BRITISH STANDARD

BS EN ISO 21570:2005 +A1:2013

Incorporating corrigendum no. 1

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

ICS 67.050

NO COPYING WITHOUT BSI PERMISSION EXCEPT AS PERMITTED BY COPYRIGHT LAW

National foreword

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 It supersedes BS EN ISO 21570:2005, which is withdrawn.

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

The start and finish of text introduced or altered by corrigendum is indicated in the text by tags. Text altered by ISO corrigendum December 2006 is indicated in the text by [AC1⟩ ⟨AC1].

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

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

The publication does not purport to include all the necessary provisions of a contract. Users are responsible for its correct application.

Compliance with a British Standard cannot confer immunity from legal obligations.

This British Standard was published under the authority of the Standards Policy and Strategy Committee on 23 January 2006

© The British Standards Institution 2013.
Published by BSI Standards Limited 2013

ISBN 978 0 580 76308 3

Amendments/corrigenda issued since publication

Amd. No.	Date	Comments
17383 Corrigendum No. 1	31 October 2007	See national foreword
	30 June 2013	Implementation of ISO amendment 1:2013 with CEN endorsement A1:2013

EUROPEAN STANDARD

NORME EUROPÉENNE

EUROPÄISCHE NORM

EN ISO 21570:2005+A1

April 2013

ICS 67.050

Incorporating corrigendum February 2007

English Version

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

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

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

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

CEN members are bound to comply with the CEN/CENELEC Internal Regulations which stipulate the conditions for giving this European Standard the status of a national standard without any alteration. Up-to-date lists and bibliographical references concerning such national standards may be obtained on application to the Central Secretariat or to any CEN member.

This European Standard exists in three official versions (English, French, German). A version in any other language made by translation under the responsibility of a CEN member into its own language and notified to the Central Secretariat has the same status as the official versions.

CEN members are the national standards bodies of Austria, Belgium, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Slovakia, Slovenia, Spain, Sweden, Switzerland and United Kingdom.

EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

Management Centre: rue de Stassart, 36 B-1050 Brussels

© 2005 CEN All rights of exploitation in any form and by any means reserved worldwide for CEN national Members.

Ref. No. EN ISO 21570:2005: E

Foreword

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

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

According to the CEN/CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard: Austria, Belgium, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Slovakia, Slovenia, Spain, Sweden, Switzerland and United Kingdom.

Foreword to amendment 1

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

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

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

According to the CEN-CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, Former Yugoslav Republic of Macedonia, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and the United Kingdom.

Endorsement notice

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

Contents

Page

Foreword ... iii
Introduction ... iv
1 Scope ... 1
2 Normative references ... 1
3 Terms and definitions ... 1
4 Principle ... 1
4.1 General ... 1
4.2 Amplification, detection and confirmation of PCR products ... 2
4.3 Quantitation of PCR products ... 2
5 Reagents ... 2
6 Apparatus and equipment ... 2
7 Guidelines concerning the procedure ... 2
7.1 General ... 2
7.2 Target sequence stability ... 3
7.3 Calibration of the analysis ... 3
7.4 Quantitation considerations ... 3
7.5 Quality assurance requirements ... 3
8 Interpretation ... 4
9 Expression of results ... 4
10 Test report ... 5
Annex A (informative) Target taxon-specific methods ... 6
A.1 Target taxon-specific method for the absolute quantitation of the *adh*1 gene DNA of maize using real-time PCR ... 6
A.2 Target-taxon-specific method for the detection of DNA derived from rice ... 12
A.3 Target-taxon-specific method for the detection of components derived from tomato (Lycopersicon exculentum ... 19
Annex B (informative) Screening methods ... 26
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 ... 26
Annex C (informative) Construct-specific methods ... 34
C.1 Construct-specific method for the quantitation of soya bean line GTS 40-3-2 DNA using real-time PCR (Method 1) ... 34
C.2 Construct-specific method for the quantitation of soya bean line GTS 40-3-2 DNA using real-time PCR (Method 2) ... 41
C.3 Construct-specific method for the quantitation of Event176 maize DNA using real-time PCR ... 48
C.4 Construct-specific method for the quantitation of soya bean line GTS 40-3-2 DNA using real-time PCR ... 55
C.5 Construct-specific method for the quantitation of maize line MON 810 DNA using real-time PCR ... 63

C.6 Construct-specific method for the quantitation of maize line Event176 DNA using real-time PCR ... 70

C.7 Construct-specific method for the quantitation of maize line Bt11 DNA using real-time PCR ... 77

C.8 Construct-specific method for the quantitation of maize line GA21 DNA using real-time PCR ... 85

C.9 Construct-specific method for the quantitation of maize line T25 DNA using real-time PCR ... 92

Annex D (informative) Event-specific methods ... 101

D.1 Event-specific method for the absolute and relative quantitation of maize line Bt11 DNA based on real-time PCR ... 101

D.2 Event-specific method for the relative quantitation of maize line MON 810 DNA using real-time PCR ... 107

Bibliography ... 114

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.

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

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

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

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

3 Terms and definitions

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

4 Principle

4.1 General

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

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

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

The analysis generally consists of

— amplification of one or more specific target sequences,

— detection and confirmation of the specificity of the PCR product(s), and

— quantitation of the amplified fragments relative to calibrants.

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

4.2 Amplification, detection and confirmation of PCR products

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

4.3 Quantitation of PCR products

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

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

5 Reagents

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

6 Apparatus and equipment

See Annexes A to D and ISO 24276.

7 Guidelines concerning the procedure

7.1 General

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

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

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

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

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

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

7.2 Target sequence stability

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

7.3 Calibration of the analysis

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

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

7.4 Quantitation considerations

PCR methods should be appropriately designed to minimize the variability.

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

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

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

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

7.5 Quality assurance requirements

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

a) within test portion consistency:

- through rejection of measurements $<$LOQ, and
- through maximum deviation observed between dilutions and individual measurements equals the value expected from the corresponding dilution factor ± 33 %;

b) between test portion consistency:

- estimated relative GMO-derived DNA concentrations obtained under a) for each test portion should not differ by values greater than –50 % to +100 % of the estimated quantity value (equal to a ΔC_t of 1 in real-time PCR) (i.e. for two test portions, measurements of 1,0 % and 2,0 % are acceptable, measurements of 0,9 % and 2,1 % are not).

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

A1 8 Interpretation

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

a) Fit for quantification of the target sequence provided:

- the result is positive according to ISO 21569:2005, 8.1;
- the observable inhibition of the reaction is negligible;
- the analysis produces an unambiguous measurement value;
- the target sequence content is within the dynamic range of the method;
- the analysis is calibrated in an acceptable way (see 7.3).

b) Unfit for quantification of the target sequence if any of the conditions listed in a) are not fulfilled.

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

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

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

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

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

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

9 Expression of results

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

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

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

A1 10 Test report

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

a) the LOQ of the method and the matrix used to establish it;

b) the practical LOQ;

c) a reference to the method which has been used for the extraction of DNA;

d) a reference to the methods used for the amplification of the DNA target sequences;

e) the reference material used;

f) the results expressed according to Clause 9;

g) the PCR target and whether considered "event specific" or "construct specific" or "screening";

h) the definition of the measurement uncertainty used.

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

Table 1 — Expression of results

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

A1

Annex A
(informative)

Target taxon-specific methods

A.1 Target taxon-specific method for the absolute quantitation of the *adh*1 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*) *adh*1 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/DDBJ[2)] accession number X04050. This sequence is unique to *Zea mays* (maize/corn) and *Zea mays* subsp. *diploperennis* (teosinte) [11].

A.1.2.3.2 Theoretical specificities

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

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

A.1.2.3.3 Experimental determination of specificity

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

A.1.2.4 Optimization

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

A.1.2.5 Limit of detection (LOD)

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

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

A.1.2.6 Limit of quantitation (LOQ)

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

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

A.1.3 Adaptation

No specific information is available.

A.1.4 Principle

A 134 bp fragment of the *adh*1 gene is amplified using two maize *adh*1-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 *adh*1-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 *adh*1-DNA in an unknown sample, the C_t-value is converted into a corresponding copy number value by comparison with a calibration curve whose C_t-values are directly linked with known copy numbers (regression analysis).

A.1.5 Reagents

A.1.5.1 General

For quality of reagents to be used, see [A1⟩ ISO 24276:2006 ⟨A1], 6.6.

A.1.5.2 Water.

A.1.5.3 PCR buffer (without $MgCl_2$), 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 $MgCl_2$ solution, $c(MgCl_2)$ = 25 mmol/l.

A.1.5.5 dNTP solution, c(dNTP) = 2,5 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 *adh*1 amplicon is 134 bp.

A.1.5.7 Thermostable DNA polymerase

AmpliTaq Gold® DNA polymerase [4)].

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 *N*-glycosylase)		
Reaction buffer (containing passive reference ROX) [a]		
dNTP mix		
Primers	see Table A.2	see A.1.5.6
Probe	see Table A.2	see A.1.5.6
[a] ROX = carboxy-X-rhodamine.		

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

A.1.6 Apparatus

A.1.6.1 General

Standard laboratory apparatus should be used throughout unless otherwise specified.

A.1.6.2 Thermal cycler

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

A.1.6.3 Reaction vials

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

A.1.7 Procedure: PCR set-up

A.1.7.1 General

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

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

A.1.7.2 PCR controls

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

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

A.1.7.3 Temperature-time programme

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

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

Table A.4 — Procedure: Reaction conditions

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

A.1.8 Limitations and interpretation of the results

The presence of PCR inhibitors may have a strong impact on the accuracy of the estimated copy number of the *adh*1 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 *adh*1 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 Excel[6], or directly by options available with the sequence detection system software.

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

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

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

A.2.1 Principle

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

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

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

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

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

— ISO 5725;[51]–[56]

— the IUPAC protocol for the design, conduct and interpretation of method-performance studies (Reference [12]).

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

A.2.2 Scope

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

A.2.3 Validation status and performance criteria

A.2.3.1 Robustness of the method

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

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

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

A.2.3.2 Intralaboratory trial

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

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

A.2.3.3 Collaborative trial

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

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

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

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

A1) A.2.3.4 Molecular selectivity

A.2.3.4.1 General

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

Table A.6 — Oligonucleotide primers and probe sequences

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

A.2.3.4.2 Experimental

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

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

A.2.3.4.3 Theoretical

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

A.2.4 Principle and summary

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

A.2.5 Terms and definitions

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

A.2.6 Sample type and amounts

DNA samples extracted from the grains of four rice cultivars, were used to construct the standard curves in this collaborative study. Then, eight blind samples were analysed using the four standard curves constructed. (A1

A1 The participants received the following samples.

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

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

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

A.2.8 Estimation of measurement uncertainty

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

A.2.9 Interferences

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

A.2.10 Physical and environmental conditions

The procedures require experience of working under sterile conditions.

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

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

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

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

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

Pipettes should be calibrated regularly, if necessary. A1

[A1) A.2.11 Apparatus and equipment

Usual laboratory equipment and in particular the following.

A.2.11.1 **Microcentrifuge**.

A.2.11.2 **Freezer** maintained at −20 °C and **refrigerator** maintained at 4 °C.

A.2.11.3 **Micropipettes**.

A.2.11.4 **Vortex mixer**.

A.2.11.5 **Tubes**, of capacities 0,2 ml, 1,5 ml, 2,0 ml.

A.2.11.6 **Tips** and **filter tips** for micropipettes.

A.2.11.7 **Rack** for reaction tubes.

A.2.11.8 **Vinyl** or **latex gloves**.

A.2.11.9 **Vacuum dryer** suitable for drying DNA pellets,optional.

A.2.11.10 **Real-time PCR system** with plastic reaction vials suitable for fluorescence measurement.

A.2.11.11 **Software**: Sequence Detection System[1)] version 1.7 (Applied Biosystems Part No 4311876[1)]) or equivalent versions.

A.2.11.12 **Optical 96 well reaction plates**, MicroAmp®[1)] (Applied Biosystems Part No N801-0560[1)]).

A.2.11.13 **Optical adhesive covers**, MicroAmp®[1)] (Applied Biosystems Part No 4311971[1)]).

A.2.11.14 **Optical caps**, MicroAmp®[1)] (Applied Biosystems Part No. No 801-0935[1)]).

A.2.12 Reagents and materials

A.2.12.1 General

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

A.2.12.2 DNA extraction

A.2.12.2.1 **Cetyltrimethylammonium bromide** (CTAB), ultrapure grade.

A.2.12.2.2 ***Tris*-(hydroxymethyl)aminomethane hydrochloride** (tris), molecular biology grade.

A.2.12.2.3 **Ethylenediaminetetraacetic acid disodium salt** (EDTA), titration 99,9 % mass fraction.

A.2.12.2.4 **Ethanol**, $\varphi[CH_3CH_2OH]$ at least 96 % volume fraction.

A.2.12.2.5 **Isopropanol**, $\varphi[CH_3CH(OH)CH_3]$ at least 99,7 % volume fraction.

A.2.12.2.6 **Chloroform**, $\varphi(CHCl_3)$ at least 99 % volume fraction.

A.2.12.2.7 **Sodium chloride**, $w(NaCl)$ at least 99 % mass fraction.

A.2.12.2.8 **Sodium hydroxide**, anhydrous, $w(NaOH)$ at least 98 % mass fraction.

A.2.12.2.9 **RNase A solution**, 10 mg/ml.

A.2.12.2.10 **Proteinase K solution**, 20 mg/ml. (A1]

A1 **A.2.12.2.10 Proteinase K solution**, 20 mg/ml.

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

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

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

A.2.12.3 Quantitative real-time PCR

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

A.2.13 Sample collection, transport, preservation and storage

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

A.2.14 Preparation of test sample

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

A.2.15 Instrument calibration

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

A.2.16 Analysis steps

A.2.16.1 General

A.2.16.1.1 Preparation of the DNA for standard curve construction

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

For specific requirements, see ISO 21571.

A.2.16.1.2 PCR reagents

A.2.16.1.3 PCR master mix

See A.2.12.3.

A.2.16.1.4 Primers and probe

See Table A.6.

A.2.16.2 Procedure

A.2.16.2.1 General

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

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

A1 Distribute 20 µl/tube of the master mixture to 200 µl PCR reaction tubes (A.2.11.5). Add 5 µl of DNA solution samples, rice positive control, negative control, and blank control (H_2O) to the tubes, respectively.

Mix the PCR tubes gently and centrifuge in the microcentrifuge (A.2.11.1) at 1 000 × *g* for 10 s.

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

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

A.2.16.2.2 PCR controls

See A.2.6.

A.2.16.2.3 Preparation of standards

Calibration curves are produced by plotting C_t values against the logarithm of the quantity of target DNA, in nanograms. This can be done by use of spreadsheet software [e.g. Microsoft Excel[1]], or directly by options available with the sequence detection system software (A.2.11.11).

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

A.2.16.2.4 Temperature–time programme (PCR)

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

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

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

A.2.16.2.5 Accept or reject criteria

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

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

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

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

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

A.2.16.2.6 Identification

The use of a TaqMan®[1] probe allows the unambiguous identification of PCR products. A1

[A1) A.2.17 Sample identification

See A.2.6.

A.2.18 Interpretation and calculations of the results

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

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

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

A.2.19 Record keeping

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

A.2.20 Reporting

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

A.2.21 Safety measures

No specific requirements. See ISO 24276.

A.2.22 Pollution prevention and waste disposal

No specific requirements. See ISO 24276.

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

A.3.1 Principle

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

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

— Quantitative real-time PCR for the quantitative standard curve construction using tomato DNA samples from four different tomato cultivars.

— Quantitative real-time PCR for the quantification of blind tomato DNA samples using the constructed standard curves. (A1]

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

— ISO 5725,[51]–[56] especially considered in relation to the measure of precision (i.e. repeatability and reproducibility) and trueness;

— the IUPAC protocol for the design, conduct and interpretation of method-performance studies (Reference [12]).

A.3.2 Scope

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

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

A.3.3 Validation status and performance criteria

A.3.3.1 Robustness of the method

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

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

A.3.3.2 Intralaboratory trial

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

A.3.3.3 Collaborative trial

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

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

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

A1⟩ **Table A.8 — Validation data of the *LAT52* real-time PCR system**

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

A.3.3.4 Molecular selectivity

A.3.3.4.1 General

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

Table A.9 — Oligonucleotide primers and probe sequences,

Name	Oligonucleotide DNA Sequence (5′ to 3′)
Quantitative real time PCR primer and probe sequence	
LAT52 primer F	AgACCACgAGAACgATATTTgC
LAT52 primer R	TTCTTgCCTTTTCATATCCAgACA
LAT52 probe	HEX- CTCTTTgCAgTCCTCCCTTgggCT-BHQ

A.3.3.4.2 Experimental

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

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

A.3.3.4.3 Theoretical

The theoretical specificity of the *LAT52* primers and probe was analysed by a similarity search using the BLASTN 2.0MP-WashU program (Reference [64], search date: 2010-01-20). The 92 bp *LAT52* sequence used as query is part of the NCBI accession number X15855 (nucleotides 1385–1476), and the blast analysis result confirmed the complete identity of the query sequence with tomato anther specific *LAT52* gene sequences. ⟨A1

A1) A.3.4 Principle and summary

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

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

A.3.5 Terms and definitions

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

A.3.6 Sample type and amounts

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

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

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

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

A.3.8 Estimation of measurement uncertainty

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

A.3.9 Interferences

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

A.3.10 Physical and environmental conditions

The procedures should be performed under sterile working conditions.

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

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

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

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

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

Pipettes should be checked for precision and calibrated frequently.

A.3.11 Apparatus and equipment

Usual laboratory equipment and in particular the following. (A1

[A1) **A.3.11.1** **Microcentrifuge**.

A.3.11.2 **Freezer** maintained at −20 °C and **refrigerator** maintained at 4 °C.

A.3.11.3 **Micropipettes**.

A.3.11.4 **Vortex mixer**.

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

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

A.3.11.7 **Rack** for reaction tubes.

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

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

A.3.11.10 **Real-time PCR instrument**.

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

A.3.11.12 **Software**: Sequence Detection System[1)] version 1.7 (Applied Biosystems Part No 4311876[1)]) or equivalent versions.

A.3.12 Reagents and materials

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

A.3.12.1 Real-time PCR master mixture.

A.3.12.2 Oligonucleotides (primers and probe).

A.3.13 Sample collection, transport, preservation and storage

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

A.3.14 Test sample preparation

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

A.3.15 Instrument calibration

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

A.3.16 Analysis steps

A.3.16.1 General

A.3.16.1.1 Preparation of the DNA for standard curves construction

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

Each of the 50 ng/µl tomato DNA sample was diluted to 10 ng/µl, 1 ng/µl, 0,1 ng/µl, 0,01 ng/µl, 0,002 ng/µl using the 0,1 × TE, respectively. (A1]

A1 A.3.16.1.2 PCR reagents

A.3.16.1.3 Real-time PCR master mixture

See A.3.12.

A.3.16.1.4 Primers

See Table A.9.

A.3.16.2 Procedure

A.3.16.2.1 General

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

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

Distribute 20 µl per tube of the master mixture to 200 µl PCR reaction tubes (A.3.11.7). Add 5 µl of DNA solution samples, tomato positive control, negative control, and blank control (H_2O) to the tubes, respectively.

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

Place the plate into the instrument.

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

A.3.16.2.2 PCR controls

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

A.3.16.2.3 Preparation of standards

Calibration curves are generated by plotting C_t values against the logarithm of the target DNA amount, in nanograms. This can be done by use of spreadsheet software [e.g. Microsoft Excel[1)]], or directly by options available with the sequence detection system software (A.3.11.11).

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

A.3.16.2.4 Temperature–time programme (PCR)

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

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

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

A1

[A1) A.3.16.2.5 Accept or reject criteria

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

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

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

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

A.3.16.2.6 Identification

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

A.3.17 Sample identification

See A.3.6.

A.3.18 Interpretation and calculations of the results

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

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

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

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

A.3.19 Record keeping

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

A.3.20 Reporting

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

A.3.21 Safety measures

No specific requirements. Refer to ISO 24276.

A.3.22 Pollution prevention and waste disposal

No specific requirements. Refer to ISO 24276. (A1]

Annex B
(informative)

Screening methods

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

B.1.1 Introduction

This annex describes a method for the specific amplification and detection of a taxon-specific soya bean gene (lectin gene, *le*1) 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 databases [9)] using the nucleotide sequences as query sequences with the BLASTN programme 2.2.3 [9)] [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 International [9)] 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 system [9)] (SDS) using TaqMan® chemistry [9)].

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® chemistry [10)] is employed.

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

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

B.1.5 Reagents

B.1.5.1 General

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

B.1.5.2 Water.

B.1.5.3 PCR buffer (without $MgCl_2$), 10-fold.

B.1.5.4 $MgCl_2$ solution, $c(MgCl_2)$ = 25 mmol/l.

B.1.5.5 dNTP solution, c(dNTP) = 2,5 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 polymerase [11)].

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

B.1.6 Apparatus

B.1.6.1 General

Standard laboratory apparatus should be used throughout unless otherwise specified.

B.1.6.2 Thermal cycler

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

B.1.6.3 Reaction vials

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

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

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 *N*-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 N-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.

---

12) 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 ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

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 Ct (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)

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

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, *le*1) 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

13) 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 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 isgiven 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 $\times$ 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® chemistry [16] 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® probe [16] (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 [A1) ISO 24276:2006 (A1], 6.6.

C.1.5.2 Water.

C.1.5.3 PCR buffer (without $MgCl_2$), 10-fold.

C.1.5.4 $MgCl_2$ solution, $c(MgCl_2)$ = 25 mmol/l.

C.1.5.5 dNTP solution, c(dNTP) = 2,5 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 adaption 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 AmpErase uracil *N*-glycosylase	400 µmol/l 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 AmpErase uracil N-glycosylase	400 µmol/l 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 series [18)]) 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 polymerase [18)]. 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):

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

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, *le*1) 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-410R [20])) [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-410R [20])) 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® system [20]) (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® system [20]) (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 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 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® chemistry 23) [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® 7700 23).

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® chemistry 23) is employed.

A 74 bp fragment of the taxon-specific soya bean *lectin* gene (*le*1) is amplified by PCR in a separate real-time PCR using two soya bean *le*1 gene specific primers, and the PCR products are measured during each PCR cycle by means of a *le*1-specific TaqMan® probe 23).

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 [A1) ISO 24276:2006, (A1] 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.

[AC1) **C.2.5.5 dNTP solution**, c(dNTP) = 2,5 mmol/l. (AC1]

C.2.5.6 Oligonucleotides

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

[AC1) 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 TTg 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 AgC AgA TCT TTC-TAMRA-3'[a]	125 nmol/l

[a] FAM: 6-carboxyfluorescein; TAMRA: 6-carboxytetramethylrhodamine.

(AC1]

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 uracil *N*-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	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_2O (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 *N*-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_2O (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-410R [27]. At each of the four calibration points, duplicate (ABI PRISM® 7700 SDS [27] and GeneAmp® 5700) [27] or single determinations (LightCycler® system [27]) 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® system [27].

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 Excel [27], 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 *cry*1A(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 411 [28])) 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 411 [28] 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 SDS [28] and 7 laboratories using the ABI GeneAmp® 5700 SDS [28].

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 (*Lycopersicum 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-411 30), 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 SDS 30) using the TaqMan® chemistry [30]. No additional optimization was carried out for the GeneAmp® 5700 SDS 30) because the thermal cycler in this unit is identical to the cycler of the ABI PRISM® 7700 SDS 30).

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® chemistry 30) 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 [A1) ISO 24276:2006 (A1], 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.

[AC1) **C.3.5.5 dNTP solution**, c(dNTP) = 2,5 mmol/l (each). (AC1]

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 Biosystems [31)]. 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 Biosystems [31)]).

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 *N*-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 *N*-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 s	Temperature °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 series 33). 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 Excel 33) 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, *le*1) 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 pMulSL2 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 apparatus 33) using plasmid pMulSL2 as the reference material [33]. The pMulSL2 plasmid includes in particular PCR products amplified from the PCR systems to specifically amplify soya bean taxon sequence (*Le*1), 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 (*Le*1) 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 (α = 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 (α = 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 *Le*1 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. DDBJ [35)] 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 DDBJ [35)] 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 2.2.3 [36)]. 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 NFRI [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® chemistry [36)] [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® chemistry [37)] is employed.

A 118 bp fragment of the taxon-specific soya bean lectin gene (*Le*1) is amplified by PCR in a separate real-time PCR reaction using two soya bean *Le*1 specific primers, and the PCR products are measured during each PCR cycle by means of a *Le*1-specific TaqMan® probe [37)].

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 *Le*1 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 *Le*1 gene and construct-specific Cf 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 A1) ISO 24276:2006 (A1, 6.6.

C.4.5.2 Water.

C.4.5.3 TaqMan® Universal Master Mix [37)], 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		
*Le*1n02-5’	5’-gCC CTC TAC TCC ACC CCC A-3’	500 nmol/l
*Le*1n02-3’	5’-gCC CAT CTg CAA gCC TTT TT-3’	500 nmol/l
*Le*1-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 *Le*1 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 *Le*1 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 *Le*1 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	*Le1*n02-5' and *Le1*n02-3' (see Table C.18)	see Table C.18
Probe	*Le1*-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.

a) 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.

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 Soybean (RRS) Detection Plasmid Set (Fasmac No. PS-2 and Nippon Gene No. 310-04981 [39]) [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 Mix [39]. 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 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.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 (Cf) 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:

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

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 apparatus [40)] using plasmid pMul5 [40)] as the reference material [33]. The pMul5 plasmid includes in particular PCR products amplified from the PCR systems to specifically amplify maize taxon sequence (z*SSIIb*), 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 kit [41)] 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 (α = 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 kit [41)]. The data submitted from the retained laboratories by outlier tests were used to calculate mean and confidence interval (α = 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 x 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 2.2.3 [44)]. 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® chemistry [44)] [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® probe [44)].

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 pMul5 45) [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 *zSSIIb* 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 *zSSIIb* 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 [A1⟩ ISO 24276:2006 ⟨A1], 6.6.

C.5.5.2 Water.

C.5.5.3 TaqMan® Universal Master Mix 45), 2-fold.

C.5.5.4 Reference material (Plasmid)

The reference material used to develop and validate the method was the plasmid pMul5 45) [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		
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		
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 SSIIb 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) respectively[46]. 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:

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

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 apparatus 48) using plasmid [AC1) pMul5 (AC1] 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.

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 (α = 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 the calculate mean and confidence interval (α = 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 *zSSIIb* 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

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.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® probe[50)] 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 DDBJ [51)] 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 [50)]. 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 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 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® chemistry [50] [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® chemistry [52)] 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® probe [52)].

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 pMul5 [52)]. 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 Cf 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 [A1⟩ ISO 24276:2006 ⟨A1], 6.6.

C.6.5.2 Water.

C.6.5.3 TaqMan® Universal Master Mix [52)], 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.

a) 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.

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) [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 Mix [54)]. 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 Cf 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:

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

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.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 (Cf) 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 apparatus [55)] 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 terminatior 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 kit [56)] 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 *zSSIIb* 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 (α = 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 (α = 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 *zSSIIb* 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 × 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

[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.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® probe [57)] 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 DDBJ [58)] 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 [57)]. 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 SDS 59) using TaqMan® chemistry 59) [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 SDS 59) 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 pMul5 59) [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 Cf 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 [A1) ISO 24276:2006 (A1], 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 *zSSIIb* 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 *zSSIIb* and in Table C.38 for Bt11.

Table C.37 — Amplification reaction mixture for taxon-specific zSSIIb 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	SSIIb1-5’ and SSIIb1-3’ (see Table C.36)	see Table C.36
Probe	SSIIb-Taq (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-Taq (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 Mix [62)]. 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 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.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 Cf 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:

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

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.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 region 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 (Cf) that is the ratio of copy numbers for the construct-specific and taxon-specific DNA sequences in the representative genuine GA21 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 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 apparatus 63) 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 (z*SSIIb*), 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 (α = 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 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 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® chemistry [67)] 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® probe [67)].

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 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 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 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 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 Cf 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 [A1⟩ ISO 24276:2006 ⟨A1], 6.6.

C.8.5.2 Water.

C.8.5.3 TaqMan® Universal Master Mix [67)], 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 pMul5 [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		
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		
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 *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	12,5 µl
Primers	SSIIb1-5' and SSIIb1-3' (see Table C.42)	see Table C.42
Probe	SSIIb-Taq (see Table C.42)	see Table C.42

Table C.44 — Amplification reaction mixture for GA21specific 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.

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 GA21 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 [89)]). 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 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	AC1 60 AC1

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. 319-04981 [69)]). See also Reference [33].

The Cf 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:

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

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.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 instruments 69) 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 (z*SSIIb*), 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 (α = 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 (α = 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.

70) 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.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 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 MON 810, Event176, Bt11 and GA21.

C.9.2.4 Optimization

Optimization of reagent was carried out on the ABI PRISM® 7700 SDS 73) using TaqMan® chemistry 73) [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® chemistry 73) 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® probe 73).

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,

---

73) 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.

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 [74)] in the same analytical run.

The C_t values determined for the calibration points in the *zSSIIb* or T25 construct-specific target, respectively, is plotted against the logarithm of the copy number of plasmid DNA of pMul5 to establish a calibration curve. See also Reference [33]. The copy numbers measured for the test sample DNA are obtained by interpolation from the standard curves. For the determination of the amount of T25 in the test sample, the copy number of the T25 construct is divided by the copy number of the z*SSIIb* gene and construct-specific Cf of T25, multiplied by 100 to obtain the percentage as described in C.9.9.

C.9.5 Reagents

C.9.5.1 General

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

C.9.5.2 Water.

C.9.5.3 TaqMan® Universal Master Mix [74)], 2-fold.

C.9.5.4 Reference material (Plasmid)

The sequence of the reference material used to develop and validate the method was the plasmid pMul5 which is included in the GM Maize Detection Plasmid Set (Fasmac No. PM-2 and Nippon Gene No. 319-04981)[74)]. See also Reference [33]. Other reference materials may be used provided that the performance can be demonstrated to be equal or better.

C.9.5.5 Oligonucleotides

The sequences of oligonucleotide of primers and probes for T25 construct-specific and maize taxon-specific genes are listed in Table C.48, in which primers and probes are mixed as the primer-probe mix.

Table C.48 — 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		
T25 1-5’	5'-gCC AgT TAg gCC AgT TAC CCA- 3'	500 nmol/l
T25 1-3’	5'-TgA gCg AAA CCC TAT AAg AAC CCT- 3'	500 nmol/l
T25-2-Taq	5'-FAM- TgC Agg CAT gCC CgC TgA AAT C-TAMRA- 3'	200 nmol/l
[a] FAM: 6-carboxyfluorescein, TAMRA: 6-carboxytetramethylrhodamine.		

The length of the SSIIb PCR product is 151 bp; the length of the T25 PCR product is 149 bp.

74) 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.9.6 Apparatus

C.9.6.1 General

Standard laboratory apparatus should be used throughout unless specified otherwise.

C.9.6.2 Thermal cycler

The indicated temperature-time profile was tested with the ABI PRISM® 7700 SDS (Applied Biosystems) [75] during the collaborative trial. Other real-time PCR instruments may be used after adaptation of the reaction conditions.

C.9.6.3 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) [75], respectively. Other reaction plates, vials or caps may also be used if they can be shown to lead to equivalent or better results.

C.9.7 Procedure: PCR set-up

C.9.7.1 General

The PCR set-up for the taxon-specific *zSSIIb* target sequence and for the T25-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.49 for *zSSIIb* and in Table C.50 for T25.

Table C.49 — Amplification reaction mixture for taxon-specific *zSSIIb* 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	SSIIb1-5’ and SSIIb1-3’ (see Table C.48)	see Table C.48
Probe	SSIIb-Taq (see Table C.48)	see Table C.48

Table C.50 — Amplification reaction mixture for T25 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	T25 1-5’ and T25 1-3’ (see Table C.48)	see Table C.48
Probe	T25-2-Taq (see Table C.48)	see Table C.48

75) 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.9.7.2 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 T25 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) [76)]. See also Reference [33].

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

C.9.7.3 Temperature-time programme

The temperature-time programme as outlined in Table C.51 was optimized for the ABI PRISM® [76)] 7700 SDS (Applied Biosystems) [76)]. In the collaborative trial, it was used in combination with the TaqMan® Universal Master Mix [76)]. The use of other thermal cyclers may require specific adaptation. The time for activation/initial denaturation depends on the Master Mix used.

Table C.51 — 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 (40 cycles)			
Step 1	Denaturation	30	95
Step 2	Annealing and elongation	60	59

C.9.8 Limitations and interpretation of the results

If GM maize other than T25 contains the same construct-specific DNA sequences, the method is only suitable for the quantitation of T25 DNA in the absence of GMOs other than T25 maize.

The described method is suitable for measuring the ratio of T25 the construct-specific sequence to the taxon-specific *zSSIIb* sequence of maize. This ratio reflects the amount of T25 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].

76) 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.9.9 Calibration and calculation of results

The threshold value to determine 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 [76)]). See also Reference [33].

The Cf for T25 construct-specific quantitation and the reference plasmid used in the collaborative trial is 0,34. The amount of GM maize in the sample shall be calculated by the following equation:

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

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.

Annex D
(informative)

Event-specific methods

D.1 Event-specific method for the absolute and relative quantitation of maize line Bt11 DNA based on real-time PCR

D.1.1 Introduction

This annex describes a method for the specific amplification and quantitation of genetically modified Bt11 maize (*Zea mays*) derived DNA in foods. The method yields the concentration in absolute copy numbers, and shall be combined with a target taxon-specific method for maize (e.g. A.1) for the calculation of the relative concentration of Bt11 DNA in the foods. The calculation of the relative value is also described.

For limitations, see D.1.8.

D.1.2 Validation status and performance characteristics

D.1.2.1 General

The method has been optimized for DNA extracted from pure ground Bt11 maize kernels, Bt11 maize leaves and certified reference material of Bt11 maize (IRMM-412 77) [10]) dried powder from ground kernels containing mixtures of genetically modified Bt11 and conventional maize.

The reproducibility of the described method has been tested in a collaborative trial using twelve unknown samples (six pairs of blind duplicates) consisting of mixtures of 100 % Bt11 DNA and wild type maize DNA at different corresponding copy numbers of the target sequence.

The copy number of the target sequence per haploid genome has been determined to be 1 [47].

The method originally developed for LightCycler® thermal cyclers (Roche Diagnostics) 77) has been published [47] and was later modified [15] to adapt it to the ABI PRISM® 7700/7900 SDS prior to validation in a collaborative trial. The modified protocol is presented here.

D.1.2.2 Collaborative trial

The method has been validated in a collaborative trial by the European Commission, Joint Research Centre, Institute for Health and Consumer Protection (IHCP), and the Community Reference Laboratory (CRL) [16]. The collaborative trial included the use of the maize *adh*1 reference gene system described in A.1.

Samples containing wild type sweet maize and Bt11 sweet maize at different DNA concentrations were used. The stocks of non-GM maize and 100 % Bt11 sweet maize DNA were obtained from Syngenta. The DNA extraction method used was based on a Magnesil lysis solution and magnetic separation of DNA. The details of the DNA are as follows:

- wild type leaf genomic DNA from hybrid field maize (Brasco);
- Bt11 sweet maize grain genomic DNA (GH0937).

---

77) 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 standard curve, control and unknown samples were produced by the IHCP. Maize materal containing 2,5 % of Bt11 maize was used to prepare five samples (S1 to S5) containing known concentrations of the Bt11 maize target-specific sequence in the following absolute copy numbers S1: 2367; S2: 1183; S3: 592; S4: 148; S5: 49 were used to establish a calibration curve for the absolute quantitation of Bt11 copy numbers in unknown samples. For details of the validation dealing with *adh*1, see also D.1. The absolute copy numbers in the known samples were determined by dividing the sample DNA mass (determined by fluorometric quantitation of dsDNA by PicoGreen, Molecular Probes, cat. number P-7589) by the published average 1C value for maize genomes (2,725 pg) [14].

Twelve unknown samples (six pairs of blind duplicates, U1 to U12) of maize DNA containing between 77 and 1541 copies of maize Bt11 were analysed on two PCR-plates by the participants. The copy numbers correspond to the Bt11 content of 0,1 % to 2,0 %. Samples were prepared by mixing 100 % of Bt11 maize DNA and wild type maize DNA at different corresponding copy numbers of the target sequence. In addition, two negative DNA target controls (wild type maize and Bt176 maize) and an amplification reagent control (nucleic acid free water) were used.

The real-time PCR amplification was triplicated for standard curve and control samples and quadruplicated for unknown samples.

Originally, 14 laboratories from nine different EU countries participated in the collaborative trial. The ABI PRISM® 7700/7900 SDS and ABI PRISM® 7000 equipment were used. The results of one laboratory were excluded from the data analysis due to the fact that the master-mix as described in the protocol was not used in the analysis. Thereafter, the outliers were treated by using Cochran test and Grubb's tests according to the IUPAC harmonized protocol [12].

For relative quantitation in combination with the maize *adh*1 system (see A.1), the Bt11-specific detection system resulted in a reproducibilty relative standard deviation in the range of 12,7 to 33,5 % for the relative quantitation (Table D.1). Details of the collaborative trial are listed in Table D.1.

Since the samples U1 to U12 were mixtures of the same DNA as the DNA used to prepare samples S1 to S5, the results obtained with these samples could not be used for the evaluation of the trueness of the applied real-time PCR method.

Table D.1 — Validation data for Bt11 maize specific relative quantitation with *adh*1 as reference gene

	Mixing level					
	0,1 %	**0,3 %**	**0,7 %**	**1,0 %**	**1,3 %**	**2,0 %**
Year of interlaboratory test	2003	2003	2003	2003	2003	2003
Number of laboratories having returned results	13	13	13	13	13	13
Number of samples per laboratory	2	2	2	2	2	2
Number of excluded laboratories	2	2	1	3	1	3
Number of laboratories retained after exclusion	11	11	12	10	12	10
Number of accepted samples	22	22	24	20	24	20
Expected value (%)	0,1	0,3	0,7	1,0	1,3	2,0
Mean value (%)	0,1	0,3	0,7	1,0	1,2	1,8
Median value (%)	0,1	0,3	0,7	1,0	1,2	1,9
Reproducibility relative standard deviation (%)	33,5	19,0	24,4	12,7	27,0	18,4
Reproducibility limit R (R = 2,8 s_R)	0,11	0,17	0,48	0,36	0,92	0,95

D.1.2.3 Molecular specificity

D.1.2.3.1 General

The method has been designed to target a sequence described in EMBL/GenBank/DDBJ [78] accession number AY 123624 [47]. This sequence is composed of a part of the *Zea mays* (maize) genome and a part of the inserted genetic construct integrated in GM maize event Bt11.

D.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 [78] using the nucleotide sequences as query sequences with the BLASTN programme on 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. The forward primer and the TaqMan® probe [78] match 100% with an internal part of the pUC18 plasmid (accession number L08752), and consequently retrieved many synthetic DNA sequences. The reverse primer spans the Bt11 3' integration border region, i.e. the junction between the insert (pUC18 derived sequence) and the host genomic DNA (high homology with accession number AF030935, a maize-specific 180 bp knob-specific repeat). This primer retrieved only the target accession number AY123624 [47] with 100% match, and four mouse and human clones with 17 nucleotides matches out of 20 nucleotides.

D.1.2.3.3 Experimental determination of specificity

The specificity was tested against DNA extracted from other available certified reference materials of genetically modified plants (IRMM-410R [GTS 40-3-2 soybean], IRMM-411 [Bt 176 maize] and IRMM-413 [Mon810 maize] series [78]), and against pure pUC18 cloning vector DNA in concentrations up to 1×10^9. None of the certified reference materials yielded detectable amplification, and the pUC18 DNA was not detectable with less than 1×10^9 template copies in the PCR [47].

The target sequence is a single copy sequence in the haploid Bt11 genome.

D.1.2.4 Optimization

The method was originally described for LightCycler® and TaqMan® chemistry [78], and adapted and optimized for the ABI PRISM® 7700/7900 SDS prior to conducting the collaborative trial validation [15]. Primer and probe design were carried out using the OLIGO-Applet software (TIB-MOLBIOL, Germany) [78].

D.1.2.5 Limit of detection (LOD)

According to the method developer, the absolute LOD is 10 copies of the target sequence [47].

The lowest number of target sequence copies included in the collaborative trial was 77.

The lowest relative concentration of the target sequence included in the collaborative trial was 0,1 %.

D.1.2.6 Limit of quantitation (LOQ)

According to the method developer the absolute LOQ is between 40 and 100 copies of the target sequence [47].

The lowest number of target sequence copies included in the collaborative trial was 77.

The lowest relative concentration of the target sequence included in the collaborative trial was 0,1%.

---

78) 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.

D.1.3 Adaptation

No specific information is available.

D.1.4 Principle

A 70 bp fragment of the Bt11 maize event-specific 3' integration border region is amplified using two specific primers: the forward primer targets a pUC18 derived sequence (internal to the inserted DNA sequence) and the reverse primer targets the 3' integration border region (11 nucleotides from pUC18 derived sequence, 9 nucleotides from the maize genome). Accumulation of PCR products is measured at the end of each PCR cycle (real-time) by means of a specific oligonucleotide probe (matching 100 % with a pUC18 derived sequence) labelled with two fluorescent dyes: FAM as reporter dye and TAMRA as quencher. For that purpose TaqMan® chemistry [79)] was employed.

The measured fluorescence signal exceeds 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 Bt11-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 similar amplification is done for a target sequence representing maize, e.g. *adh*1 (see A.1). For relative quantitation, the copy number of the Bt11 and maize target sequences are compared, see D.1.9.

D.1.5 Reagents

D.1.5.1 General

For the quality of the reagents used, see [A1⟩ ISO 24276:2006 ⟨A1], 6.6.

D.1.5.2 Water.

D.1.5.3 PCR buffer (without $MgCl_2$), 10-fold.

D.1.5.4 $MgCl_2$ solution, $c(MgCl_2)$ = 25 mmol/l.

D.1.5.5 dNTP solution, c(dNTP) = 2,5 mmol/l (each).

D.1.5.6 Oligonucleotides

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

Table D.2 — Oligonucleotides

Name	Oligonucleotide DNA sequence	Final concentration in PCR
Bt113JFor	5'-gCg gAA CCC CTA TTT gTT TA-3'	750 nmol/l
Bt113Jrev	5'-TCC AAg AAT CCC TCC ATg Ag-3'	750 nmol/l
Bt113JFT	5'-FAM- AAA TAC ATT CAA ATA TgT ATC CgC TCA-TAMRA-3'[a]	250 nmol/l
[a] FAM: 6-carboxyfluorescein; TAMRA: 6-carboxytetramethylrhodamine.		
The length of the Bt11 maize amplicon is 70 bp.		

A separate set of primers and probe is necessary for the reference gene, see e.g. A.1 for maize *adh*1.

79) 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.

D.1.5.7 Thermostable DNA polymerase

AmpliTaq Gold® DNA polymerase [80]).

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

D.1.5.9 Amplification reaction mixture

Details of the amplifications reaction mixture are listed in Table D.3.

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

Total reaction volume		25 µl
Template DNA (⩽ 250 ng maize DNA for standards, maximum 200 ng for unknowns)		5 µl
DNA polymerase	AmpliTaq Gold ®	1,5 U
Decontamination System	dUTP AmpErase uracil *N*-glycosylase	200 µmol/l 0,3 U
Reaction buffer	TaqMan® buffer A (containing passive reference ROX) [a]	1 ×
	$MgCl_2$	4 mmol/l
Primers	see D.1.6.1	see D.1.6.1
dNTP	dATP, dCTP, dGTP	200 µmol/l each
Probe	see D.1.6.1	see D.1.5.6

[a] ROX = carboxy-X-rhodamine.

A separate reaction is necessary for the reference gene, see e.g. A.1 for maize *adh*1.

D.1.6 Apparatus

D.1.6.1 General

Standard laboratory apparatus should be used throughout unless otherwise specified.

D.1.6.2 Thermal cycler

The indicated temperature-time profile was originally tested with the ABI PRISM® 7700/7900 SDS (Applied Biosystems). Other real-time PCR detection systems may be used after adaptation of the reaction conditions. The method was originally developed and published for LightCycler® [80]) [47].

D.1.6.3 Reaction vials

The reaction vials shall be suitable for PCR amplification on a real-time thermal cycler, e.g. MicroAmp® optical tubes 96-well reaction plate (Applied Biosystems). The method was originally developed and published for LightCycler® and glass capillaries [80]) [47].

---

80) 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.

[AC1) D.1.7 Procedure: PCR set-up (AC1]

D.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 25 µl per reaction mixture with the reagents as listed in Table D.3.

It has been optimized for the ABI PRISM® 7700/7900 SDS [81)] (Applied Biosystems). Details are presented in Table D.3.

D.1.7.2 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 one alternative is available, as follows.

— High quality, pure genomic DNA extracted from Bt11 maize (e.g. CRM IRMM-412 series [81)] may be used if the quantity of DNA is known (e.g. determined with PicoGreen as described in A.1.2.2), on the basis of calculating copy numbers of the target sequence from the genome size of maize [15].

D.1.7.3 Temperature-time programme

The temperature-time programme as outlined in Table D.4 was adapted to the ABI PRISM® 7700/7900 SDS, and was modified from the programme originally published for LightCycler® [81)]. For a suggestion of suitable reagents and concentrations on LightCycler® [81)] confer the original publication [47].

Table D.4 — 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 (50 cycles)			
Step 1	Denaturation	15	95
Step 2	Annealing elongation	60	60

D.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 Bt11 in the analysed samples. Therefore the absence of detectable PCR inhibitors should be verified (see

81) 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.

also ISO 21571 for DNA extraction methods), e.g. by setting up serial dilutions of the template DNA and examining the correspondence between dilutions and differences in C_t-values (i.e. one C_t corresponds to a doubling of template concentration).

A suitable target taxon-specific method [see e.g. A.1 (maize *adh*1)] may be used to perform PCR inhibition testing prior to setting up a Bt11 quantitation.

For the relative quantitation of GMO in unknown samples, i.e. the use of this method in combination with a target taxon-specific method such as that found in A.1, it is important that the absolute amount of template DNA (ng) is the same for both the target taxon-specific 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.

D.1.9 Calibration and calculation of results

Calibration points are produced with standards consisting of a defined amount in absolute copy numbers of genomic Bt11 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 Excel [82]), or directly by options available with the sequence detection system software.

The calibration curve is used to determine the Bt11 maize concentration (in absolute 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 Bt11 maize concentration (in absolute copy numbers) of the unknown samples. For relative quantitation, the haploid GMO content, w, is determined with the following formula:

$$w = \frac{N_{\text{Bt11}}}{N_{\text{maize}}} \times 100\ \%$$

where

N_{Bt11} designates the absolute copy number of the Bt11-specific target (determined with the present method);

N_{maize} designates the absolute copy number of the target taxon-specific target (determined with a method for maize, e.g. A.1).

D.2 Event-specific method for the relative quantitation of maize line MON 810 DNA using real-time PCR

D.2.1 Introduction

This annex describes a method for the specific amplification and quantitation of a specific part from the taxon specific maize (*Zea mays*) gene (high mobility group protein gene [*hmg*] [48] and of the single copy DNA integration-border region of the genomic sequence and the inserted sequence element originating from CaMV (35S promoter), as a result of *in vitro* recombination present in the genetically modified insect-protected MON 810 ("YieldGuard") maize (Monsanto) in order to quantify the relative amount of the maize line MON 810 DNA.

For limitations, see D.2.8.

---

82) 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.

D.2.2 Validation status and performance characteristics

D.2.2.1 General

The method has been optimized for certified reference materials (CRM IRMM-413) [49] consisting of dried maize powder containing mixtures of genetically modified MON 810 maize and conventional maize.

The reproducibility and accuracy of the described method were tested through a collaborative trial using the CRM IRMM-413 series mentioned above.

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

The method was originally developed for the ABI PRISM® 7700 SDS [83)].

D.2.2.2 Collaborative trial

This method has been validated in a collaborative trial by the Federal Institute for Risk Assessment (BfR) in collaboration with the American Association of Cereal Chemists (AACC), Joint Research Centre (JRC) of the European Commission (EC), Institute for Reference Material and Measurement (IRMM), the Institute for Health and Consumer Protection (IHCP) and GeneScan, Berlin. The study was designed to meet the critieria laid down in the IUPAC harmonized protocol [12].

The study was undertaken with 15 laboratories using either the ABI PRISM® 7700, ABI PRISM® 7900 (Applied Biosystems) [83)] or the iCycler iQ Real-Time PCR detection system (Bio-Rad Laboratories).

Fourteen laboratories reported results.

For DNA extraction, the GENE*Spin* DNA extraction system (GeneScan) [83)] was used according to the manufacturer's instructions.

For each unknown sample one DNA extraction has been carried out. Each test sample was analyzed by PCR in three replicates.

Each participant received 12 unknown samples. The samples consist of six certified reference materials (CRM IRMM-413) between $<$ 0,02 % and 5 % GM MON 810 (mass fraction) in conventional maize.

It was designed as a blind duplicate collaborative trial. Each laboratory received each level of GM MON 810 CRM in two unknown samples.

Details of the collaborative trial are listed in Table D.5.

---

83) 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 D.5 — Validation data for the MON 810 event specific quantitation

	Sample 1 < 0,02 %	Sample 2 0,10 % ± 0,03 %	Sample 3 0,50 % ± 0,04 %	Sample 4 1,00 % ± 0,05 %	Sample 5 2,0 % ± 0,1 %	Sample 6 5 %
Year of the collaborative trial	2003/04	2003/04	2003/04	2003/04	2003/04	2003/04
Number of laboratories reporting results	11	14	14	14	14	14
Number of outliers[a]	1	1	0	2	0	0
Number of laboratories retained after eliminating outliers	10	13	14	12	14	14
Mean value (%)	0,028	0,1023	0,4613	0,8327	1,7814	4,5154
Repeatability standard deviation s_r	0,00736	0,03641	0,9606	0,13744	0,28385	1,29374
Repeatability relative standard deviation (%)	26,27	35,60	20,82	16,51	15,93	28,65
Repeatability limit r (r = 2,8 s_r)	0,0206	0,1019	0,269	0,3848	0,7948	3,6225
Reproducibility standard deviation s_R	0,02326	0,04646	0,20068	0,26534	0,56609	1,65451
Reproducibility relative standard deviation (%)	83,03	45,43	43,5	31,86	31,78	36,64
Reproducibility limit R (R = 2,8 s_R)	0,0651	0,1301	0,5619	0,743	1,5851	4,6326

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

D.2.2.3 Molecular specificity

D.2.2.3.1 General

The primer sets were designed to amplify a DNA sequence specific for the artificial junction (the integration-border region) between the integrated genetic construct and the host genome, which does not occur in nature.

D.2.2.3.2 Theoretical specificities

No sequence homology with DNA sequences of non-GM maize and other crop plants has been found in data bank searches (GenBank® database; BlastN® 2.2.1 search from 2003-10-01 [84]). A search with BLASTN [84] programme on http://www.ncbi.nlm.nih.gov/blast [September 14, 2004] results in 100 % similarity to accession number AF434709 describing the MON 810 specific integration site in the maize genome.

D.2.2.3.3 Experimental determination of specificity

CRM IRMM-413 dried maize powders [84] containing < 0,02 % to 5 % genetically modified maize line MON 810 were identified.

Specificity tests prior to the study showed no cross-reactivity of the detection systems to the following non-target species/sample: soya bean DNA.

No cross-reactivity has been observed with the following GM maize events: Event176, Bt11, T25, GA21 and GTS 40-3-2 soya bean.

---

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

D.2.2.4 Optimization

Optimization of reagent concentrations was carried out on an ABI Prism® 7700 SDS and on the GenAmp 5700 SDS system using the TaqMan® chemistry [85].

Primer and probe design were carried out applying the Primer Express® software (Applied Biosystems) [85].

D.2.2.5 Limit of detection (LOD)

The absolute LOD was not assessed in a collaborative trial. According to the method developer, the absolute LOD as defined in ISO 24276 has been determined to be 5 copies of the target sequence.

The relative LOD was not assessed in a collaborative trial. According to the method developer, the relative LOD, as defined in ISO 24276 has been demonstrated to be at least 0,1 %.

D.2.2.6 Limit of quantitation (LOQ)

The absolute LOD was not assessed in a collaborative trial. According to the method developer, the absolute limit of quantitation has been determined to be 10 copies of the target sequence.

The relative LOD was not assessed in a collaborative trial. According to the method developer, the relative limit of quantitation has been determined to be at least, 0,1% (equal to the lowest concentration point of the calibration curve used).

D.2.3 Adaptation

No specific information is available.

D.2.4 Principle

A 92 bp fragment specific for the maize line MON 810 is amplified by PCR. Accumulated PCR products are measured over each cycle (real-time) by means of a target sequence specific oligonucleotide probe labelled with two fluorescent dyes: FAM as reporter dye and TAMRA as quencher [30]

For relative quantitation of the maize line MON 810 DNA content, a maize specific reference system amplifies a 79 bp fragment of the maize *hmg* gene using a gene specific combination of primers and probe.

D.2.5 Reagents

D.2.5.1 General

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

85) 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.

D.2.5.2 Water.

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

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

[AC1) **D.2.5.5 dNTP solution**, c(dNTP) = 2,5 mmol/l (each). (AC1]

D.2.5.6 Oligonucleotides

Details of the oligonucleotides are listed in Table D.6.

Table D.6 — 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		
Mail-F1	5´-TCg AAg gAC gAA ggA CTC TAA CgT-3´	300 nmol/l
Mail-R1	5´-gCC ACC TTC CTT TTC CAC TAT CTT-3´	300 nmol/l
Probe Mail-S2	5´-FAM-AAC ATC CTT TgC CAT TgC CCA gC-TAMRA P-3´	180 nmol/l
[a] FAM: 6-carboxylfluorecein; TAMRA: 6-carboxytetramethylrhodamine.		

The length of the *hmg* gene-specific PCR product is 79 bp; the length of the MON 810-specific PCR product is 92 bp.

D.2.5.7 Thermostable DNA polymerase

AmpliTaq Gold® DNA polymerase [86)].

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

D.2.5.9 Amplification reaction mixture

Details of the amplification reaction mixture are listed in Table D.7.

D.2.6 Apparatus

D.2.6.1 General

Standard laboratory apparatus should be used throughout unless otherwise specified.

---

86) 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.

D.2.6.2 Thermal cycler

The indicated temperature-time profile was originally tested with the ABI PRISM® 7700 SDS and GeneAmp® 5700 SDS (Applied Biosystems) [87]. Other real-time PCR detection systems may be used after adaption of the reaction conditions.

D.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) [87].

D.2.7 Procedure: PCR set-up

D.2.7.1 General

The PCR set-up for the taxon-specific 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 Table D.7.

Table D.7 — Amplification reaction mixture in the final volume/concentration per reaction vial

Total reaction volume		25 µl
Template DNA added (2,3 ng to 150 ng maize DNA)		5 µl
DNA polymerase	AmpliTaq Gold®	1,25 U
Decontamination system	dUTP	400 µmol/l
	AmpErase uracil *N*-glycosylase	0,5 U
Reaction buffer	TaqMan® buffer A (containing passive reference ROX) [a]	1 fold
	$MgCl_2$	6,5 mol/l
Primers	see Table D.6	see Table D.6
dNTP	dATP, dCTP, dGTP	200 µmol/l each
Probe	see Table D.6	see Table D.6

[a] ROX = carboxy-X-rhodamine.

D.2.7.2 PCR controls

As a positive control and as calibrant reference material, certified reference materials of MON 810 (material containing < 0,02 % to 5 % of genetically modified maize) produced by IRMM, Geel, Belgium (IRMM-413 series) may be used.

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

87) 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.

D.2.7.3 Temperature-time programme

The temperature-time programme as outlined in Table D.8 was optimized for the ABI PRISM® 7700 SDS (Applied Biosystems) [88]. In the validation study, it was used in combination with the AmpliTaq Gold® DNA polymerase [88]. The use of other thermal cyclers may require specific adaptation. The time for activation/initial denaturation depends on the polymerase used.

Table D.8 describes the reaction conditions.

Table D.8 — 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	95
Step 2	Annealing elongation	60	60

D.2.8 Limitations and interpretation of the results

The described method is suitable for measuring the ratio of MON 810-specific DNA to maize DNA. This ratio reflects the relative amount of MON 810 in the maize ingredient of the investigated foodstuff.

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.

D.2.9 Calibration and calculation of results

After defining a threshold value within the logarithmic phase of amplification [e.g. 0,01 to 0,1 normalized reporter dye fluorescence (R_n)], the instruments software calculates the C_t-values for each reaction. The C_t-values measured for the calibration points prepared from CRMs intended for quantitative analyses (CRM IRMM-413) in the taxon-specific maize or MON 810-specific PCR system, respectively, are plotted against the natural logarithm of the DNA copy numbers. The copy numbers measured for the unknown sample DNA are obtained by interpolation from the standard curves.

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 Excel [88], or directly by options available with the sequence detection system software.

For the determination of the amount of MON 810 DNA in the test sample, the MON 810 copy number is divided by the number of maize genome equivalents and multiplied by 100 to get the percentage value. For 1 C value, see Reference [14].

In addition to a quantitative analysis run, an optional so-called ‘monitor-run’ should be performed. A monitor run should also be included in the collaborative trial and would further improve the analysis procedure. The monitor run is most important when analysing matrices for which little knowledge on DNA yield and DNA quality is available. 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 inhibitor(s) can be identified and also a suitable dilution of the nucleic acid extract obtained from the test sample fitting into the calibration range of the quantitative analysis can be determined.

---

88) 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.

Bibliography

[1] DIVIACCO, S., NORIO, P., ZENTILIN, L., MENZO, S., CLEMENTI, M., BIAMONTI, G., RIVA, S., FALASCHI, A., and GIACCA, M. A novel procedure for quantitative polymerase chain reaction by coamplification of competitive templates. *Gene*, 1992, **122**(2), 313-320

[2] PANNETIER, C., DELASSUS, S., DARCHE, S., SAUCIER, C. and KOURILSKY, P. Quantitative titration of nucleic acids by enzymatic amplification reactions run to saturation. *Nucleic Acids Res*., 1993, **21**(3), 577-583

[3] ORLANDO, C., PINZANI, P. and PAZZAGLI, M. Developments in quantitative PCR. *Clin. Chem. Lab. Med*.,1998, **36**(5), 255-269

[4] YANG, B., YOLKEN, R., and VISCIDI, R. Quantitative polymerase chain reaction by monitoring enzymatic activity of DNA polymerase. *Anal. Biochem*., 1993, **208**(1): 110-116

[5] THOMPSON, M., and WOOD, R. Harmonized Guidelines for Internal Quality Control in Analytical Chemistry Laboratories, *J. Pure App. Chem*., 1995, **67**(4) 649-666

[6] Alinorm 03/23 *Criteria for Evaluating Acceptable Methods of Analysis for Codex Purposes*. Codex Alimentarius Twenty-sixth Session, Rome, Italy, 30 June - 5 July 2003

[7] http://www.eurachem.ul.pt/guides/mval.htm ISBN 0-948926-12-0

[8] Eurachem Guide: “*The fitness for Purpose of Analytical methods – A laboratory Guide to Method Validation and Related Topics*”, Eurachem Working Group, Dec 1998

[9] Eurachem/CITAG Guide: *Quantifying Uncertainty in Analytical Measurement*, Ellisson, S.L.R., Rosslein, M., Williams, A., 2000, 2nd Ed.

[10] *IRMM Certified Reference Material Reports and Certificates*. http://www.irmm.jrc.be/rm/cert-reports.html

[11] HERNANDEZ, M., DUPLAN, M.-N., BERTHIER, G., VAÏTILINGOM, M., HAUSER, W., FREYER, R., PLA, M. and BERTHEAU, Y. Development and comparison of four real-time polymerase chain reaction systems for specific detection and quantification of *Zea mays* L. *Journal of Agricultural and Food Chemistry,* 2004, **52**: 4632-4637

[12] HORWITZ, W. Protocol for the design, conduct and interpretation of method performance studies. *Pure and Appl. Chem*, 1995, **67**: 331-343

[13] DELLAPORTA, S.L., WOOD, J., HICKS, J.B. A plant DNA minipreparation: version II. *Plant Mol. Biol. Rep*. Vol. 1, 1983, 4:19-2156

[14] ARUMUGUNATHAN, K., EARLE, E.D. Nuclear DNA content of some important plant species. *Plant Mol. Biol. Rep*. 1991, **9**: 208-218

[15] Community Reference Laboratory (2004a). *GMO specific real-time PCR system*. Protocol for event-specific quantitation of Bt11 in maize. Protocol published by the European Commission, Joint Research Centre, Institute for Health and Consumer Protection. http://gmo-crl.jrc.it/detectionmethods/Bt11-protocol.pdf. 6 pp

[16] Community Reference Laboratory (2004b). *Validation of the GMO specific detection method developed by NVI/INRA for Bt11 in sweet maize*. Report published by the European Commission, Joint Research Centre, Institute for Health and Consumer Protection. http://gmo-crl.jrc.it/summaries/CRL%20Bt11%20Sweet%20maize%20validation%20report.pdf. 9 pp

[17] JAENICKE-DESPRÉS, V., BUCKLER, E.S., SMITH, B.D., GILBERT, M.T.P., COOPER, A., DOEBLEY, J. and PÄÄBO, S. Early Allelic Selection in Maize as Revealed by Ancient DNA. *Science,* 2003, **302**: 1206-1208

[18] PAUWELS, J., KRAMER, G.N., SCHIMMEL, H., ANKLAM, E., LIPP, M. BRODMANN, P. *The Certification of Reference Materials of Soya bean Powder with different Mass Fractions of RoundupReady® Soya bean*, EC certification report EUR 18683 EN, 1999, ISBN 92-828-5925-8

[19] PAULI, U., LINIGER, M., SCHROTT, M., SCHOUWEY, B., HÜBNER, Ph., BRODMANN, P., and EUGSTER, A. Quantitative Detection of Genetically Modified Soybean and Maize: Method Evaluation in a Swiss Ring Trial. *Mitt. Lebens. und Hyg*. 2001, **92**: 145-158

[20] HÜBNER, Ph., WAIBLINGER, H-U., PIETSCH, K., and BRODMANN, P. Validation of PCR Methods for Quantitation of Genetically Modified Plants in Food. *J. AOAC Int*, 2001, **84**: 1855-1864

[21] VODKIN, L.O., RHODES, P.R., GOLDBERG, R.B. Ca Lectin gene insertion has the structural features of a transposable element. *Cell*,1983, **34**: 1023-1031

[22] PADGETTE, S.R., KOLACZ, K.H., DELANNY, X., RE, D.B., LAVELLEE, B.J., TINIUS, C.N., RHODES, W.K., OTERO, Y.I., BARY, G.F., EICHHOLTZ, D.A., PESCHKE, V.M., NIDA, D.L., TAYLOR, N.B., KISHORE, G.M. Development, identification and characterization of a glyphosate-tolerant soybean line. *Crop Sci.* 1995, **35:** 1451-1461

[23] SLMB-Methode 52B/2.1.3/2000 (CD-Rom, Eidgenössische Materialzentrale, PO Box, CH 3000, Bern)

[24] GRUBBS, F.E. Procedures for detecting outlying observations in samples. *Technometrics*, 1969, **11**:1-21

[25] HEMMER, W. *Foods Derived from Genetically Modified Organisms and Detection Methods*. In: BATSreport (Agency for Biosafety research and assessment of technology Impacts of the Swiss priority, Programme Biotechnology of the Swiss National Science Foundation, Basel, Switzerland), 1997, **2**, 97

[26] BRUNT, A., CRABTREE, K., DALLWITZ, M., GIBBS, A., WATSON, L. *Viruses of Plants: Descriptions and Lists from the VIDE Database*. 1996, 1484 pp. C.A.B. International, U.K.

[27] QIU, S.G., WINTERMANTEL, W.M., SHA, Y., and SCHOELZ, J.E. Light-Dependent Systemic Infection of Solanaceous Species by Cauliflower Mosaic Virus Can Be Conditioned by a Viral Gene Encoding an Aphid Transmission Factor. *Virology*, 1997, **227**, pp 180-188

[28] WOLF, C., SCHERZINGER, M., WURZ, A., PAULI, U., HÜBNER, Ph. and LÜTHY, J. Detection of cauliflower mosaic virus by the polymerase chain reaction: testing of food components for false-positive 35S-promoter screening results. *Eur Food Res Technol*. 2000, **210**: 367-372

[29] TRAPMANN, S., LE GUERN, L., KRAMER, G.N., SCHIMMEL, H., PAUWELS, J., ANKLAM, E., VAN DEN EEDE, E., BRODMANN, P. *The Certification of a new set of Reference Materials of Soya bean Powder with different Mass Fractions of Roundup ReadyTM Soya bean*, 2000. EC certification report EUR 19573 EN, ISBN 92-828-9639-0

[30] LEE, L.G., CONNELL, C.R., and BLOCH W. Allelic discrimination by nick-translation PCR with fluorogenic probes. *Nucleic Acids Research*, 1993, **21**(16), 3761-6

[31] HUPFER, C., HOTZEL, H., SACHSE, K., ENGEL, K.-H. Detection of the genetic modification in heat treated products of Bt maize by polymerase chain reaction. *Lm. Unters. Forsch*., **206** (Band A), 1998, pp. 203-207.

[32] PAUWELS, J., KRAMER, G.N., SCHIMMEL, H., ANKLAM, E., LIPP, M., BRODMANN, P. *The Certification of Reference Materials of Maize Powder with different Mass Fractions of BT-176 Maize*, EC certification report EUR 18684 EN, ISBN 92-828-5924

[33] KURIBARA, H., SHINDO, Y., MATSUOKA, T., TAKUBO, K., FUTO, S., AOKI, M., HIRAO, T., AKIYAMA, H., GODA, Y., TOYODA, M. and HINO, A. Novel Reference Molecules for Quantitation of Genetically Modified Maize and Soybean. *J.AOAC Int.* 2002, **85**, 1077-1089

[34] SHINDO, Y., KURIBARA, H., MATSUOKA, T., FUTO, S., SAWADA, C., SHONO, J., AKIYAMA, H., GODA, Y., TOYODA, M. and HINO, A. Validation of Real-Time PCR Analyses for Line-specific Quantitation of Genetically Modified Maize and Soybean Using New Reference Molecules. *J.AOAC Int.* 2002, **85**, 1119-1126

[35] *Instruction Manual for Testing and Analysing Genetically Modified Food –Quantitative PCR*, Japanese Agricultural Standard Testing and Analysis Handbook Series (Centre for Food Quality, Labelling and Consumers Services, Saitama, Japan). 2002

[36] *Testing for Foods Produced by Recombinant DNA Techniques,* Ministry of Health, Labor and Welfare(Ministry of Health, Labour and Welfare, Japan), 2002

[37] *Guideline of Detection Methods of Genetically Modified Foods,* Korean Food and Drug Administration, Korea

[38] *Testing Manual for Genetically Modified Agricultural Products* by National Agricultural Quality Services, Korea

[39] *Official Methods of Analysis of AOAC INTERNATIONAL,* 17th Ed., 2000, AOAC INTERNATIONAL, Gaithersburg, MD, Appendix D, pp 2–11

[40] COCHRAN, W.G. The distribution of the largest of a set of estimated variances as a fraction of their total. *Annals of Eugenics,* 1949, **11**: 47-52

[41] GRUBBS, F.E. Sample criteria for testing outlying observations.. *Ann. Math. Statist. Assn.* 1950, **21:** 27-58

[42] KOPPEL, E., STADLER, M., LUTHY, J., HUBNER, P. Sensitive method for the Detection of the Genetically Engineered Soy Bean "Roundup Ready®, *Mitt. Gebiete. Hyg*. 1997, **88**: 164-175

[43] anonymous. *Relative quantitation of Gene Expression*. User Bulletin ABI Prism 7700 Sequence Detection System, 1997, **2**:1-36

[44] Eurachem Guide: "*Quantifying Uncertainty in Analytical Measurement*", Ellisson, L.R., Rosslein, M., Williams, A., 2000

[45] Eurachem/CITAG Guide: *Traceability in Chemical Measurement*, Ellisson, L.R., King, B., Rosslein, M., Salit, M., Williams, A., 2003

[46] MATSUOKA, T., KURIBARA, H., TAKUBO, K., AKIYAMA, H., MIURA, H., GODA, Y., KUSAKABE, Y., ISSHIKI, K., TOYODA, M., & HINO, A. Detection of Recombinant DNA Segments Introduced to Genetically modified Maize (Zea mays). *J. Agric. Food Chem.* 2002, **50**, 2100–2109

[47] RØNNING, S.B., VAÏTILINGOM, M., BERDAL, K.G. & HOLST-JENSEN, A. Event specific real-time quantitative PCR for genetically modified Bt11maze (*Zea mays*). *European Food Res. Technol.* 2003, **216**: 347-354

[48] KRECH, A.B., WURZ, A, STEMMER, C., FEIX, G., GRASSER, K.D. Structure of genes encoding chromosomal HMG1proteins from maize. *Gene,* 1999, **234**: 45–50

[49] Institute for Reference Materials and Measurements (IRMM): Certified Reference Materials ERM-XY000xy. CERTIFICATION REPORT. The certification of dry-mixed maize powder with different mass fractions of MON 810 maize Certified Reference Materials ERM BF413a, BF413b, BF413c, BF413d, BF413e, BF413f. http://www.erm-crm.org, 2004

[50] HOLLAND, P.M., ABRAMSON, R.D. and GELFAND D.H. Detection of specific polymerase chain reaction product by utilizing the 5' to 3' exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proc. Natl. Acad. Sci. USA*, 1991, **88**(16), 7276-80

[51] ISO 5725-1:1994, *Accuracy (trueness and precision) of measurement methods and results — Part 1: General principles and definitions*

[52] ISO 5725-2:1994, *Accuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method*

[53] ISO 5725-3:1994, *Accuracy (trueness and precision) of measurement methods and results — Part 3: Intermediate measures of the precision of a standard measurement method*

[54] ISO 5725-4:1994, *Accuracy (trueness and precision) of measurement methods and results — Part 4: Basic methods for the determination of the trueness of a standard measurement method*

[55] ISO 5725-5:1998, *Accuracy (trueness and precision) of measurement methods and results — Part 5: Alternative methods for the determination of the precision of a standard measurement method*

[56] ISO 5725-6:1994, *Accuracy (trueness and precision) of measurement methods and results — Part 6: Use in practice of accuracy values*

[57] HINO, A., MATSUOKA, T., KURIBARA, H. FUTO, S., OGAWA, M., YOSHIMURA, T., SHINDO, Y. 2000, WO02/34943 A1 (patent pending)

[58] GELFAND, D.H., HOLLAND, P.M., SAIKI, R.K, and WATSON, R.M. 1993, US Patent 5,210,015

A1 [59] DING J., JIA J., YANG L., WEN H., ZHANG C., LIU W. et al. Validation of a rice specific gene, sucrose phosphate synthase, used as the endogenous reference gene for qualitative and real-time quantitative PCR detection of transgenes. *J. Agric. Food Chem*. 2004, **52** pp. 3372–3377

[60] ENGL. *Definition of minimum performance requirements for analytical methods of GMO testing*. Brussels: European Network of GMO Laboratories. 8 p. Available (viewed 2013-03-21) at: http://gmo-crl.jrc.ec.europa.eu/doc Min_Perf_Requirements_Analytical_methods.pdf

[61] ISO/IEC 17025, *General requirements for the competence of testing and calibration laboratories*

[62] YANG L ., PAN A ., JIA J., DING J., CHEN J., HUANG C. et al. Validation of a tomato specific gene, *LAT52*, used as an endogenous reference gene in qualitative and real-time quantitative PCR detection of transgenic tomatoes. *J. Agric. Food Chem*. 2005, **53** pp. 183–190

[63] YANG L., ZHANG H., GUO J., PAN L., ZHANG D. International collaborative study for the endogenous reference gene, *LAT52*, used for qualitative and quantitative analysis of genetically modified tomato. *J. Agric. Food Chem*. 2008, **56** pp. 3438–3443

[64] Available (viewed 2013-03-21) at: http://blast.ncbi.nlm.nih.gov/Blast.cgi A1

BSI — British Standards Institution

BSI is the independent national body responsible for preparing British Standards. It presents the UK view on standards in Europe and at the international level. It is incorporated by Royal Charter.

Revisions

British Standards are updated by amendment or revision. Users of British Standards should make sure that they possess the latest amendments or editions.

It is the constant aim of BSI to improve the quality of our products and services. We would be grateful if anyone finding an inaccuracy or ambiguity while using this British Standard would inform the Secretary of the technical committee responsible, the identity of which can be found on the inside front cover. Tel: +44 (0)20 8996 9000. Fax: +44 (0)20 8996 7400.

BSI offers members an individual updating service called PLUS which ensures that subscribers automatically receive the latest editions of standards.

Buying standards

Orders for all BSI, international and foreign standards publications should be addressed to Customer Services. Tel: +44 (0)20 8996 9001. Fax: +44 (0)20 8996 7001. Email: orders@bsi-global.com. Standards are also available from the BSI website at http://www.bsi-global.com.

In response to orders for international standards, it is BSI policy to supply the BSI implementation of those that have been published as British Standards, unless otherwise requested.

Information on standards

BSI provides a wide range of information on national, European and international standards through its Library and its Technical Help to Exporters Service. Various BSI electronic information services are also available which give details on all its products and services. Contact the Information Centre. Tel: +44 (0)20 8996 7111. Fax: +44 (0)20 8996 7048. Email: info@bsi-global.com.

Subscribing members of BSI are kept up to date with standards developments and receive substantial discounts on the purchase price of standards. For details of these and other benefits contact Membership Administration. Tel: +44 (0)20 8996 7002. Fax: +44 (0)20 8996 7001. Email: membership@bsi-global.com.

Information regarding online access to British Standards via British Standards Online can be found at http://www.bsi-global.com/bsonline.

Further information about BSI is available on the BSI website at http://www.bsi-global.com.

Copyright

Copyright subsists in all BSI publications. BSI also holds the copyright, in the UK, of the publications of the international standardization bodies. Except as permitted under the Copyright, Designs and Patents Act 1988 no extract may be reproduced, stored in a retrieval system or transmitted in any form or by any means – electronic, photocopying, recording or otherwise – without prior written permission from BSI.

This does not preclude the free use, in the course of implementing the standard, of necessary details such as symbols, and size, type or grade designations. If these details are to be used for any other purpose than implementation then the prior written permission of BSI must be obtained.

Details and advice can be obtained from the Copyright & Licensing Manager. Tel: +44 (0)20 8996 7070. Fax: +44 (0)20 8996 7553. Email: copyright@bsi-global.com.

BSI
389 Chiswick High Road
London
W4 4AL