Japan, Italy, and China.

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

- qualitative PCR for validation of the heterogeneity of the *SPS* gene among rice cultivars for different geographic and phylogenetic origins;
- qualitative PCR for validation of the species specificity of *SPS* gene for rice;
- qualitative PCR for evaluation of the LOD of the established *SPS* qualitative PCR assay.

The collaborative study was carried out in accordance with Reference [44].

The results of the collaborative study as well as the related protocol are given in A.5.3.

A.5.2 Principle

The method has been optimized for rice seeds and other processed products such as seed powder. Applicability of the *SPS* gene was evaluated in this collaborative study using DNA samples extracted from rice seeds and other plant materials.

The collaborative study organizer provided method-specific reagents (primers, probes, reaction master mix), and the test DNA samples extracted from rice materials to collaborative study participants.

A.5.3 Validation status and performance criteria

A.5.3.1 Robustness of the method

Robustness has been tested on the *SPS* gene qualitative PCR system for three different annealing temperatures (i.e. 56 °C, 58 °C, and 60 °C), on three different DNA samples containing known amounts of rice DNA (10 ng, 1 ng, 0,1 ng rice genome DNA samples) and with three repetitions per sample. The qualitative PCR systems demonstrated the expected robustness and performed well at all three annealing temperatures and three concentrations of the rice DNA samples.

The *SPS* gene qualitative PCR system was also tested on different thermal cyclers (PTC-100,¹⁾ MJ Research and instruments from Bio-Rad and Applied Biosystems), on three different reaction volumes (25 µl, 30 µl, and 50 µl) and three repetitions per volume. The qualitative PCR systems had the expected robustness and performed well on different thermal cyclers and with different reaction volumes.

A.5.3.2 Intralaboratory trial

The rice *SPS* gene has been described as being suitable for use as an endogenous reference gene in rice identification and quantification (Reference [44]). The detailed technical information was modified from Reference [44].

1) Example of a suitable 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. 

A1 For sample preparation in the collaboration 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 DNA extracted was performed using a method adopted from ISO 21571:2005, B.1. After the DNA quantification, a qualitative PCR using an 18S PCR system (Reference [45]) was carried out to provide data about possible PCR inhibition.

The *SPS* gene PCR system was tested using rice genomic DNA by three researchers at the GMDL-SJTU. The results were satisfactory; in particular, for qualitative PCR, the results show that the *SPS* gene is specific for rice, and the LOD is about 0,1 %.

A.5.3.3 Collaborative trial

For the collaborative study, each participant received 12 rice DNA samples for heterogeneity testing; 10 DNA samples from plants other than rice plus one DNA sample from rice for species specificity testing; and 10 serially diluted rice samples for LOD evaluation. A negative and a positive control were also included.

The heterogeneity of the *SPS* gene among rice cultivars was evaluated using 12 rice cultivars from different geographic and phylogenetic origins in China, such as Najing14, Taibei309, Shengnong265, Jinyinbao, Minghui78, Huke3, Guangluai4, Zhe733, Hejiang19, Baizhehu, Xiangwanxian9 and Nipponbare. The results returned from 12 laboratories showed that out of a total of 144 (12 × 12) rice DNA samples, 143 positive results were obtained using the *SPS* gene PCR system. This means that the false-negative rate of the *SPS* gene PCR system for rice is 0,69 % (1/144) (see Table A.14). These data suggest that there is low heterogeneity of the *SPS* gene in the target region.

The species specificity of the *SPS* gene was validated using a rice genome DNA sample (Guangluai4) and 10 other plant DNAs that were evolutionarily related to rice, common crops or model plants, such as the fruit materials of bamboo (*Phyllostachys* spp.), green bristlegrass [*Setaria viridis* (L.) Beauv.], barley (*Hordeum vulgare*), wheat (*Triticum aestivum*), foxtail millet (*Setaria italica*), rapeseed (*Brassica napus*), tomato (*Lycopersicon esculentum*), potato (*Solanum tuberosum*), soya bean (*Glycine max*) and thale cress (*Arabidopsis thaliana*). The results returned from 12 laboratories showed that out of a total of 120 (10 × 12), non-rice plant DNA samples, 118 negative results were obtained using the *SPS* gene PCR system. This means that the false-positive rate of the *SPS* gene PCR system for other 10 plant materials was 1,67 % (2/120) (see Table A.14). These data suggest that the *SPS* gene is species specific for detection of rice.

Table A.14 — The results of heterogeneity and of specificity testing of the qualitative PCR

Parameter (collaborative study of 2007)	Value
No. laboratories	12
No. laboratories submitting results	12
No. samples per laboratory	22
No. accepted results	264
No. samples containing rice	144
No. samples not containing rice	120
False-positive results	2 (1,67 %)
False-negative results	1 (0,69 %)

A1

A1 The LOD of the *SPS* gene PCR system was validated using mixed powder containing maize and various quantities of rice seed by means of qualitative PCR: all 12 laboratories detected the *SPS* gene in the DNA sample extracted from mixed powder containing 0,1 % mass fraction or higher of rice, and two in 12 laboratories detected it from mixed powder containing 0,01 % mass fraction of rice. These data suggest that the LOD of the *SPS* gene PCR system is as low as 0,1 % mass fraction (see Table A.15).

Table A.15 — The results of the LOD test of the qualitative PCR

Parameter (collaborative study of 2007)	Rice to maize mass fraction, $m_{\text{rice}}/m_{\text{maize}}$				
	10 %	1 %	0,1 %	0,05 %	0,01 %
No. laboratories	12	12	12	12	12
No. laboratories submitting results	12	12	12	12	12
No. samples per laboratory	2	2	2	2	2
No. laboratories accepted results	12	12	12	12	12
Positive results	12 (100 %)	12 (100 %)	12 (100 %)	4 (33,33 %)	2 (16,67 %)

A.5.3.4 Molecular selectivity

A.5.3.4.1 General

For qualitative validation of the *SPS* gene as a specific rice gene, a 279 bp fragment of the conserved region of the *SPS* gene was selected and amplified using specific primers.

A.5.3.4.2 Experimental

DNA samples extracted from 11 different plant materials (including rice) were analysed by the *SPS* gene PCR system as described (Reference [44]). Among the 11 samples, only rice DNA gave positive results. The other 10 samples (see A.5.3.3) gave negative results.

The DNA samples extracted from 12 different rice cultivars were analysed by the *SPS* gene PCR system reported in Reference [44]. All 12 samples gave positive results.

A.5.3.4.3 Theoretical

The theoretical specificity of the *SPS* gene primer was assessed through a homology search using the BLASTN 2.0MP-WashU program (Reference [82], search date: 2010-01-09). The 279 bp sequence used as query is part of the NCBI accession number U33175 (nucleotides 1055–1333). The results of the basic local alignment search tool (BLAST) confirmed the complete identity of the query sequence with rice *SPS* gene sequence, and no homology with other genes and species.

A.5.4 Principle and summary

This methodology is a PCR procedure for the applicability of the *SPS* gene for use as a rice endogenous gene in qualitative detection of GM or non-GM rice. Heterogeneity, species specificity of the *SPS* gene and LOD were evaluated as part of the validation of this method. The 279 bp PCR product was visualized by agarose gel electrophoresis.

A.5.5 Terms and definitions

For the purposes of this document, the terms and definitions of ISO 5725-1^[40] and ISO 24276 apply.

A.5.6 Sample type and amounts

In the following, the data from the collaborative study are given as examples for sample types and sample amounts adequate for this method. **A1**

A1 DNA samples extracted from the seeds of 12 rice cultivars, 10 other plant materials (see A.5.3.3) and the mixed powder containing different mass fractions of rice in maize seed power, were used in this collaborative study.

The participants received the following samples.

- 12 DNA samples from 12 different rice cultivars that are widely planted in different region of China (i.e. Najing14, Taibei309, Shengnong265, Jinyinbao, Minghui78, Huke3, Guangluai4, Zhe733, Hejiang19, Baizhehu, Xiangwanxian9, and Nipponbare), 20 ng/μl, 50 μl each. These DNA samples were used to validate the heterogeneity of the *SPS* gene among rice cultivars.
- 11 DNA samples from rice (Guangluai4) and 10 other plant materials which are related to rice (i.e. bamboo, green bristlegrass, barley, wheat, foxtail millet) or common GM crops (i.e. rapeseed, tomato, potato and soya bean) or model plants (i.e. thale cress), 20 ng/μl, 50 μl each. These DNA samples were used to validate the species specific of the *SPS* gene in rice.
- 10 DNA samples from mixed powders of maize with different mass fractions of rice, 20 ng/μl, 50 μl each. These DNA samples were double blind replicates of the series of five rice concentrations used for testing the LOD of the *SPS* gene PCR system.
- Negative DNA target control (labelled N): salmon sperm DNA (20 ng/μl).
- Positive DNA target control (labelled P): rice (Guangluai4) genomic DNA (20 ng/μl). All the DNA samples were purified using the CTAB method by the GMDL-SJTU. The negative and positive DNA target controls were used for each PCR plate.
- Reaction reagents, primers for the *SPS* gene PCR system as follows:
 - primer pair for conventional PCR: SPS-F/SPS-R;
 - DNA dilution solution [0,1× tris–EDTA (TE), 1,2 ml].

A.5.7 Limit of detection and range of use

DNA was extracted from five mixed powder samples containing different amounts of rice. These samples were analysed by the *SPS* PCR system as described (Reference [44]). Positive results were obtained with samples containing mass fractions of 10 %, 1 %, and 0,1 % rice. The other two samples (containing mass fractions of 0,05 % and 0,01 %) gave negative results.

According to the developed method, the relative LOD of the qualitative PCR method is about 0,1 % mass fraction. The *SPS* gene PCR system can be used for specific detection and identification of rice materials in other plant materials.

A.5.8 Estimation of measurement uncertainty

The reproducibility of the method is given by the results of the collaborative trial (see A.5.3.3).

A.5.9 Interferences

In the studies performed, no additional information about interferences have been observed.

A.5.10 Physical and environmental conditions

See ISO 24276 for details. For example:.

- maintain strictly separated working areas for DNA preparation, PCR set-up, PCR amplification and electrophoresis;
- any residual DNA should be removed from all equipment prior to its use; **A1**

- A₁** — in order to avoid contamination, use filter pipette tips protected against aerosol;
— use only powder-free gloves and change them frequently.

A.5.11 Apparatus and equipment

A.5.11.1 Microcentrifuge.

A.5.11.2 Freezer operating at -20 °C and refrigerator operating at 4 °C.

A.5.11.3 Micropipettes.

A.5.11.4 Mixer, e.g. vortex mixer.

A.5.11.5 Microcentrifuge tubes, capacities: 0,2 ml, 1,5 ml, and 2,0 ml.

A.5.11.6 Tips and aerosol-resistant tips for micropipettes.

A.5.11.7 Rack for reaction tubes.

A.5.11.8 PVC or latex gloves.

A.5.11.9 DNA amplifying equipment (thermal cycler or equivalent apparatus).

A.5.11.10 Electrophoresis equipment, with power supply.

A.5.11.11 Imaging system for gel analysis.

A.5.11.12 Microwave oven (optional).

A.5.12 Reagents and materials

A.5.12.1 General

Unless otherwise stated, only reagents that conformed to the specifications of ISO 24276 and only molecular biology grade water or water of equivalent purity were used.

A.5.12.2 Qualitative PCR

A.5.12.2.1 PCR buffer (without MgCl₂) 10×.

A.5.12.2.2 MgCl₂ solution 25 mmol/l.

A.5.12.2.3 dNTP solution 2,5 mmol/l each.

A.5.12.2.4 Primer (see Table A.16).

A.5.12.2.5 DNA polymerase, thermostable.

A.5.12.3 Electrophoresis

For details see e.g. ISO 21571:2005, B.1. **A₁**

A.5.12.3.1 Loading buffer (10 g/l sodium dodecyl sulfate, 500 g/l glycerol, 0,5 g/l bromophenol blue), 10×.

A.5.12.3.2 DNA size standard.

A.5.13 Sample collection, transportation, 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.5.14 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 ISO 21571 and ISO 24276.

A.5.15 Instrument calibration

Instruments, e.g. thermal cyclers and pipettes should be calibrated as per ISO/IEC 17025.^[41]

A.5.16 Analysis steps

A.5.16.1 Preparation of the DNA for qualitative PCR

Extract DNA from the samples by using an adequate extraction method, e.g. ISO 21571:2005, A.3, CTAB extraction method. Thaw, mix gently and centrifuge the DNA samples needed for the PCR run. Keep thawed reagents at 1 °C to 4 °C on ice.

A.5.16.2 PCR reagents

A.5.16.2.1 Conventional PCR master mix

A conventional PCR reaction mixture containing the following: 1× PCR buffer, 200 µmol/l each of dNTPs, 2,5 mmol/l Mg²⁺, 330 nmol/l forward/reverse primer, 1 unit *Taq* DNA polymerase.

A.5.16.2.2 Primers

See Table A.16.

Table A.16 — Oligonucleotide primer sequences for qualitative PCR

Name	Oligonucleotide DNA sequence (5' to 3')
Qualitative PCR primer sequence	
SPS primer F	TTg CgC CTg AAC ggA TAT
SPS primer R	ggA gAA gCA CTg gAC gAgg

A.5.16.3 Procedure

A.5.16.3.1 General

The qualitative PCR for rice *SPS* gene was developed for a total volume of 30 µl per reaction mixture. The use of 100 ng of template DNA per reaction well is recommended.

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

A1 Distribute 25 µl/tube of the master mixture to 200 µl PCR reaction tubes. 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, centrifuge in the microcentrifuge at 1 000 × g for 10 s.

Insert the plate into the instrument.

Run the PCR with qualitative PCR cycling conditions.

A.5.16.4 PCR controls

See 7.5 and ISO 24276.

A.5.16.5 Temperature–time programme

The PCR assay has been optimized for use in a PTC-100¹⁾ (MJ Research) and an ABI 2720¹⁾ (Applied Biosystems) thermal cycler PCR machine. Although other PCR machines may be used, the thermal cycling conditions may need to be verified. The qualitative PCR cycling parameters are indicated in Table A.17.

Table A.17 — Qualitative PCR temperature–time programme

Step	Stage		Temperature °C	Time s	No. cycles
1	Activation and initial denaturation		94	900	1×
2a	Amplification	Denaturation	94	30	35×
2b		Annealing	58	30	
2c		Elongation	72	30	
3	Final elongation		72	420	1×

A.5.16.6 Detection

After the PCR programme has finished, transfer 3 µl of 10× loading buffer to each reaction tube and mix with the PCR products.

Load 10 µl of each PCR product on to electrophoresis gel (20 g/l agarose, 0,5 µg/ml ethidium bromide), respectively.

Run the gel in the electrophoresis equipment under 5 V/cm, 20 min.

Record gel image with an UV gel documentation or similar system.

A fragment of 279 bp should be the specific product; other bands existing in the agarose electrophoresis are unexpected products.

A.5.16.7 Accept or reject criteria

Method performance requirements used to evaluate the results from the collaborative study are as follows. A fragment of 279 bp should be detected in the rice positive control (sample P), and no target fragment should be detected in negative control (sample N) and blank. The detection of fragments with a size of 279 bp indicates that the sample DNA solution contains amplifiable DNA of *SPS*, and the result is positive, otherwise the result is negative.

A.5.17 Sample identification

All samples should be identified unambiguously. **A1**

A.5.18 Interpretation and calculations of the results

The expected amplicon length of *SPS* is 279 bp in size.

A fragment of 279 bp should be detected in the rice positive control (sample P), and no target fragment should be detected in negative control (sample N) and blank. The detection of fragments with a size of 279 bp indicates that the sample DNA solution contains amplifiable DNA of *SPS*, and the result is positive, otherwise the result is negative.

A.6 Target taxon-specific method for the detection of components derived from tomato

A.6.1 Purpose, relevance and scientific basis

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 being used as species-specific gene in GM tomato identification and quantification (Reference [46]). The GMO Detection Laboratory of Shanghai Jiao Tong University (GMDL-SJTU) organized the collaborative trial for validation of the applicability of the tomato *LAT52* gene as species-specific gene for qualitative analysis of genetically modified (GM) or non-GM tomato. The study involved 13 laboratories from the US, Singapore, Korea, Lithuania, Slovenia, Norway, Italy, and China (Reference [47]). The results are given in Table A.18 and Table A.19.

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

- a) qualitative PCR for the validation of the heterogeneity of the *LAT52* gene among tomato cultivars for different geographic and phylogenetic origin;
- b) qualitative PCR for the validation of the species-specificity of the *LAT52* gene for tomato;
- c) qualitative PCR for the evaluation of the LOD of the established *LAT52* qualitative PCR assay.

The collaborative study was carried out in accordance with the following internationally accepted guidelines:

- ISO 5725-2^[39] 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 [48]).

A.6.2 Principle

This method describes the detection of tomato DNA by using qualitative PCR.

The method has been optimized for tomato seeds, tomato fruits, tomato ketchup, tomato juice, and other processed products derived from tomato. The applicability of the *LAT52* gene was tested through collaborative trial using DNA samples extracted from tomato seeds and other plant materials.

A.6.3 Validation status and performance criteria

A.6.3.1 Robustness of the method

The robustness of the *LAT52* qualitative PCR system was tested by the method developer using three different annealing temperatures (i.e. 56 °C, 58 °C, and 60 °C), on three different DNA samples containing known amounts of tomato seed DNA (10 ng, 1 ng, 0,1 ng tomato genome DNA samples, and three repetitions per sample). The qualitative PCR systems showed the expected robustness and performed satisfactorily at all three annealing temperatures and three concentrations of the tomato DNA samples. ^[A1]

A₁ The *LAT52* qualitative PCR system has also been tested by the method developer on different thermal cyclers [PTC-100,¹⁾ MJ Research; S1000,¹⁾ Bio-Rad; and ABI 9700,¹⁾ Applied Biosystems] and with three different reaction volumes (25 µl, 30 µl and 50 µl, and three repetitions per volume). The qualitative PCR system showed the expected robustness when used at different thermal cyclers and different reaction volumes.

A.6.3.2 Intralaboratory trial

The tomato *LAT52* gene has been validated suitable for use as species-specific gene in GM tomato identification and quantification (Reference [46]). The detailed technical information given here is modified from Reference [46].

For sample preparation for the validation study, the DNA samples were extracted by the GMDL-SJTU using the CTAB method adopted from ISO 21571:2005, A.3. 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 qualitative PCR run applying the 18S PCR system was carried out to provide data about possible PCR inhibition (Reference [49]).

The *LAT52* PCR system was tested by three operators by the GMDL-SJTU using tomato genomic DNA providing satisfactory and consistent results; in particular, in qualitative PCR, the results showed the *LAT52* gene is specific for tomato, and the relative LOD is at least 0,1 % mass fraction.

A.6.3.3 Collaborative trial

The heterogeneity of *LAT52* gene among tomato cultivars was evaluated using 12 tomato cultivars from different geographic and phylogenetic origins in China, such as Shengnong2, Jifan4, Zhongsu5, Yashu6, Jiafen1, Shenfeng2, Hongza9, R144, Nongyou30, Dongnong704, Lichun, and Zaokui. The results returned from 13 laboratories showed that from the total of 156 (12 × 13) tomato DNA samples, 155 positive results were obtained using the *LAT52* gene PCR system. Thus, the false-negative rate of the *LAT52* PCR system for tomato is 0,64 % (1/156) (see Table A.18). These data suggest that the *LAT52* gene has low heterogeneity among tomato cultivars from China.

The species specificity of the *LAT52* gene was validated using a tomato genome DNA sample (Jiafen1) and 10 other plant DNAs that were evolutionarily related to tomato or common GM crops or model plants, such as the fruit materials of aubergine (*Solanum melongena*), potato (*Solanum tuberosum*), sweet pepper (*Capsicum annuum*); maize (*Zea mays*), soya bean (*Glycine max*), rapeseed (*Brassica rapa*), rice (*Oryza sativa*); leaf materials of petunia (*Petunia hybrida*), tobacco (*Nicotiana tabacum*), and thale cress (*Arabidopsis thaliana*). The results returned from 13 laboratories show that from the total of 130 (10 × 13, without tomato DNA sample) various plant DNA, 126 negative results were obtained using the *LAT52* gene PCR system. Thus the false-positive rate of the *LAT52* gene PCR system was 3,08 % (4/130) (see Table A.18). The false-positive results might come from the contamination of the PCR operation. These data suggest that the *LAT52* gene is species-specific for the detection of tomato.

Table A.18 — Results of the qualitative PCR

Parameter (collaborative trial of 2007)	Value
No. laboratories	13
No. laboratories submitting results	13
No. samples per laboratory	22
No. accepted results	286
No. samples containing tomato	156
No. samples not containing tomato	130
False-positive results	4 (3,08 %)
False-negative results	1 (0,64 %)

A₁

- A₁** The LOD of the *LAT52* PCR system was validated using mixed powder containing maize and tomato seeds by means of qualitative PCR. All 13 laboratories were able to detect the DNA sample extracted from 0,1 % mass fraction or higher tomato contents in the mixed powder, while two detected the 0,01 % mass fraction tomato in the mixed powder. These data suggest that the LOD of the *LAT52* PCR system is as low as 0,1 % mass fraction (see Table A.19).

Table A.19 — Results of the LOD test of the qualitative PCR

Parameter (collaborative trial of 2007)	Tomato to maize mass fraction, $m_{\text{tomato}}/m_{\text{maize}}$				
	2 %	0,5 %	0,1 %	0,05 %	0,01 %
No. laboratories	13	13	13	13	13
No. laboratories submitting results	13	13	13	13	13
No. samples per laboratory	2	2	2	2	2
No. samples	26	26	26	26	26
Positive results	25 (96, 2 %)	25 (96, 2 %)	26 (100 %)	0 (0 %)	2 (15, 4 %)

A.6.3.4 Molecular selectivity

A.6.3.4.1 General

The *LAT52* method targets the tomato *LAT52* gene which is stably present with a single copy per haploid genome of different tomato cultivars. The specific primers (Table A.20) amplify a 92 bp long amplicon.

A.6.3.4.2 Experimental

DNA samples extracted from 11 different plant materials (including tomato) were analysed with the *LAT52* PCR system by the method developer. Out of the 11 samples, only tomato DNA gave positive results. The other 10 samples (see A.6.3.3) gave negative results.

DNA samples extracted from 12 different tomato cultivars were analysed with the *LAT52* PCR system by the method developer (see A.6.6). All samples gave positive results.

A.6.3.4.3 Theoretical

The theoretical specificity of the *LAT52* primers was assessed through a homology search using the BLASTN 2.0MP-WashU program (Reference [82], search date: 2010-01-20). The 92 bp sequence used as query is part of the NCBI accession number X15855 (nucleotides 1385–1476). The results of the BLAST confirmed the complete identity of the query sequence with the tomato anther-specific *LAT52* gene sequences, and showed no homology with sequences of other genes and species.

A.6.4 Principle and summary

The methodology is a qualitative PCR procedure using the *LAT52* gene as a tomato species-specific gene for qualitative detection of GM or non-GM tomato. The detection of the 92 bp long PCR product is carried out by agarose gel electrophoresis.

A.6.5 Terms and definitions

For the purposes of this document, the terms and definitions of ISO 5725-1^[40] and ISO 24276 apply.

A.6.6 Sample type and amounts

For the collaborative study, the following samples were used: **A₁**

- A1** — 12 DNA samples from seeds of 12 different tomato cultivars that were widely planted in different regions of China (i.e. Shengnong2, Jifan4, Zhongsu5, Yashu6, Jiafen1, Shenfeng2, Hongza9, Nongyou30, R144, Dongnong704, Lichun, and Zaokui), 20 ng/μl, 50 μl each. These DNA samples were used to validate the heterogeneity of the *LAT52* target sequence among tomato cultivars.
- 11 DNA samples from tomato (Jiafen1) seeds and the leaves of 10 other plant materials which were evolutionarily related to tomato (i.e. aubergine, potato, petunia, and capsicum) or common GM crops (i.e. maize, soya bean, rapeseed, and rice) or model plants (i.e. tobacco and thale cress), 20 ng/μl, 50 μl each. These DNA samples were used to validate the specificity of the *LAT52* method.
- 10 DNA samples from the mixed powder with maize and varied contents of tomato, 20 ng/μl, 50 μl each. These DNA samples were prepared as double-blind replicates of the series of five tomato concentrations and used for testing the LOD of *LAT52* PCR system.

A.6.7 Limit of detection and range of use

The LOD of the method was about 0,1 % mass fraction of tomato material. The *LAT52* method can be used to specifically detect and identify tomato materials in a sample.

DNA samples extracted from five mixed powders containing different mass fractions of tomato seed material were analysed with the *LAT52* PCR system. Only powder samples containing mass fractions of 0,1 % or above (i.e. 2 %, 0,5 % and 0,1 %) gave positive results. The other two samples gave negative results (i.e. mass fractions of 0,05 % and 0,01 %).

A.6.8 Estimation of measurement uncertainty

The global uncertainty of the method is given by the results of the collaborative trial (see A.6.3.3).

A.6.9 Interferences

In the studies performed, no additional information is given about interferences observed.

A.6.10 Physical and environmental conditions

See ISO 24276 for details.

A.6.11 Apparatus and equipment

A.6.11.1 DNA amplifying equipment (thermal cycler or equal apparatus).

A.6.11.2 Electrophoresis equipment, with power supply.

A.6.11.3 Documentation system for gel analysis.

A.6.11.4 Microwave oven (optional).

A.6.12 Reagents and materials

Unless otherwise stated, only reagents that conformed to the specifications of ISO 24276 and only molecular biology grade water or water of equivalent purity were used.

A.6.12.1 Conventional PCR master mix (see A 6.16.2). **A1**

A.6.12.2 Oligonucleotides (see Table A.20)."**A.6.12.3 Loading buffer.****A.6.12.4 Electrophoresis buffer.****A.6.12.5 Agarose.****A.6.12.6 DNA size standard.****A.6.13 Sample collection, transportation, preservation and storage**

DNA solutions should be stored at 4 °C for a maximum of 1 week, or at -20 °C for long-term storage.

A.6.14 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.

The DNA samples were extracted by the GMDL-SJTU using the CTAB method according to ISO 21571:2005, A.3.

A.6.15 Instrument calibration

Instruments, e.g. thermocyclers and pipettes should be calibrated e.g. according to ISO/IEC 17025.^[41]

A.6.16 Analysis steps**A.6.16.1 Preparation of the DNA for qualitative PCR**

Concerning the extraction of DNA from the test sample, the general instructions and measures described in ISO 21571 should be followed. It is recommended that one of the DNA extraction methods described in ISO 21571:2005, Annex A be chosen.

A.6.16.2 Conventional PCR master mix

The conventional PCR master mix included 1× PCR buffer, 200 µmol/l each of dNTPs, 400 nmol/l each of the forward and reverse primers (see Table A.20), and 1 unit of HotStar¹) *Taq* DNA polymerase.

Table A.20 — Oligonucleotide primers sequences for qualitative PCR

Name	Oligonucleotide DNA Sequence (5' to 3')
LAT52 primer F	A gAC CAC gAg AAC gAT ATT TgC
LAT52 primer R	TT CTT gCC TTT TCA TAT CCAg ACA

A.6.16.3 Procedure

The PCR set-up is developed for a total volume of 30 µl per reaction mixture. The use of 100 ng of template DNA per reaction well is recommended.

Thaw, mix gently and centrifuge the conventional PCR master mix needed for the run. Keep thawed reagents at 1 °C to 4 °C on ice.

Distribute 25 µl/tube of the master mixture to 200 µl PCR reaction tubes. Add 5 µl of DNA solution samples, tomato positive control, negative control, and blank control (H₂O) to the tubes, respectively. Mix the PCR tubes gently, centrifuge briefly in the microcentrifuge in order to collect all drops of the solution together. ^[A1]

A1 Insert the plate into the instrument.

Run the PCR with qualitative PCR cycling conditions described in A.6.16.5.

A.6.16.4 PCR controls

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

A.6.16.5 Temperature–time programme

The PCR assay is optimized for use in a PTC-100¹⁾ (MJ Research) and an ABI 2720¹⁾ (Applied Biosystems) thermal cycler PCR machine. Although other PCR machine may be used, thermal cycling conditions should be verified. The temperature–time programme is given in Table A.21.

Table A.21 — Qualitative PCR temperature–time programme

Step	Stage		Temperature °C	Time s	No. cycles
1	Activation and initial denaturation		94	900	1×
2a	Amplification	Denaturation	94	30	35×
2b		Annealing	56	30	
2c		Elongation	72	30	
3	Final elongation		72	420	1×

A.6.16.6 Detection

After the PCR, transfer 2 µl of loading buffer to each of reaction tube and mix with the PCR product.

Load 10 µl of each PCR product on to the electrophoresis gel (30 g/l agarose, 0,5 µg/ml ethidium bromide), respectively.

Run the gel in the electrophoresis equipment under 5 V/cm, 20 min.

The gel is imaged and recorded by an appropriate imaging system for gel analysis.

TBE should be used as the electrophoresis buffer because of the short amplicon.

A fragment of 92 bp should be the specific product; existence of other DNA fragments indicates non- specific amplification.

A.6.16.7 Accept or reject criteria

A fragment of 92 bp should be detected in the tomato positive control (sample P), and no PCR product should be detected in the negative control (sample N) and the blank. The detection of fragments with a size of 92 bp indicates that the sample DNA solution contains amplifiable DNA of *LAT52*, and the result is positive, otherwise, the result is negative.

A.6.17 Sample identification

Samples shall be unambiguously identified as detected or not detected.

A.6.18 Interpretation and calculations of the results

The detection of fragments with a size of 92 bp indicates that the sample DNA solution contains amplifiable DNA of *LAT52*, and the result is “detected”, otherwise, the result is “not detected”.

The 92 bp PCR product can be verified using DNA sequencing analysis. **A1**

Annex B (informative)

Screening methods

B.1 Screening method for the detection of genetically modified plant DNA (CaMV 35S promoter)

B.1.1 General

This is a method for the detection of a variable copy number DNA sequence from the cauliflower mosaic virus (CaMV) 35S promoter. Due to the presence of CaMV 35S promoter in many genetically modified plants, this method may be used to screen for the presence of GM-plant-derived DNA [22], [23].

B.1.2 Validation status and performance criteria

B.1.2.1 Collaborative study

The method has been validated in several collaborative studies with different raw and processed food matrices [24], [25].

The method has been validated in a collaborative study [24] under the coordination of the German Federal Institute for Health Protection of Consumers and Veterinary Medicine (BgVV) by the working group “Development of methods for identifying foodstuffs produced by means of genetic engineering techniques”. The number of participants as well the number of samples followed the criteria according to ISO 5725-2. For DNA extraction, the CTAB method as outlined in ISO 21571:2005, A.3 was used (but with a test portion of 100 mg).

The data of the collaborative study are listed in Table B.1

Table B.1 — Results of the collaborative study

Year	1999
Number of laboratories	27
Number of laboratories submitting results	23
Number of samples per laboratory	5
Number of accepted results	115
Number of samples containing GTS 40-3-2	59
Number of samples containing non-GM soya beans	56
False positive results	0 (0 %)
False negative results	0 (0 %)

B.1.2.2 Molecular specificity

B.1.2.2.1 General

This annex fulfils the requirements as outlined in Clause 7.

The method has been designed to target a sequence described in e.g. GenBank® database accession No. V00141.

A list of genetically modified plants containing the CaMV 35S promoter is provided in the annex of Reference [24].

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 derived from samples of *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 can be used [6].

B.1.2.2.2 Theoretical

No sequence homology with DNA sequences of non-GM crop plants has been found in databank searches (NCBI BlastN® search, EMBL database, September 28th, 2001). However, both primers match one accession that is not referable to either cauliflower mosaic virus or recombinant vectors or patents: S70105 cp (coat protein) [cucumber mosaic virus]. The primers also match more than 100 entries referable to cauliflower mosaic virus and recombinant vectors and patents.

B.1.2.2.3 Experimental

Amplification has not been observed with DNA from non-genetically modified crop-plants in the absence of DNA from the virus itself [22], [24], [25], [28].

Amplification has been observed with DNA from many genetically modified plants, e.g. GTS 40-3-2 (Roundup Ready® soya beans), the maize lines Event 176 (Bt 176), Bt 11, MON 810, MON 809, and ripening delayed tomatoes (Zeneca) [22], [24], [25], [28].

The number of copies of the DNA sequence vary.

B.1.2.3 Limit of detection (LOD)

The absolute LOD has not been determined. A relative LOD of 0,1 % genetically modified soya beans in soya bean flour IRMM-410 and of 0,1 % genetically modified maize Event 176 (Bt 176) IRMM-411 in maize-flour (mass fraction) (certified reference materials, CRMs) has been demonstrated [25].

B.1.3 Adaptation

No specific information is available.

B.1.4 Principle

A 195 bp DNA fragment from the CaMV 35S promoter sequence is amplified by PCR and detected after separation by agarose gel electrophoresis. For identification of the PCR product, a verification step should be performed.

Promoters are recognition or binding sequences for RNA-polymerases, which are responsible for the expression of genes. The constitutive 35S promoter from CaMV is frequently used in genetically modified plants [24].

B.1.5 Reagents

For the quality of the reagents used, see ISO 24276.

B.1.5.1 Water

B.1.5.2 PCR buffer (without MgCl_2), 10 $\times$.

B.1.5.3 MgCl_2 solution, $c(\text{MgCl}_2) = 25 \text{ mmol/l}$.

B.1.5.4 dNTP solution, $c(\text{dNTP}) = 2,5 \text{ mmol/l}$ (each).

B.1.5.5 Oligonucleotides

B.1.5.5.1 Forward primer

CaMV 35S promoter, 35s-1: [24], [28] 5'- gCT CCT ACA AAT gCC ATC A -3'.

Designed, together with a corresponding reverse primer, to amplify sequences such as described in accession No. V00141.

B.1.5.5.2 Reverse primer

CaMV 35S promoter, 35s-2: [24], [28] 5'- gAT AgT ggg ATT gTg CgT CA -3'.

Designed, together with a corresponding forward primer, to amplify sequences such as described in accession No. V00141.

B.1.5.6 Thermostable DNA polymerase (for hot-start PCR), 5 IU/ μl .

B.1.5.7 Restriction enzyme: *Xmn* I (= *Asp* 700).

B.1.6 Apparatus and equipment

B.1.6.1 Thermal cycler

B.1.6.2 Gel electrophoresis chamber, with power supply.

B.1.7 Procedure (PCR set-up)

B.1.7.1 General

The method is described for a total PCR volume of 25 μl per reaction mixture with the reagents as listed in Table B.2. The PCR may also be carried out in a larger volume if the solutions are adjusted appropriately. The final concentrations of reagents as outlined in Table B.2 have proven to be suitable.