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

*Produits alimentaires — Méthodes d'analyse pour la détection des
organismes génétiquement modifiés et des produits dérivés —
Méthodes quantitatives basées sur l'utilisation des acides nucléiques*

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Foreword

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

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

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

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

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

Introduction

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

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

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

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

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

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

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

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

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

and

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

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

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

1 Scope

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

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

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

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

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

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

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

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

ISO 24276:—¹⁾, *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — General requirements and definitions*

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

3 Terms and definitions

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

1) To be published.

4 Principle

4.1 General

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

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

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

The analysis generally consists of

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

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

4.2 Amplification, detection and confirmation of PCR products

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

4.3 Quantitation of PCR products

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

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

5 Reagents

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

6 Apparatus and equipment

See Annexes A to D and ISO 24276.

7 Guidelines concerning the procedure

7.1 General

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

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

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

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

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

Appropriate controls shall be included (see ISO 24276:—, Table 1).

7.2 Target sequence stability

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

7.3 Calibration of the analysis

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

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

7.4 Quantitation considerations

PCR methods should be appropriately designed to minimize the variability.

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

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

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

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

7.5 Quality assurance requirements

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

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

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

8 Interpretation

The PCR result will be either

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

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

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

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

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

9 Expression of results

The results shall clearly state the quantity of the GM target sequence relative to the target taxon-specific sequence. The results should also provide values for the measurement uncertainty, such as the standard

deviation or relative standard deviation. Furthermore, the LOD and LOQ of the method and the practical LOD and LOQ should be reported.

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

Table 1 — Expression of results

Result	Expression of the result
Target taxon-specific sequence is not detected.	See ISO 21569. "For species x, DNA was not detected."
Target taxon-specific sequence is detected but GM target sequence is not detected.	According to ISO 21569. "For species x, GMO-derived DNA was not detected." In addition, if applicable, add: "The practical limit of detection is X %." (Specify unit used.)
The target taxon-specific sequence and the GM target sequence are both detected but the quantity is below the LOQ of at least one of the target sequences.	For each GMO, state: "GMO (specify the GMO) derived DNA as determined by detection of (specify target sequence) derived from (specify species) was detected." In addition, if applicable: "The practical limit of quantitation is X %." (Specify unit used.)
The target taxon-specific sequence and the GM target sequence are both detected and the quantity is above the LOQ for both target sequences.	For each GMO, state: "The content of GMO (specify the GMO) derived DNA as determined by detection of (specify target sequence) derived from (specify species) is $X \pm$ uncertainty %." (Specify unit used.)

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

10 Test report

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

- a) LOQ of the method and the matrix used to establish it;
- b) the practical LOQ;
- c) a reference to the method which has been used for the extraction of DNA;
- d) the reference material used;
- e) the results expressed according to Clause 9.

Annex A (informative)

Target taxon-specific methods

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

A.1.1 Introduction

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

For limitations, see A.1.8.

A.1.2 Validation status and performance characteristics

A.1.2.1 General

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

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

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

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

A.1.2.2 Collaborative trial

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

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

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

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

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

Table A.1 — Validation data

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

A.1.2.3 Molecular specificity

A.1.2.3.1 General

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

A.1.2.3.2 Theoretical specificities

The theoretical specificities of the primers and probes were assessed through a search of the GenBank/EMBL/DDBJ databases²⁾ using the nucleotide sequences as query sequences with the BLASTN programme²⁾ at <http://www.ncbi.nlm.nih.gov/blast/> [October 9, 2003]. The result of the search confirmed a complete identity only with the expected target sequences.

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

A.1.2.3.3 Experimental determination of specificity

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

A.1.2.4 Optimization

This was carried out for the ABI PRISM 7700[®] sequence detection system (SDS)³⁾ and TaqMan[®] chemistry³⁾. Primer and probe design were done with Primer Express[®] software (Applied Biosystems)³⁾.

A.1.2.5 Limit of detection (LOD)

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

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

A.1.2.6 Limit of quantitation (LOQ)

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

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

A.1.3 Adaptation

No specific information is available.

A.1.4 Principle

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

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

A.1.5 Reagents

A.1.5.1 General

For quality of reagents to be used, see ISO 24276:—, 6.6.

A.1.5.2 Water.

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

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

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

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

A.1.5.6 Oligonucleotides

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

Table A.2 — Oligonucleotides

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

^a FAM: 6-carboxyfluorescein; TAMRA: 6-carboxytetramethylrhodamine.

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

A.1.5.7 Thermostable DNA polymerase

AmpliTaq Gold[®] DNA polymerase⁴⁾.

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

A.1.5.9 Amplification reaction mixture

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

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

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

^a ROX = carboxy-X-rhodamine.

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

A.1.6 Apparatus

A.1.6.1 General

Standard laboratory apparatus should be used throughout unless otherwise specified.

A.1.6.2 Thermal cycler

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

A.1.6.3 Reaction vials

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

A.1.7 Procedure: PCR set-up

A.1.7.1 General

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

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

A.1.7.2 PCR controls

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

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

A.1.7.3 Temperature-time programme

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

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

Table A.4 — Procedure: Reaction conditions

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

A.1.8 Limitations and interpretation of the results

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

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

A.1.9 Calibration and calculation of results

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

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

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

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

Annex B (informative)

Screening methods

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

B.1.1 Introduction

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

For limitations, see B.1.8.

B.1.2 Validation status and performance characteristics

B.1.2.1 General

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

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

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

The method has been published in Reference [23].

B.1.2.2 Collaborative trial

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

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

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

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

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

Samples were analysed in duplicate.

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

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

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

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

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

⁸⁾ These are examples of suitable products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

B.1.2.3 Molecular specificity

B.1.2.3.1 General

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

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

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

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

B.1.2.3.2 Theoretical specificities

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

B.1.2.3.3 Experimental determination of specificity

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

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

B.1.2.4 Optimization

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

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

B.1.2.5 Limit of detection (LOD)

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

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

B.1.2.6 Limit of quantitation (LOQ)

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

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

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

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

B.1.3 Adaptation

No specific information is available.

B.1.4 Principle

An 82 bp fragment of the CaMV 35S promoter sequence is amplified by PCR using two 35S-promoter-specific primers. PCR products are measured during each PCR cycle (real-time) by means of a 35S-promoter-specific oligonucleotide probe labelled with two fluorescent dyes: FAM as a reporter dye and TAMRA as a quencher. For that purpose, TaqMan[®] chemistry¹⁰⁾ is employed.

An 81 bp fragment of the soya bean lectin gene sequence is amplified by PCR in a separate real-time PCR reaction using two soya bean lectin gene specific primers, and the PCR products are measured over each PCR cycle by means of a soya bean lectin gene specific TaqMan[®] probe¹⁰⁾.

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

B.1.5 Reagents**B.1.5.1 General**

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

B.1.5.2 Water.**B.1.5.3 PCR buffer (without MgCl₂), 10-fold.****B.1.5.4 MgCl₂ solution, $c(\text{MgCl}_2) = 25 \text{ mmol/l}$.****B.1.5.5 dNTP solution, $c(\text{dNTP}) = 2,5 \text{ mmol/l}$ (each).****B.1.5.6 Oligonucleotides**

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

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

Table B.2 — Oligonucleotides

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

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

B.1.5.7 Thermostable DNA polymerase

AmpliTaq Gold[®] DNA polymerase¹¹⁾.

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

B.1.6 Apparatus

B.1.6.1 General

Standard laboratory apparatus should be used throughout unless otherwise specified.

B.1.6.2 Thermal cycler

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

B.1.6.3 Reaction vials

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

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

B.1.7 Procedure: PCR set-up

B.1.7.1 General

The PCR set-up for the reference gene target sequence and for the GMO target sequence should be carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.

The method is described for a total PCR volume of 50 µl per reaction mixture with the reagents as listed in Table B.3.

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

Reference gene target sequence		
Total volume		50 µl
Template DNA (maximal amount 200 ng)		10 µl
DNA polymerase	AmpliTaq Gold®	1,25 U
Decontamination system	dUTP	400 µmol/l
	AmpErase® uracil <i>N</i> -glycosylase	0,5 U
Reaction buffer	TaqMan® buffer A (containing passive reference ROX) ^a	1 X
	MgCl ₂	5 mmol/l
Primers	Lectin-F and Lectin-R (see Table B.2)	see Table B.2
dNTP	dATP, dCTP, dGTP	200 µmol/l each
Probe	Lectin-TMP (see Table B.2)	see Table B.2
GMO target sequence		
Total volume		50 µl
Template DNA (maximal amount 200 ng)		10 µl
DNA polymerase	AmpliTaq Gold™	1,25 U
Decontamination system	dUTP	400 µmol/l
	AmpErase uracil <i>N</i> -glycosylase	0,5 U
Reaction buffer	TaqMan® buffer A (containing passive reference ROX) ^a	1 X
	MgCl ₂	5 mmol/l
Primers	35S-F and 35S-R (see Table B.2)	see Table B.2
dNTP	dATP, dCTP, dGTP	200 µmol/l each
Probe	35S-TMP (see Table B.2)	see Table B.2
^a ROX = carboxy-X-rhodamine.		

B.1.7.2 PCR controls

As a positive control and as calibrant reference material, certified reference materials of GTS 40-3-2 (material containing 0,1 % to 5 % of genetically modified soya bean) produced by IRMM, Geel, Belgium (IRMM-410 series) ¹²⁾ may be used ^[18].

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.4 was optimized for the ABI PRISM[®] 7700 sequence detection system (SDS) (Applied Biosystems) ¹²⁾. In the validation study, it was used in combination with the AmpliTaq Gold[®] DNA polymerase ¹²⁾. The use of other thermal cyclers may require specific adaptation. The time for activation/initial denaturation depends on the polymerase used.

Table B.4 describes the reaction conditions.

Table B.4 — Procedure: Reaction conditions

		Time s	Temperature °C
Pre-PCR: decontamination		120	50
Pre-PCR: activation of DNA polymerase and denaturation of template DNA		600	95
PCR (45 cycles)			
Step 1	Denaturation	15	95
Step 2	Annealing and elongation	60	60

B.1.8 Limitations and interpretation of the results

Since GMOs other than soya bean line GTS 40-3-2 contain 35S-promoter DNA sequences, the method is only suited for quantitation of GTS 40-3-2 DNA in the absence of GMOs other than GTS 40-3-2 soya bean. In all other instances the method may only be applied for screening and control purposes.

The described method is suitable for measuring the ratio of 35S-promoter DNA to soya bean DNA in the absence of other GMO and *Cauliflower mosaic viruses*. This ratio reflects the amount of GTS 40-3-2 soya bean in the soya bean ingredient of the investigated foodstuff. Contents of 1 % GTS 40-3-2 soya bean can be determined if the amount of the soya bean ingredient exceeds 5 % in the investigated foodstuff.

NOTE If food processing has lead to degradation or removal of DNA (e.g. refined soya bean oil, refined soya bean lecithins) the described method does not yield reliable results.

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B.1.9 Calibration and calculation of results

After defining a threshold value (e.g. 0,01 to 0,1 normalized reporter dye fluorescence (R_n)) the sequence detection system calculates the C_t (cycle of threshold) values for each PCR ($\Delta\Delta C_t$ -method). The differences between the 35S-specific and the lectin-specific C_t -values of the samples ($\Delta C_{t,sam}$) and of the reference samples ($\Delta C_{t,ref}$) are calculated. The relative amount of 35S DNA in the sample, w , in percent with respect to the reference material is calculated according to Equation (B.1)

$$w = 2^{-(\Delta C_{t,sam} - \Delta C_{t,ref})} \times c_{ref} \quad (B.1)$$

where c_{ref} is the concentration of the reference sample.

Alternatively, a calibration curve is calculated ($\log [c]$ vs. C_t) by the sequence detection system based on standards consisting of GMO mixtures of defined concentrations of genetically modified soya bean line GTS 40-3-2 (e.g. 0,1 %; 0,5 %, 1 %, 2 % and 5 %) or standards consisting of suitable dilutions of test solutions obtained from GMO mixtures of defined concentrations of GTS 40-3-2 (e.g. 5 %) (double calibration curve method). This calibration curve is used to determine the GTS 40-3-2 line concentration of the unknown samples. Since the sample DNA can be degraded due to food processing or since the sample can contain other ingredients than soya bean, the calculated GTS 40-3-2 concentration shall be normalized with the amount of amplifiable soya bean DNA present in the sample. This amount is determined by means of the soya bean lectin gene specific real-time PCR using as standard DNA mixtures of defined concentration of soya bean DNA [e. g. 100 % (by mass), 50 % (by mass), 25 % (by mass), 10 % (by mass) and 1 % (by mass)] soya bean DNA from IRMM 410 series diluted with suitable carrier DNA. For normalization, the measured amount of DNA of GTS 40-3-2 is divided by the measured amount of soya bean DNA.

For this alternative procedure for the calculation of results, it is important that the absolute amount of template DNA (ng) be the same for each PCR used in the calibration.

Another alternative procedure is described in C.2.

Annex C (informative)

Construct-specific methods

C.1 Construct-specific method for the quantitation of soya bean line GTS 40-3-2 DNA using real-time PCR (Method 1)

C.1.1 Introduction

This annex describes a method for the specific amplification and detection of a taxon-specific soya bean gene (lectin gene, *le1*) and of DNA originating from the specific gene construct present in the genetically modified soya bean line GTS 40-3-2. The method is suitable for quantitative determination of the amount of DNA originating from genetically modified soya bean line GTS 40-3-2 in soya bean ingredients containing genetically modified soya bean line GTS 40-3-2 (Roundup Ready[®] 13).

For limitations, see C.1.7.

C.1.2 Validation status and performance characteristics

C.1.2.1 General

The method has been optimized for certified reference materials (CRM IRMM-410 13) [18] consisting of dried soya bean powder containing mixtures of GTS 40-3-2 and conventional soya bean.

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

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

The method has been published in Reference [23].

C.1.2.2 Collaborative trial

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

The GTS 40-3-2 construct-specific detection method resulted in a reproducibility relative standard deviation in the range of 16 % to 28 % (see Table C.1). During the initial experiments of the collaborative trial, it was determined that the 1 % soya bean CRM was inconsistent with its designated value. Investigations revealed that the diverging reference materials were manufactured using different procedures at different time points, leading to different degrees of DNA degradation. The collaborative trial participants were advised during the course of the collaborative trial to use the 2 % CRM and dilute the DNA to obtain a 1 % reference DNA solution for use in quantitation.

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

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3 h. After an RNase digestion step, 500 µl of the cleared extract was mixed with 1 ml of Wizard[®] 14) silica resin and the resin with bound DNA was recovered by aspiration through a Wizard[®] 14) filter column. After isopropanol washing, the DNA was eluted from the silica resin in 10 mmol/l Tris buffer pH 9,0 at 70 °C. The DNA concentration was estimated by OD measurement at 260 nm and adjusted to 20 µg/ml. For subsequent PCR analysis, 200 ng of DNA from each sample was analysed in two independent reactions.

Samples have been analysed in duplicate.

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

The exclusion of laboratories is based on the real-time PCR apparatus used and on the statistical calculation of outliers. The method was developed for block thermal cyclers. Therefore, two laboratories using a LightCycler[®] 14) system (Roche Diagnostics) were excluded before the calculation of outliers. The reason for the exclusion of a specific type of PCR apparatus was the observation that the applied method needs careful adaptation and optimization if run on other real-time PCR apparatus than those specified in the method. The retained laboratories were in addition examined for outliers according to Grubbs [24]. Details of the collaborative trial are listed in Table C.1.

Table C.1 — Validation data for 35S-promotor-specific GMO detection

	Sample				
	SA	SB	SC	SD	SE
Year of interlaboratory test	1999	1999	1999	1999	1999
Number of laboratories having returned results	11	11	11	11	11
Number of samples per laboratory	1	1	1	1	1
Number of excluded laboratories	3	2	3	3	3
Number of laboratories retained after exclusion	8	9	8	8	8
Number of accepted samples	8	9	8	8	8
Expected value (% GMO)	1,4	1,8	3	0,7	1
Mean value (% GMO)	1,70	1,89	3,65	0,86	1,58
Median value (% GMO)	1,71	1,90	3,68	0,85	1,49
Reproducibility standard deviation s_R (% GMO)	0,27	0,53	0,57	0,15	0,38
Reproducibility relative standard deviation (%)	16	28	16	17	24
Reproducibility limit R ($R = 2,8 s_R$)	0,77	1,48	1,60	0,42	1,06

In addition, four unknown commercially available food samples containing genetically modified soya bean line GTS 40-3-2 between 0,3 % and 36 % (by mass) (mean values) were analysed by four participants. The GTS 40-3-2 construct specific detection method resulted in reproducibility relative standard deviation in the range of 23 % to 36 % [20].

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

C.1.2.3 Molecular specificity

C.1.2.3.1 General

The method has been designed to target a sequence described in e.g. GenBank® database ¹⁵⁾, accession number AX033493.

C.1.2.3.2 Theoretical specificities

The theoretical specificities of the primers and probes were assessed through a search of the GenBank/EMBL/DDBJ databases ¹⁵⁾ using the nucleotide sequences as query sequences with the BLASTN programme 2.2.3 ¹⁵⁾ [24th April 2002]. The result of the search confirmed a complete identity only with the expected target sequences.

C.1.2.3.3 Experimental determination of specificity

The experimental specificity of the method was assessed by analysis of CRM from IRMM-410 from IRMM dried soya bean powder containing 0 % to 5 % of GTS 40-3-2 soya bean and of reference material from Leatherhead Food Research Association International ¹⁵⁾ full fat soya bean flour (Lot No. 2/99-01) containing 0 %, 0,3 %, 1,25 % and 2 % soybean GTS 40-3-2 soya bean line, respectively.

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

C.1.2.4 Optimization

Optimization was carried out on the ABI PRISM® 7700 sequence detection system ¹⁵⁾ (SDS) using TaqMan® chemistry ¹⁵⁾.

Primer and probe design was carried out applying the Primer Express® software (Applied Biosystems) ¹⁵⁾.

C.1.2.5 Limit of detection (LOD)

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

C.1.2.6 Limit of quantitation (LOQ)

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

According to the method developer, the limit of quantitation is 50 genome copies of soya bean line GTS 40-3-2 in 82 000 genome copies of conventional soya bean flour. For 1 C value, see Reference [20].

This yields an estimated relative LOQ of 0,06 (= 50 copies/82 000 copies × 100 %).

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

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

¹⁵⁾ These are examples of suitable products available commercially. This information is only given for the convenience of users of this International Standard and does not mean an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

C.1.3 Adaptation

No specific information is available.

C.1.4 Principle

An 83 bp fragment of the transgene used for construction of the soya bean line GTS 40-3-2 is amplified by PCR using two primers specific to the CTP gene from *Petunia hybrida* (RRS-F, see Table C.2) and to 35S promoter (RRS-R, see Table C.2). PCR products are measured over each PCR cycle (real-time) by means of an oligonucleotide probe specific for the junction between the CTP gene and the 35S promoter (RRS-TMP, see Table C.2). This oligonucleotide probe is labelled with two fluorescent dyes: FAM as a reporter dye and TAMRA as a quencher. For that purpose TaqMan[®] chemistry¹⁶⁾ is employed.

An 81 bp fragment of the soya bean lectin gene sequence is amplified by PCR in a separate real-time PCR reaction using two soya bean lectin gene specific primers, and the PCR products are measured during each PCR cycle by means of a soya bean lectin gene specific TaqMan[®] probe¹⁶⁾ (Lectin-TMP, see Table C.2).

Quantitation is performed using either the $\Delta\Delta C_t$ method or the double calibration curve method (see C.1.8).

C.1.5 Reagents

C.1.5.1 General

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

C.1.5.2 Water.

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

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

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

C.1.5.6 Oligonucleotides

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

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

Table C.2 — Oligonucleotides

Name	Oligonucleotide DNA sequence	Final concentration in PCR
Reference gene target sequence		
Lectin-F	5'-TCC ACC CCC ATC CAC ATT T-3'	900 nmol/l
Lectin-R	5'-ggC ATA gAA ggT gAA gTT gAA ggA-3'	900 nmol/l
Lectin-TMP	5'-FAM-AAC Cgg TAg CgT TgC CAg CTT Cg-TAMRA-3' ^a	100 nmol/l
GMO target sequence		
RRS-F	5'-gCC ATg TTg TTA ATT TgT gCC AT-3'	900 nmol/l
RRS-R	5'-gAA gTT CAT TTC ATT Tgg AgA ggA C-3'	900 nmol/l
RRS-TMP	5'-FAM-CTT gAA AgA TCT gCT AgA gTC AgC TTg TCA gCg-TAMRA-3' ^a	100 nmol/l
^a FAM: 6-carboxyfluorescein; TAMRA: 6-carboxytetramethylrhodamine.		

The length of the lectin PCR product is 81 bp; the length of the RRS PCR product is 83 bp.

C.1.5.7 Thermostable DNA polymerase

AmpliTaq Gold[®] DNA polymerase. ¹⁷⁾

C.1.5.8 Uracil N-glycosylase (optional).

C.1.6 Apparatus

C.1.6.1 General

Standard laboratory apparatus should be used throughout unless specified otherwise.

C.1.6.2 Thermal cycler

The indicated temperature-time profile was originally tested with ABI PRISM[®] 7700 SDS ¹⁷⁾ and GeneAmp[®] 5700 SDS (Applied Biosystems) ¹⁷⁾. Other real-time PCR detection systems may be used after adaptation of the reaction conditions.

C.1.6.3 Reaction vials

The reaction vials shall be suitable for PCR amplification on a thermal cycler, e.g. ABI PRISM[®] 96-Well Optical Reaction Plate, or MicroAmp[®] Optical Caps (8 caps/strip, flat) (Applied Biosystems) ¹⁷⁾.

C.1.6.4 Procedure: PCR set-up

The PCR set-up for the reference gene target sequence and for the GMO target sequence should be carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.

The method is described for a total PCR volume of 50 µl per reaction mixture with the reagents as listed in Table C.3.

¹⁷⁾ These are examples of suitable products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

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

Reference gene target sequence		
Total volume		50 µl
Template DNA (maximal amount 200 ng)		10 µl
DNA polymerase	AmpliTaq Gold®	1,25 U
Decontamination system	dUTP	400 µmol/l
	AmpErase uracil <i>N</i> -glycosylase	0,5 U
Reaction buffer	TaqMan® buffer A (containing passive reference ROX) ^a	1 X
	MgCl ₂	5 mmol/l
Primers	Lectin-F and Lectin-R (see Table C.2)	see Table C.2
dNTP	dATP, dCTP, dGTP	200 µmol/l each
Probe	Lectin-TMP (see Table C.2)	see Table C.2
GMO target sequence		
Total volume		50 µl
Template DNA (maximal amount 200 ng)		10 µl
DNA polymerase	AmpliTaq Gold®	1,25 U
Decontamination system	dUTP	400 µmol/l
	AmpErase uracil <i>N</i> -glycosylase	0,5 U
Reaction buffer	TaqMan® buffer A (containing passive reference ROX) ^a	1 X
	MgCl ₂	5 mmol/l
Primers	RRS-F and RRS-R (see Table C.2)	see Table C.2
dNTP	dATP, dCTP, dGTP	200 µmol/l each
Probe	RRS-TMP (see Table C.2)	see Table C.2
^a ROX = carboxy-X-rhodamine.		

C.1.6.5 PCR controls

As a positive control and as calibrant reference material, certified reference materials of GTS 40-3-2 (material containing 0,1 % to 5 % of genetically modified soya bean) produced by IRMM, Geel, Belgium (IRMM-410 series¹⁸⁾) may be used [18].

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

C.1.6.6 Temperature-time programme

The temperature-time programme as outlined in Table C.4 was optimized for the ABI PRISM® 7700 sequence detection system (SDS)¹⁸⁾ (Applied Biosystems). In the validation study, it was used in combination with the AmpliTaq Gold® DNA polymerase¹⁸⁾. The use of other thermal cyclers may require specific adaptation. The time for activation/initial denaturation depends on the polymerase used.

Table C.4 describes the reaction conditions.

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

Table C.4 — Procedure: Reaction conditions

		Time s	Temperature °C
Pre-PCR: decontamination		120	50
Pre-PCR: activation of DNA polymerase and denaturation of template DNA		600	95
PCR (45 cycles)			
Step 1	Denaturation	15	95
Step 2	Annealing and elongation	60	60

C.1.7 Limitations and interpretation of the results

Since GMOs other than soya bean line GTS 40-3-2 may contain part of the transgene used for construction of the soya bean line GTS 40-3-2, especially the 35S promoter, the CTP sequence from *Petunia hybrida* and the specific junction in between these two genetic elements, the method is suited for quantitation of GTS 40-3-2 DNA in the absence of other GMOs, as specified above.

The described method is suitable for measuring the ratio of DNA from soya bean line GTS 40-3-2 to soya bean DNA. This ratio reflects the amount of GTS 40-3-2 soya bean in the soya bean ingredient of the investigated foodstuff.

If the amount of the soya bean ingredient is less than 5 % in the investigated foodstuff, it is unlikely that a level of 1 % GTS 40-3-2 soya bean can be determined.

NOTE If food processing has lead to degradation or removal of DNA (e.g. refined soya bean oil, refined soya bean lecithins), the described method does not yield reliable results.

C.1.8 Calibration and calculation of results

After defining a threshold value (e.g. 0,01 to 0,1 normalized reporter dye fluorescence (R_n)) the sequence detection system calculates the C_t (cycle of threshold) values for each PCR ($\Delta\Delta C_t$ -method). The differences between the RRS-specific and the lectin-specific C_t -values of the samples ($\Delta C_{t,sam}$) and of the reference samples ($\Delta C_{t,ref}$) are calculated. The relative amount of DNA from soya bean line GTS 40-3-2 in the sample, w , in percent, with respect to the reference material is calculated according to Equation (C.1):

$$w = 2^{-(\Delta C_{t,sam} - \Delta C_{t,ref})} \times c_{ref} \tag{C.1}$$

Alternatively, a calibration curve is calculated (log [c] vs C_t) by the sequence detection system based on standards consisting of GMO mixtures of defined concentrations of genetically modified soya bean line GTS 40-3-2 (e.g. 0,1 %, 0,5 %, 1 %; 2 % and 5 %), or standards consisting of suitable dilutions of test solutions obtained from GMO mixtures of defined concentrations of GTS 40-3-2 (e.g. 5 %) (double calibration curve method). This calibration curve is used to determine the GTS 40-3-2 line concentration of the unknown samples. Since the sample DNA can be degraded due to food processing, or since the sample may contain ingredients other than soya bean, the calculated GTS 40-3-2 concentration shall be normalized with the amount of amplifiable soya bean DNA present in the sample. This amount is determined by means of the soya bean lectin gene specific real-time PCR using as standards DNA mixtures of defined concentration of soya bean DNA [e.g. 100 % (by mass), 50 % (by mass), 25 % (by mass), 10 % (by mass) and 1 % (by mass)] soya bean DNA from IRMM 410 or 410R ¹⁹⁾ diluted with suitable carrier DNA. For normalization, the measured amount of DNA of GTS 40-3-2 is divided by the measured amount of soya bean DNA.

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

For this alternative procedure of calculation of results, it is important that the absolute amount of template DNA (ng) be the same for each PCR used in the calibration.

Another alternative is described in C.2.

C.2 Construct-specific method for the quantitation of soya bean line GTS 40-3-2 DNA using real-time PCR (Method 2)

C.2.1 Introduction

This annex describes a method for the specific amplification and detection of a taxon-specific soya bean gene (*lectin* gene, *le1*) and of the specific gene construct junction region between the 35S-promoter DNA from the cauliflower mosaic virus and the chloroplast targeting signal of *Petunia hybrida* preceding the sequence of *Agrobacterium* 5-enolpyruvylshikimate-3-phosphate synthase gene (*epsps*) present in the genetically modified soya bean line GTS 40-3-2 (Roundup Ready[®] 20) in order to quantify the relative amount of soya bean line GTS 40-3-2 DNA.

For limitations, see C.2.8.

C.2.2 Validation status and performance characteristics

C.2.2.1 General

The method has been optimized for real-time PCR instruments using certified reference materials (CRM IRMM-410R²⁰) [18] consisting of dried soya bean powder containing mixtures of GTS 40-3-2 and conventional soya beans.

The reproducibility and accuracy of the described method have been tested in collaborative studies using unknown samples consisting of the reference materials mentioned above. In addition, a processed reference material of texturized vegetable protein-type (TVP) was tested.

The copy numbers of each of the target sequences per genome have not been assessed in detail.

C.2.2.2 Collaborative trial

Six unknown samples containing between 0,1 % and 5 % (by mass) of the above-mentioned certified reference materials (CRM IRMM-410R²⁰) and TVP containing 2 % (by mass) of the soya bean line GTS 40-3-2 were analysed by

- 14 laboratories using the ABI PRISM[®] 7700 SDS,
- 6 laboratories using the ABI GeneAmp[®] 5700 SDS, and
- 12 laboratories using the LightCycler[®] system²⁰ (Roche Diagnostics).

The number of participants as well the number of samples followed the criteria according to the ISO 5725 series.

The GTS 40-3-2 construct-specific detection system resulted in a reproducibility relative standard deviation in the range of 27 % to 44 % using the ABI PRISM[®] 7700 SDS and ABI GeneAmp[®] 5700 SDS machines, respectively, and in the range of 27 % to 64 % using the LightCycler[®] system²⁰ (Roche Diagnostics).

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

For DNA extraction, the procedure described in ISO 21571:2005, A.3, was used.

For each sample, two DNA extractions were analysed in parallel. Each test sample was analysed in three replicates.

The method precision, accuracy and limit of quantitation were determined by collaborative trial. Data on the specificity, linearity and on the detection and quantitation limit were determined prior to the collaborative trial. The DNA content of the test samples covered these values.

A summary of the validation data for trueness and precision is given in Tables C.5 and C.6.

Table C.5 — Validation data for ABI PRISM® 7700 SDS and GeneAmp® 5700 SDS ²¹⁾

	Sample 1 0,10 % ± 0,03 %	Sample 2 0,50 % ± 0,06 %	Sample 3 1,0 % ± 0,1 %	Sample 4 2,0 % ± 0,2 %	Sample 5 5,0 % ± 0,2 %	Sample 6 2 % TVP
Year of interlaboratory test	2000	2000	2000	2000	2000	2000
Number of laboratories	19	19	19	19	19	19
Number of outliers ^a	0	2	1	0	1	0
Number of laboratories retained after eliminating outliers	19	17	18	19	18	19
Mean value (%)	0,11	0,49	1,00	2,27	5,11	1,71
Repeatability standard deviation s_r	0,04	0,12	0,21	0,25	0,53	0,48
Repeatability relative standard deviation (%)	33	24	21	11	10	28
Repeatability limit r ($r = 2,8 s_r$)	0,10	0,33	0,59	0,71	1,48	1,34
Reproducibility standard deviation s_R	0,05	0,3	0,28	0,71	1,38	0,55
Reproducibility relative standard deviation (%)	44	27	28	32	27	32
Reproducibility limit R ($R = 2,8 s_R$)	0,13	0,37	0,77	2,00	3,87	1,54
^a Outliers were identified with the Grubbs and Cochran tests. [24]						

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

Table C.6 — Validation data for LightCycler® 22) system

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
	0,10 % ± 0,03 %	0,50 % ± 0,06 %	1,0 % ± 0,1 %	2,0 % ± 0,2 %	5,0 % ± 0,2 %	2 % TVP
Year of the collaborative trial	2000	2000	2000	2000	2000	2000
Number of laboratories	7	7	7	7	7	5
Number of outliers ^a	1	0	0	0	0	1
Number of laboratories retained after eliminating outliers	6	7	7	7	7	4
Mean value (%)	0,13	0,55	0,95	2,01	5,43	1,82
Repeatability standard deviation s_r	0,07	0,23	0,28	0,56	1,10	0,20
Repeatability relative standard deviation (%)	55	42	30	28	20	11
Repeatability limit r ($r = 2,8 s_r$)	0,19	0,66	0,79	1,57	3,07	0,56
Reproducibility standard deviation s_R	0,08	0,31	0,34	0,64	1,94	0,50
Reproducibility relative standard deviation (%)	64	55	35	32	36	27
Reproducibility limit R ($R = 2,8 s_R$)	0,22	0,86	0,95	1,79	5,43	1,40
^a Outliers were identified with the Grubbs and Cochran tests. [24]						

The results as listed in Table C.6 illustrate the variability observed for the LightCycler® system 22) (Roche Diagnostics). It is noted that the number of laboratories returning results was not sufficient according to ISO 5725.

C.2.2.3 Molecular specificity

C.2.2.3.1 General

The method has been designed to target a sequence described in, for example, GenBank® database 22) accession No. AY596948.

C.2.2.3.2 Theoretical specificities

The theoretical specificities of the primers and probes were assessed through a search of the GenBank/EMBL/DDBJ databases 22) using the nucleotide sequences as query sequences with the BLASTN programme 2.2.3 22) [24th April 2002]. The result of the search confirmed a complete identity only with the expected target sequences.

C.2.2.3.3 Experimental determination of specificity

CRM IRMM-410R 22) dried soya bean powders containing 0 % to 5 % of GTS 40-3-2 soya bean were identified. The commercial food matrices tested were soya bean flour and soya bean protein isolates.

Specificity tests prior to the collaborative trial showed no cross reactivity of the detection systems to the following non-target species/samples: rice (*Oryza sativa*), rye (*Secale cereale*), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), millet (*Panicum miliaceum*), lentil (*Lens culinaris*), white bean (*Phaseolus vulgaris*), mung bean (*Phaseolus aureus*), yellow lupin (*Lupinus luteus*), teosinte (*Zea mays* ssp. *mexicana*), tomato (*Lycopersicon esculentum*), potato (*Solanum tuberosum* ssp. *tuberosum*), sorghum (*Sorghum vulgare*), maize

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

(*Zea mays*), rape (*Brassica napus*), oats (*Avena sativa*), spelt (*Triticum spelta*), flax (*Linum usitatissimum*), buckwheat (*Fagopyrum esculentum*), sesame (*Sesamum sp.*), human (*Homo sapiens sapiens*), salmon (*Salmo salar*), beef (*Bos taurus*), and *Bacillus subtilis*. Furthermore, no cross reactivity has been observed with MS8xRF3 (SeedLink) rapeseed or with the following GM corn events: Bt176, Bt11, T25, MON 810, CBH351, DBT418, GA21.

The allelic and copy number stability of the soya bean reference gene has been assessed using at least eight different cultivars of soya bean.

C.2.2.4 Optimization

Optimization of reagent concentrations was carried out on an ABI PRISM[®] 7700 sequence detection system (SDS) and on the LightCycler[®] instrument using TaqMan[®] chemistry²³⁾ [3]. No additional optimization was carried out for the GeneAmp[®]5700 because the thermal cycler in this instrument is identical to the cycler of the ABI PRISM[®] 7700²³⁾.

Primer and probe design were carried out applying the Primer Express[®] software (Applied Biosystems)²³⁾.

C.2.2.5 Limit of detection (LOD)

The limit of detection was not assessed during the collaborative trial.

The limit of detection for PCR was calculated by measuring serial target DNA dilutions.

According to the method developer, the LOD has been determined to be 5 copies of the target sequence (determined with plasmids).

C.2.2.6 Limit of quantitation (LOQ)

The limit of quantitation was assessed by measuring serial target DNA dilutions.

According to the method developer, the limit of quantitation is at least 50 genome copies of soya bean line GTS 40-3-2. For 1 C value, see Reference [20].

The concentrations tested in the collaborative trial are listed in Tables C.5 and C.6.

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

C.2.3 Adaptation

No specific information is available.

C.2.4 Principle

A 74 bp fragment of the construct-specific sequence of GTS 40-3-2 is amplified by PCR using a specific primer pair for GTS 40-3-2. PCR products are measured over each PCR cycle (real time) by means of a GTS 40-3-2 construct-specific oligonucleotide probe labelled with two fluorescent dyes: FAM as a reporter dye and TAMRA as a quencher. For that purpose TaqMan[®] chemistry²³⁾ is employed.

A 74 bp fragment of the taxon-specific soya bean *lectin* gene (*le1*) is amplified by PCR in a separate real-time PCR using two soya bean *le1* gene specific primers, and the PCR products are measured during each PCR cycle by means of a *le1*-specific TaqMan[®] probe²³⁾.

The calibration curve method has been used for quantitation of unknown test sample DNA extracts.

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

NOTE Prior to the quantitative PCR analysis, the extracted DNA solutions were analysed using a qualitative PCR run on the real-time PCR machine. The qualitative PCR run ("monitor run") was conducted to determine the "cycle threshold" (C_t -value) of each sample for which no experimental experience exists. By testing two different dilutions of the extracted nucleic acid (e.g. a 1:10 and 1:40 dilution of the DNA solution) in the monitor run, the possible presence of amplification inhibition can be identified. Additionally, a suitable dilution of the nucleic acid extract obtained from the test sample that fits into the calibration range of the quantitative analysis can be determined.

C.2.5 Reagents

C.2.5.1 General

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

C.2.5.2 Water.

C.2.5.3 PCR buffer (without $MgCl_2$), 10-fold.

C.2.5.4 $MgCl_2$ solution, $c(MgCl_2) = 25$ mmol/l.

C.2.5.5 DNTP solution, $c(DNTP) = 2,5$ mmol/l.

C.2.5.6 Oligonucleotides

Details of the oligonucleotides are listed in Table C.7.

Table C.7 — Oligonucleotides

Name	Oligonucleotide DNA sequence	Final concentration in PCR
Reference gene target sequence		
GM1-F	5'-CCA gCT TCg CCg CTT CCT TC-3'	600 nmol/l
GM1-R	5'-Gaa ggC AAg CCC ATC TgC AAg CC-3'	600 nmol/l
Probe GM1	5'-FAM-CTT CAC CTT CTA TgC CCC TgA CAC-TAMRA-3' ^a	120 nmol/l
GMO target sequence		
RR1-F	5'-CAT Tg gAg Agg ACA CgC TgA-3'	600 nmol/l
RR1-R	5'-gAg CCA TgT TgT TAA TTT gTg CC-3'	600 nmol/l
Probe RR1	5'-FAM-CAA gCT gAC TCT TTC-TAMRA-3' ^a	125 nmol/l
^a FAM: 6-carboxyfluorescein; TAMRA: 6-carboxytetramethylrhodamine.		

The length of the lectin and of the GTS 40-3-2 PCR products are 74 bp.

C.2.5.7 Thermostable DNA polymerase

AmpliTaq Gold[®] DNA polymerase ²⁴⁾

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

C.2.5.8 Uracil *N*-glycosylase (optional).

C.2.6 Apparatus

C.2.6.1 General

Standard laboratory apparatus should be used throughout unless otherwise specified.

C.2.6.2 Thermal cycler

The indicated temperature-time profiles were originally tested with ABI PRISM® 7700 SDS or GeneAmp®5700 SDS (Applied Biosystems), LightCycler®system (Roche Diagnostics) ²⁵⁾. Other real-time PCR instruments may also be used if they can be shown to lead to equivalent or better results.

C.2.6.3 Reaction vials

The reaction vials shall be suitable for PCR amplification on a thermal cycler, e.g. ABI PRISM® 96-Well Optical Reaction Plate, or MicroAmp® Optical Caps (8 caps/strip, flat) (Applied Biosystems) and LightCycler® capillaries (Roche Diagnostics) ²⁵⁾.

C.2.7 Procedure: PCR set-up

C.2.7.1 General

The PCR set-up for the reference gene target sequence and for the GMO target sequence should be carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.

Before the “quantitation run” a real-time PCR run is performed with at least two dilutions of the DNA extracted from test samples for both PCR-systems in order to monitor amplifiability. The data generated by this “monitor run” are used to control the quality of the DNA (inhibition). The C_t values obtained from two linear dilutions should be proportional to a certain C_t value difference (ΔC_t), e.g. a one-to-four dilution results in a ΔC_t value of approximately 2. A smaller ΔC_t value indicates non-linear amplification which might be caused by PCR inhibitors. The C_t value of an unknown DNA determined in a monitor run also gives information about the quantity of the target DNA. In this way, an appropriate DNA concentration of the unknown test sample is selected which shall be in the range of the standard curve.

The method is described for a total PCR volume of 50 µl per reaction mixture with the reagents as listed in Tables C.8 and C.9.

Table C.8 — Amplification reaction mixture in the final volume/ concentration per reaction vial for the target taxon sequence

Total reaction volume		50 µl
Template DNA added (1,7 ng to 108 ng soya bean DNA)		5 µl
DNA polymerase	AmpliTaq Gold® DNA polymerase ^a	1,25 U
Decontamination system	dUTP	400 µmol/l
	uracil <i>N</i> -glycosylase	0,5 U
Reaction buffer	TaqMan® buffer A (containing passive reference ROX) ^b	1-fold
	MgCl ₂	4,5 mmol/l
Primers	GM1-F and GM1-R (see Table C.7)	see Table C.7
dNTP	dATP, dCTP, dGTP	200 µmol/l each
Probe	GM1 (see Table C.7)	see Table C.7
H ₂ O (molecular biology grade)		add to 50 µl
^a When using the LightCycler® system, an alternative buffer and Taq-Polymerase is recommended, e.g. Platinum Taq or FastStart Taq within the reaction buffer of the manufacturer.		
^b ROX = carboxy-X-rhodamine.		

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

**Table C.9 — Amplification reaction mixture in the final volume/
concentration per reaction vial for the GMO target sequence**

Total reaction volume		50 µl
Template DNA added (1,7 ng to 108 ng soya bean DNA)		5 µl
DNA polymerase	AmpliTaq Gold® DNA polymerase ^a	1,25 U
Decontamination system	dUTP uracil <i>N</i> -glycosylase	400 µmol/l 0,5 U
Reaction buffer	TaqMan® buffer A (containing passive reference ROX) ^b	1 fold
	MgCl ₂	4,5 mmol/l
Primers	RR1-F and RR1-R (see Table C.7)	see Table C.7
dNTP	dATP, dCTP, dGTP	200 µmol/l each
Probe	RR1 (see Table C.7)	see Table C.7
H ₂ O (molecular biology grade)		add to 50 µl
^a When using the LightCycler® system, an alternative buffer and Taq-Polymerase is recommended, e.g. Platinum Taq or FastStart Taq within the reaction buffer of the manufacturer.		
^b ROX = carboxy-X-rhodamine.		

C.2.7.2 PCR controls

As a positive control, certified reference materials of GTS 40-3-2 genetically modified soya beans produced by IRMM, Geel, Belgium (IRMM-410 series ²⁶⁾) may be used [18].

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.10 was optimized for the ABI PRISM® 7700 SDS (Applied Biosystems) and the LightCycler® system (Roche Diagnostics) ²⁶⁾. In the validation study, it was used in combination with the AmpliTaq Gold® DNA polymerase ²⁶⁾. The use of other thermal cyclers may require specific adaptation. The time for activation/initial denaturation depends on the polymerase used.

Table C.10 describes the reaction conditions.

Table C.10 — Procedure: Reaction conditions

		Time s	Temperature °C
Pre-PCR: decontamination (optional)		120	50
Pre-PCR: activation of DNA polymerase and denaturation of template DNA		600	95
PCR (45 cycles)			
Step 1	Denaturation	15 ^a / 5 ^b	95
Step 2	Annealing elongation	60 ^a / 25 ^b	60
^a Optimized for ABI PRISM® 7700 SDS (Applied Biosystems) with software version 1.6.3.			
^b Optimized for the LightCycler® system. Fluorescence parameter in the LightCycler® are channel 1 (gain 4) and single acquisition during denaturation with software version 3.			

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

C.2.8 Limitations and interpretation of the results

Since GMOs other than soya bean line GTS 40-3-2 may contain part of the genetic construct used for development of the soya bean line GTS 40-3-2, especially the specific junction between the 35S-promoter and the CTP signal sequence from *Petunia hybrida* elements, the method is suitable for quantitation of GTS 40-3-2 DNA in the absence of other GMOs as specified above.

The described method is suitable for measuring the ratio of GTS 40-3-2 construct-specific DNA to soya bean DNA. This ratio reflects the relative amount of GTS 40-3-2 in the soya bean ingredient of the investigated foodstuff.

This method is validated for soya bean flour and texturized vegetable protein.

NOTE If the soya bean DNA is removed or highly degraded during food processing (e.g. refined oil) or if soya bean is only a very minor component of the sample analysed, the amount of soya bean reference and/or GM specific copies will be at or below the limit of quantitation and the described methods will not be applicable.

C.2.9 Calibration and calculation of results

Separate calibration curves with each primer/probe system are generated in the same analytical amplification run. The calibration curves comprise four dilutions of DNA extracted from the 5 % CRM IRMM-410R²⁷⁾. At each of the four calibration points, duplicate (ABI PRISM[®] 7700 SDS²⁷⁾ and GeneAmp[®] 5700²⁷⁾ or single determinations (LightCycler[®] system²⁷⁾) are performed. Triplicate reactions using an appropriate dilution of the DNA extracted from the unknown sample are measured in the ABI instruments, while single determinations using two different dilutions of the sample DNA extract were performed in the LightCycler[®] system²⁷⁾.

A calibration curve is produced by plotting C_T -values against the logarithm of the target copy number for the calibration points. This can be carried out, for example, by use of spread-sheet software such as Microsoft Excel²⁷⁾, or directly by options available with the sequence detection system software.

The copy numbers measured for the unknown sample DNA are obtained by interpolation from the standard curves. For determination of the amount of GTS 40-3-2 DNA in the unknown sample, the copy number of the GTS 40-3-2 target is divided by the copy number of the *lectin* gene and multiplied by 100 and is then expressed as a percentage.

C.3 Construct-specific method for the quantitation of Event176 maize DNA using real-time PCR

C.3.1 Introduction

This annex describes a method for the specific amplification and detection of the taxon-specific maize (*Zea mays*) high mobility group protein gene (*hmg*) and of a specific part of the synthetic *cry1A(b)* gene derived from *Bacillus thuringiensis* [synthetic *cry1A(b)*] present in the genome GM maize Event176 (Bt176; Maximizer[™]) in order to quantify the relative amount of Event176 DNA.

For limitations, see C.3.8.

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

C.3.2 Validation status and performance characteristics

C.3.2.1 General

The method has been optimized for certified reference materials (CRM IRMM 411²⁸⁾) consisting of dried maize powder containing mixtures of Event176 and conventional maize.

The reproducibility and accuracy of the described method were tested in a collaborative trial using unknown samples of the CRM IRMM 411²⁸⁾ from 0,1 % to 5 % Event176 in conventional maize, as well as using reference materials produced from heat-sterilized maize kernels (HSK) containing 2 % of Event176 maize in conventional maize.

The copy number of the target genes per genome has not been assessed in detail.

C.3.2.2 Collaborative trial

The collaborative trial was organized by the former Federal Institute for Health Protection of Consumers and Veterinary Medicine (BgVV now BfR, Germany) together with GeneScan Europe (Teltow, Germany). The number of participants as well as the number of samples followed the criteria according to the ISO 5725 series.

The study was undertaken with 10 laboratories using the ABI PRISM[®] 7700 SDS²⁸⁾ and 7 laboratories using the ABI GeneAmp[®] 5700 SDS²⁸⁾.

For DNA extraction, the procedure described in ISO 21571:2005, A.3, was used.

For each sample, two DNA extractions were analysed in parallel. Each test sample was analysed in three replicates.

Each participant received six unknown samples. The samples consisted of four certified reference materials: CRM IRMM 411 between 0,1 % and 2 % Event176 maize in maize powder (by mass), a maize flour sample containing a mixture (by mass) of 50 % soya bean line GTS 40-3-2 (Roundup ready), 49 % maize (non-GM) and 1 % Event176 maize (labelled Mix A) and a dried powder produced from heat sterilized kernels (labelled HSK) containing 2 % Event176 maize in non-GM maize (powder).

The method precision, accuracy and limit of quantitation were determined by a collaborative trial. Data on specificity, linearity and on the detection and quantitation limit which exceeded the range of the test samples had been determined prior to the collaborative trial.

A summary of the validation data for trueness and precision is given in Table C.11.

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

Table C.11 — Validation data for the Bt 176 construct-specific quantitation

	Sample 1 0,100 % ± 0,002 %	Sample 2 0,50 % ± 0,01 %	Sample 3 1,00 % ± 0,02 %	Sample 4 2,00 % ± 0,04 %	Sample 5 Mix A (2 %)	Sample 6 2 % HSK
Year of the collaborative trial	2 000	2 000	2 000	2 000	2 000	2 000
Number of laboratories	17	17	17	17	17	17
Number of outliers ^a	1	0	0	0	2	2
Number of laboratories retained after eliminating outliers	16	17	17	17	15	15
Mean value (%)	0,13	0,67	1,47	2,27	2,04	0,75
Repeatability standard deviation s_r	0,03	0,13	0,28	0,45	0,37	0,21
Repeatability relative standard deviation (%)	22,28	19,48	18,83	19,88	18,22	27,71
Repeatability limit r ($r = 2.8 s_r$)	0,08	0,37	0,78	1,26	1,04	0,58
Reproducibility standard deviation s_R	0,05	0,26	0,55	0,92	0,85	0,25
Reproducibility relative standard deviation (%)	36,13	39,23	37,19	40,72	41,62	32,90
Reproducibility limit R ($R = 2,8 s_R$)	0,13	0,74	1,54	2,59	2,38	0,69

^a Outliers were identified with the Grubbs and Cochran tests. [24]

C.3.2.3 Molecular specificity

C.3.2.3.1 General

Information about the genetic construct introduced into the maize genome is available in Reference [31].

C.3.2.3.2 Theoretical specificity

The theoretical specificities of the primers and probes were assessed through a search of the GenBank/EMBL/DDBJ databases ²⁹⁾ using the nucleotide sequences as query sequences with the BLASTN programme 2.2.4. ²⁹⁾ [8th December 2002]. The result of the search confirmed a complete identity only with the expected target sequences.

C.3.2.3.3 Experimental determination of specificity

CRM from IRMM-411 ²⁹⁾ dried maize powder containing 0,1 % to 5 % Event176 maize were identified.

With the Event176 specific primer/probe system, no specific amplification products were observed with DNA from 20 different maize lines (*Zea mays* cultivars), or rice (*Oryza sativa*), rye (*Secale cereale*), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), millet (*Panicum miliaceum*), lentil (*Lens culinaris*), white bean (*Phaseolus vulgaris*), mung bean (*Phaseolus aureus*), yellow lupin (*Lupinus luteus*), teosinte (*Zea mays*, ssp. *mexicana*), tomato (*Lycopersicon esculentum*), potato (*Solanum tuberosum* ssp. *tuberosum*), sorghum (*Sorghum vulgare*), maize (*Zea mays*), rape (*Brassica napus*), oats (*Avena sativa*), spelt (*Triticum spelta*), flax (*Linum usitatissimum*), buckwheat (*Fagopyrum esculentum*), sesame (*Sesamum spec.*), human (*Homo sapiens sapiens*), salmon (*Salmo salar*), beef (*Bos taurus*). Furthermore, no cross reactivity has been observed with genetically modified soybean event GTS 40-3-2 or the following GM maize events: Bt11, T25, MON 810, CBH351, DBT418 and GA21.

²⁹⁾ These are examples of suitable products available commercially. This information is only given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

For the maize specific *hmg* gene primer/probe system, amplification products were detected only with DNA from the different non-GM maize lines and with DNA extracted from CRM IRMM-411³⁰⁾, but not with one of the other above-mentioned species except for teosinte (*Zea mays*, ssp. *mexicana*), which is a close maize relative.

C.3.2.4 Optimization

Optimization was carried out on an ABI PRISM® 7700 SDS³⁰⁾ using the TaqMan® chemistry^[30]. No additional optimization was carried out for the GeneAmp® 5700 SDS³⁰⁾ because the thermal cycler in this unit is identical to the cycler of the ABI PRISM® 7700 SDS³⁰⁾.

Primer and probe design was carried out applying the Primer Express software (Applied Biosystems)³⁰⁾

C.3.2.5 Limit of detection (LOD)

The LOD was not assessed during the collaborative trial.

The LOD for PCR was calculated by measuring serial target DNA dilutions.

According to the method developer, the LOD has been determined to be 5 copies of the target sequence (determined with plasmids).

C.3.2.6 Limit of quantitation (LOQ)

The LOQ for the PCR stage was calculated by measuring serial target DNA dilutions.

The LOQ was calculated by the method provider to be 50 genome copies of Event176 maize in 82 000 genome copies based on the theoretical genome size of 2,725 pg per haploid genome^[14].

The concentrations tested in the collaborative trial are listed in Table C.11.

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

C.3.3 Adaptation

No specific information is available.

C.3.4 Principle

An 129 bp fragment of the synthetic *cry1A (b)* gene is amplified by PCR using two specific primers. PCR products are measured over each PCR cycle (real-time) by means of a synthetic *cry1A (b)* gene specific oligonucleotide probe labelled with two fluorescent dyes: FAM as reporter dye and TAMRA as quencher. For that purpose, TaqMan® chemistry³⁰⁾ is employed.

An 79 bp fragment of the maize specific *hmg* gene is amplified by PCR in a separate real-time PCR using specific primers and the PCR products are measured during each PCR cycle by means of a *hmg* gene specific oligonucleotide probe labelled with two fluorescent dyes: FAM as reporter dye and TAMRA as quencher.

NOTE Event Bt176 (Maximizer™) maize contains two copies of a synthetic *cry1A(b)* gene under the control of the maize *calcium dependent protein kinase* (CDPK6) gene promotor and the maize *phosphoenolpyruvate carboxylase* (PEPC) gene promotor, respectively.

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

The standard curve method is used for quantitation of unknown test sample DNA extracts. Separate calibration curves with each primer/probe system are generated in the same analytical amplification run. The calibration curves comprise four dilutions of DNA extracted from the 5 % CRM IRMM-411, which was used as calibration standard material. At each of the four calibration points, duplicate determinations are performed. Triplicate reactions on each of the two extracts of template DNA from the unknown test sample are measured, using appropriate dilutions of the DNA.

Before the “quantitation run” a real-time PCR run is performed with at least two dilutions of the DNA extracted from test samples (e.g. a 1:10 and 1:40 dilution of the DNA solution) in order to monitor amplification capability. The data generated by this “monitor run” are used to determine the presence of any PCR inhibitors. The C_t values obtained from two linear dilutions should be proportional to a certain C_t value difference (ΔC_t), e.g. a one-to-four dilution results in a ΔC_t of approximately 2. A smaller ΔC_t value indicates non-linear amplification which might be caused by PCR inhibitors. The C_t value of an unknown DNA determined in a monitor run also gives information about the quantity of the target DNA. In this way an appropriate DNA concentration of the unknown test sample is determined which shall be in the dynamic range of the standard curve.

C.3.5 Reagents

C.3.5.1 General

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

C.3.5.2 Water.

C.3.5.3 PCR buffer (without $MgCl_2$), 10-fold.

C.3.5.4 $MgCl_2$ solution, $c(MgCl_2) = 25$ mmol/l.

C.3.5.5 DNTP solution, $c(dNTP) = 2,5$ mmol/l (each).

C.3.5.6 Oligonucleotides

Details of the oligonucleotides are listed in Table C.12.

Table C.12 — Oligonucleotides

Name	Oligonucleotide DNA sequence	Final concentration in PCR
Reference gene target sequence		
ZM1-F	5'-TTg gAC TAg AAA TCT CgT gCT gA-3'	300 nmol/l
ZM1-R	5'-gCT ACA TAg ggA gCC TTg TCC T-3'	300 nmol/l
Probe ZM1	5'-FAM-CAA TCC ACA CAA ACg CAC gCg TA-TAMRA-3' ^a	160 nmol/l
GMO target sequence		
CRY2-F	5'-CCC ATC gAC ATC AgC CTg AgC-3'	300 nmol/l
CRY2-R	5'-CAg gAA ggC gTC CCA CTg gC-3'	300 nmol/l
Probe BTSYN	5'-FAM-ATg TCC ACC Agg CCC AgC ACg-TAMRA-3' ^a	160 nmol/l

^a FAM: 6-carboxyfluorescein, TAMRA: 6-carboxytetramethylrhodamine.

The length of taxon-specific PCR product is 79 bp; the length of Event176 specific PCR product is 129 bp.

C.3.5.7 Thermostable DNA polymerase

AmpliTaq Gold® DNA polymerase.³¹⁾

C.3.5.8 Uracil *N*-glycosylase (optional).**C.3.6 Apparatus****C.3.6.1 General**

Standard laboratory apparatus should be used throughout unless otherwise specified.

C.3.6.2 Thermal cycler

The indicated temperature-time profile was originally determined with ABI PRISM® 7700 SDS and the GeneAmp® 5700 SDS, Applied Biosystems³¹⁾. Other real-time PCR detection systems may be used after adaptation of the reaction conditions.

C.3.6.3 Reaction vials

The reaction vials shall be suitable for PCR amplification on a thermal cycler, e.g. ABI PRISM® 96-Well Optical Reaction Plate, or MicroAmp® Optical Caps (8 caps/strip, flat) (Applied Biosystems³¹⁾).

C.3.7 Procedure: PCR set-up**C.3.7.1 General**

The PCR set-up for the reference gene target sequence and for the GMO target sequence should be carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.

The method is described for a total PCR volume of 25 µl per reaction mixture with the reagents as listed in Tables C.13 and C.14.

**Table C.13 — Amplification reaction mixture in the final volume/
concentration per reaction vial for target taxon sequence**

Total reaction volume		25 µl
Template DNA (2,3 ng to 150 ng maize DNA)		5 µl
DNA polymerase	AmpliTaq Gold® DNA polymerase	1,25 U
Decontamination system	dUTP uracil <i>N</i> -glycosylase (optional)	400 µmol/l 0,5 U
Reaction buffer	TaqMan® buffer A (containing passive reference ROX) ^a	1 X
	MgCl ₂	4,5 mmol/l
Primers	ZM1-F and ZM1-R (see Table C.12)	see C.3.5.6
dNTP	dATP, dCTP, dGTP	200 µmol/l each
Probe	Probe ZM1 (see Table C.12)	see C.3.5.6
^a ROX = carboxy-X-rhodamine		

³¹⁾ These are examples of suitable products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

Table C.14 — Amplification reaction mixture in the final volume/ concentration per reaction vial for GMO target sequence

Total reaction volume		25 µl
Template DNA (2,3 ng to 150 ng maize DNA)		5 µl
DNA polymerase	AmpliTaq Gold® DNA polymerase	1,25 U
Decontamination system	dUTP uracil <i>N</i> -glycosylase (optional)	400 µmol/l 0,5 U
Reaction buffer	TaqMan® buffer A (containing passive reference ROX) ^a	1 X
	MgCl ₂	4,5 mmol/l
Primers	CRY2-F and CRY2-R (see C.3.5.6)	see C.3.5.6
dNTP	dATP, dCTP, dGTP	200 µmol/l each
Probe	Probe BTSYN (see C.3.5.6)	see C.3.5.6
^a ROX = carboxy-X-rhodamine.		

C.3.7.2 PCR controls

As a positive control, certified reference materials of Event176 material produced by IRMM, Geel, Belgium (IRMM-411 series ³²) may be used ^[5].

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

C.3.7.3 Temperature-time programme

The temperature-time programme as outlined in Table C.15 was optimized for the ABI PRISM® 7700 sequence detection system (SDS) ³²) (Applied Biosystems). In the validation study, it was used in combination with the AmpliTaq Gold® DNA polymerase ³²). The use of other thermal cyclers may require specific adaptation. The time for activation/initial denaturation depends on the polymerase used.

Table C.15 describes the reaction conditions.

Table C.15 — Procedure: Reaction conditions

		Time	Temperature
		s	°C
Pre-PCR: decontamination (optional when UNG is added)		120	50
Pre-PCR: activation of DNA polymerase and denaturation of template DNA		600	95
PCR (45 cycles)			
Step 1	Denaturation	15	95
Step 2	Annealing elongation	60	60

C.3.8 Limitations and interpretation of the results

The described method is suitable for measuring the ratio of Event176 construct-specific DNA to maize DNA. This ratio reflects the relative amount of Event176 in the maize ingredient of the investigated foodstuff.

³²) These are examples of suitable products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

NOTE If the maize DNA is removed or highly degraded during food processing (e.g. refined oil) or if maize is only a very minor component of the sample analysed, the amount of maize reference and/or GM specific copies will be at or below the limit of quantitation and the described methods will not be applicable.

C.3.9 Calibration and calculation of results

Separate calibration curves with each primer/probe system are generated in the same analytical amplification run. The calibration curves comprise four dilutions of DNA extracted from the 5 % CRM IRMM-411 series³³⁾. At each of the four calibration points, duplicate reactions (ABI PRISM[®] 7700 SDS and GeneAmp[®] 5700)³³⁾ are performed. A series of one-to-four dilution intervals at a starting concentration of 40 000 genome copies is used (one maize genome is assumed to correlate to 2,725 pg per haploid maize genomic DNA^[4]). Triplicate reactions using an appropriate dilution of the DNA extracted from the unknown sample are measured in the ABI instruments.

A calibration curve is produced by plotting C_T -values against the logarithm of the target copy number for the calibration points. This can be carried out, for example, by use of spread-sheet software such as Microsoft Excel³³⁾ or directly by options available with the sequence detection system software.

The copy numbers measured for the unknown sample DNA are obtained by interpolation from the standard curves. For the determination of the amount of Event176 DNA in the unknown sample, the copy number of the Event176 target is divided by the copy number of the *hmg* gene then multiplied by 100 and then expressed as a percentage.

C.4 Construct-specific method for the quantitation of soya bean line GTS 40-3-2 DNA using real-time PCR

C.4.1 Introduction

This annex describes a method for the detection and quantitation of a taxon-specific soya bean gene (lectin gene, *le1*) and of the specific DNA construct junction region between the *Petunia hybrida* chloroplast transit peptide sequence and the *Agrobacterium* 5-enolpyruvylshikimate-3-phosphate synthase gene (*epsps*) sequence presented in the genetically modified (GM) soya bean line GTS 40-3-2 [Roundup Ready[®] soya bean (RRS)]. The method is based on real-time PCR using plasmid pMuSL2 as a reference material in order to quantify the relative amount of GTS 40-3-2 in soya bean using a conversion factor (Cf) that is the ratio of copy numbers between the GTS 40-3-2 specific and taxon-specific DNA sequences in the representative genuine GTS 40-3-2 seeds.

NOTE Cf is used for the calculation of GMO content (% by mass) from the GMO DNA copy numbers of the target-specific and the taxon-specific sequence. Cf could be measured as the ratio of the copy numbers for the target-specific sequence and the taxon-specific sequence from an appropriate reference material.

For limitations, see C.4.8.

C.4.2 Validation status and performance characteristics

C.4.2.1 General

This method has been optimized for ABI PRISM[®] 7700 SDS real-time PCR apparatus³³⁾ using plasmid pMuSL2 as the reference material^[33]. The pMuSL2 plasmid includes in particular PCR products amplified from the PCR systems to specifically amplify soya bean taxon sequence (*Le1*), and construct-specific sequence of GTS 40-3-2.

NOTE The plasmid is used as a calibrator to determine the GMO contents calculated from relative copy numbers between the GM-specific and taxon-specific DNA sequence.

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

The repeatability and reproducibility of the described method have been tested in collaborative trials using the reference material and unknown dried soya bean seed powder containing mixtures of GTS 40-3-2 and conventional soya bean [34].

The copy numbers of taxon specific sequences (*Le1*) per genome have assessed for 10 representative varieties of soya bean.

The method has been published in Japanese and Korean national standards [35],[36],[37],[38].

C.4.2.2 Collaborative trial

Six pairs of soya bean unknown samples containing between 0 % and 10 % of dried soya bean powder derived from line GTS 40-3-2 were analysed by fifteen participants.

NOTE 1 The unknown samples of mixture of soya bean powder for the validation were prepared for 0 %; 0,1 %; 0,5 %; 1 %; 5 % and 10 % (by mass) of dried soya bean powder derived from line GTS 40-3-2. The homogeneity of the samples at each level was tested by using this quantitative method according to the AOAC protocol [39].

The method was validated for GTS 40-3-2 in a collaborative trial according to the AOAC protocol [34], [39]. The collaborative trial was organized by the National Food Research Institute (NFRI, Tsukuba, Japan) together with the Centre for Food Quality Labelling and Consumers Services, Saitama, Japan, and the National Institute of Health Sciences, Japan. Fifteen participants from Japan, Republic of Korea and United States performed the collaborative trials using ABI PRISM® 7700 SDS (Applied Biosystems) 34) in two separate stages. All participants were requested to follow the procedures, DNA extraction and quantitative PCR. The first stage was aiming to determine the Cf of GTS 40-3-2. All participants received the set of primers, probes, reference material and the DNAs extracted from the seeds of GTS 40-3-2, which were prepared by the Qiagen DNA Easy Plant Maxikit 34). The DNAs were used to measure copy numbers of each construct-specific and a soya bean taxon-specific DNA sequence. All measurements in this stage were repeated three times. A total of 135 data was submitted from the participants. The correlations of calibration curves of which data were submitted from all participants were acceptable ($r > 0,990$). Under the AOAC protocol [39], outlier laboratories were removed by an extreme variation (Cochran's test, $p < 0,025$) and extreme average level (Grubbs's test, $p < 0,025$). No outliers were observed in either test, as shown in Table C.16.

Table C.16 — Summary of the Cf

Target sequence	GTS 40-3-2 construct-specific
Number of participating laboratories	15
Number of Cochran outliers	0
Number of Grubbs outliers	0
Number of retained laboratories	15
Cf ^a	0,95 ± 0,02
^a Expressed as mean ± confidence interval ($\alpha = 0,05$).	

The Cf may be redetermined by analysts using appropriate reference materials of GTS 40-3-2 soya bean.

Blind tests were performed as the second stage. The unknown samples of soya bean powder were designed as 6 pairs of blind duplicates, which include 0 %, 0,1 %, 0,5 %, 1 %, 5 % and 10 % (by mass) of dried powder of GM soya bean line GTS 40-3-2 in conventional soya bean. The sample of 0 % of soya bean line

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

GTS 40-3-2 as blank sample was used in order to remove the invalid laboratories prior to statistical analysis. The participants were instructed to extract DNA from the samples using the Qiagen kit. The data submitted from the retained laboratories by outlier tests were used to calculate mean and confidence interval ($\alpha = 0,05$). The mean values were defined as the Cf for calculation of the GMO amount (%) in the blind test. The mean value of Cf for GTS 40-3-2 construct-specific quantitation was 0,95.

Thirteen laboratories that participated in the second stage analysed 156 samples by amplification of *Le1* and construct-specific sequence. Laboratories that failed to report blank samples as 0 % were judged as invalid and all their data were rejected before the outlier tests. In all experiments, the correlations of calibration curves were acceptable ($r > 0,990$). The laboratories showing an extreme variation and extreme average of data in the blind duplicate pair of the GTS 40-3-2 level were removed as Cochran [40] and Grubbs outliers [41], respectively, prior to the statistical analysis of accuracy and precision. No Cochran outlier and one Grubbs outlier was detected in the data. The calculated mean, bias, repeatability relative standard deviation (%) and reproducibility relative standard deviation (%) at each mixing level are shown in Table C.17.

NOTE 2 Collaborators did not calculate the final results using Cf determined by the first collaboration study. The copy numbers of each target sequence obtained in the Cf determinations and blind tests were reported to NFRI and unit of % GMO of blind test samples were converted to the final results using the Cf.

Table C.17 — Summary of validation for construct-specific GTS 40-3-2 soya bean quantitation

	Mixing level (%)				
	0,1	0,5	1	5	10
Number of participating laboratories	13	13	13	13	13
Number of invalid laboratories	1	1	1	1	1
Number of Cochran outliers	0	0	0	0	0
Number of Grubbs outliers	1	0	0	0	0
Number of retained laboratories	11	12	12	12	12
Mean of GMO amount (%)	0,1	0,6	1,2	5,8	11,7
Bias of true value (%)	+8,1	+14,3	+16,1	+15,1	+17,2
Repeatability standard deviation s_r ^a	0,015	0,068	0,129	0,435	0,993
Repeatability limit r ^a ($r = 2,8 s_r$)	0,041	0,191	0,362	1,219	2,779
Repeatability relative standard deviation (%) ^b	13,4	12,0	11,2	7,6	8,5
Reproducibility standard deviation s_R ^a	0,015	0,091	0,161	0,660	1,246
Reproducibility limit R ^a ($R = 2,8 s_R$)	0,041	0,255	0,451	1,849	3,489
Reproducibility relative standard deviation (%) ^b	13,4	15,9	13,9	11,5	10,6
Below 20 copies ^c (Absolute Detection Limit in this method)	4/22	0/24	0/24	0/24	0/24
^a Expressed in units of % GMO. ^b Expressed as percentage of the mean value. ^c Below 20 copies are expressed as the ratio of the number of retained data below 20 copies and the total number of retained data.					

C.4.2.3 Molecular specificity

C.4.2.3.1 General

The method has been described in Reference [33]. Information on the genetic construct introduced into the soya bean genome is available in References [33] and [22]. Sequences of DNA for the development of this method have been obtained in e.g. DDBJ³⁵⁾ database accession number X04879, a report [42] and US patent number 5633435.

If the construct DNA introduced into the GTS 40-3-2 is used to other GM events, a false positive result could occur since the amplified sequence is derived from the construct.

C.4.2.3.2 Theoretical specificities

The theoretical specificities of the primers and probes were assessed through a search of the DDBJ³⁵⁾ databases [3rd December 1999] and the safety evaluation documents disclosed by the Ministry of Health Labour and Welfare in Japan, and by the Ministry of Agriculture, Forestry and Fisheries in Japan, using the nucleotide sequence as query sequence with the BLASTN program 2.2.3³⁶⁾. The result of the search confirmed a complete identity only with the expected target sequence.

C.4.2.3.3 Experimental determination of specificity

Amplification with the primers and probes resulted in the expected PCR products when tested with the dried soya bean powders containing 0 % to 10 % (by mass) GM soya bean line GTS 40-3-2, which were prepared for this method by NFR I [33],[34].

Specificity tests prior to the collaborative trial showed no cross reactivity of the detection system to the following non-target species/samples: rice (*Oryza sativa*), wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*). No cross reactivity had been observed with the following GM maize: MON 810, Event176, Bt11, GA21 and T25.

C.4.2.4 Optimization

Optimization of reagent was carried out on the ABI PRISM[®] 7700 SDS (Applied Biosystems) using TaqMan[®] chemistry³⁶⁾ [43].

Primer and probe design was carried out applying the Primer Express[®] software (Applied Biosystems).

C.4.2.5 Limit of detection (LOD)

Absolute LOD according to the developer: 20 plasmid copies of reference material [33].

Relative LOD validated in collaborative trial: 0,1 % of GTS 40-3-2.

C.4.2.6 Limit of quantitation (LOQ)

Absolute LOQ according to the method developer: 20 plasmid copies of reference material.

Relative LOQ validated in collaborative trial: 0,1 % of GTS 40-3-2.

35) DDBJ: DNA Data Bank of Japan (<http://www.ddbj.nig.ac.jp/searches-e.html>)

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

C.4.3 Adaptation

No specific information is available.

C.4.4 Principle

A 121 bp fragment of the construct-specific sequence of GTS 40-3-2 is amplified by PCR using a specific primer pair for GTS 40-3-2. PCR products are measured over each PCR cycle (real time) by means of a GTS 40-3-2 specific oligonucleotide probe labelled with two fluorescent dyes: FAM as a reporter dye and TAMRA as a quencher. For that purpose, TaqMan[®] chemistry³⁷⁾ is employed.

A 118 bp fragment of the taxon-specific soya bean lectin gene (*Le1*) is amplified by PCR in a separate real-time PCR reaction using two soya bean *Le1* specific primers, and the PCR products are measured during each PCR cycle by means of a *Le1*-specific TaqMan[®] probe³⁷⁾.

The calibration curve method has been used for quantitation of copy number in extracted DNA from unknown test sample DNA extracts. Separate calibration curves with each primer/probe system are generated in the same analytical amplification run. The calibration curves are composed of five concentrations including 20, 125, 1 500, 20 000, 250 000 copies of plasmid DNA of pMulSL2. At each of the five calibration points, triplicate measurement is performed. Triplicate reactions using an appropriate dilution of the DNA extracted from the unknown sample are also measured in the ABI PRISM[®] 7700 SDS (Applied Biosystems)³⁷⁾ in the same analytical run.

The C_t (cycle of threshold) values determined for the calibration points in the *Le1* or GTS 40-3-2 construct specific target, respectively, are plotted against the logarithm of the copy number of plasmid DNA of pMulSL2^[33] to establish a calibration curve. The copy numbers measured for the test sample DNA is obtained by interpolation from the standard curves. For the determination of the amount of GTS 40-3-2 in the test sample, the copy number of the GTS 40-3-2 construct is divided by the copy number of the *Le1* gene and construct-specific C_f of GTS 40-3-2, multiplied by 100 to obtain the percentage as described in C.4.9.

C.4.5 Reagents

C.4.5.1 General

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

C.4.5.2 Water.

C.4.5.3 TaqMan[®] Universal Master Mix³⁷⁾, 2-fold.

C.4.5.4 Reference material (Plasmid)

The reference material used to develop and validate the method was the plasmid pMulSL2^[33] which is included in the GM Soybean (RRS) Detection Plasmid Set (Fasmac No. PS-2 and Nippon Gene No. 310-04981)³⁷⁾.

C.4.5.5 Oligonucleotides

The sequences of the primers and probes for the soya bean line GTS 40-3-2 construct-specific and the taxon-specific genes are listed in Table C.18.

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

Table C.18 — Oligonucleotides

Name	Oligonucleotide DNA sequence	Final concentration in PCR
Taxon-specific gene target sequence		
Le1n02-5'	5'-gCC CTC TAC TCC ACC CCC A-3'	500 nmol/l
Le1n02-3'	5'-gCC CAT CTg CAA gCC TTT TT-3'	500 nmol/l
Le1-Taq	5'-FAM-AgC TTC gCC gCT TCC TTC AAC TTC AC-TAMRA- 3' ^a	200 nmol/l
GMO target sequence		
RRS 01-5'	5'-CCT TTA ggA TTT CAg CAT CAg Tgg-3'	500 nmol/l
RRS 01-3'	5'-gAC TTg TCg CCg ggA ATg-3'	500 nmol/l
RRS-Taq	5'-FAM- CgC AAC CgC CCg CAA ATC C-TAMRA -3' ^a	200 nmol/l
^a FAM: 6-carboxyfluorescein; TAMRA: 6-carboxytetramethylrhodamine.		

The length of the lectin PCR product is 118 bp; the length of the GTS 40-3-2 PCR product is 121 bp.

C.4.6 Apparatus

C.4.6.1 General

Standard laboratory apparatus should be used throughout unless otherwise specified.

C.4.6.2 Thermal cycler

The indicated temperature-time profile was tested with an ABI PRISM[®] 7700 SDS (Applied Biosystems) ³⁸⁾ during the collaborative trial. Other real-time PCR detection systems may be used after the adaptation of the reaction condition.

C.4.6.3 Reaction plate and caps

The reaction plate and caps shall be suitable for PCR amplification on a thermal cycler, e.g. ABI PRISM[®] 96-Well Optical Reaction Plate, or MicroAmp[®] Optical Caps (8 caps/strip, flat) (Applied Biosystems) ³⁸⁾, respectively.

C.4.7 Procedure: PCR set-up

C.4.7.1 General

The PCR set-up for the taxon specific *Le1* target sequence and for the GTS 40-3-2 specific target sequence should be carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.

The method is described for a total PCR volume of 25 µl per reaction mixture with the reagents as listed in Table C.19 for *Le1* and in Table C.20 for GTS 40-3-2.

³⁸⁾ These are examples of suitable products available commercially. This information is only given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

Table C.19 — Amplification reaction mixture for taxon specific *Le1* sequence in the final volume per reaction vial

Total volume		25 µl
Template DNA (50 ng soya bean genomic DNA)		2,5 µl
Reaction buffer (including DNA polymerase and dNTP)	TaqMan® Universal PCR Master Mix (ABI)	12,5 µl
Primers	<i>Le1n02-5'</i> and <i>Le1n02-3'</i> (see Table C.18)	see Table C.18
Probe	<i>Le1</i> -Taq (see Table C.18)	see Table C.18

Table C.20 — Amplification reaction mixture for GTS 40-3-2 specific sequence in the final volume per reaction vial

Total volume		25 µl
Template DNA (50 ng soya bean genomic DNA)		2,5 µl
Reaction buffer (including DNA polymerase and dNTP)	TaqMan® Universal PCR Master Mix (ABI)	12,5 µl
Primers	RRS 01-5' and RRS 01-3' (see Table C.18)	see Table C.18
Probe	RRS -Taq (see Table C.18)	see Table C.18

C.4.7.2 PCR controls

Each test series shall include the controls as stated in ISO 24276.

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

As a positive control/calibration reference material, at least two alternatives are available, as follows.

- High quality, pure genomic DNA extracted from soya bean may be used if the quantity of DNA is known, on the basis of calculating copy numbers of the target sequence from the genome size of soya bean.
- A plasmid containing the target sequence(s) may be added in different concentrations with known copy numbers. Such a plasmid is available from the GM Soybean (RRS) Detection Plasmid Set (Fasmac No. PS-2 and Nippon Gene No. 310-04981³⁹⁾ [33].

According to quality assurance requirements, the positive controls should preferably not be the same as the calibration reference materials.

C.4.7.3 Temperature-time programme

The temperature-time programme as outlined in Table C.21 was optimized for the ABI PRISM® 7700 SDS (Applied Biosystems)³⁹⁾. In the collaborative trial, it was used in combination with the TaqMan® Universal Master Mix³⁹⁾. The use of other thermal cyclers may require specific adaptation. The time for activation/initial denaturation depends on the Master Mix used.

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

Table C.21 — Procedure: Reaction conditions

		Time	Temperature
		s	°C
Pre-PCR: decontamination		120	50
Pre-PCR: activation of DNA polymerase and denaturation of template DNA		600	95
PCR (40 cycles)			
Step 1	Denaturation	30	95
Step 2	Annealing and elongation	60	59

C.4.8 Limitations and interpretation of the results

If GM soya bean other than GTS 40-3-2 contains the same construct-specific DNA sequences, the method is only suitable for the quantitation of GTS 40-3-2 DNA in the absence of GMOs other than GTS 40-3-2 soya bean.

The described method is only suitable for quantitation of the soya bean line GTS 40-3-2 in the absence of other GM events containing this construct. This ratio reflects the amount of GTS 40-3-2 in the investigated soya beans. This method is only validated for soya beans.

The collaborative trial is a valuable source of data to support an uncertainty estimation. It is also necessary to identify any sources of uncertainty that are not covered by the collaborative trial data, such as sampling and others, according to the framework of international fora [44],[45].

C.4.9 Calibration and calculation of results

The threshold value to determine the cycle of threshold (C_t) shall be defined by the analyst.

An example of the procedures after PCR analysis is available in the manufacturer’s Guide to the GM Soybean (RRS) Detection Plasmid Set (Fasmac No. PS-2 and Nippon Gene No. 310-04981). See also Reference [33].

The conversion factor (C_f) for GTS 40-3-2 construct specific quantitation and reference plasmid used in the collaborative trial is 0,95. Calculate the amount of RRS in soya bean, w , by the following equation:

$$w = \frac{N_{GM}}{N_{TX}} \times \frac{100}{C_f} \%$$

where

N_{GM} is the number of copies of GM-specific target sequence in the test sample DNA;

N_{TX} is the number of copies of taxon-specific target sequence in the test sample DNA.

C.5 Construct-specific method for the quantitation of maize line MON 810 DNA using real-time PCR

C.5.1 Introduction

This annex describes a method for the detection and quantitation of a taxon-specific maize gene (maize starch synthase IIb: *zSSIIb*) and of the specific DNA construct junction region between the intron sequence of maize heat shock protein 70 gene and synthetic *cryIA(b)* gene derived from *Bacillus thuringiensis* presented in the genetically modified (GM) maize MON 810 based on real-time PCR using plasmid as a reference material in order to quantify the relative amount of MON 810 using a conversion factor (Cf) that is the ratio of copy numbers between construct-specific and taxon-specific DNA sequences in the representative genuine MON 810 seeds.

NOTE Cf is used for the calculation of GMO content (% by mass) from the GMO DNA copy numbers of the target-specific and the taxon-specific sequence. Cf could be measured as the ratio of the copy numbers for the target-specific sequence and the taxon-specific sequence from an appropriate reference material.

For limitations, see C.5.8.

C.5.2 Validation status and performance characteristics

C.5.2.1 General

This method has been optimized for ABI PRISM® 7700 SDS real-time PCR apparatus⁴⁰⁾ using plasmid pMul5⁴⁰⁾ as the reference material^[33]. The pMul5 plasmid includes in particular PCR products amplified from the PCR systems to specifically amplify maize taxon sequence (*zSSIIb*), cauliflower mosaic virus 35S promoter sequence (p35S), nopaline synthase terminator sequence (tNOS), and construct specific sequence of MON 810, Event176, Bt11, GA21 and T25.

NOTE The plasmid is used as a calibrator to determine the GM contents calculated from the relative copy numbers for the GM-specific and taxon-specific DNA sequences.

The repeatability and reproducibility of the described method has been tested in collaborative trial using the reference material and unknown dried maize seed powder containing mixtures of MON 810 and conventional maize kernel^[34].

The copy numbers of taxon-specific sequences (*zSSIIb*) per genome have been assessed for 20 representative varieties of maize.

The method has been published in Japanese and Korean national standards^{[35],[36],[37],[38]}.

C.5.2.2 Collaborative trial

A total of 12 maize unknown samples containing between 0 % and 10 % (by mass) of dried maize powder derived from line MON 810 were analysed by fifteen participants.

NOTE 1 Seeds of a representative MON 810 variety heterozygous for the GM-trait were used to determine the Cf values and the preparation of unknown samples for the collaborative trials. The unknown samples of mixtures of maize powder for the validation were prepared for 0 %, 0,1 %, 0,5 %, 1 %, 5 %, and 10 % (by mass) of dried maize powder derived from the variety. The homogeneity of the samples at each level was tested by using this quantitative method according to AOAC protocols^{[34], [39]}.

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

The method was validated for MON 810 maize in a collaborative trial according to the AOAC protocol^[39]. The collaborative trial was organized by the National Food Research Institute (NFRI, Tsukuba, Japan) together with the Centre for Food Quality Labelling and Consumers Services, Saitama, Japan, and the National Institute of Health Sciences, Tokyo, Japan. Fifteen laboratories including those from Japan, Republic of Korea and United States, performed the collaborative trials using the ABI PRISM® 7700 SDS (Applied Biosystems)⁴¹⁾ in two separate stages. All participants were requested to follow the procedures, DNA extraction and quantitative PCR.

The first stage aimed to determine the Cf of MON 810. All participants received the set of primers, probes, reference material and the DNAs extracted from the seeds of MON 810, which were prepared by Qiagen DNeasy Plant Maxi kit⁴¹⁾ and whose pertinence was tested at the NFRI prior to the study. The DNAs were used to measure copy numbers of MON 810 construct-specific and a maize taxon-specific *zSSIIb* DNA sequence. All measurements in this stage were repeated three times. A total of 90 data sets was submitted from the participants. The correlations of calibration curves for which data were submitted from all participants were acceptable ($r > 0,990$). Under the AOAC protocol^[39], outlier laboratories were removed by an extreme variation (Cochran's test, $p < 0,025$) and extreme average level (Grubbs's test, $p < 0,025$). After both tests, one laboratory was detected as a Cochran outlier at the ratio of the construct-specific sequence of MON 810 and taxon-specific *zSSIIb*. No outliers were observed at the other ratios as shown in Table C.22.

Table C.22 — Summary of the Cf for MON 810

Target sequence	MON 810 construct-specific
Number of participant laboratory	15
Number of Cochran outlier	1
Number of Grubbs outlier	0
Number of retained laboratory	14
Cf ^a	0,38 ± 0,01
a Cf are expressed as mean ± confidence interval ($\alpha = 0,05$).	

The Cf may be redetermined using appropriate reference materials of MON 810 maize by analysts.

Blind tests were performed as the second stage. The unknown samples of maize powder were designed as six pairs of blind duplicates, which included 0 %, 0,1 %, 0,5 %, 1,5 %, and 10,0 % (mass fraction) of dried powder of GM maize line MON 810 in conventional maize. The sample with 0 % of maize line MON 810 as blank sample was used in order to remove the invalid laboratories prior to statistical analysis. The participants were instructed to extract DNA from the samples using the Qiagen kit⁴¹⁾. The data submitted from the retained laboratories by outlier tests were used to calculate mean and confidence interval ($\alpha = 0,05$). The mean values were defined as the Cf for calculation of the GMO amount (%) in blind tests. The mean value of Cf for MON 810 construct specific quantitation was 0,38.

Fourteen laboratories that participated in the second stage analysed 168 samples by amplifying *zSSIIb* and construct specific sequence of MON 810. Laboratories that failed to report blank samples as 0 % were judged as invalid and all their data were rejected before outlier tests. In all experiments, the correlations of calibration curves were acceptable ($r > 0,990$). The laboratories showing extreme variation and extreme average of data in the blind duplicate pair of the MON 810 level were removed as Cochran and Grubbs outliers^{[40], [41]}, respectively, prior to the statistical analysis of accuracy and precision. Five Cochran outliers and one Grubbs outlier were detected in the data. The calculated mean, bias, the repeatability relative standard deviation (%) and the reproducibility relative standard deviation (%) at each mixing level are shown in Table C.23^[34].

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

NOTE 2 Collaborators did not calculate the final results using Cf determined by the first collaboration study. The copy numbers of each target sequence obtained in the Cf determinations and blind tests were reported to NFRI and unit of % GMO of blind test samples were converted to the final results using the Cf.

Table C.23 — Summary of validation for construct-specific MON 810 maize quantitation

	Mixing level (%)				
	0,1	0,5	1	5	10
Number of participant laboratories	14	14	14	14	14
Number of invalid laboratories	0	0	0	0	0
Number of Cochran outliers	2	1	0	1	1
Number of Grubbs outliers	1	0	0	0	0
Number of retained laboratories	11	13	14	13	13
Mean of GMO amount (%)	0,1	0,5	1,0	4,8	9,8
Bias of true value (%)	+25,0	+9,4	+4,6	-4,3	-1,8
Repeatability standard deviation s_r ^a	0,040	0,082	0,124	0,647	1,028
Repeatability limit r ^a ($r = 2,8 s_r$)	0,113	0,231	0,347	1,813	2,879
Repeatability relative standard deviation (%) ^b	32,3	15,1	11,8	13,5	10,5
Reproducibility standard deviation s_R ^a	0,040	0,107	0,158	0,647	1,140
Reproducibility limit R ^a ($R = 2,8 \times s_R$)	0,113	0,301	0,443	1,813	3,191
Reproducibility relative standard deviation (%) ^b	32,3	19,6	15,1	13,5	11,6
Below 20 copies ^c (Absolute Detection Limit in this method)	19/22	0/26	0/28	0/26	0/26
^a Expressed in units of % GMO.					
^b Expressed as percentage of the mean value.					
^c Below 20 copies are expressed as the ratio of the number of retained data below 20 copies to the total number of retained data.					

C.5.2.3 Molecular specificity

C.5.2.3.1 General

The method has been described in Reference [33]. Information on the genetic construct introduced into the maize genome is available in References [33] and [46]. Primers and TaqMan[®] 42) probe for the development of this method has been designed by the information described in Reference [46].

If the construct DNA introduced into the MON 810 is used for other GM events, a false positive result could occur since the amplified sequence is derived from the construct.

C.5.2.3.2 Theoretical specificities

The theoretical specificities of the primers and probes were assessed through a search of the DDBJ 43) databases (December 3rd, 1999) and the safety evaluation documents disclosed by the Ministry of Health, Labour and Welfare in Japan, and by the Ministry of Agriculture, Forestry and Fisheries in Japan, using the

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

43) DDBJ: DNA Data Bank of Japan (<http://www.ddbj.nig.ac.jp/searches-e.html>)

nucleotide sequence as query sequence with the BLASTN program 2.2.3⁴⁴⁾. The result of the search confirmed a complete identity only with the expected target sequence.

C.5.2.3.3 Experimental determination of specificity

Amplification with the primers and probes resulted in the expected PCR products when tested with the dried maize powders containing 0 % to 10 % (mass fraction) GM maize line MON 810, which were prepared for this method by NFRI^[33],^[34].

Tested matrices were maize kernel, maize grits, maize flour and maize meal.

Specificity tests prior to the collaborative trial showed no cross reactivity of the detection system to the following non-target species/samples: rice (*Oryza sativa*), wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*). No cross reactivity had been observed with the GM soya bean line GTS 40-3-2, the GM maize line Event176, Bt11, GA21 and T25.

C.5.2.4 Optimization

Optimization of reagent was carried out on the ABI PRISM[®] 7700 SDS (ABI)⁴⁴⁾ using TaqMan[®] chemistry⁴⁴⁾^[43].

Primer and probe design was carried out applying the Primer Express[®] software (Applied Biosystems)⁴⁴⁾.

C.5.2.5 Limit of detection (LOD)

Absolute LOD according to the developer: 20 plasmid copies of reference material^[33].

Relative LOD validated in collaborative trial: 0,5 % of MON 810.

C.5.2.6 Limit of quantitation (LOQ)

Absolute LOQ according to the method developer: 20 plasmid copies of reference material^[33].

Relative LOQ validated in collaborative trial: reference material 0,5 % of MON 810.

C.5.3 Adaptation

No specific information is available.

C.5.4 Principle

A 113 bp fragment of the construct-specific sequence of MON 810 is amplified by PCR using a specific primer pair for MON 810. PCR products are measured over each PCR cycle (real time) by means of a MON 810 specific oligonucleotide probe labelled with two fluorescent dyes: FAM as a reporter dye and TAMRA as a quencher. For that purpose TaqMan[®]⁴⁴⁾ chemistry is employed.

A 151 bp fragment of the taxon-specific *zSSIIb* is amplified by PCR in a separate real-time PCR reaction using two maize *zSSIIb* specific primers, and the PCR products are measured during each PCR cycle by means of a *zSSIIb* specific TaqMan[®] probe⁴⁴⁾.

The calibration curve method has been used for quantitation of copy numbers in extracted DNA from unknown test sample DNA extracts. Separate calibration curves with each primer/probe system are generated in the

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

same analytical amplification run. The calibration curves are composed of five concentrations including 20, 125, 1 500, 20 000, 250 000 copies of DNA of plasmid pMul5⁴⁵⁾ [33]. At each of the five calibration points, triplicate measurement is performed. Triplicate reactions using an appropriate dilution of the DNA extracted from the unknown sample were also measured in the ABI PRISM[®] 7700 SDS (Applied Biosystems)⁴⁵⁾ in the same analytical run.

The C_t (cycle of threshold) values determined for the calibration points in the *zSSIb* or MON 810 construct-specific target, respectively, are plotted against the logarithm of the copy number of plasmid DNA of pMul5^[33] to establish a calibration curve. The copy numbers measured for the test sample DNA are obtained by interpolation from the standard curves. For the determination of the amount of MON 810 in the test sample, the copy number of the MON 810 construct is divided by the copy number of the *zSSIb* gene and the construct-specific Cf of MON 810, multiplied by 100 to obtain the percentage as described in C.5.9.

C.5.5 Reagents

C.5.5.1 General

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

C.5.5.2 Water.

C.5.5.3 TaqMan[®] Universal Master Mix⁴⁵⁾, 2-fold.

C.5.5.4 Reference material (Plasmid)

The reference material used to develop and validate the method was the plasmid pMul5⁴⁵⁾ [33] which is included in the GM Maize Detection Plasmid Set (Fasmac No. PM-2 and Nippon Gene No. 319-04981)⁴⁵⁾. Other reference materials may be used provided that the performance can be demonstrated to be equal or better.

C.5.5.5 Oligonucleotides

The sequences of the primers and probes for MON 810 construct-specific and maize taxon-specific genes are listed in Table C.24.

Table C.24 — Oligonucleotides

Name	Oligonucleotide DNA sequence	Final concentration in PCR
Taxon-specific gene target sequence		
<i>SSIb</i> 1-5'	5'-CTC CCA ATC CTT TgA CAT CTg C-3'	500 nmol/l
<i>SSIb</i> 1-3'	5'-TCg ATT TCT CTC TTg gTg ACA gg-3'	500 nmol/l
<i>SSIb</i> –Taq	5'-FAM-AgC AAA gTC AgA gCg CTg CAA TgC A-TAMRA-3' ^a	200 nmol/l
GMO target sequence		
MON810 2-5'	5'-gAT gCC TTC TCC CTA gTg TTg A-3'	500 nmol/l
MON810 2-3'	5'-ggA TgC ACT CgT TgA TgT TTg-3'	500 nmol/l
MON810-Taq	5'-FAM- AgA TAC CAA gCg gCC ATg gAC AAC AA-TAMRA-3' ^a	200 nmol/l

^a FAM: 6-carboxyfluorescein; TAMRA: 6-carboxytetramethylrhodamine.

The length of the *SSIb* PCR product is 151 bp; the length of the MON 810 PCR product is 113 bp.

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

C.5.6 Apparatus

C.5.6.1 General

Standard laboratory apparatus should be used throughout unless otherwise specified.

C.5.6.2 Thermal cycler

The indicated temperature-time profile was tested with the ABI PRISM® 7700 SDS (Applied Biosystems)⁴⁶⁾ during the collaborative trial. Other real-time PCR instruments may be used after adaptation of the reaction conditions.

C.5.6.3 Reaction plate and caps

The reaction plate and caps shall be suitable for PCR amplification on a thermal cycler, e.g. ABI PRISM® 96-Well Optical Reaction Plates, or MicroAmp® Optical Caps (8 caps/strip, flat) (Applied Biosystems) respectively⁴⁶⁾. Other reaction plates, vials or caps may also be used if they can be shown to lead to equivalent or better results.

C.5.7 Procedure: PCR set-up

C.5.7.1 General

The PCR set-up for the taxon-specific *zSSIIb* target sequence and for the MON 810-specific target sequence should be carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.

The method is described for a total PCR volume of 25 µl per reaction mixture, with the reagents as listed in Table C.25 for *zSSIIb* and in Table C.26 for MON 810.

Table C.25 — Amplification reaction mixture for taxon-specific *zSSIIb* sequence in the final volume per reaction vial

Total reaction volume		25 µl
Template DNA (50ng maize genomic DNA)		2,5 µl
Reaction buffer (including DNA polymerase and dNTP)	TaqMan® Universal PCR Master Mix (ABI)	12,5 µl
Primers	SSIIb1-5' and SSIIb1-3' (see Table C.24)	see Table C.24
Probe	SSIIb-Taq (see Table C.24)	see Table C.24

Table C.26 — Amplification reaction mixture for MON 810-specific sequence in the final volume per reaction vial

Total reaction volume		25 µl
Template DNA (50 ng maize genomic DNA)		2,5 µl
Reaction buffer (including DNA polymerase and dNTP)	TaqMan® Universal PCR Master Mix (ABI)	12,5 µl
Primers	MON810 2-5' and MON810 2-3' (see Table C.24)	see Table C.24
Probe	MON810-Taq (see Table C.24)	see Table C.24

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

C.5.7.2 PCR controls

Each test series shall include the controls as stated in ISO 24276.

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

As a positive control/calibration reference material, at least two alternatives are available, as follows.

- a) High quality, pure genomic DNA extracted from maize kernel may be used if the quantity of DNA is known, on the basis of calculating copy numbers of the target sequence from the genome size of MON 810 maize.
- b) A plasmid containing the target sequence(s) may be added in different concentrations with known copy numbers. Such a plasmid is available from the GM Maize Detection Plasmid Set (Fasmac No. PS-2 and Nippon Gene No. 310-04981) ⁴⁷⁾ [33].

According to quality assurance requirements, the positive controls should preferably not be the same as the calibration reference materials.

C.5.7.3 Temperature-time programme

The temperature-time programme as outlined in Table C.27 was optimized for the ABI PRISM[®] 7700 SDS (Applied Biosystems) ⁴⁷⁾. In the collaborative trial, it was used in combination with the TaqMan[®] Universal Master Mix ⁴⁷⁾. The use of other thermal cyclers may require specific adaptation. The time for activation/initial denaturation depends on the Master Mix used.

Table C.27 — Reaction conditions

		Time s	Temperature °C
Pre-PCR: decontamination		120	50
Pre-PCR: activation of DNA polymerase and denaturation of template DNA		600	95
PCR (40 cycles)			
Step 1	Denaturation	30	95
Step 2	Annealing and elongation	60	59

C.5.8 Limitations and interpretation of the results

If GM maize other than MON 810 contains the same construct-specific DNA sequences, the method is only suitable for the quantitation of MON 810 DNA in the absence of GMOs other than MON 810 maize.

The described method is suitable for measuring the ratio of MON 810 construct-specific sequence to the taxon-specific *zSSIIb* sequence of maize. This ratio reflects the amount of MON 810 in the investigated maize. This method has only been validated for maize kernels.

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

The collaborative trial is a valuable source of data to support an uncertainty estimation. It is also necessary to identify any sources of uncertainty that are not covered by the collaborative trial data, such as sampling and others, according to the framework of international fora^[44],^[45].

C.5.9 Calibration and calculation of results

The threshold value to determine the cycle of threshold (C_t) shall be defined by the analyst.

Example of the procedures after PCR analysis is available in the manufacturer's Guide in the GM Maize Detection Plasmid Set (Fasmac No. PM-2 and Nippon Gene No. 319-04981)⁴⁸⁾. See also Reference [33]

The Cf for MON 810 construct-specific quantitation and reference plasmid used in the collaborative trial is 0,38. Calculate the amount of GM maize in the matrix, w , by the following equation:

$$w = \frac{N_{GM}}{N_{TX}} \times \frac{100}{Cf} \%$$

where

N_{GM} is the copy number of the GM-specific target sequence in the test sample DNA;

N_{TX} is the copy number of the taxon-specific target sequence in the test sample DNA.

C.6 Construct-specific method for the quantitation of maize line Event176 DNA using real-time PCR

C.6.1 Introduction

This annex describes a method for the detection and quantitation of a taxon-specific maize gene (maize starch synthase IIb: *zSSIIb*) and of the specific DNA construct junction region between the synthetic *cryIA(b)* gene derived from *Bacillus thuringiensis* and the intron sequence No. 9 of maize phosphoenol pyruvate carboxylase presented in the GM maize Event176 based on real-time PCR using plasmid as a reference material in order to quantify the relative amount of Event176 using a conversion factor (Cf) that is the ratio of copy numbers between construct-specific and taxon-specific DNA sequences in the representative genuine Event176 seeds.

NOTE Cf is used for the calculation of GMO content (% by mass) from the GMO DNA copy numbers of the target-specific and the taxon-specific sequences. Cf could be measured as the ratio of the copy numbers for the target-specific sequence and the taxon-specific sequence from an appropriate reference material.

For limitations, see C.6.8.

C.6.2 Validation status and performance characteristics

C.6.2.1 General

This method has been optimized for the ABI PRISM[®] 7700 SDS real-time PCR apparatus⁴⁸⁾ using plasmid pMuSL2 as the reference material^[33]. The pMu5 plasmid includes in particular PCR products amplified from the PCR systems to specifically amplify maize taxon sequence (*zSSIIb*) cauliflower mosaic virus 35S promoter sequence (p35S), nopaline synthase terminator sequence (tNOS), and the construct-specific sequence of MON 810, Event176, Bt11, GA21 and T25.

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

NOTE The plasmid is used as a calibrator to determine the GM contents calculated from the relative copy numbers for the GM-specific and taxon-specific DNA sequences.

The repeatability and reproducibility of the described method has been tested in a collaborative trial using the reference material and unknown dried maize seed powder containing mixtures of Event176 and conventional maize kernels [34].

The copy numbers of taxon-specific sequences (*zSSIIb*) per genome have been assessed for 20 representative varieties of maize.

The method has been published in Japanese and Korean national standards [35],[36],[37],[38].

C.6.2.2 Collaborative trial

A total of 12 unknown samples of maize containing between 0 % and 10 % (mass fraction) of dried maize powder derived from line Event176 were analysed by fifteen participants.

NOTE 1 Seeds of a representative Bt176 variety of heterozygous for the GM-trait was used to determine the Cf values and the preparation of unknown samples for the collaborative trials. The unknown samples of a mixture of maize powder for the validation were prepared for 0 %, 0,1 %, 0,5 %, 1 %, 5 %, and 10 % (mass fraction) of dried maize powder derived from the variety. The homogeneity of the samples at each level was tested by using this quantitative method according to AOAC protocols [34],[39].

The method was validated for Event176 maize in a collaborative trial according to the AOAC protocol [39]. The collaborative trial was organized by the National Food Research Institute (NFRI, Tsukuba, Japan), together with the Centre for Food Quality Labelling and Consumers Services, Saitama, Japan, and the National Institute of Health Sciences, Tokyo, Japan. Fifteen laboratories including those from Japan, Republic of Korea and United States performed the collaborative trials using the ABI PRISM®7700 SDS 49) (Applied Biosystems) in two separate stages. All participants were requested to follow the procedures, DNA extraction and quantitative PCR.

The first stage aimed to determine the Cf of Event176. All participants received the set of primers, probes, reference material and the DNAs extracted from the seeds of Event176 which are prepared by Qiagen DNeasy Plant Maxi kit 49) and whose pertinence was tested at the NFRI prior to the study. The DNAs were used to measure copy numbers of Event176 construct-specific and a maize taxon-specific *zSSIIb* DNA sequence. All measurements in this stage were repeated three times. A total of 90 data sets were submitted by the participants. The correlations of calibration curves of which data were submitted by all participants were acceptable ($r > 0,990$). Under the AOAC protocol [39], outlier laboratories were removed by an extreme variation (Cochran's test, $p < 0,025$) and extreme average level (Grubbs's test, $p < 0,025$). No outliers were observed in either test, as shown in Table C.28.

Table C.28 — Summary of the Cf^a for Event176

Target sequence	Event176 construct-specific
Number of participating laboratories	15
Number of Cochran outliers	0
Number of Grubbs outliers	0
Number of retained laboratories	15
Cf ^a	2,05 ± 0,04
^a Expressed as mean ± confidence interval ($\alpha = 0,05$).	

The Cf may be redetermined by analysts using appropriate reference materials of Event176 maize.

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

Blind tests were performed as the second stage. The unknown samples of maize powder were designed as six pairs of blind duplicates, which include 0 % 0,1 %, 0,5 %, 1 %, 5 % and 10 % (mass fraction) of dried powder of GM maize line Event176 in conventional maize. The sample of 0 % of maize line Event176 as blank sample was used in order to remove the invalid laboratories prior to statistical analysis. The participants were instructed to extract DNA from the samples using the Qiagen kit. The data submitted from the retained laboratories by outlier tests were used to calculate the mean and confidence interval ($\alpha = 0,05$). The mean values were defined as the Cf for calculation of the GMO content (%) in blind tests. The mean value of Cf for Event176 construct-specific quantitation was 2,05.

Fourteen laboratories that participated in the second stage analysed 168 samples by amplifying *zSSI**b*** and construct-specific sequence of Event176. Laboratories that failed to report blank samples as 0 % were judged as invalid and all their data were rejected before outlier tests. In all experiments, the correlations of calibration curves were acceptable ($r > 0,990$). The laboratories showing an extreme variation and extreme average of data in the blind duplicate pair of the Event176 level were removed as Cochran and Grubbs outliers [40], [41], respectively, prior to the statistical analysis of accuracy and precision. Four Cochran outliers were detected in the data. The calculated mean, bias, the repeatability relative standard deviation (%) and the reproducibility relative standard deviation (%) at each mixing level are shown in Table C.29.

NOTE 2 Collaborators did not calculate the final results using Cf determined by the first collaboration study. The copy numbers of each target sequence obtained in the Cf determinations and blind tests were reported to NFRI and unit of % GMO of blind test samples were converted to the final results using the Cf.

Table C.29 — Summary of validation for construct-specific Event176 maize quantitation

	Mixing level (%)				
	0,1	0,5	1	5	10
Number of participating laboratories	14	14	14	14	14
Number of invalid laboratories	1	1	1	1	1
Number of Cochran outliers	1	2	0	0	1
Number of Grubbs outliers	0	0	0	0	0
Number of retained laboratories	12	11	13	13	12
Mean of GMO amount (%)	0,1	0,5	0,9	5,0	9,6
Bias of true value (%)	+11,3	-1,6	-7,7	0,0	-3,8
Repeatability standard deviation s_r^a	0,018	0,029	0,066	0,406	0,554
Repeatability limit r^a ($r = 2,8 \times s_r$)	0,051	0,080	0,184	1,137	1,552
Repeatability relative standard deviation (%) ^b	16,3	5,8	7,1	8,1	5,8
Reproducibility standard deviation s_R^a	0,024	0,051	0,106	0,559	0,917
Reproducibility limit R^a ($R = 2,8 s_R$)	0,066	0,142	0,296	1,565	2,566
Reproducibility relative standard deviation (%) ^b	21,3	10,3	11,4	11,2	9,5
Below 20 copies ^c (Absolute Detection Limit in this method)	1/24	0/22	0/26	0/26	0/24
<p>^a Expressed in units of % GMO.</p> <p>^b Expressed as percentage of the mean value.</p> <p>^c Below 20 copies are expressed as the ratio of the number of retained data below 20 copies to the total number of retained data.</p>					

C.6.2.3 Molecular specificity

C.6.2.3.1 General

The method has been described in Reference [33]. Information on the genetic construct introduced into the maize genome is available in References [33] and [46]. Primers and TaqMan[®] probe⁵⁰⁾ for the development of this method were designed according to the information described in Reference [46].

If the construct DNA introduced into the Event176 is used to other GM events, a false positive result could occur since the amplified sequence is derived from the construct.

C.6.2.3.2 Theoretical specificities

The theoretical specificities of the primers and probes were assessed through a search of the DDBJ⁵¹⁾ databases (December 3rd, 1999) and the safety evaluation documents disclosed by the Ministry of Health, Labour and Welfare in Japan, and by the Ministry of Agriculture, Forestry and Fisheries in Japan, using the nucleotide sequence as query sequence with the BLASTN program 2.2.3⁵⁰⁾. The result of the search confirmed a complete identity only with the expected target sequence.

C.6.2.3.3 Experimental determination of specificity

Amplification with the primers and probes resulted in the expected PCR products when tested with the dried maize powders containing 0 % to 10 % (by mass) GM maize line Event176, which were prepared for this method by NFR1 [33],[34].

Tested matrices were maize kernel, maize grits, maize flour and maize meal.

Specificity tests prior to the collaborative trial showed no cross reactivity of the detection system to the following non-target species/samples: rice (*Oryza sativa*), wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*). No cross reactivity had been observed with the GM soya bean line GTS 40-3-2, the GM maize line MON 810, Bt11, GA21 and T25.

C.6.2.4 Optimization

Optimization of reagent was carried out on the ABI PRISM[®] 7700 SDS (ABI) using TaqMan[®] chemistry⁵⁰⁾ [43].

Primer and probe design was carried out applying the Primer Express[®] software (Applied Biosystems).

C.6.2.5 Limit of detection (LOD)

Absolute LOD according to the developer: 20 plasmid copies of reference material [33].

Relative LOD validated in collaborative trial: 0,1 % of Event176.

C.6.2.6 Limit of quantitation (LOQ)

Absolute LOQ according to the method developer: 20 plasmid copies reference material [33].

Relative LOQ validated in collaborative trial: 0,1 % of Event176.

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

51) DDBJ: DNA Data Bank of Japan (<http://www.ddbj.nig.ac.jp/searches-e.html>)

C.6.3 Adaptation

No specific information is available.

C.6.4 Principle

A 100 bp fragments of the construct-specific sequence of Event176 is amplified by PCR using a specific primer pair for Event176. PCR products are measured over each PCR cycle (real time) by means of an Event176 specific oligonucleotide probe labelled with two fluorescent dyes: FAM as a reporter dye and TAMRA as a quencher. For that purpose TaqMan[®] chemistry⁵²⁾ is employed.

A 151 bp fragment of the taxon-specific *zSSIIb* is amplified by PCR in a separate real-time PCR reaction using a specific primer pair for *zSSIIb*, and the PCR products are measured over each PCR cycle by means of a *zSSIIb*-specific TaqMan[®] probe⁵²⁾.

The calibration curve method has been used for quantitation of copy number in extracted DNA from unknown test sample DNA extracts. Separate calibration curves with each primer/probe system are generated in the same analytical amplification run. The calibration curves are composed of five concentrations including 20, 125, 1 500, 20 000, 250 000 copies of DNA of plasmid pMul5⁵²⁾. At each of the five calibration points, triplicate measurement is performed. Triplicate reactions using an appropriate dilution of the DNA extracted from the unknown sample are also measured in the ABI PRISM[®] 7700 SDS (Applied Biosystems) in the same analytical run.

The C_t values determined for the calibration points in the *zSSIIb* or Event176 construct-specific target, respectively, are plotted against the logarithm of the copy number of plasmid DNA of pMul5^[33] to establish a calibration curve. The copy numbers measured for the test sample DNA are obtained by interpolation from the standard curves. For determination of the amount of Event176 in the test sample, the copy number of the Event176 construct is divided by the copy number of the *zSSIIb* gene and the construct-specific C_f of Event176, multiplied by 100 to obtain the percentage as described in C.6.9.

C.6.5 Reagents

C.6.5.1 General

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

C.6.5.2 Water.

C.6.5.3 TaqMan[®] Universal Master Mix⁵²⁾, 2-fold.

C.6.5.4 Reference material (Plasmid)

The reference material used to develop and validate the method was the plasmid pMul5^[33] which is included in the GM Maize Detection Plasmid Set (Fasmac No. PM-2 and Nippon Gene No. 319-04981)⁵²⁾. Other reference materials may be used provided that the performance can be demonstrated to be equal or better.

C.6.5.5 Oligonucleotides

The sequences of the primers and probes for Event176 construct-specific and maize taxon-specific genes are listed in Table C.30.

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

Table C.30 — Oligonucleotides

Name	Oligonucleotide DNA sequence	Final concentration in PCR
Taxon-specific gene target sequence		
SSIIb 1-5'	5'-CTC CCA ATC CTT TgA CAT CTg C-3'	500 nmol/l
SSIIb 1-3'	5'-TCg ATT TCT CTC TTg gTg ACA gg-3'	500 nmol/l
SSIIb -Taq	5'-FAM-AgC AAA gTC AgA gCg CTg CAA TgC A-TAMRA-3' ^a	200 nmol/l
GMO target sequence		
E176 2-5'	5'-TgT TCA CCA gCA gCA ACC Ag-3'	500 nmol/l
E176 2-3'	5'-ACT CCA CTT TgT gCA gAA CAg ATC T-3'	500 nmol/l
E176-Taq	5'-FAM- CCg ACg TgA CCg ACT ACC ACA TCg A-TAMRA-3' ^a	200 nmol/l
^a FAM: 6-carboxyfluorescein; TAMRA: 6-carboxytetramethylrhodamine.		

The length of the SSIIb PCR product is 151 bp; the length of the Event176 PCR product is 100 bp.

C.6.6 Apparatus

C.6.6.1 General

Standard laboratory apparatus should be used throughout unless otherwise specified.

C.6.6.2 Thermal cycler

The indicated temperature-time profile was tested with ABI PRISM[®] 7700 SDS (Applied Biosystems)⁵³⁾ during the collaborative trial. Other real-time PCR instruments may be used after adaptation of the reaction conditions.

C.6.6.3 Reaction plates and caps

The reaction plate and caps shall be suitable for PCR amplification on a thermal cycler, e.g. ABI PRISM[®] 96-Well Optical Reaction Plate, or MicroAmp[®] Optical Caps (8 caps/strip, flat) (Applied Biosystems)⁵³⁾, respectively. Other reaction plate, vials or caps may also be used if they can be shown to lead to equivalent or better results.

C.6.7 Procedure: PCR set-up

C.6.7.1 General

The PCR set-up for the taxon-specific zSSIIb target sequence and for the Event176 specific target sequence should be carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.

The method is described for a total PCR volume of 25 µl per reaction mixture with the reagents as listed in Table C.31 for zSSIIb and in Table C.32 for Event176.

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

Table C.31 — Amplification reaction mixture for taxon-specific zSSIIB sequence in the final volume per reaction vial

Total reaction volume		25 µl
Template DNA (50ng maize genomic DNA)		2,5µl
Reaction buffer (including DNA polymerase and dNTP)	TaqMan® Universal PCR Master Mix (ABI)	12,5 µl
Primers	SSIIB1-5' and SSIIB1-3' (see Table C.30)	see Table C.30
Probe	SSIIB-Taq (see Table C.30)	see Table C.30

Table C.32 — Amplification reaction mixture for Event176 specific sequence in the final volume per reaction vial

Total reaction volume		25 µl
Template DNA (50ng maize genomic DNA)		2,5 µl
Reaction buffer (including DNA polymerase and dNTP)	TaqMan® Universal PCR Master Mix (ABI)	12,5 µl
Primers	E176 2-5' and E176 2-3' (see Table C.30)	see Table C.30
Probe	E176-Taq (see Table C.30)	see Table C.30

C.6.7.2 PCR controls

Each test series shall include the controls as stated in ISO 24276.

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

As a positive control/calibration reference material, at least two alternatives are available, as follows.

- High quality, pure genomic DNA extracted from maize kernel can be used if the quantity of DNA is known, on the basis of calculating copy numbers of the target sequence from the genome size of Event176 maize.
- A plasmid containing the target sequence(s) may be added in different concentrations with known copy numbers. Such a plasmid is available from the GM Maize Detection Plasmid Set (Fasmac No. PS-2 and Nippon Gene No. 310-04981)⁵⁴⁾ [33].

According to quality assurance requirements, the positive controls should preferably not be the same as the calibration reference materials.

C.6.7.3 Temperature-time programme

The temperature-time programme as outlined in Table C.33 was optimized for the ABI PRISM® 7700 SDS (Applied Biosystems)⁵⁴⁾. In the collaborative trial it was used in combination with the TaqMan® Universal Master Mix⁵⁴⁾. The use of other thermal cyclers may require specific adaptation. The time for activation/initial denaturation depends on the Master Mix used.

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

Table C.33 — Reaction conditions

		Time s	Temperature °C
Pre-PCR: decontamination		120	50
Pre-PCR: activation of DNA polymerase and denaturation of template DNA		600	95
PCR (40 cycles)			
Step 1	Denaturation	30	95
Step 2	Annealing and elongation	60	59

C.6.8 Limitations and interpretation of the results

If GM maize other than Event176 contains the same construct-specific DNA sequences, the method is only suitable for the quantitation of Event176 DNA in the absence of GMOs other than Event176 maize.

The described method is suitable for measuring the ratio of Event176 construct-specific sequence to the taxon-specific *zSSIIb* sequence of maize. This ratio reflects the amount of Event176 in the investigated maize. This method has only been validated for maize kernels.

The collaborative trial is a valuable source of data to support an uncertainty estimation. It is also necessary to identify any sources of uncertainty that are not covered by the collaborative trial data, such as sampling and others, according to the framework of international fora [44], [45].

C.6.9 Calibration and calculation of results

The threshold value to determine the cycle of threshold (C_t) shall be defined by the analyst.

Examples of the procedures after PCR analysis is available in the manufacturer's Guide in the GM Maize Detection Plasmid Set (Fasmac No. PM-2 and Nippon Gene No. 319-04981). See also Reference [33].

The C_f for Event176 construct-specific quantitation and reference plasmid used in the collaborative trial is 2,05. The amount of GM maize in the matrix shall be calculated by the following equation:

$$w = \frac{N_{GM}}{N_{TX}} \times \frac{100}{C_f} \%$$

where

N_{GM} is the copy number of the GM-specific target sequence in the test sample DNA;

N_{TX} is the copy number of the taxon-specific target sequence in the test sample DNA.

C.7 Construct-specific method for the quantitation of maize line Bt11 DNA using real-time PCR

C.7.1 Introduction

This annex describes a method for the detection and quantitation of a taxon-specific maize gene (maize starch synthase IIb: *zSSIIb*) and of the specific DNA construct junction region between the synthetic *cryIA(b)* gene derived from *Bacillus thuringiensis* and the intron sequence of maize alcohol dehydrogenase *Adh1* present in the GM maize Bt11, based on real-time PCR using plasmid as a reference material in order to quantify the relative amount of Bt11 using a conversion factor (C_f) that is the ratio of copy numbers between the construct-specific and taxon-specific DNA sequences in the representative genuine Bt11 seeds.

NOTE Cf is used for the calculation of GMO content (% by mass) from the GMO DNA copy numbers of the target-specific and taxon-specific sequences. Cf could be measured as the ratio of the copy numbers for the target-specific sequence to the taxon-specific sequence from an appropriate reference material.

For limitations, see C.7.8.

C.7.2 Validation status and performance characteristics

C.7.2.1 General

This method has been optimized for ABI PRISM[®] 7700 SDS real-time PCR apparatus⁵⁵⁾ using plasmid pMul5 as reference material^[33]. The pMul5 plasmid includes in particular PCR products amplified from the PCR systems to specifically amplify maize taxon sequence (*zSSIIb*), cauliflower mosaic virus 35S promoter sequence (p35S), nopaline synthase terminator sequence (tNOS) and the construct-specific sequence of MON 810, Event176, Bt11, GA21 and T25.

NOTE The plasmid is used as a calibrator to determine the GM contents calculated from the relative copy numbers for the GM-specific and taxon-specific DNA sequences.

The repeatability and reproducibility of the described method has been tested in a collaborative trial using the reference material and unknown dried maize seed powder containing mixtures of Bt11 and conventional maize kernels^[34].

The copy numbers of taxon-specific sequences (*zSSIIb*) per genome have been assessed for 20 representative varieties of maize.

The method has been published in Japanese and Korean national standards^{[35],[36],[37],[38]}.

C.7.2.2 Collaborative trial

A total of 12 unknown dried maize powder samples containing between 0 % and 10 % (w/w) of dried maize powder derived from line Bt11 were analysed by fifteen participants.

NOTE 1 Seeds of a representative Bt11 variety of heterozygous for the GM-trait was used to determine the Cf values and the preparation of unknown samples for the collaborative trials. The unknown samples of mixture of maize powder for the validation were prepared for 0 %, 0,1 %, 0,5 %, 1 %, 5 %, and 10 (w/w) of dried maize powder derived from the variety. The homogeneity the samples was tested at each level by using this quantitative method according to AOAC protocols^{[34],[39]}.

The method was validated for Bt11 maize in a collaborative trial according to AOAC protocol^[39]. The collaborative trial was organized by the National Food Research Institute (NFRI, Tsukuba, Japan), together with the Centre for Food Quality, Labelling and Consumer Services, Saitama, Japan, and the National Institute of Health Sciences, Tokyo, Japan. Fifteen laboratories including those from Japan, Republic of Korea and United States performed the collaborative trials using the ABI PRISM[®] 7700 SDS (Applied Biosystems)⁵⁵⁾ in two separate stages. All participants were requested to follow the procedures, DNA extraction and quantitative PCR.

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

The first stage aimed to determine the Cf of Bt11. All participants received the set of primers, probes, reference material and the extracted DNAs from the seeds of Bt11, which are prepared by Qiagen DNeasy Plant Maxi kit⁵⁶⁾ and whose pertinence was tested at the NFRI prior to the study^[34]. The DNAs were used to measure copy numbers of the Bt11 construct-specific and the maize taxon-specific *zSSI/b* DNA sequences. All measurements in this stage were repeated three times. A total of 135 data sets were submitted by the participants. The correlations of calibration curves of which data were submitted by all participants were acceptable ($r > 0,990$). Under the AOAC protocol^[39], outlier laboratories were removed by an extreme variation (Cochran's test, $p < 0,025$) and extreme average level (Grubbs's test, $p < 0,025$). No outliers were observed in either test, as shown in Table C.34.

Table C.34 — Summary of the Cf for Bt11

Target sequence	Bt11 construct-specific
Number of participating laboratories	15
Number of Cochran outliers	0
Number of Grubbs outliers	0
Number of retained laboratories	15
Cf ^a	0,50 ± 0,01
^a Expressed as mean ± confidence interval ($\alpha = 0,05$).	

The Cf may be redetermined by analysts using appropriate reference materials of Bt11 maize.

Blind tests were performed as the second stage. The unknown samples of maize powder were designed as six pairs of blind duplicates, which include 0 %, 0,1 %, 0,5 %, 1,0 %, 5,0 % and 10,0 % (mass fraction) of dried powder of the GM maize line Bt11 in conventional maize. The sample of 0 % of maize line Bt11 as blank sample was used in order to remove the invalid laboratories prior to statistical analysis. The participants were instructed to extract DNA from the samples using the Qiagen kit. The data submitted from the retained laboratories by outlier tests were used to calculate the mean and confidence interval ($\alpha = 0,05$). The mean values were defined as the Cf for calculation of the GMO amount (%) in blind tests. The mean value of Cf for Bt11 construct-specific quantitation was 0,5.

Fourteen laboratories that participated in the second stage analysed 168 samples by amplifying *zSSI/b* and the construct-specific sequence of Bt11. Laboratories that failed to report blank samples as 0 % were judged as invalid and all their data were rejected before outlier tests. In all experiments, the correlations of calibration curves were acceptable ($r > 0,990$). The laboratories showing extreme variation and extreme average of data in the blind duplicate pair of the Bt11 level were removed as Cochran and Grubbs outliers^{[40], [41]}, respectively, prior to the statistical analysis of accuracy and precision. Two Cochran outliers and one Grubbs outlier were detected in the data. The calculated mean, bias, the repeatability relative standard deviation (%) and the reproducibility relative standard deviation (%) at each mixing level are shown in Table C.35.

NOTE 2 Collaborators did not calculate the final results using Cf determined by the first collaboration study. The copy numbers of each target sequence obtained in the Cf determinations and blind tests were reported to NFRI and unit of % GMO of blind test samples were converted to the final results using the Cf.

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

Table C.35 — Summary of accuracy and precision statistics for real-time quantitative PCR for Bt11

	Mixing level (%)				
	0,1	0,5	1	5	10
Number of participating laboratories	14	14	14	14	14
Number of invalid laboratories	0	0	0	0	0
Number of Cochran outliers	2	0	0	0	0
Number of Grubbs outliers	1	0	0	0	0
Number of retained laboratories	11	14	14	14	14
Mean of GMO amount (%)	0,1	0,5	1,2	6,1	12,1
Bias of true value (%)	-9,0	+2,0	+14,7	+21,6	+21,1
Repeatability standard deviation s_r^a	0,020	0,121	0,216	0,830	1,258
Repeatability limit r^a ($r = 2,8 s_r$)	0,057	0,338	0,606	2,325	3,524
Repeatability relative standard deviation (%) ^b	22,3	23,7	18,9	13,7	10,4
Reproducibility standard deviation s_R^a	0,020	0,121	0,216	0,830	1,389
Reproducibility limit R^a ($R = 2,8 \times s_R$)	0,057	0,338	0,606	2,325	3,889
Reproducibility relative standard deviation (%) ^b	22,3	23,7	18,9	13,7	11,5
Below 20 copies ^c (absolute detection limit in this method)	21/22	0/28	0/28	0/28	0/28
<p>^a Expressed in units of % GMO.</p> <p>^b Expressed as percentage of the mean value.</p> <p>^c Below 20 copies are expressed as the ratio of the number of retained data below 20 copies to the total number of retained data.</p>					

C.7.2.3 Molecular specificity

C.7.2.3.1 General

The method has been described in Reference [33]. Information on the genetic construct introduced into the maize genome is available in References [33] and [46]. Primers and TaqMan[®] probe⁵⁷⁾ for the development of this method have been designed by the information described in Reference [46].

If the construct DNA introduced into the Bt11 is used for other GM events, a false positive result could occur since the amplified sequence is derived from the construct.

C.7.2.3.2 Theoretical specificities

The theoretical specificities of the primers and probes were assessed through a search of the DDBJ⁵⁸⁾ databases (December 3rd, 1999) and the safety evaluation documents disclosed by the Ministry of Health, Labour and Welfare in Japan, and by the Ministry of Agriculture, Forestry and Fisheries in Japan, using the nucleotide sequence as query sequence with the BLASTN program 2.2.3⁵⁷⁾. The result of the search confirmed a complete identity only with the expected target sequence.

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

58) DDBJ: DNA Data Bank of Japan (<http://www.ddbj.nig.ac.jp/searches-e.html>)

C.7.2.3.3 Experimental determination of specificity

Amplification with the primers and probes resulted in the expected PCR products when tested with the dried maize powders containing 0 % to 10 % (by mass) GM maize line Bt11, which were prepared for this method by NFRI [33].

Tested matrices were maize kernel, maize grits, maize flour and maize meal.

Specificity tests prior to the collaborative trial showed no cross reactivity of the detection system to the following non-target species/samples: rice (*Oryza sativa*), wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*). No cross reactivity had been observed with the GM soya bean line GTS 40-3-2, the GM maize line MON 810, Event176, GA21 and T25.

C.7.2.4 Optimization

Optimization of reagent was carried out on ABI PRISM[®] 7700 SDS⁵⁹⁾ using TaqMan[®] chemistry⁵⁹⁾ [43].

Primer and probe design was carried out applying the Primer Express[®] software (Applied Biosystems)⁵⁹⁾.

C.7.2.5 Limit of detection (LOD)

Absolute LOD according to the method developer: 20 plasmid copies of reference material [33].

Relative LOD validated in collaborative trial: 0,5 % of Bt11.

C.7.2.6 Limit of quantitation (LOQ)

Absolute LOQ according to the method developer: 20 plasmid copies of reference material [33].

Relative LOQ validated in collaborative trial: 0,5 % of Bt11.

C.7.3 Adaptation

No specific information is available.

C.7.4 Principle

A 127 bp fragment of the construct-specific sequence of Bt11 is amplified by PCR using a specific primer pair for Bt11. PCR products are measured over each PCR cycle (real time) by means of a Bt11 specific oligonucleotide probe labelled with two fluorescent dyes: FAM as a reporter dye and TAMRA as a quencher. For that purpose TaqMan[®] ⁵⁹⁾ chemistry is employed.

A 151 bp fragment of the taxon-specific *zSSIIb* is amplified by PCR in a separate real-time PCR reaction using a specific primer pair for *zSSIIb*, and the PCR products are measured over each PCR cycle by means of a *zSSIIb*-specific TaqMan[®] probe⁵⁹⁾.

The calibration curve method has been used for quantitation of the copy number in extracted DNA from unknown test sample DNA extracts. Separate calibration curves with each primer/probe system are generated in the same analytical amplification run. The calibration curves are composed of five concentrations including 20, 125, 1 500, 20 000, 250 000 copies of plasmid DNA of pMul5. At each of the five calibration points, triplicate measurement is performed. Triplicate reactions using an appropriate dilution of the DNA extracted from the unknown sample are also measured in the ABI PRISM[®] 7700 SDS⁵⁹⁾ in the same analytical run.

The C_t -values determined for the calibration points in the *zSSIIb* or Bt11 construct-specific target, respectively, are plotted against the logarithm of the copy number of DNA of plasmid pMul5⁵⁹⁾ [33] to establish a calibration curve. The copy numbers measured for the test sample DNA are obtained by interpolation from the standard curves. For the determination of the amount of Bt11 in the test sample, the copy number of the Bt11 construct is divided by the copy number of the *zSSIIb* gene and the construct-specific C_f of Bt11, multiplied by 100 to obtain the percentage as described in C.7.9.

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

C.7.5 Reagents

C.7.5.1 General

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

C.7.5.2 Water.

C.7.5.3 TaqMan® Universal Master Mix ⁶⁰⁾, 2-fold.

C.7.5.4 Reference material (Plasmid)

The reference material used to develop and validate the method was the plasmid pMul5 ^[33] which is included in the GM Maize Detection Plasmid Set (Fasmac No. PM-2 and Nippon Gene No. 319-04981) ^{[33] 60)}. Other reference materials may be used provided that the performance can be demonstrated to be equal or better.

C.7.5.5 Oligonucleotides

The sequence of the oligonucleotides for the primers and probes for Bt11 construct-specific and maize taxon-specific genes are listed in Table C.36.

Table C.36 — Oligonucleotides

Name	Oligonucleotide DNA sequence	Final concentration in PCR
Taxon-specific gene target sequence		
SSIIb 1-5'	5'-CTC CCA ATC CTT TgA CAT CTg C-3'	500 nmol/l
SSIIb 1-3'	5'-TCg ATT TCT CTC TTg gTg ACA gg-3'	500 nmol/l
SSIIb –Taq	5'-FAM-AgC AAA gTC AgA gCg CTg CAA TgC A-TAMRA-3' ^a	200 nmol/l
GMO target sequence		
Bt11 3-5'	5'-AAA AgA CCA CAA CAA gCC gC-3'	500 nmol/l
Bt11 3-3'	5'-CAA TgC gTT CTC CAC CAA gTA CT-3'	500 nmol/l
Bt11-2-Taq	5'-FAM- CgA CCA Tgg ACA ACA ACC CAA ACA TCA-TAMRA-3' ^a	200 nmol/l

^a FAM: 6-carboxyfluorescein; TAMRA: 6-carboxytetramethylrhodamine.

The length of the SSIIb PCR product is 151 bp; the length of the Bt11 PCR product is 127 bp.

C.7.6 Apparatus

C.7.6.1 General

Standard laboratory apparatus should be used throughout unless otherwise specified.

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

C.7.6.2 Thermal cycler

The indicated temperature-time profile was tested with the ABI PRISM® 7700 SDS (Applied Biosystems)⁶¹⁾ during the collaborative trial. Other real-time PCR instruments may be used after adaptation of the reaction conditions.

C.7.6.3 Reaction plate and caps

The reaction plate and caps shall be suitable for PCR amplification on a thermal cycler, e.g. ABI PRISM® 96-Well Optical Reaction Plate, or MicroAmp® Optical Caps (8 caps/strip, flat) (Applied Biosystems)⁶¹⁾, respectively. Other reaction plates, vials or caps may also be used if they can be shown to lead to equivalent or better results.

C.7.7 Procedure: PCR set-up

C.7.7.1 General

The PCR set-up for the taxon-specific *zSSI**l**b* target sequence and for the Bt11-specific target sequence should be carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.

The method is described for a total PCR volume of 25 µl per reaction mixture with the reagents as listed in Table C.37 for *zSSI**l**b* and in Table C.38 for Bt11.

Table C.37 — Amplification reaction mixture for taxon-specific *zSSIl**b* sequence in the final volume per reaction vial**

Total reaction volume		25 µl
Template DNA (50 ng maize genomic DNA)		2,5 µl
Reaction buffer (including DNA polymerase and dNTP)	TaqMan® Universal PCR Master Mix (ABI)	12,5 µl
Primers	SSI l b1-5' and SSI l b1-3' (see Table C.36)	see Table C.36
Probe	SSI l b-Ta q (see Table C.36)	see Table C.36

Table C.38 — Amplification reaction mixture for Bt11-specific sequence in the final volume per reaction vial

Total reaction volume		25 µl
Template DNA (50 ng maize genomic DNA)		2,5 µl
Reaction buffer (including DNA polymerase and dNTP)	TaqMan® Universal PCR Master Mix (ABI)	12,5 µl
Primers	Bt11 3-5' and Bt11 3-3' (see Table C.36)	see Table C.36
Probe	Bt11-2-Ta q (see Table C.36)	see Table C.36

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

C.7.7.2 PCR controls

Each test series shall include the controls as stated in ISO 24276.

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

As a positive control/calibration reference material, at least two alternatives are available, as follows.

- a) High quality, pure genomic DNA extracted from maize kernel may be used if the quantity of DNA is known, on the basis of calculating copy numbers of the target sequence from the genome size of Bt11 maize.
- b) A plasmid containing the target sequence(s) may be added in different concentrations with known copy numbers. Such a plasmid is available from the GM Maize Detection Plasmid Set (Fasmac No. PS-2 and Nippon Gene No. 310-04981)⁶²⁾ [33].

According to quality assurance requirements, the positive controls should preferably not be the same as the calibration reference materials.

C.7.7.3 Temperature-time programme

The temperature-time programme as outlined in Table C.39 was optimized for the ABI PRISM® 7700 SDS (Applied Biosystems)⁶²⁾. In the collaborative trial, it was used in combination with the TaqMan® Universal Master Mix⁶²⁾. The use of other thermal cyclers may require specific adaptation. The time for activation/initial denaturation depends on the Master Mix used.

Table C.39 — Procedure: Reaction conditions

		Time	Temperature
		s	°C
Pre-PCR: decontamination		120	50
Pre-PCR: activation of DNA polymerase and denaturation of template DNA		600	95
PCR (40 cycles)			
Step 1	Denaturation	30	95
Step 2	Annealing and elongation	60	59

C.7.8 Limitations and interpretation of the results

If GM maize other than Bt11 contains the same construct-specific DNA sequences, the method is only suitable for the quantitation of Bt11 DNA in the absence of GMOs other than Bt11 maize.

The described method is suitable for measuring the ratio of Bt11 construct-specific sequence to the taxon-specific *zSSIIb* sequence of maize. This ratio reflects the amount of Bt11 in the investigated maize. This method has only been validated for maize kernels.

The collaborative trial is a valuable source of data to support an uncertainty estimation. It is also necessary to identify any sources of uncertainty that are not covered by the collaborative trial data, such as sampling and others, according to the framework of international fora^{[44],[45]}.

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

C.7.9 Calibration and calculation of results

The threshold value to determine the cycle of threshold (C_t) shall be defined by the analyst.

An example of the procedures after PCR analysis is available in the manufacturer's Guide in the GM Maize Detection Plasmid Set (Fasmac No. PM-2 and Nippon Gene No. 319-04981)⁶³⁾. See also Reference [33].

The C_f for Bt11 construct-specific quantitation and reference plasmid used in the collaborative trial is 0,50. The amount of GM maize in the matrix shall be calculated by the following equation:

$$w = \frac{N_{GM}}{N_{TX}} \times \frac{100}{C_f} \%$$

where

N_{GM} is the copy number of the GM-specific target sequence in the test sample DNA;

N_{TX} is the copy number of the taxon-specific target sequence in the test sample DNA.

C.8 Construct-specific method for the quantitation of maize line GA21 DNA using real-time PCR

C.8.1 Introduction

This annex describes a method for the detection and quantitation of a taxon-specific maize gene (maize starch synthase IIb: *zSSIIb*) and of the specific DNA construct junction between the optimized transit peptide sequence and the maize 5-enolpyruvylshikimate-3-phosphate synthase gene (*m-epsps*) presented in the GM maize GA21, based on real-time PCR using plasmid as a reference material in order to quantify the relative amount of maize line GA21 using a conversion factor (C_f) that is the ratio of copy numbers for the construct-specific and taxon-specific DNA sequences in the representative genuine GA21 seeds.

NOTE C_f is used for the calculation of GMO content (% by mass) from the GMO DNA copy numbers of the target-specific and the taxon-specific sequences. C_f could be measured as the ratio of the copy numbers for the target-specific sequence to the taxon specific sequence from an appropriate reference material.

For limitations, see C.8.8.

C.8.2 Validation status and performance characteristics

C.8.2.1 General

This method has been optimized for the ABI PRISM® 7700 SDS real-time PCR apparatus⁶³⁾ using plasmid pMul5 as the reference material^[33]. The pMul5 plasmid includes, in particular, PCR products amplified from the PCR systems to specifically amplify maize taxon sequence (*zSSIIb*), cauliflower mosaic virus 35S promoter sequence (p35S), nopaline synthase terminator sequence (tNOS), and the construct-specific sequence of MON 810, Event176, Bt11, GA21 and T25.

NOTE The plasmid is used as a calibrator to determine the GM contents calculated from the relative copy numbers for the GM-specific and taxon-specific DNA sequences.

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

The repeatability and reproducibility of the described method have been tested in a collaborative trial using the reference material and an unknown dried maize seed powder containing mixtures of GA21 and conventional maize kernel [34].

The copy numbers of taxon-specific sequences (*zSSIIb*) per genome have been assessed for 20 representative varieties of maize.

The method has been published in Japanese and Korean national standards [35],[36],[37],[38].

C.8.2.2 Collaborative trial

A total of 12 unknown samples of maize containing between 0 % and 10 % (by mass) of dried maize powder derived from line GA21 were analysed by fifteen participants.

NOTE 1 Seeds of a representative GA21 variety of heterozygous for the GM-trait was used to determine the Cf values and the preparation of unknown samples for the collaborative trials. The unknown samples of mixture of maize powder for the validation were prepared for 0 %, 0,1 %, 0,5 %, 1 %, 5 %, and 10 % (by mass) of dried maize powder derived from the variety. The homogeneity of the samples at each level was tested by using this quantitative method according to AOAC protocols [34],[39].

The method was validated for GA21 maize in a collaborative trial according to the AOAC protocol [39]. The collaborative trial was organized by the National Food Research Institute (NFRI, Tsukuba, Japan), together with the Centre for Food Quality Labelling and Consumer Services, Saitama, Japan, and the National Institute of Health Sciences, Tokyo, Japan. Fifteen laboratories including those from Japan, Republic of Korea and United States performed the collaborative trials using the ABI PRISM®7700 SDS (Applied Biosystems) 64) in two separate stages. All participants were requested to follow the procedures, DNA extraction and quantitative PCR.

The first stage aimed to determine the Cf of GA21. All participants received the set of primers, probes, reference material and the extracted DNAs from the seeds of GA21, which were prepared by Qiagen DNeasy Plant Maxi kit 64) and whose pertinence was tested at the NFRI prior to the study. The DNAs were used to measure copy numbers of GA21 construct-specific and a maize taxon-specific *zSSIIb* DNA sequence. All measurements in this stage were repeated three times. A total of 90 data sets was submitted by the participants. The correlations of calibration curves of which data were submitted from all participants were acceptable ($r > 0,990$). Under the AOAC protocol [39], outlier laboratories were removed by an extreme variation (Cochran’s test, $p < 0,025$) and extreme average level (Grubbs’s test, $p < 0,025$). No outliers were observed in either test, as shown in Table C.40.

Table C.40 — Summary of the Cf for GA21

Target sequence	GA21 construct-specific
Number of participating laboratories	15
Number of Cochran outliers	0
Number of Grubbs outliers	0
Number of retained laboratories	15
Cf ^a	1,40 ± 0,05
^a Expressed as mean ± confidence interval ($\alpha = 0,05$).	

The Cf may be redetermined by analysts using appropriate reference materials of GA21 maize.

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

Blind tests were performed as the second stage. The unknown samples of maize powder were designed as six pairs of blind duplicates, which include 0 %, 0,1 %, 0,5 %, 1 %, 5 % and 10 % (by mass) of dried powder of the GM maize line GA21 in conventional maize. The sample of 0 % of maize line GA21 as blank sample was used in order to remove the invalid laboratories prior to statistical analysis. The participants were instructed to extract DNA from the samples using the Qiagen kit. The data submitted from the retained laboratories by outlier tests were used to calculate the mean and confidence interval ($\alpha = 0,05$). The mean values were defined as the Cf for calculation of the GMO amount (%) in blind tests. The mean value of the Cf for GA21 construct-specific quantitation was 1,40.

Fourteen laboratories that participated in the second stage analysed 168 samples by amplifying *zSSIIb* and the construct-specific sequence of GA21. Laboratories that failed to report blank samples as 0 % were judged as invalid and all their data were rejected before outlier tests. In all experiments, the correlations of calibration curves were acceptable ($r > 0,990$). The laboratories showing an extreme variation and extreme average of data in the blind duplicate pair of the GA21 level were removed as Cochran and Grubbs outliers [40], [41], respectively, prior to the statistical analysis of accuracy and precision. Two Cochran outliers were detected in the data. The calculated mean, bias, the repeatability relative standard deviation (%) and the reproducibility relative standard deviation (%) at each mixing level are shown in Table C.41. [34]

NOTE 2 Collaborators did not calculate the final results using Cf determined by the first collaboration study. The copy number of each target sequence obtained in the Cf determinations and blind tests were reported to NFRI and unit of % GMO of blind test samples were converted to the final results using the Cf.

Table C.41 — Summary of validation for construct-specific of GA21 maize quantitation

	Mixing level (%)				
	0,1	0,5	1	5	10
Number of participating laboratories	14	14	14	14	14
Number of invalid laboratories	1	1	1	1	1
Number of Cochran outliers	1	0	0	1	0
Number of Grubbs outliers	0	0	0	0	0
Number of retained laboratories	12	13	13	12	13
Mean of GMO amount (%)	0,1	0,5	1,2	5,8	11,5
Bias of true value (%)	-5,4	+7,7	+20,2	+16,6	+15,0
Repeatability standard deviation s_r^a	0,019	0,068	0,148	0,476	0,907
Repeatability limit r^a ($r = 2,8 s_r$)	0,054	0,189	0,414	1,332	2,539
Repeatability relative standard deviation (%) ^b	20,5	12,6	12,3	8,2	7,9
Reproducibility standard deviation s_R^a	0,019	0,117	0,224	0,927	1,565
Reproducibility limit R^a ($R = 2,8 \times s_R$)	0,055	0,329	0,627	2,597	4,382
Reproducibility relative standard deviation (%) ^b	20,6	21,8	18,6	15,9	13,6
Below 20 copies ^c (Absolute Detection Limit in this method)	4/24	0/26	0/26	0/24	0/26
^a Expressed in units of % GMO.					
^b Expressed as percentage of the mean value.					
^c Below 20 copies are expressed as the ratio of the number of retained data below 20 copies to the total number of retained data.					

C.8.2.3 Molecular specificity

C.8.2.3.1 General

The method has been described in Reference [33]. Information on the genetic construct introduced into the maize genome is available in References [33] and [46]. Primers and TaqMan[®] probe ⁶⁵⁾ for the development of this method have been designed by the information described in Reference [46].

If the construct DNA introduced into the GA21 is used for other GM events, a false positive result could occur since the amplified sequence is derived from the construct.

C.8.2.3.2 Theoretical specificities

The theoretical specificities of the primers and probes were assessed through a search of the DDBJ ⁶⁶⁾ databases (December 3rd, 1999) and the safety evaluation documents disclosed by the Ministry of Health, Labour and Welfare in Japan, and by the Ministry of Agriculture, Forestry and Fisheries in Japan, using the nucleotide sequence as query sequence with the BLASTN program 2.2.3 ⁶⁵⁾. The result of the search confirmed a complete identity only with the expected target sequence.

C.8.2.3.3 Experimental determination of specificity

Amplification with the primers and probes resulted in the expected PCR products when tested with the dried maize powders containing 0 % to 10 % (mass fraction) GM maize line GA21, which were prepared for this method by NFR [33], [34].

Tested matrices were maize kernel, maize grits, maize flour and maize meal.

Specificity tests prior to the collaborative trial showed no cross reactivity of the detection system to the following non-target species/samples: rice (*Oryza sativa*), wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*). No cross reactivity had also been observed with the GM soya bean line GTS 40-3-2, the GM maize line MON 810, Event176, Bt11 and T25.

C.8.2.4 Optimization

Optimization of reagent was carried out on the ABI PRISM[®] 7700 SDS using TaqMan[®] chemistry ⁶⁵⁾ [43].

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

C.8.2.5 Limit of detection (LOD)

Absolute LOD according to the developer: 20 plasmid copies of reference material ^[33].

Relative LOD validated in collaborative trial: 0,1% of GA21.

C.8.2.6 Limit of quantitation (LOQ)

Absolute LOQ according to the method developer: 20 plasmid copies of reference material.

Relative LOQ validated in collaborative trial: 0,1% of GA21.

C.8.3 Adaptation

No specific information is available.

⁶⁵⁾ These are examples of suitable products available commercially. This information is only given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

⁶⁶⁾ DDBJ: DNA Data Bank of Japan (<http://www.ddbj.nig.ac.jp/searches-e.html>).

C.8.4 Principle

A 133 bp fragment of the construct-specific sequence of GA21 is amplified by PCR using a specific primer pair for GA21. PCR products are measured over each PCR cycle (real time) by means of a GA21 specific oligonucleotide probe labelled with two fluorescent dyes: FAM as a reporter dye and TAMRA as a quencher. For that purpose TaqMan[®] chemistry⁶⁷⁾ is employed.

A 151 bp fragment of the taxon-specific *zSSIIb* is amplified by PCR in a separate real-time PCR reaction using a specific primer pair for *zSSIIb*, and the PCR products are measured over each PCR cycle by means of a *zSSIIb*-specific TaqMan[®] probe⁶⁷⁾.

The calibration curve method has been used for quantitation of the copy number for extracted DNA from unknown test sample DNA extracts. Separate calibration curves with each primer/probe system are generated in the same analytical amplification run. The calibration curves are composed of five concentrations including 20, 125, 1 500, 20 000, 250 000 copies of plasmid DNA of pMuI5. At each of the five calibration points, triplicate measurement is performed. Triplicate reactions using an appropriate dilution of the DNA extracted from the unknown sample are also measured in the ABI PRISM[®] 7700 SDS (Applied Biosystems) in the same analytical run.

The C_t (cycle of threshold) values determined for the calibration points in the *zSSIIb* or GA21 construct-specific target, respectively, are plotted against the logarithm of the copy number of plasmid DNA of pMuI5^[33] to establish a calibration curve. The copy numbers measured for the test sample DNA are obtained by interpolation from the standard curves. For the determination of the amount of GA21 in the test sample, the copy number of the GA21 construct is divided by the copy number of the *zSSIIb* gene and the construct-specific C_f of GA21, multiplied by 100 to obtain the percentage as described in C.8.9.

C.8.5 Reagents

C.8.5.1 General

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

C.8.5.2 Water.

C.8.5.3 TaqMan[®] Universal Master Mix⁶⁷⁾, 2-fold.

C.8.5.4 Reference material (Plasmid)

The sequence of the reference material used to develop and validate the method was the plasmid pMuI5^[33] which is included in the GM Maize Detection Plasmid Set (Fasmac No. PM-2 and Nippon Gene No. 319-04981)⁶⁷⁾. Other reference materials may be used provided that the performance can be demonstrated to be equal or better.

C.8.5.5 Oligonucleotides

The sequences of oligonucleotides for the primers and probes for the GA21 construct-specific and maize taxon-specific genes are listed in Table C.42.

⁶⁷⁾ These are examples of suitable products available commercially. This information is only given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

Table C.42 — Oligonucleotides

Name	Oligonucleotide DNA sequence	Final concentration in PCR
Taxon-specific gene target sequence		
<i>SSIIb</i> 1-5'	5'-CTC CCA ATC CTT TgA CAT CTg C-3'	500 nmol/l
<i>SSIIb</i> 1-3'	5'-TCg ATT TCT CTC TTg gTg ACA gg-3'	500 nmol/l
<i>SSIIb</i> –Taq	5'-FAM-AgC AAA gTC AgA gCg CTg CAA TgC A-TAMRA-3' ^a	200 nmol/l
GMO target sequence		
GA21 3-5'	5'-gAA gCC TCg gCA ACg TCA-3'	500 nmol/l
GA21 3-3'	5'-ATC Cgg TTg gAA AgC gAC TT-3'	500 nmol/l
GA21-2-Taq	5' -FAM- AAg gAT CCg gTg CAT ggC Cg-TAMRA- 3' ^a	200 nmol/l
^a FAM: 6-carboxyfluorescein; TAMRA: 6-carboxytetramethylrhodamine.		

The length of the *SSIIb* PCR product is 151 bp; the length of the GA21 PCR product is 133 bp.

C.8.6 Apparatus

C.8.6.1 General

Standard laboratory apparatus should be used throughout unless otherwise specified.

C.8.6.2 Thermal cycler

The indicated temperature-time profile was tested with the ABI PRISM[®] 7700 SDS (Applied Biosystems) ⁶⁸⁾ during the collaborative trial. Other real-time PCR instruments may be used after adaptation of the reaction conditions.

C.8.6.3 Reaction plate and caps

The reaction plate and caps shall be suitable for PCR amplification on a thermal cycler, e.g. ABI PRISM[®] 96-Well Optical Reaction Plate, or MicroAmp[®] Optical Caps (8 caps/strip, flat) (Applied Biosystems) ⁶⁸⁾, respectively. Other reaction plates, vials or caps may also be used if they can be shown to lead to equivalent or better results.

C.8.7 Procedure: PCR set-up

C.8.7.1 General

The PCR set-up for the taxon-specific *zSSIIb* target sequence and for the GA21-specific target sequence should be carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.

The method is described for a total PCR volume of 25 µl per reaction mixture with the reagents as listed in Table C.43 for *zSSIIb* and in Table C.44 for GA21.

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

Table C.43 — Amplification reaction mixture for taxon-specific zSSI*lb*** sequence in the final volume per reaction vial**

Total reaction volume		25 µl
Template DNA (50ng maize genomic DNA)		2,5 µl
Reaction buffer (including DNA polymerase and dNTP)	TaqMan® Universal PCR Master Mix	12,5 µl
Primers	SSI <i>lb</i> 1-5' and SSI <i>lb</i> 1-3' (see Table C.42)	see Table C.42
Probe	SSI <i>lb</i> -Taq (see Table C.42)	see Table C.42

Table C.44 — Amplification reaction mixture for GA21 specific sequence in the final volume per reaction vial

Total reaction volume		25 µl
Template DNA (50 ng maize genomic DNA)		2,5 µl
Reaction buffer (including DNA polymerase and dNTP)	TaqMan® Universal PCR Master Mix	12,5 µl
Primers	GA21 3-5' and GA21 3-3' (see Table C.42)	see Table C.42
Probe	GA21-2-Taq (see Table C.42)	see Table C.42

C.8.7.2 PCR controls

Each test series shall include the controls as stated in ISO 24276.

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

As a positive control/calibration reference material, at least two alternatives are available, as follows.

- High quality, pure genomic DNA extracted from maize kernel may be used if the quantity of DNA is known, on the basis of calculating copy numbers of the target sequence from the genome size of GA21 maize.
- A plasmid containing the target sequence(s) may be added in different concentrations with known copy numbers. Such a plasmid is available from the GM Maize Detection Plasmid Set (Fasmac No. PS-2 and Nippon Gene No. 310-04981⁸⁹). See also Reference [33].

According to quality assurance requirements, the positive controls should preferably not be the same as the calibration reference materials.

C.8.7.3 Temperature-time programme

The temperature-time programme as outlined in Table C.45 was optimized for the ABI PRISM® 7700 SDS (Applied Biosystems). In the collaborative trial, it was used in combination with the TaqMan® Universal Master Mix. The use of other thermal cyclers may require specific adaptation. The time for activation/initial denaturation depends on the Master Mix used.

Table C.45 — Procedure: Reaction conditions

		Time	Temperature
		s	°C
Pre-PCR: decontamination		120	50
Pre-PCR: activation of DNA polymerase and denaturation of template DNA		600	95
PCR (40 cycles)			
Step 1	Denaturation	30	95
Step 2	Annealing and elongation	60	95

C.8.8 Limitations and interpretation of the results

If GM maize other than GA21 contains the same construct-specific DNA sequences, the method is only suitable for the quantitation of GA21 DNA in the absence of GMOs other than GA21 maize.

The described method is suitable for measuring the ratio of the GA21 construct-specific sequence to the taxon-specific *zSSIIb* sequence of maize. This ratio reflects the amount of GA21 in the investigated maize. This method has only been validated for maize kernels.

The collaborative trial is a valuable source of data to support an uncertainty estimation. It is also necessary to identify any sources of uncertainty that are not covered by the collaborative trial data, such as sampling and others, according to the framework of international fora [44],[45].

C.8.9 Calibration and calculation of results

The threshold value determine cycle of threshold (C_t) shall be defined by the analyst.

An example of the procedures after PCR analysis is available in the manufacturer's Guide in the GM Maize Detection Plasmid Set (Fasmac No. PM-2 and Nippon Gene No. 319-04981⁶⁹). See also Reference [33].

The C_f for the GA21 construct-specific quantitation and reference plasmid used in the collaborative trial is 1,40. The amount of GM maize in the matrix shall be calculated by the following equation:

$$w = \frac{N_{GM}}{N_{TX}} \times \frac{100}{C_f} \%$$

where

N_{GM} is the copy number of the GM-specific target sequence in the test sample DNA;

N_{TX} is the copy number of the taxon-specific target sequence in the test sample DNA.

C.9 Construct-specific method for the quantitation of maize line T25 DNA using real-time PCR

C.9.1 Introduction

This annex describes a method for the detection and quantitation of a taxon-specific maize gene (maize starch synthase IIb: *zSSIIb*) and of the specific DNA construct junction region between the promoter sequence of cauliflower mosaic virus and the synthetic phosphinothricin acetyltransferase (*pat*) gene derived from *Streptomyces viridochromogenes* presented in the GM maize T25, based on real-time PCR using plasmid as

a reference material in order to quantify the relative amount of T25 using a conversion factor (Cf) that is the ratio of the copy numbers for the construct-specific and taxon-specific DNA sequences in the representative genuine T25 seeds.

NOTE Cf is used for the calculation of GMO content (% , mass fraction) from the GMO DNA copy numbers of the target-specific and the taxon-specific sequence. Cf could be measured as the ratio of the copy numbers for the target-specific sequence to the taxon-specific sequence from an appropriate reference material.

For limitations, see C.9.8.

C.9.2 Validation status and performance characteristics

C.9.2.1 General

This method has been optimized for the ABI PRISM[®] 7700 SDS real-time PCR apparatus instruments⁶⁹⁾ using plasmid pMul5 as the reference material^[33]. The pMul5 plasmid includes, in particular, PCR products amplified from the PCR systems to specifically amplify maize taxon-specific (*zSSIIb*), cauliflower mosaic virus 35S promoter sequence (p35S), nopaline synthase terminator sequence (tNOS), and the construct-specific sequence of MON 810, Event176, Bt11, GA21 and T25.

NOTE The plasmid is used as a calibrator to determine the GM contents calculated from relative copy numbers for the GM-specific and taxon-specific DNA sequence.

The repeatability and reproducibility of the described method has been tested in a collaborative trial using the reference material and unknown dried maize seed powder containing mixtures of T25 and conventional maize kernels^[34].

The copy numbers of the taxon-specific sequences (*zSSIIb*) per genome have been assessed for 20 representative varieties of maize.

The method has been published in Japanese and Korean national standards^{[35],[36],[37],[38]}.

C.9.2.2 Collaborative trial

A total of 12 unknown samples of maize containing between 0 % and 10 % (by mass) of dried maize powder derived from line T25 were analysed by fifteen participants.

NOTE 1 Seeds of a representative T25 variety of heterozygous for the GM-trait was used to determine the Cf values and the preparation of unknown samples for the collaborative trials. The unknown samples of the mixture of maize powder for the validation were prepared for 0 %, 0,1 %, 0,5 %, 1 %, 5 %, and 10 % (mass fraction) of dried maize powder derived from the variety. The homogeneity of the samples at each level are tested by using this quantitative method according to AOAC protocols^{[34],[39]}.

The method was validated for T25 maize in a collaborative trial according to the AOAC protocol^[39]. The collaborative trial was organized by the National Food Research Institute (NFRI, Tsukuba, Japan), together with the Centre for Food Quality Labelling and Consumer Services, Saitama, Japan, and the National Institute of Health Sciences, Japan. Fifteen laboratories including those from Japan, Republic of Korea and United States performed the collaborative trials using the ABI PRISM[®] 7700 SDS (Applied Biosystems)⁶⁹⁾ in two separate stages. All participants were requested to follow the procedures, DNA extraction and quantitative PCR.

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

The first stage aimed to determine the Cf of T25. All participants received the set of primers, probes, reference material and the extracted DNAs from the seeds of T25, which are prepared by Qiagen DNeasy Plant Maxi kit ⁷⁰⁾ and whose pertinence was tested at the NFRI prior to the study. The DNAs were used to measure copy numbers of T25 construct specific and a maize taxon specific *zSSIIb* DNA sequence. All measurements in this stage were repeated three times. A total of 90 data sets were submitted by the participants. The correlations of calibration curves of which data were submitted by all participants were acceptable ($r > 0,990$). Under the AOAC protocol ^[39], outlier laboratories were removed by an extreme variation (Cochran's test, $p < 0,025$) and extreme average level (Grubbs's test, $p < 0,025$). No outliers were observed in either test, as shown in Table C.46.

Table C.46 — Summary of the Cf for T25

Target sequence	T25 construct-specific
Number of participating laboratories	15
Number of Cochran outliers	0
Number of Grubbs outliers	0
Number of retained laboratories	15
Cf ^a	0,34 ± 0,01
^a Expressed as mean ± confidence interval ($\alpha = 0,05$).	

The Cf may be redetermined by analysts using appropriate reference materials of T25 maize.

Blind tests were performed as the second stage. The unknown samples of maize powder were designed as six pairs of blind duplicates, which included 0 %, 0,1 %, 0,5 %, 1 %, 5 % and 10,0 % (by mass) of dried powder of the GM maize line T25 in conventional maize ^[34]. The sample of 0 % of maize line T25 was used as blank sample in order to remove the invalid laboratories prior to statistical analysis. The participants were instructed to extract DNA from the samples using the Qiagen kit. The data submitted from the retained laboratories by outlier tests were used to calculate the mean and confidence interval ($\alpha = 0,05$). The mean values were defined as the Cf for calculation of the GMO amount (%) in blind tests. The mean value of Cf for T25 construct-specific quantitation was 0,34.

Fourteen laboratories that participated in the second stage analysed 168 samples by amplifying *zSSIIb* and the construct-specific sequence of T25. Laboratories that failed to report blank samples as 0 % were judged as invalid and all their data were rejected before the outlier tests. In all experiments, the correlations of calibration curves were acceptable ($r > 0,990$). The laboratories showing extreme variation and extreme average of data in the blind duplicate pair of the T25 level were removed as Cochran and Grubbs outliers ^{[40],[41]}, respectively, prior to the statistical analysis of accuracy and precision. Three Cochran outliers and one Grubbs outlier were detected in the data. The calculated mean, bias, the repeatability relative standard deviation (%) and the reproducibility relative standard deviation (%) at each mixing level are shown in Table C.47.

NOTE 2 Collaborators did not calculate the final results using Cf determined by the first collaboration study. The copy number of each target sequence obtained in the Cf determinations and blind tests were reported to NFRI and unit of % GMO of blind test samples were converted to the final results using the Cf.

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Table C.47 — Summary of validation for construct-specific T25 maize quantitation

	Mixing level (%)				
	0,1	0,5	1	5	10
Number of participating laboratories	14	14	14	14	14
Number of invalid laboratories	0	0	0	0	0
Number of Cochran outliers	2	0	1	0	0
Number of Grubbs outliers	1	0	0	0	0
Number of retained laboratories	11	14	13	14	14
Mean of GMO amount (%)	0,1	0,6	1,2	5,6	10,8
Bias of true value (%)	+38,6	+15,3	+20,0	+11,6	+8,1
Repeatability standard deviation s_r^a	0,033	0,162	0,082	0,690	1,439
Repeatability limit r^a ($r = 2,8 s_r$)	0,092	0,455	0,228	1,932	4,030
Repeatability relative standard deviation (%) ^b	23,7	28,2	6,8	12,4	13,3
Reproducibility standard deviation s_R^a	0,037	0,162	0,138	0,827	1,591
Reproducibility limit R^a ($R = 2,8 s_R$)	0,103	0,455	0,386	2,317	4,456
Reproducibility relative standard deviation (%) ^b	26,5	28,2	11,5	14,8	14,7
Below 20 copies ^c (Absolute Detection Limit in this method)	22/22	1/28	0/26	0/28	0/28
^a Expressed in units of % GMO. ^b Expressed as percentage of the mean value. ^c Below 20 copies are expressed as the ratio of the number of retained data below 20 copies to the total number of retained data.					

C.9.2.3 Molecular specificity

C.9.2.3.1 General

The method has been described in Reference [33]. Information on the genetic construct introduced into the maize genome is available in References [33] and [46]. Primers and TaqMan[®] probe ⁷¹⁾ for the development of this method have been designed by the information described in Reference [46].

If the construct DNA introduced into the T25 is used for other GM events, a false positive result could occur since the amplified sequence is derived from the construct.

C.9.2.3.2 Theoretical specificities

The theoretical specificities of the primers and probes were assessed through a search of the DDBJ ⁷²⁾ databases ⁷¹⁾ (December 3rd, 1999) and the safety evaluation documents disclosed by the Ministry of Health, Labour and Welfare in Japan, and by the Ministry of Agriculture, Forestry and Fisheries in Japan, using the nucleotide sequence as query sequence with the BLASTN program 2.2.3 ⁷¹⁾. The result of the search confirmed a complete identity only with the expected target sequence.

⁷¹⁾ These are examples of suitable products available commercially. This information is only given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

⁷²⁾ DDBJ: DNA Data Bank of Japan (<http://www.ddbj.nig.ac.jp/searches-e.html>)

C.9.2.3.3 Experimental determination of specificity

Amplification with the primers and probes resulted in the expected PCR products when tested with the dried maize powders containing 0 % to 10 % (by mass) GM maize line T25, which were prepared for this method by NFR1 [33], [34].

Tested matrices were maize kernel, maize grits, maize flour and maize meal

Specificity tests prior to the collaborative trial showed no cross reactivity of the detection system to the following non-target species/samples: rice (*Oryza sativa*), wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*). No cross reactivity had been observed with the GM soya bean line GTS 40-3-2, the GM maize line MON 810, Event176, Bt11 and GA21.

C.9.2.4 Optimization

Optimization of reagent was carried out on the ABI PRISM[®] 7700 SDS⁷³⁾ using TaqMan[®] chemistry⁷³⁾ [43].

Primer and probe design were carried out applying the Primer Express[®] software (Applied Biosystems)⁷³⁾.

C.9.2.5 Limit of detection (LOD)

Absolute LOD according to the developer: 20 plasmid copies of reference material [33].

Relative LOD validated in collaborative trial: 0,5 % of T25.

C.9.2.6 Limit of quantitation (LOQ)

Absolute LOQ according to the method developer: 20 plasmid copies of reference material [33].

Relative LOQ validated in collaborative trial: 0,5% of T25.

C.9.3 Adaptation

No specific information is available.

C.9.4 Principle

A 149 bp fragment of the construct-specific sequence of T25 is amplified by PCR using a specific primer pair for T25. PCR products are measured over each PCR cycle (real time) by means of a T25 specific oligonucleotide probe labelled with two fluorescent dyes: FAM as a reporter dye and TAMRA as a quencher. For that purpose TaqMan[®] chemistry⁷³⁾ is employed.

A 151 bp fragment of the taxon-specific *zSSIIb* is amplified by PCR in a separate real-time PCR reaction using a specific primer pair for *zSSIIb*, and the PCR products are measured over each PCR cycle by means of a *zSSIIb*-specific TaqMan[®] probe⁷³⁾.

The calibration curve method has been used for quantitation of the copy number in extracted DNA from unknown test sample DNA extracts. Separate calibration curves with each primer/probe system are generated in the same analytical amplification run. The calibration curves are composed of five concentrations including 20, 125, 1 500, 20 000, 250 000 copies of plasmid DNA of pMul5. At each of the five calibration points,

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