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

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

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Foreword

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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).

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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*:

— *Sampling* (ISO 21568);

— *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
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and

Roche Molecular Systems, Inc.
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1145 Atlantic Avenue
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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:—[1], *Foodstuffs — Nucleic acid based methods of analysis for the detection of genetically modified organisms and derived products — General requirements and definitions*

3 Terms and definitions

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

1) To be published.

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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.

A qualitative result shall clearly demonstrate the presence or absence of the genetic element under study, relative to appropriate controls and within the detection limits of the analytical method used and test portion analysed.

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.

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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 $\geqslant$ 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.

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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 ≈ 60 °C (should be established experimentally), i.e. estimated melting temperature ⩽ 65 °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.

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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 a representative 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.

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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.

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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:—, Table 2, or

b) negative if a specific PCR product has not been detected, and all the controls give results as specified in ISO 24276:—, 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).”

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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

Results from both test portions shall be consistent. If one test portion gives a positive result and the other gives a negative result, then the analysis shall be repeated (see ISO 24276), if possible by increasing the quantity of template nucleic acid in the reaction so as to obtain consistent results for both test portions. Moreover, as a minimum, the purity of the template nucleic acid should be checked by including a PCR inhibition control. Other controls to check the length and integrity of the template nucleic acid may be useful.

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;

— description of the specificity of the analytical method;

— the result expressed according to Clause 9.

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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.

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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 *Le*1 [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 $MgCl_2$), 10× [4)].

A.1.5.3 $MgCl_2$ solution, $c(MgCl_2)$ = 25 mmol/l.

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

A.1.5.5 Oligonucleotides

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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.

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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 Reagent[5)] 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.

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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 polymerase[6)]. 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.

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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).

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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.

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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 $MgCl_2$), 10×

A.2.5.3 $MgCl_2$ solution, $c(MgCl_2)$ = 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.

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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 polymerase[7]. 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.

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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.

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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 *Lycopersicon esculentum* Mill cv. Ailsa Craig strain Nema 282F (Zeneca)	43
Number of samples containing *Lycopersicon esculentum* Mill cv. Ailsa Craig strain Nema 282C (non-genetically modified)	47
False positive results	0 (0 %)
False negative results	0 (0 %)

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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 *Lycopersicon esculentum* Mill cv. Ailsa Craig strain Nema 282F (Zeneca)	88
Number of samples containing *Lycopersicon esculentum* 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.

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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 $MgCl_2$), 10×

A.3.5.3 $MgCl_2$ solution, $c(MgCl_2)$ = 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.

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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 available[8)].

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 polymerase[9)]. 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.

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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.

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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-System[10].

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.

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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 ⩽ 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.

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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, $c(MgCl_2)$ = 25 mmol/l.

A.4.5.4 dNTP solution, c(dNTP) = 2,5 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 µmol/l	0,5 µmol/l	1,25
Primer IVR1-R, 10 µmol/l	0,5 µmol/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.

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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 polymerase[11]. 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.

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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.

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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].

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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×.

B.1.5.3 $MgCl_2$ solution, $c(MgCl_2)$ = 25 mmol/l.

B.1.5.4 dNTP solution, c(dNTP) = 2,5 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.

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Table B.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 35s-1, 5 µmol/l	0,2 µmol/l	1
Primer 35S-2, 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.

B.1.7.2 PCR controls

As a positive control, certified reference materials of GTS 40-3-2 (material containing 0,1 % of genetically modified plant ingredients), produced by IRMM Geel, Belgium (IRMM-410), may be used.

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

B.1.7.3 Temperature-time programme

The temperature-time programme as outlined in Table B.3 has been used for the validation study using thermal cyclers GeneAmp® PCR-systems 2400 or GeneAmp® 9600 and AmpliTaq Gold® DNA polymerase[12]. 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 carefully adhered to unless the protocol states otherwise.

Table B.3 — Temperature-time programme

Activation/initial denaturation	10 min/95 °C
Amplification	20 s/94 °C 40 s/54 °C 60 s/72 °C
Number of cycles	40
Final extension	3 min/72 °C

B.1.8 Identification

The identity of the PCR product may be verified by restriction analysis of the PCR product with *Xmn* I, which is expected to yield two fragments (115 bp and 80 bp) [22], [24].

12) GeneAmp® PCR systems 2400 and 9600 and AmpliTaq Gold® DNA polymerase are examples of suitable products available commercially from Applied Biosystems, previously known as 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.

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B.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 material (prepared from, for example, IRMM-410 series [GTS 40-3-2] from IRMM, Geel, Belgium).

For identification purposes, see B.1.8.

The detection of fragments with a size of 195 bp indicates that the sample DNA solution contains amplifiable DNA of CaMV or GM origin within the assessed limitations of specificity described in B.1.2.2.

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

B.2 Alternative screening method for the detection of genetically modified plant DNA (CaMV 35S promoter)

B.2.1 General

This is a method for the detection of a variable copy number DNA sequence from cauliflower mosaic virus (CaMV) 35S promoter in processed food matrices. Due to the presence of CaMV 35S promoter in many genetically modified plants, this method may be used to screen for GM-plant-derived DNA. [22], [23], [29]

No tool to verify the identity of the PCR product is described. Therefore this method cannot be considered as an identification method. It may be used to assess the amplifiability of DNA containing the target sequence.

B.2.2 Validation status and performance criteria

B.2.2.1 Collaborative study

The method has been validated following the criteria specified in ISO 5725-2. The collaborative study involved 23 European laboratories and was coordinated by the EC JRC [29], [30]. The method has been evaluated for detection of GMOs in various processed food matrices (cooked maize grit, infant formula, biscuits, meal of acidified soya beans) containing each 0 %, 2 %, and 100 % (10 % instead of 100 % in the case of biscuits) of either GTS 40-3-2 or Event 176. Each participant received 4 control samples and 30 unknown independent duplicates, of which 10 corresponded to 0 % GMO samples and 20 contained various percentages of the genetically modified events. All participants received a detailed method description for DNA extraction with either the CTAB method or a commercially available kit. However, the laboratories were free to apply their method of choice for DNA extraction while the PCR conditions had to be optimized specifically for their local equipment. The laboratories were asked to analyse each sample once and to specify whether it was considered to be GMO positive or negative. Since most laboratories returned correct results (14 laboratories reported between 90 % to 100 % of correct scores; and 3 between 80 % to 90 % of correct scores) and none provided correct results in the range of 70 % to 80 %, the cut-off level was set at 80 % of correctly reported results. As a consequence, 5 laboratories were excluded from further statistical analysis. An average of 96,1 % correct results was obtained for non-GMO containing samples (3,9 % false positive results) and an average of 98,1 % correct results for GMO-containing samples (1,9 % false negative results) [30]. The results are given in Table B.4.

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Table B.4 — Results of the collaborative study

Year	1999
Number of laboratories	30
Number of laboratories submitting results	18
Number of samples per laboratory	12
Number of total samples	360
Number of accepted results	540
False positive results	3,9 %
False negative results	1,9 %

B.2.2.2 Molecular specificity

B.2.2.2.1 General

This annex fulfils the requirements as outlined in Clause 7.

The method has been designed to target the sequence described in, for example, GenBank® database accession No. V00141. For a list of genetically modified plants containing the CaMV 35S promoter, see References [23] and [24].

A false positive result can occur since the amplified sequence is derived from a promoter of the 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 a proof for the presence of GM-plant-derived products without additional confirmation.

In order to distinguish between a viral infection and GM material, methods for the detection of cauliflower mosaic virus may be used [6].

B.2.2.2.2 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). The primers match an extensive list of accessions referable to cauliflower mosaic virus and recombinant vectors and patents.

B.2.2.2.3 Experimental

No amplification has been observed using DNA from non-GM soya bean in performance tests before the collaborative study [29].

B.2.2.3 Limit of detection (LOD)

The absolute limit of detection with this method has not been determined, but it has been demonstrated to detect at least 50 copies of GTS 40-3-2 DNA [29].

The relative LOD was not determined, but in the collaborative study [28], 2 % GMO [GTS 40-3-2 (Roundup Ready® soya beans) and/or Event 176 maize (Bt 176 maize)] could be detected in biscuits, infant formula and acidified soya beans with 100 % correct results [30].

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B.2.3 Adaptation

No specific information is available

B.2.4 Principle

A 123 bp DNA fragment from the CaMV 35S promoter sequence is amplified by PCR and detected by gel electrophoresis. The identity of the PCR product can be verified, for example by DNA sequencing. However, no verification procedure has been validated.

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 [22].

B.2.5 Reagents

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

B.2.5.1 Water

B.2.5.2 PCR buffer, $c(\mathrm{MgCl_2})$ = 15 mmol/l, 10×.

B.2.5.3 dNTP solution, $c(\mathrm{dNTP})$ = 4 mmol/l (each).

B.2.5.4 Oligonucleotides

B.2.5.4.1 Forward primer

CaMV 35S promoter, 35s-cf3: 5'- CCA CgT CTT CAA AgC AAg Tgg-3'.

Designed to amplify the CaMV 35S promoter, e.g. accession No. V00141.

B.2.5.4.2 Reverse primer

CaMV 35S promoter, 35s-cr4: 5'-TCC TCT CCA AAT gAA ATg AAC TTC C-3'.

Designed to amplify the CaMV 35S promoter, e.g. accession No. V00141.

B.2.5.5 Thermostable DNA polymerase (for hot-start PCR), 5 IU/µl.

B.2.6 Apparatus and equipment

As specified in B.1.6.

B.2.7 Procedure

B.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 B.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 B.5 have proven to be suitable.

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Table B.5 — Addition of reagents

Reagent	Final concentration	Volume per sample (µl)
Sample-DNA		5
Water		14,84
10 × PCR buffer (with $MgCl_2$ 15 mmol/l)[a]	1 ×	2,5
dNTP solution, 16 mmol/l	0,64 mmol/l	1
Primer 35s-cf3, 20 µmol/l	0,6 µmol/l	0,75
Primer 35S-cr4, 20 µmol/l	0,6 µmol/l	0,75
Taq DNA polymerase, 5 IU/µl	0,8 IU	0,16

[a] If PCR buffer without $MgCl_2$ is used, the volumes and concentrations should be adjusted accordingly.

B.2.7.2 PCR controls

As a positive control, certified reference materials of GTS 40-3-2 (material containing 0,1 % of genetically modified plant ingredients), 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.

B.2.7.3 Temperature-time programme

The temperature-time programme as outlined in Table B.6 has been used for the validation study using the Perkin Elmer 2400/9600/9700 thermal cycler systems and AmpliTaq Gold® DNA polymerase[13]. The use of other thermal cyclers might make an adaptation necessary. The time for activation/initial denaturation depends from the polymerase used. If using a hot-start polymerase, the recommendation of the manufacturer should be carefully adhered to unless the protocol states otherwise.

Table B.6 — Temperature-time programme

Activation/initial denaturation	10 min/95 °C
Amplification	25 s/95 °C 30 s/62 °C 45 s/72 °C
Number of cycles	50
Final extension	7 min/72 °C

B.2.8 Identification

It is recommended to verify the identity of the PCR product derived from the unknown sample by, for example, restriction, DNA sequencing or DNA hybridization.

13) GeneAmp® 2400, 9600 and 9700, 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.

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B.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, determined by comparison with products derived from certified reference material containing the target DNA sequence (e.g. IRMM-410 series from IRMM, Geel, Belgium).

For identification purposes see B.2.8.

The detection of fragments with a size of 123 bp indicates that the sample DNA solution contains amplifiable DNA of CaMV 35S promoter origin within the assessed limitations of specificity described in B.2.2.2.

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

B.3 Screening method for the detection of genetically modified plant DNA (*Agrobacterium tumefaciens* NOS-terminator)

B.3.1 General

This is a method for the detection of a variable copy number DNA sequence from the *Agrobacterium tumefaciens* nopaline synthase (NOS) terminator. Due to the presence of the NOS-terminator in many genetically modified plants, this method may be used to screen for the presence of GM-plant-derived components [22], [23], [29], [30].

No tool to verify the identity of the PCR product is described. Therefore this method cannot be considered as an identification method. It may be used to assess the amplifiability of DNA containing the target sequence.

B.3.2 Validation status and performance criteria

B.3.2.1 Collaborative study

The method has been validated following the criteria specified in ISO 5725-2. The collaborative study involved 23 European laboratories and was coordinated by the EU JRC [29], [30]. The method has been evaluated for detection of GMOs in various processed food matrices (cooked maize grit, infant formula, biscuits, acidified soya bean meal) containing each 0 %, 2 %, and 100 % (10 % instead of 100 % in the case of biscuits) of either GTS 40-3-2 or Event 176. Since Event 176 does not contain NOS-terminator sequences, samples containing Event 176 should not be evaluated with this method. However since the collaborative study was organized in conjunction with the 35S method, all samples were submitted and evaluated by the laboratories. The cooked maize grits results were excluded from statistical analysis at a later stage.

Each participant received 4 control samples and 30 unknown independent duplicates, of which 10 corresponded to 0 % GMO samples and 20 contained various percentages of the genetically modified events. All participants received a detailed method description for DNA extraction with either the CTAB method or a commercially available kit. However, the laboratories were free to apply their method of choice for DNA extraction while the PCR conditions had to be optimized specifically for their local equipment. The laboratories were asked to analyse each sample once and to specify whether it was considered to be GMO positive or negative. Since most laboratories returned correct results (14 laboratories reported between 90 % and 100 % of correct scores; and 3 between 80 % and 90 % of correct scores) and none provided correct results in the range of 70 % to 80 %, the cut-off level was set at 80 % of correctly reported results. As a consequence, 5 laboratories were excluded from further statistical analysis. Since Event 176 does not contain NOS-terminator sequences, all analytical results from the cooked maize grits preparations should be negative. The results from the cooked maize grits samples had a high percentage (100 %) of correct results and were excluded from statistical evaluation.

An average of 98,2 % correct results was obtained for non-GMO-containing samples (1,8 % false positive results) and an average of 97,9 % correct results for GMO-containing samples (2,1 % false negative results) [29]. The data are listed in Table B.7.

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Table B.7 — Results of the collaborative study

Year	1999
Number of laboratories	30
Number of laboratories submitting results	18
Number of samples per laboratory	12
Number of total samples	360
Number of accepted results	540
False positive results	1,8 %
False negative results	2,1 %

B.3.2.2 Molecular specificity

B.3.2.2.1 General

This annex fulfils the requirements as outlined in Clause 7.

The method has been designed to target the *Agrobacterium tumefaciens* nopaline synthase terminator sequence described in GenBank® database accession No. V00087.

A false positive result can occur since the amplified sequence is derived from *Agrobacterium,* which is a soil bacterium present in nature. Positive results may indicate the presence of a GM-plant-derived product but shall not be interpreted without additional confirmation. The potential contamination of the material with *Agrobacterium* or related bacteria should be considered.

B.3.2.2.2 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). Note that reverse primer match 100 % to AF015682 Rice ragged stunt virus polymerase. Both primers match a long list of accessions referable to cloning vectors and patents, as well as nopaline synthase.

B.3.2.2.3 Experimental

No amplification has been observed using DNA from non-GM crop-plants and derived processed food matrices in performance tests before the collaborative study [30].

B.3.2.3 Limit of detection (LOD)

The absolute limit of detection has not been determined, but this method has been demonstrated to detect 50 copies of GTS 40-3-2 DNA [29].

In a collaborative study, 2 % of GTS 40-3-2 (Roundup Ready® soya beans) were detected in biscuits, infant formula and acidified soya beans with at least 96,4 % of correct results [29].

B.3.3 Adaptation

No specific information is available

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B.3.4 Principle

A 118 bp DNA fragment from the NOS-terminator sequence is amplified by PCR and detected by gel electrophoresis. The identity of the PCR product can be verified, for example by DNA sequencing. However, no verification procedure has been validated.

B.3.5 Reagents

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

B.3.5.1 Water

B.3.5.2 PCR buffer, $c(MgCl_2)$ = 15 mmol/l, 10×.

B.3.5.3 dNTP solution, c(dNTP) = 4 mmol/l (each).

B.3.5.4 Oligonucleotides

B.3.5.4.1 Forward primer

Agrobacterium tumefaciens NOS-terminator, HA-nos118f: 5'- gCA TgA CgT TAT TTA TgA gAT ggg-3'.

Designed to amplify a sequence described in accession No. V00087.

B.3.5.4.2 Reverse primer

Agrobacterium tumefaciens NOS-terminator, HA-nos118r: 5'-gAC ACC gCg CgC gAT AAT TTA TCC-3'.

Designed to amplify a sequence described in accession No. V00087.

B.3.5.5 Thermostable DNA polymerase (for hot-start PCR), 5 IU/µl.

B.3.6 Apparatus and equipment

As specified in B.1.6.

B.3.7 Procedure (PCR set-up)

B.3.7.1 General

The method is described for a total PCR volume of 25 µl per reaction mixture with the reagents as listed in Table B.8. 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.8 have proven to be suitable.

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Table B.8 — Addition of reagents

Reagent	Final concentration	Volume per sample (µl)
Sample-DNA		5
Water		14,84
10 × PCR buffer (with $MgCl_2$, 15 mmol/l)[a]	1 ×	2,5
dNTP solution, 16 mmol/l	0,64 mmol/l	1
Primer HA-nos118f, 20 µmol/l	0,6 µmol/l	0,75
Primer HA-nos118r, 20 µmol/l	0,6 µmol/l	0,75
Taq DNA polymerase, 5 IU/µl	0,8 IU	0,16

[a] If PCR buffer without $MgCl_2$ is used, the volumes and concentrations should be adjusted accordingly.

B.3.7.2 PCR controls

As a positive control, certified reference materials of GTS 40-3-2 (material containing 0,1 % of genetically modified plant ingredients), 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.

B.3.7.3 Temperature-time programme

The temperature-time programme as outlined in Table B.9 has been used for the validation study using the Perkin Elmer 2400/9600/9700 thermal cycler systems and AmpliTaq Gold® DNA polymerase[14]. 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 carefully adhered to unless the protocol states otherwise.

Table B.9 — Temperature-time programme

Activation/initial denaturation	10 min/95 °C
Amplification	25 s/95 °C 30 s/62 °C 45 s/72 °C
Number of cycles	50
Final extension	7 min/72 °C

B.3.8 Identification

It is recommended to verify the identity of the PCR product derived from the unknown sample by, for example, restriction, DNA sequencing or DNA hybridization.

14) GeneAmp® 2400, 9600 and 9700, and AmpliTaq Gold® polymerase are examples of suitable products available commercially from Applied Biosystems, previously known as 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.

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B.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 certified reference material containing the target sequence (e.g. IRMM-410 series from IRMM, Geel, Belgium).

For identification purposes, see B.3.8.

The detection of fragments with a size of 118 bp indicates that the sample DNA solution contains amplifiable DNA of NOS-terminator origin within the assessed limitations of specificity described in B.3.2.2.

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

B.4 Screening method for the detection of genetically modified plant DNA (*npt* II gene)

B.4.1 General

This is a method for the detection of a gene coding for the neomycin phosphotransferase (*npt* II). Due to insertion of this gene in the integrated constructs in many genetically modified plants, this genetic element may be used to screen for GMO-derived plant materials.

B.4.2 Validation status and performance criteria

B.4.2.1 Collaborative study

The method was validated in an collaborative study with raw material [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.10.

Table B.10 — Results of the collaborative study

Sample	Zeneca tomato
Primer	APH2 short/APH2 reverse
Year	1998
Number of laboratories	10
Number of laboratories submitting results	9
Number of samples per laboratory	5
Number of accepted results	45
Number of samples containing the neomycin phosphotransferase gene (Zeneca tomato)	22
False positive results	0 (0 %)
False negative results	0 (0 %)

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B.4.2.2 Molecular specificity

B.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® database accession No. AF269238.

The neomycin phosphotransferase derived from *E. coli* K12 and is present in several genetically modified organisms.

The *npt* II gene is derived from *E. coli* K12 and is present in several genetically modified organisms.

A false positive result can occur since the target sequence is derived from *E. coli* K12. Positive results should not be interpreted as proof of the presence of a GM-plant-derived product.

B.4.2.2.2 Theoretical

No sequence homology with DNA sequences of non-genetically modified crop plants was found in databank searches (NCBI BlastN® search, EMBL database, September 28th, 2001). The primers retrieved only the Tn5 transposon, and synthetic and patented sequences.

B.4.2.2.3 Experimental

No amplification has been observed using DNA from non-genetically modified crop plants and derived processed food matrices.

B.4.2.3 Limit of detection (LOD)

Validation has only been performed with 0 % and 100 % GM material.

B.4.3 Adaptation

No specific information is available.

B.4.4 Principle

A 215 bp DNA fragment from the neomycin phosphotransferase gene sequence is amplified by PCR and detected by gel electrophoresis. The identity of the PCR product may be verified by, for example, restriction.

Neomycin phosphotransferase yields bacterial resistance to neomycin/kanamycin antibiotics and the gene has been introduced only as a marker gene.

B.4.5 Reagents

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

B.4.5.1 Water

B.4.5.2 PCR buffer, $c(MgCl_2)$ = 15 mmol/l, 10×.

B.4.5.3 dNTP solution, c(dNTP) = 2,5 mmol/l (each).

B.4.5.4 Oligonucleotides

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B.4.5.4.1 Forward primer

APH2 short: 5'-CTC ACC TTg CTC CTg CCg AgA-3'.

B.4.5.4.2 Reverse primer

APH2 reverse: 5'-CgC CTT gAg CCT ggC gAA CAg -3'.

B.4.5.5 Thermostable DNA polymerase (for hot-start PCR), 5 IU/µl.

B.4.5.6 Restriction enzyme: *Rsa* I

B.4.6 Apparatus and equipment

As specified in B.1.6.

B.4.7 Procedure (PCR set-up)

B.4.7.1 General

The method is described for a total PCR volume of 25 µl per reaction mixture with, for example, the volumes as listed in Table B.11. 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.11 have proven to be suitable.

Table B.11 — Addition of reagents

Reagent	Final concentration	Volume per sample (µl)
Sample-DNA		5
Water		14,6
10 × PCR buffer (with $MgCl_2$ 15 mmol/l)	1 ×	2,5
dNTP solution, 10 mmol/l	0,2 mmol/l	0,5
Primer APH2 short, 10 µmol/l	0,4 µmol/l	1
Primer APH2 reverse, 10 µmol/l	0,4 µmol/l	1
Taq DNA polymerase, 5 IU/µl	2 IU	0,4

B.4.7.2 PCR controls

No reference material is commercially available.[15)]

B.4.7.3 Temperature-time programme

The temperature-time programme as outlined in Table B.12 has been used for the validation study using thermal cyclers GeneAmp® 2400 or GeneAmp® 9600 and AmpliTaq Gold® DNA polymerase[16)]. 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.

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

16) GeneAmp® 2400 and 9600 and AmpliTaq Gold® polymerase are examples of suitable products available commercially from Applied Biosystems, previously known as 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.

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Table B.12 — Temperature-time programme

Activation/initial denaturation	**10 min/95 °C**
Amplification	25 s/95 °C 30 s/60 °C 45 s/72 °C
Number of cycles	35
Final extension	7 min/72 °C

B.4.8 Identification

It is recommended to verify the identity of the PCR product derived from the unknown DNA-sample by, for example, restriction, DNA sequencing or DNA hybridization. Restriction of the PCR product with *Rsa* I should yield two fragments (122 and 93 bp, respectively) [24].

B.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 appropriate reference material (e.g. a commercial plasmid containing the target DNA sequence).

For identification purposes, see B.4.8.

The detection of fragments with a size of 215 bp indicates that the sample DNA solution contains amplifiable DNA of *npt* II origin within the assessed limitations of specificity described in B.4.2.2.

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

B.5 Screening method for the detection of DNA derived from genetically modified tomato (Zeneca® F282)

This method is described in detail in A.3.

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Annex C
(informative)

Construct-specific methods

C.1 Construct-specific method for the detection of modified DNA sequences from genetically modified GTS 40-3-2 (Roundup Ready® soya beans)

C.1.1 General

This is a method for the detection of genetically modified glyphosate resistant GTS 40-3-2 (Roundup Ready®17)) soya beans in raw/processed materials [8], [11] by amplification of a 172 bp single copy sequence representing the junction region between the CaMV 35S promoter and the *Petunia hybrida* chloroplast targeting signal preceding the *Agrobacterium* EPSPS sequence.

The same construct has been used in other GMOs.

It is not possible to use this method to distinguish between GTS 40 3-2 and gene-stacked cultivars originating from a cross between GTS 40 3-2 and other soyabean event(s), except on single kernels and plants where the presence/absence of sequences derived from other events can be verified.

C.1.2 Validation status and performance criteria

C.1.2.1 Collaborative study

The method has been validated in a collaborative study [8] under 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 from the collaborative study are listed in Table C.1.

Table C.1 — Results of the collaborative study

Year	1998
Number of laboratories	25
Number of laboratories submitting results	24
Number of samples per laboratory	5
Number of accepted results	105
Number of samples containing GTS 40-3-2	56
Number of samples containing non-GM soya beans	49
False positive results	0 (0 %)
False negative results	0 (0 %)

17) Roundup Ready is a registered trademark of Monsanto. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

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C.1.2.2 Molecular specificity

C.1.2.2.1 General

This annex fulfils the requirements as outlined in Clause 7.

The method is described in Reference [8]. Information on the genetic construct introduced into the soya bean genome is available in Reference [31].

C.1.2.2.2 Theoretical

No sequence homology with DNA sequences of non-genetically modified soya beans and other crop plants has been found in databank searches (GenBank® database; BlastN® 2.2.1 search July 1st, 2001). Moreover the primer set was designed to amplify a DNA sequence specific for an artificial junction region not expected to occur in nature.

C.1.2.2.3 Experimental

No amplification has been observed using DNA from non-GM soya beans, potatoes, tomatoes, maize and sugar beets [32] or from the genetically modified maize-lines Event 176 (Bt 176), Bt 11, T 25 and MON 810.

C.1.2.3 Limit of detection (LOD)

Based on the assumption that there is only one copy of the genetic construct per haploid genome (AGBIOS database: http://www.agbios.com/) and that the haploid genome size of soya bean is $1,13 \times 10^9$ bp (see Reference [5]), the absolute LOD with 50 ng DNA from soya bean, with a relative GMO content of a mass fraction of 0,1 % in ground seeds is 40 haploid genome equivalents [32].

The relative LOD has been determined to be better than or equal to a mass fraction of 0,1 % with soya beans in soya bean flour (certified reference materials IRMM-410R produced by IRMM, Geel, Belgium) [9]. With this method, soya bean flour containing 0,45 % GTS 40-3-2 has also been shown to be detectable after baking [33].

C.1.3 Adaptation

No specific information is available.

C.1.4 Principle

The glyphosate tolerance of GTS 40-3-2 soya bean is due to a genetic construct coding for enolpyruvylshikimi-3-phosphate synthase (EPSPS) from the *Agrobacterium* sp. strain CP4 joined to the chloroplast transfer peptide sequence originating from *Petunia hybrida* (transit-signal sequence, CTP for transition of EPSPS into the chloroplasts). Glyphosate inhibits the EPSPS in plants. A 172 bp DNA fragment, spanning the junction between the CaMV 35S promoter sequence and the CTP sequence is amplified by PCR and detected by gel electrophoresis. For identification of the PCR product, a hybridization probe is described and may be used.

C.1.5 Reagents

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

C.1.5.1 Water

C.1.5.2 PCR buffer (without $MgCl_2$), 10×.

C.1.5.3 $MgCl_2$ solution, $c(MgCl_2)$ = 25 mmol/l.

C.1.5.4 dNTP solution, $c(dNTP)$ = 2,5 mmol/l (each).

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C.1.5.5 Oligonucleotides

C.1.5.5.1 Forward primer

35s-f2: 5'- TgA TgT gAT ATC TCC ACT gAC g -3'.

Accession No. (GenBank®): V00141, J02048.

C.1.5.5.2 Reverse primer

petu-r1: 5’- TgT ATC CCT TgA gCC ATg TTg T -3'.

Accession No. (GenBank®): M21084, J03227.

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

C.1.5.7 Hybridization probe

H-35s-ar1: 5'- ggg TCT TgC gAA ggA TAg Tg-3'.

C.1.5.8 Prehybridization solution, containing 5 × SSC, 0,1 % (mass concentration) *N*-lauroylsarcosine, 0,02 % (mass concentration) SDS, 1 % Blocking Reagent.[8]

C.1.5.9 Hybridization solution, containing 10 pmol hybridization probe in 2,5 ml prehybridization solution (C.1.5.8). The hybridization temperature is 50 °C. Further information on conditions for hybridization are given in Reference [12].

C.1.6 Apparatus and equipment

C.1.6.1 Thermal cycler

C.1.6.2 Gel electrophoresis chamber, with power supply.

C.1.6.3 Hybridization apparatus

C.1.7 Procedure

C.1.7.1 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 C.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 C.2 have proven to be suitable.

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Table C.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 35s-f2, 5 µmol/l	0,2 µmol/l	1
Primer petu-r1, 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.

C.1.7.2 PCR controls

As a positive control, certified reference materials of GTS 40-3-2 (material containing 0,1 % of genetically modified plant ingredients), 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.

C.1.7.3 Temperature-time programme

The temperature-time programme as outlined in Table C.3 has been used for the validation study using thermal cyclers GeneAmp™ 2400 or GeneAmp® 9600 and AmpliTaq Gold® DNA polymerase[18]. The use of other thermal cyclers might make an adaptation necessary. The time for activation/initial denaturation depends on the polymerase used.

Table C.3 — Temperature-time programme

Activation/initial denaturation	10 min/95 °C
Amplification	30 s/95 °C 30 s/60 °C 25 s/72 °C
Number of cycles	35 to 40
Final extension	3 min/72 °C

C.1.8 Identification

The specificity of the amplified product can be demonstrated by Southern hybridization using a fluorescein-labelled oligonucleotide probe H35s-ar1 (C.1.5.7 to C.1.5.9). Non-genetically modified samples shall be negative in the hybridization assay [8].

18) GeneAmp® 2400 and 9600 and AmpliTaq Gold® polymerase are examples of suitable products available commercially from Applied Biosystems, previously known as 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.

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C.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 material prepared from GTS 40-3-2 (e.g. IRMM-410 series from IRMM, Geel, Belgium).

For identification purposes, see C.1.8.

The detection of fragments with a size of 172 bp indicates that the sample DNA solution contains amplifiable DNA of GTS 40-3-2 origin within the assessed limitations of specificity described in C.1.2.2. For details on electrophoretic stages, see a ISO 21571:2005, B.2.

C.2 Construct-specific method for the detection of modified DNA sequences from genetically modified tomatoes (Zeneca® F282)

C.2.1 General

This is a method for the detection of genetically modified delayed ripening tomatoes (Zeneca) in raw materials by PCR amplification of the junction region between single copy sequence elements originating from the *Agrobacterium tumefaciens* (NOS-terminator) and the polygalacturonase (PG) gene from *Lycopersicon esculentum* Mill which have been joined by means of *in vitro* recombination.

The same construct might be used in other GMOs in the future.

It is not possible to use this method to distinguish between Zeneca 282F tomato and gene-stacked cultivars originating from a cross between Zeneca 282F and other tomato event(s), except on single kernels and plants where the presence/absence of sequences derived from other events can be verified.

C.2.2 Validation status and performance criteria

C.2.2.1 Collaborative study

The method was validated in a collaborative study [15], under 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 SO 21571:2005, A.3, was used (but with a test portion of 100 mg).

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

Table C.4 — Results of collaborative study

Year	**1999**
Number of laboratories	18
Number of laboratories submitting results	18
Number of samples per laboratory	5
Number of accepted results	90
Number of samples containing genetically modified tomatoes (Zeneca 282F)	43
Number of samples containing non-GM tomatoes (Zeneca 282C)	47
False positive results	0 (0 %)
False negative results	0 (0 %)

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C.2.2.2 Molecular specificity

C.2.2.2.1 General

This annex fulfils the requirements as outlined in Clause 7.

The method is described in References [15] and [16]. Information on the genetic construct introduced into the tomato genome is available in Reference [18].

C.2.2.2.2 Theoretical

No significant similarity with sequences of non-GM tomatoes or other crop plants has been observed in databank searches (GenBank® database; BlastN® 2.2.1 search, July 1st, 2001). Moreover, the primer set was designed to amplify a DNA sequence specific for an artificial junction region not expected to occur in nature.

C.2.2.2.3 Experimental

No amplification was observed using DNA from non-GM tomatoes with similar phenotypes: *Long-Life*-tomatoes of type Selfesta F1, Seduro F1, Lioba F1 and Harzglut F1 (seed production Quedlinburg, Germany)[15].

C.2.2.3 Limit of detection (LOD)

Based on the assumption that there is only one copy of the genetic construct per haploid genome (AGBIOS database: http://www.agbios.com/) and that the haploid genome size of tomato is 1,0 x 10^9 bp (see Reference [5]), the absolute LOD with 10 pg DNA from tomato, with a relative GMO content of a mass fraction of 100 % in (raw tomatoes) is 10 haploid genome equivalents [19].

C.2.3 Adaptation

For the identification of a genetic alteration in tomato paste, it is advisable to extract nucleic acid from five times the quantity specified in ISO 21571. After combination of the extracted nucleic acid, a subsequent purification step using the QIAquick PCR Purification Kit[19)] will result in a sufficient amount of DNA suitable for PCR.

C.2.4 Principle

The trait of the genetically modified tomato (*Lycopersicon esculentum* Mill.) developed by Zeneca is the fruit ripening delay on the basis of the inhibited production of polygalacturonase (PG).

The genetic modification from the genetically modified tomatoes is based on the introduction of an additional incomplete polygalacturonase gene (PG) as cDNA in the genome. The presence of this gene results in the drastic reduction of the endogenous tomato PG-enzyme. This enzyme is principally responsible for the softening of tomatoes [17].

This method amplifies a 350 bp DNA fragment spanning the artificial junction between a segment of the cDNA fragment of the PG gene and the adjacent NOS-terminator sequence that is only present in genetically modified tomatoes. The resulting PCR product is detected by gel electrophoresis. For identification purposes, a specific hybridization probe is described and may be used.

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19) QIAquick PCR Purification Kit is the trade name of a product supplied by QIAGEN, Hilden, Germany. 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 lead to the same results.

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C.2.5 Reagents

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

C.2.5.1 Water

C.2.5.2 PCR buffer (without $MgCl_2$), 10×.

C.2.5.3 $MgCl_2$ solution, $c(MgCl_2)$ = 25 mmol/l.

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

C.2.5.5 Oligonucleotides

C.2.5.5.1 Forward primer

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

Accession No. X04583.

C.2.5.5.2 Reverse primer

t-NOS: 5'- CAT CgC AAg ACC ggC AAC Ag-3'.

Accession No. NC002147.

C.2.5.6 Thermostable DNA polymerase (for hot-start PCR), 5 IU/µl.

C.2.5.7 Hybridization probe, Tomate-2

The Digoxigenin (Dig)-labelled DNA probe (Tomate-2) has the following sequence:

5'-Dig-CCT CTA gAg Tcg ACC TgC Agg TCg-3'.

C.2.5.8 Prehybridization solution, containing 5 × SSC, 0,1 % (mass concentration) of *N*-lauroyl-sarcosine, 0,02 % (mass concentration) of SDS, and 1 % Blocking Reagent [15].

C.2.5.9 Hybridization solution, containing 10 pmol of hybridization probe in 2,5 ml of prehybridization solution (C.2.5.8).

The hybridization temperature is 60 °C. Further information on conditions for hybridization are given in Reference [12].

C.2.5.10 Restriction enzyme: *Eae* I or *Mwo* I.

C.2.6 Apparatus and equipment

As specified in C.1.6.

C.2.7 Procedure

C.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 C.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 C.5 have proven to be suitable.

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Table C.5 — Addition of reagents

Reagent	Final concentration	Volume per sample (µl)
Sample-DNA	10 ng to 50 ng	0,5
Water		17,3
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 each	0,4 mmol/l	1,0
Primer PG34L, 10 µmol/l	0,4 µmol/l	1,0
Primer t-NOS, 10 µmol/l	0,4 µmol/l	1,0
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.

C.2.7.2 PCR controls

No reference material to be used as a positive control is commercially available.[20)]

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

C.2.7.3 Temperature-time programme

The temperature-time programme as outlined in Table C.6 has been used for the validation study using thermal cyclers GeneAmp® 2400 or GeneAmp® 9600 and AmpliTaq Gold® DNA polymerase[21)]. The use of other thermal cyclers might make an adaptation necessary. The time for activation/initial denaturation depends on the polymerase used.

Table C.6 — 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

C.2.8 Identification

The specificity of the amplified product can be demonstrated by Southern hybridization using a digoxigenine-labelled oligonucleotide probe Tomate-2 (C.2.5.7 to C.2.5.9). Non-genetically modified samples shall be negative in the hybridization assay [15].

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

21) GeneAmp® 2400 and 9600 and AmpliTaq Gold® DNA polymerase are examples of suitable products available commercially from Applied Biosystems, previously known as 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.

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The specificity of the amplified product can be demonstrated by restriction analysis using either *Eae* I or *Mwo* I. Digestion with *Eae* I yields two fragments of 126 bp and 224 bp, respectively. Digestion with *Mwo* I yields three fragments of 8 bp, 164 bp and 178 bp, respectively.

C.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, determined by comparison with products derived from appropriate reference material.

For identification purposes, see C.2.8.

The detection of fragments with a size of 350 bp indicates that the sample DNA solution contains amplifiable DNA of Zeneca genetically modified tomato origin within the assessed limitations of specificity described in C.2.2.2. For details on electrophoretic stages, see ISO 21571:2005, B.2.

C.3 Construct-specific method for the detection of modified DNA sequences from genetically modified Bt 11 maize

C.3.1 General

This is a method for the detection of genetically modified *Bacillus thuringiensis* toxin-producing Bt 11 maize (Syngenta, former Novartis) in raw materials by PCR amplification of the junction region of single copy sequence elements originating from the maize adh 1S-Intron2 (IVS2) and the *pat* gene from *Streptomyces viridochromogenes*.

The same construct might be used in the future in other GMOs.

It is not possible to use this method to distinguish between BT11 maize and gene-stacked cultivars originating from a cross between BT11 and other maize event(s), except on single kernels and plants where the presence/absence of sequences derived from other events can be verified.

C.3.2 Validation status and performance criteria

C.3.2.1 Collaborative study

The method has been validated in different collaborative studies [20] under 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 as the number of samples were chosen conforming to the criteria specified in ISO 5725-2. 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-System[22].

22) 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.

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The data from the collaborative study are listed in Table C.7.

Table C.7 — Results of collaborative study

Year	2000
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 11	32
Number of samples containing Event 176 maize	32
Number of samples containing non-GM maize	32
False positive results	3 (5 %)
False negative results	3 (10 %)

In addition, 14 laboratories received DNA samples extracted from genetically modified Bt 11 maize containing 50 ng, 5 ng, 0,5 ng and 0,05 ng DNA. The results are outlined in Table C.8 [20]:

Table C.8 — Results of the collaborative study

DNA amount	Result		Comment
	Correct	False	
50 ng	14	—	
5 ng	14	—	
0,5 ng	12	1	False negative
		1	Ambiguous
0,05 ng	7	5	False negative
		2	Ambiguous

C.3.2.2 Molecular specificity

C.3.2.2.1 General

This annex fulfils the requirements as outlined in Clause 7.

The method has been described in Reference [20].

Information on the DNA construct introduced into the maize genome is available in Reference [34]. The DNA construct is the construct described in EMBL/GenBank® accession No. AR110602 (patented), which contains all the same elements in the same order as reported for Bt 11.

C.3.2.2.2 Theoretical

No sequence homology with DNA sequences of non-genetically modified maize and other crop plants has been found in databank searches (GenBank® database; BlastN® 2.2.1 search July 1st, 2001). Moreover the primer set was designed to amplify a DNA sequence specific for an artificial junction region not occurring in nature.

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C.3.2.2.3 Experimental

No amplification was observed using DNA from non-GM maize, or from genetically modified GTS 40-3-2 (Roundup Ready® soya beans) or maize-lines Event 176 maize (Bt 176), T25 and MON 810.

The number of target sequences is one.

C.3.2.3 Limit of detection (LOD)

Based on the assumption that there is only one copy of the genetic construct per genome (AGBIOS database: http://www.agbios.com/) and that the genome size of maize is 2,65 x 10^9 bp (see Reference [5]), the absolute LOD with 50 ng DNA from maize with a relative GMO content of 0,1 % in ground seeds is 20 genome equivalents [34]. The relative LOD is better than or equal to 0,1 % in ground maize seeds [34].

C.3.3 Adaptation

No specific information is available.

C.3.4 Principle

The Bt gene originates from the soil bacterium *Bacillus thuringiensis*; the protein thus produced in the plant tissue protects it from being attacked by European corn borer larvae. The Bt protein becomes active in the intestine of these insects, causes pores to be formed in the cell membrane, and leads to a disruption in the osmotic balance resulting in cell lysis.

The *pat* gene originates from the soil bacterium *Streptomyces viridochromogenes* and codes for the enzyme phosphinothricin-*N*-acetyltransferase which renders the plant tolerant to the herbicide glufosinate ammonium. Glufosinate ammonium disrupts the synthesis of glutamine in plants. A 189 bp DNA fragment spanning the junction between the *adh* intron IVS2 and the *pat* gene sequence is amplified by PCR and detected by gel electrophoresis. For identification of the PCR product, a hybridization probe is described and may be used.

C.3.5 Reagents

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

C.3.5.1 Water

C.3.5.2 PCR buffer (without $MgCl_2$), 10×.

C.3.5.3 $MgCl_2$ solution, $c(MgCl_2)$ = 25 mmol/l.

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

C.3.5.5 Oligonucleotides

C.3.5.5.1 Forward primer

Intron IVS2-2: 5'-CTg ggA ggC CAA ggT ATC TAA T-3'.

Accession No. AR110602.

C.3.5.5.2 Reverse primer

PAT protein coding region, PAT-B: 5'-gCT gCT gTA gCT ggC CTA ATC T-3'.

Accession No. AR110602.

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

C.3.5.7 Hybridization probe, 5'-labelled (e.g. digoxygenine-labelled) probe Bt: 5'-TAT CTg TCT CAg ggg CAg ACT C-3'; c = 20 µmol/l.

C.3.5.8 Prehybridization solution, containing 5 × SSC, 0,1 % (mass concentration) of *N*-lauroyl-sarcosine, 0,02 % (mass concentration) of SDS, and 1 % Blocking Reagent.

C.3.5.9 Hybridization solution, containing 10 pmol of hybridization probe in 2,5 ml of prehybridization solution (C.3.5.7). The hybridization temperature is 60 °C. Further information on conditions for hybridization are given in Reference [12].

C.3.5.10 Restriction enzyme

*Hin*f I.

C.3.6 Apparatus and equipment

As specified in C.1.6.

C.3.7 Procedure

C.3.7.1 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 C.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 C.9 have proven to be suitable.

Table C.9 — Addition of reagents

Reagent	Final concentration	Volume per sample (µl)
Sample DNA	10 ng to 50 ng	1
Water		15,8
10 × PCR buffer (without $MgCl_2$)	1 ×	2,5
$MgCl_2$-solution[a], 25 mmol/l	2 mmol/l	2,0
dNTP solution, 10 mmol/l	0,4 mmol/l	1,0
Primer IVS2-2, 10 µmol/l	0,5 µmol/l	1,25
Primer PAT-B, 10 µmol/l	0,5 µmol/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 $MgCl_2$ reaction mixture is adjusted to 2 mmol/l.

C.3.7.2 PCR controls

As a positive control, certified reference material, for example, material containing 1 % genetically modified Bt 11 maize produced by IRMM, Geel, Belgium (IRMM-412), may be used.

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

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C.3.7.3 Temperature-time programme

The temperature-time programme as outlined in Table C.10 has been used for the validation study using thermal cyclers GeneAmp® 2400 or GeneAmp® 9600 and AmpliTaq Gold® DNA polymerase[23)]. The use of other thermal cyclers might make an adaptation necessary. The time for activation/initial denaturation depends on the polymerase used.

Table C.10 — Temperature-time programme

Activation/initial denaturation	**12 min/95 °C**
Amplification	30 s/95 °C 30 s/64 °C 30 s/72 °C
Number of cycles	38
Final extension	10 min/72 °C

C.3.8 Identification

The identity of the amplified product can be verified by Southern hybridization using a digoxygenine-labelled oligonucleotide probe Bt (C.3.5.7 to C.3.5.9). Non-genetically modified samples shall be negative in the hybridization assay [20].

The identity of the amplified product may be demonstrated by restriction analysis using *Hin*f I yielding two fragments of 116 bp and 73 bp, respectively [20].

C.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 certified reference material prepared from Bt11 maize (e.g. IRMM-412 series from IRMM, Geel, Belgium).

For identification purposes, see C.3.8.

The detection of fragments with a size of 189 bp indicates that the sample DNA solution contains amplifiable DNA of Bt11 maize origin within the assessed limitations of specificity described in C.3.2.2.

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

C.4 Construct-specific method for the detection of modified DNA sequences from genetically modified Event 176 maize (Bt 176 maize)

C.4.1 General

This is a method for the detection of genetically modified Event 176 maize (Syngenta) in raw/processed materials by PCR amplification of an artificial junction region between two copies of the genetic construct integrated into the plant genome. The maize has been modified to produce the Bt toxin (type cryIA(b)) from *Bacillus thuringiensis* by insertion of a synthetic Bt gene regulated by a CDPK promoter from *Zea mays*.

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23) GeneAmp® 2400 and 9600 and AmpliTaq Gold® polymerase are examples of suitable products available commercially from Applied Biosystems, previously known as 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.

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The same construct might be used in the future in other GMOs.

Gene-stacked cultivars cannot be distinguished by this method except on single kernels and plants.

C.4.2 Validation status and performance criteria

C.4.2.1 Collaborative study

The method has been validated in different collaborative studies [20] under 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 as the number of samples followed the criteria of ISO 5725-2. 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-System[24].

The data from the collaborative study are listed in Table C.11.

Table C.11 — Results of collaborative study

Year	2000
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 Event 176 maize	32
Number of samples containing Bt 11 maize	32
Number of samples containing non-GM maize	32
False positive results	0 (0 %)
False negative results	0 (0 %)

In addition, 13 laboratories received DNA samples extracted from a mass fraction of 0,1 % genetically modified Event 176 maize (Bt 176 maize) in dried maize powder [mass fraction] (certified reference materials, CRMs, prepared by the IRMM, Geel, Belgium). Twelve laboratories determined the sample as positive for Event 176 (Bt 176) and one laboratory obtained ambiguous results in duplicate determination [20].

C.4.2.2 Molecular specificity

C.4.2.2.1 General

This annex fulfils the requirements as outlined in Clause 7.

The method has been described in References [20] and [35].

Information on the genetic construct introduced into the maize genome is available in Reference [35].

24) 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.

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C.4.2.2.2 Theoretical

No sequence homology with DNA sequences of non-GM maize and other crop plants has been found in databank searches (GenBank® database; BlastN® 2.2.1 search July 1st, 2001). Moreover the primer set was designed to amplify a DNA sequence specific for an artificial junction region not occurring in nature.

C.4.2.2.3 Experimental

No amplification has been observed using DNA from GM soya beans (GTS 40-3-2) and maize lines Bt 11, T25 and MON810 [11], or from non-GM maize [11], [20].

The number of sequence copies is two.

C.4.2.3 Limit of detection (LOD)

Based on the assumption that there are two copies of the genetic construct per genome (AGBIOS database: http://www.agbios.com/) and that the genome size of maize is $2,65 \times 10^9$ bp (see Reference [5]), the absolute LOD with 50 ng DNA from maize, with a relative GMO content of 0,1 % in ground seeds is 20 genome equivalents [34]. The relative LOD is better than or equal to 0,1 % in ground maize seeds [34].

C.4.3 Adaptation

No specific information is available.

C.4.4 Principle

Bacillus thuringiensis toxin (Bt) is an insecticide of bacterial origin. Genetically modified plants containing the Bt gene produce the gene product as an endogeneous pesticide. Event 176 maize (Bt 176 maize) contains a synthetic Bt gene of the type CryIA(b) under the control of the CDPK6 promoter. A 211 bp DNA fragment, spanning the junction between the CDPK6 promoter and the Bt gene sequence is amplified by PCR and the PCR product is detected by gel electrophoresis. For identification purposes, a hybridization probe is described and may be used.

C.4.5 Reagents

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

C.4.5.1 Water

C.4.5.2 PCR buffer (without $MgCl_2$), 10×.

C.4.5.3 $MgCl_2$ solution, $c(MgCl_2)$ = 25 mmol/l.

C.4.5.4 dNTP solution, c(dNTP) = 2,5 mmol/l (each).

C.4.5.5 Oligonucleotides

C.4.5.5.1 Forward primer

Cry03: 5'-CTC TCg CCg TTC ATg TCC gT-3'.

Accession No. is not available. Primer is located in the CDPK6 promoter. Primer matches 100 % with the maize CDPK, accession No. L27484.1.

C.4.5.5.2 Reverse primer

Cry04: 5'-ggT CAg gCT Cag gCT gAT gT-3'.

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Accession No. is 41419 (according to Reference [36]). Primer is located in the synthetic CryIA(b) gene.

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

C.4.5.7 Hybridization probe (Cry01)

5'-ATg gAC AAC AAC CCC AAC ATC-3.'

C.4.5.8 Prehybridization solution, containing 5 × SSC, 0,1 % (mass concentration) of *N*-lauroyl-sarcosine, 0,02 % (mass concentration) of SDS, and 1 % of Blocking Reagent.

C.4.5.9 Hybridization solution, containing 10 pmol of hybridization probe in 2,5 ml of prehybridization solution (C.4.5.8).

The hybridization temperature is 50 °C. Further information on conditions for hybridization are given in Reference [12].

C.4.6 Apparatus and equipment

As specified in C.1.6.

C.4.7 Procedure

C.4.7.1 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 C.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 C.12 have proven to be suitable.

Table C.12 — Addition of reagents

Reagent	Final concentration	Volume per sample (µl)
Sample DNA	10 ng to 50 ng	2
Water		15,4
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	1,0
Primer Cry03, 5 µmol/l	0,25 µmol/l	1,25
Primer Cry04, 5 µmol/l	0,25 µmol/l	1,25
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.

C.4.7.2 PCR controls

As a positive control, certified reference material [0,1 % genetically modified Event 176 maize (Bt 176)], for example, produced by IRMM, Geel, Belgium (IRMM-411, MZ-0,1), may be used.

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

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C.4.7.3 Temperature-time programme

The temperature-time programme as outlined in Table C.13 has been used for the validation study using thermal cyclers GeneAmp® 2400, 9600 and AmpliTaq Gold® DNA polymerase[25]. 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 recommendations of the manufacturer should be adhered to unless the protocol states otherwise.

Table C.13 — Temperature-time programme

Activation/initial denaturation	**12 min/95 °C**
Amplification	30 s/95 °C 30 s/63 °C 30 s/72 °C
Number of cycles	38
Final extension	6 min/72 °C

C.4.8 Identification

The identity of the amplified product may be verified by Southern hybridization using a digoxygenine-labelled oligonucleotide probe Cry01 (C.4.5.7 to C.4.5.9). Non-genetically modified samples shall be negative in the hybridization assay [20].

The identity of the amplified product may be verified by restriction analysis using either *Hae* III, *Taq* I or *Dde* I. Digestion with *Hae* III yields two fragments of 162 and 49 bp, respectively. Digestion with *Taq* I yields three fragments of 168, 22 and 21 bp, respectively [20]. Digestion with *Dde* I yields three fragments of 128, 72 and 11 bp, respectively [20].

C.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 Event 176 (Bt 176) maize (e.g. IRMM-411 series from IRMM, Geel, Belgium).

For identification purposes, see C.4.8.

The detection of fragments with a size of 211 bp indicates that the sample DNA solution contains amplifiable DNA of Event 176 (Bt 176) maize origin within the assessed limitations of specificity described in C.4.2.2.

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

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25) GeneAmp® 2400 and 9600 and AmpliTaq Gold® polymerase are examples of suitable products available commercially from Applied Biosystems, previously known as 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.

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C.5 Construct-specific method for the detection of modified DNA sequences from genetically modified T 25 maize

C.5.1 General

This is a method for the detection of genetically modified herbicide-resistant T25 maize/“LibertyLink” in raw materials by PCR amplification of the single copy junction region of DNA sequences originating from the CaMV 35S promoter and the *pat* gene that have been joined by means of *in vitro* recombination.

The same construct might be used in the future in other GMOs.

Gene-stacked cultivars cannot be distinguished by this method except on single kernels and plants.

C.5.2 Validation status and performance criteria

C.5.2.1 Collaborative study

The method has been validated in a collaborative study [20] under 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.

Samples from flour (ground kernels) of T25 (0,1 %, 1 %), MON 810 (0,1 %, 1 %) and non-GMO maize were prepared for the collaborative study.

The data of the collaborative study are listed in Tables C.14 and 15.

Table C.14 — Results of collaborative study

Year	**2001**
Number of laboratories	16
Number of laboratories submitting results	16
Number of samples per laboratory	5
Number of accepted results	75
Number of samples containing T25	33
Number of samples containing MON810	31
Number of samples containing non-GM maize	11
False positive results	0 (0 %)
False negative results	4 (12 %)

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Table C.15 — Detailed results of the collaborative study

Kind of sample	Number of samples	Correct	False
T25 negative samples:			
0 % GMO	11	11	0
0,1 % MON 810	13	13	0
1 % MON 810	18	18	0
T25 positive samples:			
0,1 % T25	18	15	3 (neg)[a]
1 % T25	15	14	1 (neg)

[a] All false negative results were obtained in one laboratory.

C.5.2.2 Molecular specificity

C.5.2.2.1 General

This annex fulfils the requirements as outlined in Clause 7.

The method has been described in Reference [20].

NOTE Sequence information for the development of this method was provided by Bayer Crop Science (formerly Aventis CropScience).

Information on the genetic construct introduced into the maize genome is available in Reference [37].

C.5.2.2.2 Theoretical

No sequence homology with DNA sequences of non-GM maize and other crop plants has been found in databank searches (GenBank® database; BlastN® 2.2.1 search July 1st, 2001). Moreover the primer set was designed to amplify a DNA sequence specific for an artificial junction region not occurring in nature.

C.5.2.2.3 Experimental

No amplification has been observed using DNA from non-GM maize, from genetically modified GTS 40-3-2 (Roundup Ready®) soya beans, or from Event 176 (Bt 176), Bt 11 and MON 810 GM maize lines.

The number of sequence copies is one.

C.5.2.3 Limit of detection (LOD)

Based on the assumption that there is only one copy of the genetic construct per genome (AGBIOS database: http://www.agbios.com/) and that the genome size of maize is $2,65 \times 10^9$ bp (see Reference [5]) the absolute LOD with 50 ng DNA from maize, with a relative GMO content of 0,1 % in ground seeds is 20 genome equivalents [34]. The relative LOD is better than or equal to 0,1 % in ground maize seeds [34].

C.5.3 Adaptation

No specific information is available.

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C.5.4 Principle

The *pat* gene originates from the soil bacterium *Streptomyces viridochromogenes* and codes for the enzyme phosphinothricin-*N*-acetyltransferase, which renders the plant tolerant to the herbicide glufosinate ammonium. Glufosinate ammonium disrupts the synthesis of glutamine in plants.

A 209 bp DNA fragment spanning the junction between the CaMV 35S promoter and the *pat* gene sequences is amplified by PCR and detected by gel electrophoresis. For identification of the PCR product, a restriction procedure is described and may be used.

C.5.5 Reagents

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

C.5.5.1 Water

C.5.5.2 PCR buffer (without $MgCl_2$), 10×.

C.5.5.3 $MgCl_2$ solution, $c(MgCl_2)$ = 25 mmol/l.

C.5.5.4 dNTP solution, c(dNTP) = 2,5 mmol/l (each).

C.5.5.5 Oligonucleotides

C.5.5.5.1 Forward primer

T25-F7: 5'-ATg gTg gAT ggC ATg ATg TTg-3'.

Accession No. (GenBank®) is NC001497. Primer is located in the CaMV 35S promoter.

C.5.5.5.2 Reverse primer

T25-R3: 5'- TgA gCg AAA CCC TAT AAg AAC CC -3'.

Accession No. is not available. Primer is located in the PAT protein coding region.

C.5.5.6 Thermostable DNA polymerase (for hot-start PCR), 5 IU/µl.

C.5.5.7 Restriction enzymes: *Hin*f I and *Mw*o I.

C.5.6 Apparatus and equipment

As for C.1.6.1 and C.1.6.2.

C.5.7 Procedure

C.5.7.1 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 C.16. 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 C.16 have proven to be suitable.

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Table C.16 — Addition of reagents

Reagent	Final concentration	Volume per sample (µl)
Sample DNA	10 ng to 50 ng	2
Water		14,8
10 × PCR buffer (without $MgCl_2$)	1 ×	2,5
$MgCl_2$ solution[a], 25 mmol/l	2 mmol/l	2,0
dNTP solution, 10 mmol/l	0,4 mmol/l	1,0
Primer T25-F7, 10 µmol/l	0,5 µmol/l	1,25
Primer T25-R3, 10 µmol/l	0,5 µmol/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 $MgCl_2$ reaction mixture is adjusted to 2 mmol/l.

C.5.7.2 PCR controls

Reference material is not commercially available.[26)]

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

C.5.7.3 Temperature-time programme

The temperature-time programme as outlined in Table C.17 has been used for the validation study using thermal cycler GeneAmp® 2400, 9600 and AmpliTaq Gold® DNA polymerase[27)]. 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 C.17 — Temperature-time programme

Activation/initial denaturation	12 min/95 °C
Amplification	30 s/95 °C 30 s/64 °C 30 s/72 °C
Number of cycles	40
Final extension	10 min/72 °C

C.5.8 Identification

The identity of the amplified product may be verified by restriction analysis using either *Hin*f I or *Mwo* I. Digestion with *Hin*f I yields two fragments of 121 and 88 bp, respectively. Digestion with *Mwo* I yields two fragments of 141 and 68 bp, respectively [20].

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

27) GeneAmp® 2400 and 9600 and AmpliTaq Gold® polymerase are examples of suitable products available commercially from Applied Biosystems, previously known as 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.

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C.5.9 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 appropriate reference material prepared from T25 maize.

For identification purposes, see C.5.8.

The detection of fragments with a size of 209 bp indicates that the sample DNA solution contains amplifiable DNA of T25 maize origin within the assessed limitations of specificity described in C.5.2.2.

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

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Annex D
(informative)

Event-specific methods

D.1 Event-specific method for the detection of modified DNA sequences from genetically modified MON 810 maize

D.1.1 General

This is a method for the detection of genetically modified insect-protected MON 810/“YieldGuard” maize in raw materials by amplification of the single copy DNA integration-border region of the genomic sequence and the inserted sequence element originating from the CaMV 35S promoter as a result of *in vitro* recombination.

Gene-stacked cultivars cannot be distinguished by this method except on single kernels and plants.

D.1.2 Validation status and performance criteria

D.1.2.1 Collaborative studies

The method has been validated in a collaborative study [20] under 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.

Samples from flour (ground kernels) of “MON 810 DK 513/59179” (0,1 %, 1 %), T25 (0,1 %, 1 %) and non-GMO maize were prepared for the collaborative study. The data of the collaborative study are listed in Tables D.1 and D.2.

Table D.1 — Results of collaborative study

Year	2001
Number of laboratories	16
Number of laboratories submitting results	16
Number of samples per laboratory	5
Number of accepted results	75
Number of samples containing MON810	31
Number of samples containing T25	33
Number of samples containing non-GM maize	11
False positive results	0 (0 %)
False negative results	0 (0 %)

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Table D.2 — Detailed results of collaborative study

Sample	Number of samples	Correct	False
MON 810 negative samples:			
0 % GMO	11	11	0
0,1 % T25	18	18	0
1 % T25	15	15	0
MON 810 positive samples:			
0,1 % MON 810	13	13	0
1 % MON 810	18	18	0

D.1.2.2 Molecular specificity

D.1.2.2.1 General

This annex fulfils the requirements as outlined in Clause 7.

The method has been described in Reference [20].

NOTE Sequence information for the development of this method was provided by Monsanto.

Information on the genetic construct introduced into the maize genome is available in Reference [38].

D.1.2.2.2 Theoretical

No sequence homology with DNA sequences of non-GM maize and other crop plants has been found in databank searches (GenBank® database; BlastN® 2.2.1 search from 2001-07-01). Moreover the primer set was designed to amplify a DNA sequence specific for an artificial junction (the integration-border region) not occurring in nature.

D.1.2.2.3 Experimental

No amplification has been observed using DNA from non-GM maize, from genetically modified GTS 40-3-2 (Roundup Ready® soya beans), or Event 176 (Bt 176), Bt 11 and T25 GM maize lines.

The number of sequence copies is one.

D.1.2.3 Limit of detection (LOD)

Based on the assumption that there is only one copy of the target sequence per genome (AGBIOS database: http://www.agbios.com/) and that the genome size of maize is 2,65 x 10^9 bp (see Reference [5]) the absolute LOD with 50 ng DNA from maize, with a relative GMO content of 0,1 % in ground seeds is 20 genome equivalents [34]. The relative LOD is better than or equal to 0,1 % in ground maize seeds [34].

D.1.3 Adaptation

No specific information is available.

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D.1.4 Principle

The Bt gene originates from the soil bacterium *Bacillus thuringiensis* subsp. *kurstaki.* The protein thus produced in the plant tissue protects it from being attacked by European corn borer larvae. The Bt protein becomes active in the intestine of these insects, causes pores to be formed in the cell membrane and leads to a disruption in the osmotic balance resulting in cell lysis.

A 170 bp DNA fragment spanning the integration-border region between the CaMV 35S promoter and the maize genomic DNA sequence is amplified by PCR and the amplification product is detected by gel electrophoresis. For identification purposes, restriction digest profiles are described and should be used (see D.1.8).

D.1.5 Reagents

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

D.1.5.1 Water

D.1.5.2 PCR buffer (without $MgCl_2$), 10 ×.

D.1.5.3 $MgCl_2$ solution, c = 25 mmol/l.

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

D.1.5.5 Oligonucleotides

The integration-border region has been published [38] with accession No. AF434709.

D.1.5.5.1 Forward primer

VW01: 5'-TCg AAg gAC gAA ggA CTC TAA Cg-3'.

Accession No. is AF434709. Primer is located in the maize genome.

D.1.5.5.2 Reverse primer

VW03: 5'-TCC ATC TTT ggg ACC ACT gTC g-3'.

Accession No. (GenBank®) is V00141, J02048. Primer is located in the CaMV 35S promoter.

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

D.1.5.7 Restriction enzymes: M*wo* I and H*ae* III.

D.1.6 Apparatus and equipment

D.1.6.1 Thermal cycler

D.1.6.2 Gel electrophoresis chamber, with power supply.

D.1.7 Procedure

D.1.7.1 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 D.3. 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 D.3 have proven to be suitable.

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Table D.3 — Addition of reagents

Reagent	Final concentration	Volume per sample (µl)
Sample DNA	10 ng to 50 ng	2
Water		14,8
10 × PCR buffer (without $MgCl_2$)	1 ×	2,5
$MgCl_2$ solution[a], 25 mmol/l	2 mmol/l	2,0
dNTP solution, 10 mmol/l	0,4 mmol/l	1,0
Primer VW01, 10 µmol/l	0,5 µmol/l	1,25
Primer VW03, 10 µmol/l	0,5 µmol/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 $MgCl_2$ reaction mixture is adjusted to 2 mmol/l.

D.1.7.2 PCR controls

As positive controls, IRMM certified reference material IRMM-413 may be used.

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

D.1.7.3 Temperature-time programme

The temperature-time programme as outlined in Table D.4 has been used for the validation study using thermal cyclers GeneAmp® 2400, 9600 and AmpliTaq Gold® DNA polymerase[28)]. 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 D.4 — Temperature-time programme

Activation/initial denaturation	12 min/95 °C
Amplification	30 s/95 °C 30 s/64 °C 30 s/72 °C
Number of cycles	40
Final extension	10 min/72 °C

D.1.8 Identification

The identity of the amplified product may be verified by restriction analysis using either *Hae* III or *Mwo* I. Digestion with *Hae* III yields two fragments of 126 and 44 bp, respectively. Digestion with *Mwo* I yields two fragments of 109 and 61 bp, respectively.

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28) GeneAmp® 2400 and 9600 and AmpliTaq Gold® polymerase are examples of suitable products available commercially from Applied Biosystems, previously known as 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.

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D.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 material prepared from Mon810 maize (e.g. IRMM-413-series from IRMM, Geel, Belgium).

For identification purposes, see D.1.8.

The detection of fragments with a size of 170 bp indicates that the sample DNA solution contains amplifiable DNA of Mon810 maize origin within the assessed limitations of specificity described in D.1.2.2.

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

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