INTERNATIONAL STANDARD

ISO 21569

First edition
2005-06-15

AMENDMENT 1
2013-04-01

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

AMENDMENT 1

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

AMENDEMENT 1

Reference number
ISO 21569:2005/Amd.1:2013(E)

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Published in Switzerland

Foreword

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

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

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.

Amendment 1 to ISO 21569:2005was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 16, *Horizontal methods for molecular biomarker analysis*.

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

AMENDMENT 1

No attempt has been made in this amendment to update the footnote numbering to fit in with the scheme adopted in ISO 21569:2005. The footnote numbers given are for use refer solely within this amendment.

Page v, Introduction, paragraph 1

Delete "— *Sampling* (ISO 21568)".

Page 2, Clause 2, ISO 24276

Delete the footnote and update the entry to read:

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

Page 2, 4.1, paragraph 2

Delete the existing text and insert the following.

> A qualitative result shall clearly demonstrate the presence or absence of the genetic element under study, relative to appropriate controls.
>
> NOTE Detection limits and size of the test portion are critical aspects of a method.

Page 2, 7.3.3.3.3, paragraph 2

Delete "a representative" and insert "an appropriate" so that the text reads as follows.

> Primers designed to detect taxon-specific target sequences should be shown to detect these sequences reliably in an appropriate number of different members of the taxon.

Page 6, 8.1 a) and b)

In both cases, delete "ISO 24276:—", and insert "ISO 24276:2006.

Page 7, 9.4

Delete the existing text and insert the following.

> Results within the same test portion shall be consistent. In case of +/− results for the two replicates, repeat the two PCR for the respective test portion. If the two novel replicates are tested +/− or −/−, the test portion is considered as negative.
>
> Results from all test portions shall be consistent. When at least one test portion gives a positive result and at least one gives a negative result, the analysis shall be repeated.
>
> If at least one repetition of the procedure, beginning with the nucleic acid extraction, gives ambiguous results such as a positive and a negative result, the report should state that the sample is negative at the limit of detection (LOD).

Page 7, Clause 10, list item 2

Delete the existing text and insert the following.

> — the specificity of the analytical method (event specific, construct specific, or screening method);

Page 23, Annex A

Insert A.5 and A.6 after the existing text.

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

A.5.1 Purpose, relevance and scientific basis

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

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

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

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

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

A.5.2 Principle

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

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

A.5.3 Validation status and performance criteria

A.5.3.1 Robustness of the method

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

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

A.5.3.2 Intralaboratory trial

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

For sample preparation in the collaboration study, all the DNA samples were extracted by the GMDL-SJTU using the CTAB method adopted from ISO 21571:2005, A.3. Spectrophotometric quantification of DNA extracted was performed using a method adopted from ISO 21571:2005, B.1. After the DNA quantification, a qualitative PCR using an 18S PCR system (Reference [45]) was carried out to provide data about possible PCR inhibition.

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

A.5.3.3 Collaborative trial

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

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

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

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[1] Example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

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

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

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

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

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

A.5.3.4 Molecular selectivity

A.5.3.4.1 General

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

A.5.3.4.2 Experimental

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

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

A.5.3.4.3 Theoretical

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

A.5.4 Principle and summary

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

A.5.5 Terms and definitions

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

A.5.6 Sample type and amounts

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

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

The participants received the following samples.

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

A.5.7 Limit of detection and range of use

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

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

A.5.8 Estimation of measurement uncertainty

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

A.5.9 Interferences

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

A.5.10 Physical and environmental conditions

See ISO 24276 for details. For example:.

- maintain strictly separated working areas for DNA preparation, PCR set-up, PCR amplification and electrophoresis;
- any residual DNA should be removed from all equipment prior to its use;
- in order to avoid contamination, use filter pipette tips protected against aerosol;
- use only powder-free gloves and change them frequently.

A.5.11 Apparatus and equipment

A.5.11.1 Microcentrifuge.

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

A.5.11.3 Micropipettes.

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

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

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

A.5.11.7 Rack for reaction tubes.

A.5.11.8 PVC or **latex gloves**.

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

A.5.11.10 Electrophoresis equipment, with power supply.

A.5.11.11 Imaging system for gel analysis.

A.5.11.12 Microwave oven (optional).

A.5.12 Reagents and materials

A.5.12.1 General

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

A.5.12.2 Qualitative PCR

A.5.12.2.1 PCR buffer (without $MgCl_2$) 10×.

A.5.12.2.2 $MgCl_2$ solution 25 mmol/l.

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

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

A.5.12.2.5 DNA polymerase, thermostable.

A.5.12.3 Electrophoresis

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

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

A.5.12.3.2 DNA size standard.

A.5.13 Sample collection, transportation, preservation, and storage

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

A.5.14 Preparation of test sample

Ensure that the test sample is representative of the laboratory sample, e.g. by grinding or homogenization. Measures and operational steps to be taken into consideration are described in ISO 21571 and ISO 24276.

A.5.15 Instrument calibration

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

A.5.16 Analysis steps

A.5.16.1 Preparation of the DNA for qualitative PCR

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

A.5.16.2 PCR reagents

A.5.16.2.1 Conventional PCR master mix

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

A.5.16.2.2 Primers

See Table A.16.

Table A.16 — Oligonucleotide primer sequences for qualitative PCR

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

A.5.16.3 Procedure

A.5.16.3.1 General

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

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

Distribute 25 µl/tube of the master mixture to 200 µl PCR reaction tubes. Add 5 µl of DNA solution samples, rice positive control, negative control, and blank control (H_2O) to the tubes, respectively.

Mix the PCR tubes gently, centrifuge in the microcentrifuge at 1 000 × g for 10 s.

Insert the plate into the instrument.

Run the PCR with qualitative PCR cycling conditions.

A.5.16.4 PCR controls

See 7.5 and ISO 24276.

A.5.16.5 Temperature–time programme

The PCR assay has been optimized for use in a PTC-100[1)] (MJ Research) and an ABI 2720[1)] (Applied Biosystems) thermal cycler PCR machine. Although other PCR machines may be used, the thermal cycling conditions may need to be verified. The qualitative PCR cycling parameters are indicated in Table A.17.

Table A.17 — Qualitative PCR temperature–time programme

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

A.5.16.6 Detection

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

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

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

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

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

A.5.16.7 Accept or reject criteria

Method performance requirements used to evaluate the results from the collaborative study are as follows.

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

A.5.17 Sample identification

All samples should be identified unambiguously.

A.5.18 Interpretation and calculations of the results

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

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

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

A.6.1 Purpose, relevance and scientific basis

The *LAT52* gene encodes a heat-stable, glycosylated, cysteine-rich protein that is necessary for tomato pollen development. The *LAT52* detection system has been demonstrated to be suitable for being used as species-specific gene in GM tomato identification and quantification (Reference [46]). The GMO Detection Laboratory of Shanghai Jiao Tong University (GMDL-SJTU) organized the collaborative trial for validation of the applicability of the tomato *LAT52* gene as species-specific gene for qualitative analysis of genetically modified (GM) or non-GM tomato. The study involved 13 laboratories from the US, Singapore, Korea, Lithuania, Slovenia, Norway, Italy, and China (Reference [47]). The results are given in Table A.18 and Table A.19.

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

a) qualitative PCR for the validation of the heterogeneity of the LAT52 gene among tomato cultivars for different geographic and phylogenetic origin;

b) qualitative PCR for the validation of the species-specificity of the LAT52 gene for tomato;

c) qualitative PCR for the evaluation of the LOD of the established LAT52 qualitative PCR assay.

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

— ISO 5725-2[39] especially considered in relation to the measure of precision (i.e. repeatability and reproducibility) and trueness;

— The IUPAC protocol for the design, conduct and interpretation of method-performance studies (Reference [48]).

A.6.2 Principle

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

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

A.6.3 Validation status and performance criteria

A.6.3.1 Robustness of the method

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

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

A.6.3.2 Intralaboratory trial

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

For sample preparation for the validation study, the DNA samples were extracted by the GMDL-SJTU using the CTAB method adopted from ISO 21571:2005, A.3. Spectrometric quantification of the amount of total DNA extracted was performed using a method adopted from ISO 21571:2005, B.1. After the DNA quantification, a qualitative PCR run applying the 18S PCR system was carried out to provide data about possible PCR inhibition (Reference [49]).

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

A.6.3.3 Collaborative trial

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

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

Table A.18 — Results of the qualitative PCR

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

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

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

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

A.6.3.4 Molecular selectivity

A.6.3.4.1 General

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

A.6.3.4.2 Experimental

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

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

A.6.3.4.3 Theoretical

The theoretical specificity of the *LAT52* primers was assessed through a homology search using the BLASTN 2,0MP-WashU program (Reference [82], search date: 2010-01-20). The 92 bp sequence used as query is part of the NCBI accession number X15855 (nucleotides 1385–1476). The results of the BLAST confirmed the complete identity of the query sequence with the tomato anther-specific *LAT52* gene sequences, and showed no homology with sequences of other genes and species.

A.6.4 Principle and summary

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

A.6.5 Terms and definitions

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

A.6.6 Sample type and amounts

For the collaborative study, the following samples were used:

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

A.6.7 Limit of detection and range of use

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

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

A.6.8 Estimation of measurement uncertainty

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

A.6.9 Interferences

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

A.6.10 Physical and environmental conditions

See ISO 24276 for details.

A.6.11 Apparatus and equipment

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

A.6.11.2 Electrophoresis equipment, with power supply.

A.6.11.3 Documentation system for gel analysis.

A.6.11.4 Microwave oven (optional).

A.6.12 Reagents and materials

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

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

A.6.12.2 Oligonucleotides (see Table A.20).

A.6.12.3 Loading buffer.

A.6.12.4 Electrophoresis buffer.

A.6.12.5 Agarose.

A.6.12.6 DNA size standard.

A.6.13 Sample collection, transportation, preservation and storage

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

A.6.14 Preparation of test sample

Ensure that the test sample is representative of the laboratory sample, e.g. by grinding or homogenization. Measures and operational steps to be taken into consideration are described in detail in ISO 21571.

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

A.6.15 Instrument calibration

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

A.6.16 Analysis steps

A.6.16.1 Preparation of the DNA for qualitative PCR

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

A.6.16.2 Conventional PCR master mix

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

Table A.20 — Oligonucleotide primers sequences for qualitative PCR

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

A.6.16.3 Procedure

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

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

Distribute 25 µl/tube of the master mixture to 200 µl PCR reaction tubes. Add 5 µl of DNA solution samples, tomato positive control, negative control, and blank control (H_2O) to the tubes, respectively.

Mix the PCR tubes gently, centrifuge briefly in the microcentrifuge in order to collect all drops of the solution together.

Insert the plate into the instrument.

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

A.6.16.4 PCR controls

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

A.6.16.5 Temperature–time programme

The PCR assay is optimized for use in a PTC-100[1)] (MJ Research) and an ABI 2720[1)] (Applied Biosystems) thermal cycler PCR machine. Although other PCR machine may be used, thermal cycling conditions should be verified. The temperature–time programme is given in Table A.21.

Table A.21 — Qualitative PCR temperature–time programme

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

A.6.16.6 Detection

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

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

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

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

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

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

A.6.16.7 Accept or reject criteria

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

A.6.17 Sample identification

Samples shall be unambiguously identified as detected or not detected.

A.6.18 Interpretation and calculations of the results

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

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

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Insert B.6 to B.9 after the existing text.

B.6 Real-time PCR based screening method for the detection of genetically modified plant DNA (*Agrobacterium tumefaciens* nos terminator, *T-nos*)

B.6.1 Purpose, relevance and scientific basis

Testing for the presence of the *Agrobacterium tumefaciens T-nos* DNA sequence is commonly used as a screening tool for detection of genetically modified (GM) plant material since many GM events in commerce contain this element. According to a search in the CERA database (Reference [50]), at least 43 GM events contain the *T-nos* element (Reference [51]). Detailed technical information for the qualitative *T-nos* real-time PCR method and the results of the collaborative trial validation have been published (Reference [52]). The collaborative study involved 24 laboratories from Germany, Austria, and Switzerland. The study design was carried out in accordance with the IUPAC protocol (Reference [48]) using 12 blind DNA samples containing the *T-nos* sequence as target analyte at two different levels (six 0,5 % mass fraction NK603 maize and six 0,1 % mass fraction NK603 maize DNA) and six blind DNA samples that did not contain the *T-nos* target (six non-GM maize DNA). The results of the collaborative trial for the *T-nos* target were used to determine the false-positive and false-negative rates. In addition, validation data of the specificity and applicability of the methods in a practical approach to screen for GM crop plant material have been published (Reference [53]).

B.6.2 Principle

This method describes a procedure for detecting a DNA sequence from the terminator region of the nopaline synthase gene (*T-nos*) from *Agrobacterium tumefaciens* by using qualitative real-time PCR. *T-nos* is used as a regulatory element in many GM plants, therefore the method is suitable to screen for the presence of GM crop plant material. It may be used for analysing DNA extracted from foodstuffs and also other products (e.g. feedstuffs, seeds). The application of this method requires a sufficient amount of amplifiable DNA to be extractable from the relevant matrix for the purpose of analysis.

NOTE The *T-nos* DNA sequence from *Agrobacterium tumefaciens* can also be detected in samples containing DNA from these bacteria, but not containing any genetically modified DNA sequences. Analyses using construct-specific and/or event-specific methods are therefore carried out in order to confirm any positive results.

B.6.3 Validation status and performance criteria

B.6.3.1 Robustness of the method

The robustness of the method has been tested on different real-time PCR devices [ABI 5700,1) ABI 7700,1) ABI 7900,1) ABI 7500,) RotorGene 3000,1) iCycler,1) LightCycler 1.2/1.51)], with different reaction volumes (20 µl and 25 µl) and different PCR reagent kits (QuantiTect Probe1) PCR kit of Qiagen;

LightCycler TaqMan1) master kit of Roche; TaqMan1) universal master mix of Applied Biosystems). The *T-nos* qualitative real-time PCR method has the expected robustness and worked well at different PCR devices, different reaction volumes, and with different reagent kits.

B.6.3.2 Intralaboratory trial

Different DNA samples containing known amounts of genomic DNA extracted from 5 % mass fraction NK603 maize flour (50 ng, 12,5 ng, 3,1 ng and 0,78 ng) were analysed in six repetitions per sample. At all DNA contents, all six repetitions were positive in the PCR test. The coefficients of variation of repeatability, $C_{V,r}$, values of 6,9 %, 8,5 %, 9,5 %, 22 % and 24,4 % and resulting confidence intervals (CI 95 %) of 7,3 %, 8,9 %, 9,9 %, 23,1 % and 25,6 % were determined at the indicated DNA concentrations, respectively (Reference [53]).

B.6.3.3 Collaborative trial

The reliability of the method was tested in a collaborative trial in accordance with the IUPAC protocol (Reference [48]), with a total of 24 participating laboratories (Reference [52]). Participating laboratories were provided with DNA samples containing either the *T-nos* sequence as target analyte or not. Each laboratory received 18 blind DNA samples, comprising a set of six samples of DNA extracted from 0,1 % mass fraction NK603 maize flour (ERM BF-415), of a set of six samples of DNA extracted from 0,5 % mass fraction NK603 maize flour (ERM BF-415) and of a set of six samples of DNA extracted from non-GM maize flour. This design was used to obtain representative data on the false-positive and false-negative rates shown in Table B.13 In addition, participants received a positive DNA target control consisting of a DNA solution of 0,5 % mass fraction NK603 (20 ng/µl). Furthermore, participants were provided with solutions of the primers, the probe and a commercial reagent kit (QuantiTect™ Probe1) PCR kit or the LightCycler TaqMan®1) master kit). A total of 12 laboratories used real-time PCR equipment adapted for plastic vials [ABI 5700,1) ABI 7700,1) ABI 75001) or ABI 7900,1) RotorGene,1) iCycler1)] and 12 laboratories applied real-time PCR equipment adapted for glass capillaries [LightCycler 1.2/1.51)]. Each sample was analysed by the participants in a single PCR using 5 µl of the unknown sample DNA under conditions described in Tables B.15 and B.16. In addition, the amplifiability of the DNA samples required analysis by the laboratories using their respective established maize-specific reference gene real-time PCR method.

For preparation of DNA samples containing the *T-nos* DNA target sequence, CRMs (IRMM, Geel) of NK603 maize at two concentrations (BF-415b with 1 g/kg and BF-415c with 4,9 g/kg) were used. For the preparation of *T-nos* negative DNA test samples, GM-negative maize flour already used in the USDA/GIPSA proficiency programme of 2006-04 was taken. The positive DNA target control provided to the participants contained extracted DNA from 0,5 % mass fraction NK603 CRM (BF-415c). For extraction of the sample DNA a silica-membrane based kit system was applied [NucleoSpin® Food1) from Macherey-Nagel GmbH, Düren]. The quantity of the extracted DNA was measured by an ultraviolet spectrophotometric method (ISO 21571:2005, Annex B) and the final DNA solution used for preparation of the coded DNA sample aliquots was adjusted to a concentration of 20 ng/µl.

The results of the collaborative trial for the *T-nos* real-time PCR method are given in Table B.13.

Table B.13 — Results of the collaborative trial

No. participating laboratories	24
No. laboratories after eliminating the outliers	23
No. samples per laboratory	18
No. accepted results	414[a]
No. *T-nos* positive samples	276
No. *T-nos* negative samples	138
False-positive results	3[b] (2,2 %)
False-negative results	0 (0 %)

[a] The results from one laboratory were excluded, because a contamination with *T-nos* DNA causing false-positive results was reported by this laboratory.

[b] Three laboratories reported a false-positive result (at C_t 26,6, 38,9 and 39,6, respectively) for one of the *T-nos* negative DNA test samples. Real-time PCR instruments used by these laboratories were an ABI 5700 1) or the LightCycler 1.2. 1)

B.6.3.4 Molecular selectivity

B.6.3.4.1 General

For detection of the *T-nos* DNA sequence, an 84 bp fragment from the 3'-terminal region of the *Agrobacterium tumefaciens* nopaline synthase gene is selected and amplified using specific primers.

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

B.6.3.4.2 Experimental

DNA samples extracted from available reference materials (Reference [53]) were analysed and the following events tested positive in the *T-nos* real-time PCR:

- soya flour: GTS 40-3-2 (MON-04032-6);
- maize flour: Event 3272 (SYN-E3272), Bt11 (SYN-BT 011-1), CBH-351 (ACS-ZM004-3), GA21 (MON-00021-9), MIR604 (SYN-IR604-5), MON809 (PH-MON809-2), MON863 (MON-00863-5), MON 88017 (MON-88017-3), NK603 (MON-00603-6);
- rapeseed leaves: MS1×RF1 (ACS-BN004-7×ACS-BN001-4), MS8×RF8 (ACS-BN005-8 × ACS-BN003-6); OXY 235 (ACS-BN011-5);
- rice flour: Bt63;
- papaya fruit: SunUp (55-1);
- potato flour: EH92-527-1 (BPS-25271-9);
- ground cotton seeds: MON1445 (MON-01445-2), MON531 (MON-00531-6), MON15985 (MON-15985-7)

As expected, DNA extracted from reference materials (Reference [53]) of the following events gave negative results: 59122 maize (DAS-59122-7), MON810 maize (MON-00810-6), T14 maize (ACS-ZM002-1), T25 maize (ACS-ZM003-2), TC1507 maize (DAS-01507-1), Laurical 23-198 rapeseed (CGN-89465-2), GS40/90 rapeseed, T45 rapeseed (ACS-BN008-2), LL62 rice (ACS-OS002-5), LL601 rice (BCS-OS003-7), A2704-12 soya bean (ACS-GM005-3), A5547-127 soya bean (ACS-GM006-4).

B.6.3.4.3 Theoretical

The 84 bp target sequence used as query is contained in the GenBank® database (Reference [83], under NCBI accession number FN550390). The results of the BLAST confirmed the complete identity of the query sequence with several database entries that contain the *T-nos* sequence and no homology to other database entries (search date: 2010-04-22).

B.6.4 Principle and summary

An 84 bp DNA fragment from the terminator region of the nopaline synthase gene (*T-nos*) from *Agrobacterium tumefaciens* is amplified and detected by real-time PCR. Accumulated PCR products are measured over each cycle (real-time) by means of a target sequence-specific oligonucleotide probe which is labelled with two fluorescent dyes (FAM as reporter dye and TAMRA as quencher) and binds between the two primers in the DNA sequence [so-called "TaqMan®1) chemistry"] (Reference [54]).

B.6.5 Terms and definitions

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

B.6.6 Sample type and amounts (analyte and matrix)

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

DNA samples extracted from soya, maize, rice and potato flours, as well as from other plant materials, were used (Reference [53]). In the collaborative trial, participants received DNA samples extracted from 0,1 % and 0,5 % mass fraction NK603 maize flours (ERM BF-415) and from a GM-negative maize flour (Reference [54]). The DNA concentration was adjusted to 20 ng/µl. The final mass of sample DNA added per reaction did not exceed 200 ng.

B.6.7 Limit of detection and range of use

The relative LOD of the method is less than or equal to 0,1 % mass fraction, because all DNA samples extracted from the 0,1 % NK603 maize flour were detected correctly in the collaborative trial. The mass of 0,1 % mass fraction NK603 maize DNA used by the laboratories in the PCR was 100 ng. Based on the assumption that the molecular mass of the maize haploid genome is 2,72 pg (Reference [55]) per copy and that in the heterozygous NK603 maize material the *T-nos* DNA sequence is present in two copies (Reference [56]), approximately 37 copies were detected by all laboratories at an average C_t value of 34,6 (± 2,4).

Further experiments with low copy numbers of the *T-nos* sequence were performed in an interlaboratory study with five laboratories using different PCR devices [ABI 7900,1) ABI 7700,1) LightCycler 1.41)] and the reaction conditions described in Tables B.15 and B.16. Each laboratory analysed 10 replicates of 100 ng DNA extracted from non-GM maize spiked with DNA extracted from NK603 maize flour corresponding to 20 copies of the *T-nos* target sequence per reaction. Positive results were obtained in 51 of 51 reactions (100 %). In addition, lower spiking levels were analysed in an intralaboratory study using a real-time PCR device [ABI 75001)]. Detection of 10 copies of the *T-nos* target sequence occurred in 15 of 15 reactions, five copies were detected in 13 of 15 reactions. Therefore, the absolute LOD of the method using non-GM-maize DNA spiked with NK603 maize DNA is estimated to be in the range of 5 to 10 copies of the *T-nos* target sequence.

In general. the practical LOD of the method depends on the target taxon genome size and the amount of sample DNA used in the PCR.

B.6.8 Estimation of measurement uncertainty

The reproducibility of the method is given by the results of the collaborative trial (see B.6.3.3).

B.6.9 Interferences

The amount, quality, and ability to amplify the nucleic acid template influences the analytical result obtained (see ISO 21571). Therefore the nucleic acid used for the analysis should be checked, e.g. by means of a target taxon-specific PCR method.

B.6.10 Physical and environmental conditions

See ISO 24276 for details.

B.6.11 Apparatus and equipment

B.6.11.1 **Real-time PCR thermal cycler** equipped with an energy source suitable for the excitation of fluorescent molecules and an optical detection system suitable for the detection of the fluorescence signals generated during PCR.

B.6.11.2 **Reaction tubes** and **caps** or **closures** which can be repeatedly heated to 100 °C and cooled to 4 °C without damage and which do not influence the fluorescence signal generated during the amplification process.

B.6.11.3 **UV-spectrophotometer** to determine the concentration of DNA.

B.6.12 Reagents and materials

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

See also B.6.16 (Analysis steps) for specific reagents.

B.6.13 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. See also ISO 21571.

B.6.14 Test sample preparation

See ISO 21571.

B.6.15 Instrument calibration

Instruments (e.g. thermal cyclers) should be calibrated as per ISO/IEC 17025.[41]

B.6.16 Analysis steps

B.6.16.1 Preparation of the DNA extracts

B.6.16.1.1 DNA extraction

DNA is extracted from the test sample applying a suitable method (ISO 21571).

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

B.6.16.1.2 DNA quantification

Spectrophotometric quantification of the amount of total DNA extracted has been performed by using a method described in ISO 21571:2005, B.1.

B.6.16.1.3 DNA integrity evaluation

The integrity of the extracted DNA (amount, quality and amplifiability) can be determined in a real-time PCR run with a method that targets a taxon-specific (endogenous) sequence.

B.6.16.2 PCR reagents

B.6.16.2.1 General. Ready-to-use reagent mixtures or individual components can be used. Reagents and polymerases which lead to equal or better results may also be used.

B.6.16.2.2 Thermostable DNA polymerase, for hot-start PCR.

B.6.16.2.3 PCR buffer solution (contains $MgCl_2$ and deoxyribonucleoside triphosphates dATP, dCTP, dGTP and dUTP); e.g. QuantiTect Probe[1)] PCR kit (Qiagen) for real-time PCR using plastic vials or LightCycler®[1)] TaqMan®[1)] master (Roche) for using glass capillaries.

B.6.16.2.4 Primers. See Table B.14.

Table B.14 — Oligonucleotides

Name	DNA sequence of the oligonucleotides
	T-nos as target sequence
180-F	5‘- CAT gTA ATg CAT gAC gTT ATT TAT g -3‘
180-R	5‘- TTg TTT TCT ATC gCg TAT TAA ATg T –3‘
Tm-180	5‘-(FAM) – ATg ggT TTT TAT gAT TAg AgT CCC gCA A - (TAMRA) –3‘[a]

[a] FAM: 6-carboxyfluorescein, TAMRA: 6-carboxytetramethylrhodamine. Equivalent reporter dyes and/or quencher dyes can be used.

B.6.16.3 Procedure

The method description applies to a total volume of 25 µl per PCR when using plastic vials and of 20 µl when using glass capillaries. Use the reagents listed in Tables B.15 or B.16.

Table B.15 — Addition of reagents for real-time PCR using plastic vials

Reagent	Final concentration	Volume per reaction µl
2× PCR buffer solution (including $MgCl_2$, dNTPs, and DNA polymerase)[a]	1×	12,5
Primer 180-F, c = 10 µmol/l[b]	400 nmol/l	1
Primer 180-R, c = 10 µmol/l[b]	400 nmol/l	1
Probe Tm-180, c = 10 µmol/l[b]	100 nmol/l	0,25
Water	–/–	5,25
Sample DNA	up to 200 ng	5
Total reaction volume	–/–	25

[a] If QuantiTect Probe[1)] PCR master mix (Qiagen) is used.

[b] Different working concentrations may be used. If different working concentrations are used, the volumes per sample shall be adapted accordingly.

Table B.16 — Addition of reagents for real-time PCR using glass capillaries

Reagent	Final concentration	Volume per reaction µl
5× PCR buffer solution (including $MgCl_2$, dNTPs, and DNA polymerase) [a]	1×	4
Primer 180-F, c = 10 µmol/l[b]	1 µmol/l	2
Primer 180-R, c = 10 µmol/l[b]	1 µmol/l	2
Probe Tm-180, c = 10 µmol/l[b]	200 nmol/l	0,4
Water	–/–	6,6
Sample DNA	up to 200 ng	5
Total reaction volume	–/–	20

[a] If LightCycler®1) TaqMan®1) master (Roche) is used as PCR buffer.

[b] Different working concentrations may be used. If different working concentrations are used, the volumes per sample shall be adapted accordingly.

B.6.16.4 PCR controls

As a positive control, a certified reference material of GTS 40-3-2 (0,1 % mass fraction soya bean flour ERM BF410b) may be used. Any other appropriate controls should be included as described in ISO 24276.

B.6.16.5 Preparation of standards

A DNA solution with a known concentration (ng/µl) and copy number of the *T-nos* sequence calculated from this concentration can be prepared as standard for positive control.

NOTE When using genomic plant DNA as the standard DNA, the number of genome equivalents per microlitre, C_{GE}, can be calculated, as a first step, on the basis of the molecular mass of the respective haploid genome (Reference [55]) of the plant species, applying the following equation:

$$C_{GE} = \frac{\rho_{DNA} \times 1\ 000}{m_{HG}}$$

where

ρ_{DNA} is the mass concentration of DNA, in nanogram per microlitre;

m_{HG} is the mass of the haploid genome, in picogram

B.6.16.6 Temperature–time programme

A proven temperature–time programme for this PCR is given in Table B.17 when using plastic vials and in Table B.18 when using glass capillaries.

Table B.17 — Temperature–time programme for plastic reaction vessels

Step	Parameter		Temperature °C	Time	Fluorescence measurement	Cycles
1	Initial denaturation		95	15 min	no	1
2	Amplification	denaturation	94	15 s	no	45
		annealing and elongation	60	60 s	yes	

Table B.18 — Temperature–time programme for glass capillaries vessels

Step	Parameter		Temperature °C	Time	Fluorescence measurement	Cycles
1	Initial denaturation		95	10 min	no	1
2	Amplification	denaturation	94	10 s	no	45
		annealing and elongation	60	40 s	yes	

B.6.16.7 Accept or reject criteria

A corresponding real-time PCR device-specific data analysis program is used for the identification of PCR products. The amplification results may be given in a different manner, depending on the device used. In the absence of detectable PCR products (negative result), e.g. "undetermined", "no amp", or the maximum number of possible cycles is given in the report. If the amplification of the DNA target sequence occurred in a sample (positive result), the cycle number is calculated at which a predetermined fluorescence threshold value was exceeded (cycle threshold, C_t, value or crossing point, C_p, value).

If, due to atypical fluorescence measurement data, the automatic interpretation does not provide a meaningful result, it may be necessary to set the baseline and the threshold manually prior to interpreting the data. In this case, apply the device-specific instructions given in the manual regarding the use of the interpretation software.

B.6.16.8 Identification

The result of the analysis is verified by using the real-time PCR assay format with a fluorescent-labelled hybridization probe (Reference [54]) for the identification of the PCR product during the cycling process.

B.6.17 Sample identification

The target sequence is considered as detected when:

- by using the *T-nos*-specific primers 180-F and 180-R and the probe Tm-180, an increase in the measured fluorescence can be determined which is due to amplification;
- in the PCR control set-ups with no added DNA (PCR reagent control, negative extraction control), no increase in the fluorescence can be determined which is due to amplification;
- in the set-ups for the amplification control (positive DNA target control, PCR inhibition control) the expected C_t values (or C_p values) are achieved.

B.6.18 Calculations

An 84 bp fragment is generated and the increase in the measured fluorescence can be detected which is due to amplification of the target sequence. The negative control with no added DNA (PCR reagent control, negative extraction control) should result in no increase in the fluorescence. In the amplification control (positive DNA target control, PCR inhibition control) the expected C_t values (or C_p values) should be achieved.

B.7 Screening method for the detection of genetically modified organisms (*FMV 34S* promoter)

B.7.1 Purpose, relevance and scientific basis

The method is applicable to detect the figwort mosaic virus 34S (*FMV 34S*) promoter DNA sequence in RT73 rapeseed and in other GM plants which contain the *FMV 34S* promoter. It can be used to screen for the presence of DNA derived from GM plants containing this genetic element. The principle of the method is based on DNA amplification by qualitative PCR.

NOTE The detection of the PCR products by agarose gel electrophoresis only does not fulfil the requirement of a sequence-specific verification step. The necessity of a molecular verification of the PCR product resulting from a screening method depends on the analysis scheme which follows the screening method. In general, a molecular verification of the PCR product generated can be done e.g. by sequencing, by digestion with two restriction endonucleases, and/or probe-based hybridization which was not included in the validation of the method described in this annex.

B.7.2 Principle

This method describes a qualitative PCR screening method for the detection of the *FMV 34S* promoter in DNA extracted from rapeseed by amplifying a PCR product of 196 bp. Due to the presence of *FMV 34S* promoter in many GM plants, especially in GM rapeseed, potato, soya bean, cotton, tomato, and beet, this method can be used to screen for the presence of GM plant-derived DNA. Since the *FMV 34S* promoter sequence can also be detected in samples which contain DNA from the figwort mosaic virus, but no genetically modified DNA sequences, a follow-up analysis should be carried out to confirm positive results.

The method has been optimized for screening the *FMV 34S* sequences using dry rapeseed powder. The applicability of the *FMV 34S* sequences was tested through a collaborative trial using mixed powder containing different amounts of RT73 (unique identifier MON-00073-7) rapeseed in conventional rapeseed.

B.7.3 Validation status and performance criteria

B.7.3.1 Robustness of the method

Robustness of the qualitative PCR assay for detection of the *FMV 34S* promoter was tested at three different annealing temperatures (53 °C, 54 °C and 55 °C) using three DNA samples containing different amounts of genomic DNA extracted from RT73 rapeseeds (10 ng, 1 ng and 0,1 ng, corresponding to approximately 15 000, 1 500, and 150 copies of *FMV 34S* promoter, and three repetitions per sample). The qualitative PCR results showed that these PCR reactions clearly produced DNA bands of identical size irrespective of the annealing temperature and the amounts of template DNA. A final annealing temperature of 54 °C was chosen because it gave the strongest signal intensity with the lowest concentration of DNA template (0,1 ng).

The qualitative PCR assay for *FMV 34S* promoter has also been tested on different thermal cyclers [MJ Research PTC-225,[1] Applied Biosystem 2720[1] and Eppendorf Mastercycler Gradient[1]], with three different reaction volumes (25 µl, 30 µl and 50 µl, and three repetitions per volume). Qualitative PCR results from the different thermocyclers and reaction volumes were essentially equivalent. These results indicated that the qualitative PCR assay for *FMV 34S* promoter had the expected robustness.

B.7.3.2 Intralaboratory trial

The RT73 rapeseed genomic DNA was extracted by the GMO Detection Laboratory of Shanghai Entry and Exit Inspection and Quarantine Bureau of China (GMDL-SHCIQ) using the Wizard®[1] magnetic DNA purification system for food (Promega Inc.). The qualitative PCR assay for *FMV 34S* promoter was tested by three different researchers in the GMDL-SHCIQ using the RT73 rapeseed genomic DNA as template. The relative LOD was 0,1 % mass fraction of RT73 DNA in 20 ng of genomic rapeseed DNA, corresponding to approximately 16 RT73 haploid rapeseed genomic copies. The absolute LOD was 0,1 ng RT73 rapeseed genomic DNA, corresponding to approximately 81 RT73 haploid rapeseed genomic copies (Reference [57]).

B.7.3.3 Collaborative trial

GMDL-SHCIQ organized the collaborative trial for the detection method for *FMV 34S* promoter in GM RT73 rapeseed. In this study, 12 laboratories from Canada, Slovenia, the Netherlands, Germany, Argentina, and China participated.

The method developer prepared 10 double-blind samples of rapeseed containing different concentrations of RT73 rapeseed and supplied them to the participants of the collaborative trial. The rapeseed seed samples were milled with SPEX CertiPrep® 6850[1)] freezer mill. The procedure is as follows.

The dried seed samples of RT73 and conventional rapeseed were first ground into powder with the freezer mill.

Using a Sartorius BS 224S balance (uncertainty within ±0,000 3 g) calibrated as per ISO/IEC 17025,[41] 2,000 0 g, 0,400 0 g, 0,040 0 g, 0,020 0 g, 0,004 0 g genuine dried RT73 rapeseed powder and 38,000 0 g, 39,600 0 g, 39,960 0 g, 39,980 0 g, 39,996 0 g pure dried conventional rapeseed powder were weighed.

The weighed RT73 rapeseed and the corresponding mass of conventional rapeseed were put into 50 ml grinding vials simultaneously (total mass is 40,000 0 g).

The samples were ground in liquid nitrogen in the freezer mill for 10 min, and the vials were then kept at room temperature for 1 to 2 days without opening the caps of the vials.

When the outside surface of the vials was at room temperature without condensing water, these mixed powder samples were aliquoted into small bottles, 1 g for each bottle.

The resultant blending samples generated mass fractions of 5 %, 1 %, 0,1 %, 0,05 %, and 0,01 % of GM RT73 rapeseed in non-GM rapeseed. The above samples were tested to be homogeneous by randomly drawing 10 bottles from each of the 5 %, 1 % and 0,1 % mass fraction RT73 samples, respectively. Results showed that all of the DNA templates extracted from 30 bottles dry powders could be used for amplification of the target DNA fragments.

The participants received the following samples:

- 10 blinded rapeseed powder samples containing 5 %, 1 %, 0,1 %, 0,05 % and 0,01 % mass fraction RT73 rapeseed with two bottles for each mass fraction, 1 g for each bottle;
- RT73 rapeseed powder (containing 10 % mass fraction RT73 ingredient) as positive control coded with P, 1 g;
- GM phosphinothricin-tolerant male-sterile MS8 × RF3 rapeseed powder as negative control coded with M, 1 g;
- non-GM rapeseed powder for negative control coded with N, 1 g;
- Wizard®[1)] magnetic DNA purification system for food (Promega Inc.) and 1 magnetic separation stand (Promega Inc.).

Primer pair FMV 34S Primer F/R: the primer sequence and amplicon size are shown in Table B.20.

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

DNA was extracted from the dry powder samples using the Wizard®[1)] magnetic DNA purification system for food (Promega Inc.).

Spectrometry was used to quantify the amount of total extracted DNA, in accordance with ISO 21571:2005, B.1.

Qualitative PCR was employed to analyse the extracted DNAs.

Agarose gel electrophoresis was performed on the PCR products and the image recorded.

The collaborative trial was carried out in accordance with ISO 5725-2,[39] especially the section in relation to the measure of precision (i.e. repeatability and reproducibility) and trueness.

The applicability of the *FMV 34S* promoter screening detection method and the LOD were tested by using DNA samples extracted from 10 double-blind samples composed of various amounts of RT73 rapeseed and conventional rapeseed. Each laboratory received 10 double-blind dry powder samples with mass fractions of 5 %, 1 %, 0,1 %, 0,05 %, 0,01 % of RT73 rapeseed in non-GM rapeseed.

The results of this collaborative trial are reported in Table B.19. The positive rate of samples which contain 5 %, 1 %, 0,1 %, 0,05 % and 0,01 % mass fraction of GM RT73 rapeseed in non-GM rapeseed were 100 %, 100 %, 95,8 %, 70,8 % and 20,8 %, respectively. The relative LOD of the detection method for *FMV 34S* promoter has been demonstrated to be at least 0,1 % mass fraction GMO according to the internationally accepted guidelines.

Table B.19 — Results of the collaborative trial of FMV 34S promoter detection method

Parameter (collaborative trial of 2006; sample: RT73 rapeseed meal)	**Value**				
No. laboratories	12				
No. laboratories that have been evaluated	12				
No. samples per laboratory	10				
No. total samples	120				
No. accepted results	120				
No. samples containing non-GM rapeseed	120				
Target detection material content, % mass fraction	5,0	1,0	0,1	0,05	0,01
No. samples	24	24	24	24	24
No. positive	24	24	23	17	5
No. false-negative	0	0	1	7	19
No. false-positives	0	0	0	0	0
Positive %	100	100	95,8	70,8	20,8
False-negative %	0	0	4,2	29,2	79,2
False-positive %	0	0	0	0	0

B.7.3.4 Molecular selectivity

B.7.3.4.1 General

The screening detection method targets the *FMV 34S* promoter sequences, and an 196 bp fragment of the conserved region of the *FMV 34S* promoter sequence is amplified using the specific primers *FMV 34S* primer F and *FMV 34S* primer R.

B.7.3.4.2 Experimental

Amplification of FMV 34S target sequence has been observed in genomic DNA from RT73 rapeseed, MON1445, MON1698 and MON88913 cotton varieties, MON 89788 soya bean, and H7-1 sugar beet.

No amplification has been observed with DNA from non-transgenic organisms including maize, wheat, rice, soya bean, pea, cotton, barley, potato, tomato, cattle, sheep, goat, pig, duck, chicken, and fish. In addition, no amplification was observed with the DNAs from MS8×RF3, T45, Oxy235 rapeseed varieties, 40-3-2 soya bean and MON810, MON863, 1507, Bt11, GA21, NK603 and Bt176 maize varieties.

B.7.3.4.3 Theoretical

The theoretical specificity of the *FMV 34S* primer pairs was assessed through an identity search using the BLASTN 2.0MP-WashU program (Reference [82], search date: 2010-02-18). The 196 bp sequence used as query is part of the NCBI accession number AR016589. The results of the BLAST confirmed that the primers match an extensive list of accessions referable to patents, as well as figwort mosaic virus genome whose accession number is X06166.

B.7.4 Principle and summary

The methodology is a qualitative PCR screening method targeting a fragment of the *FMV 34S* promoter sequence for determination of the presence of GM ingredients in foodstuffs. The applicability of the screening method and the LOD were validated, and the 196 bp PCR product was separated by agarose gel electrophoresis.

B.7.5 Terms and definitions

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

B.7.6 Sample type and amounts

Ensure that the test sample is representative of the laboratory sample, e.g. by grinding or homogenization. Measures and operational steps to be taken into consideration are described in ISO 21571.

B.7.7 Limit of detection and range of use

The absolute LOD of the qualitative PCR assay was 0,1 ng genuine RT73 rapeseed DNA corresponding to approximately 75 haploid rapeseed genomic DNA copies and 150 copies of *FMV 34S* target sequence (Reference [57]). The relative LOD of the qualitative PCR assay was 0,1 % mass fraction RT73 DNA in 20 ng of genomic rapeseed DNA. The lowest mass fraction of the RT73 DNA in the collaborative trial was 0,01 %.

Due to the presence of *FMV 34S* promoter in many genetically modified plants, especially in rapeseed, potato, soyabean, cotton, tomato, and beet, this method can be used to screen for the presence of GM plant material with mass fractions ranging from 0,1 % to 100 %.

B.7.8 Estimation of measurement uncertainty

The global uncertainty of the method is given by the results of the collaborative trial (see B.7.3.3).

B.7.9 Interferences

The amount, quality and amplification ability of the nucleic acid template influences the analytical result obtained (see ISO 21571). Therefore the nucleic acid used for the analysis should be checked e.g. by means of a PCR specific to the target taxon.

Be aware that a false-positive result may occur since the amplified sequence is derived from figwort mosaic virus, which naturally infects plants.

B.7.10 Physical and environmental conditions

See ISO 24276 for details.

B.7.11 Apparatus and equipment

B.7.11.1 General

All the apparatus should be calibrated according to ISO/IEC 17025.[41]

B.7.11.2 Apparatus and materials for DNA extraction

B.7.11.2.1 Water bath or **heating block**.

B.7.11.2.2 Microcentrifuge.

B.7.11.2.3 Micropipettes.

B.7.11.2.4 Vortex mixer.

B.7.11.2.5 Tubes, capacity: 1,5/2,0 ml.

B.7.11.2.6 Tips and **filter tips** for micropipettes.

B.7.11.2.7 Rack for reaction tubes.

B.7.11.2.8 PVC or **latex gloves**.

B.7.11.2.9 Vacuum dryer suitable for drying DNA pellets, optional.

B.7.11.3 Apparatus and equipment for DNA quantification

B.7.11.3.1 UV spectrophotometer, single beam, double beam or photodiode array instruments are suitable, or **fluorometer** applicable for fluorescent dye-methods of DNA quantification.

B.7.11.3.2 Measurement vessels e.g. quartz cuvettes or plastic cuvettes suitable for UV detection at a wavelength of 260 nm. The size of the measurement vessels used determines the volume for measurement. This should be one of the following: half-microcuvettes (1 000 µl), microcuvettes (400 µl), ultra-microcuvettes (100 µl) and quartz capillaries (3 µl to 5 µl). The optical path of standard cuvettes is usually 1 cm.

B.7.11.4 Apparatus and equipment for qualitative PCR

B.7.11.4.1 Thermal cycler, the method was originally developed and in-house validated with MJ Research PTC-225, Applied Biosystems 2720 and Eppendorf Mastercycler Gradient thermal cyclers. Other thermal cyclers and reaction vials may also be used if they show to lead to equivalent or better results.

B.7.11.4.2 Electrophoresis chamber, with power supply.

B.7.11.4.3 Microwave oven (optional).

B.7.11.4.4 Image system for gel analysis.

B.7.11.4.5 Microcentrifuge.

B.7.11.4.6 Freezer operating at −20 °C and **refrigerator** operating at 4 °C.

B.7.11.4.7 Micropipettes.

B.7.11.4.8 Vortex mixer.

B.7.11.4.9 Tubes, capacities: 0,2 ml, 1,5 ml and 2,0 ml.

B.7.11.4.10 Tips and **filter tips** for micropipettes.

B.7.11.4.11 Rack for reaction tubes.

B.7.11.4.12 PVC or **latex gloves**.

B.7.12 Reagents and materials

B.7.12.1 General

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

B.7.12.2 DNA extraction

Wizard®1) magnetic DNA purification system for food (Promega Inc.) and 1 magnetic separation stand (Promega Inc.).

NOTE Other evaluated DNA extraction kits or other extraction methods can also be suitable.

B.7.12.3 Qualitative PCR

For quality of reagents used, see ISO 24276:2006, 5.3.5.

B.7.12.3.1 PCR buffer (without $MgCl_2$), 10×.

B.7.12.3.2 $MgCl_2$ solution, $c(MgCl_2)$ = 25 mmol/l.

B.7.12.3.3 dNTP solution, c (dNTP) = 2,5 mmol/l (each).

B.7.12.3.4 Oligonucleotides, see Table B.20.

B.7.12.3.5 Thermostable DNA polymerase, 5 IU/µl.

B.7.12.3.6 DNA size standard.

B.7.13 Sample collection, transportation, preservation and storage

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

B.7.14 Preparation of test sample

For each sample, one DNA extraction should be carried out, following the general instructions and measures described in ISO 21571.

B.7.15 Instrument calibration

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

B.7.16 Analysis steps

B.7.16.1 Preparation of the DNA extracts

B.7.16.1.1 DNA extraction

DNA extraction should be performed using Wizard®1) magnetic DNA purification system for food (Promega Inc.) or other evaluated DNA extraction kit. Other DNA extraction techniques that have been evaluated could also be used.

B.7.16.1.2 DNA quantitation

Spectrometric or fluorometric quantification of the amount of total DNA extracted should be performed using a method adopted from ISO 21571:2005, B.1.

B.7.16.1.3 DNA integrity evaluation

The integrity of the extracted DNA was evaluated by agarose gel electrophoresis.

B.7.16.2 PCR reagents

B.7.16.2.1 Thermostable DNA polymerase, buffers, etc

See B.7.12.3, thermostable DNA polymerase (with hot start enzyme property) applicable for qualitative PCR should be used. Reagents and polymerases which lead to equal or better results may also be used.

B.7.16.2.2 Primers and probe

See Table B.20.

Table B.20 — PCR primer sequences for the detection of *FMV 34S*

Name	Oligonucleotide primer sequence (5' to 3')	Amplicon length
FMV 34S primer F	AAg CCT CAA CAA ggT CAg	196 bp
FMV 34S primer R	CTg CTC gAT gTT gAC AAg	

B.7.16.3 Procedure

B.7.16.3.1 General

The qualitative PCR assay for *FMV 34S* promoter is developed for a total volume of 25 µl mixture per reaction with the reagents listed in Table B.21 Per reaction, 20 ng of template DNA was added.

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

Add the components following Table B.21. It is recommended that PCR master mix be prepared to perform the amplification.

Distribute the master mix and add the DNA samples including the blind samples, positive control, negative control, and blank control (water).

Mix the PCR tubes (or plate) gently and centrifuge for a short period using the microcentrifuge.

Transfer the tubes (or plate) into the thermal cycler.

Run the PCR with cycling conditions described in B.7.16.3.4.

After the PCR program has been finished, transfer 2 µl of loading buffer to each reaction tube and mix with the PCR products.

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

Run the gel in the electrophoresis chamber under 5 V/cm for 20 min.

The gel image is recorded by the image system for results analysis.

Table B.21 — Amplification reaction mixtures in the final volume/concentration per reaction tube

Reagent	Final concentration	Volume per reaction µl
Sample DNA	20 ng	1
Water		15,8
10× PCR buffer (without $MgCl_2$)	1×	2,5
$MgCl_2$-solution[a], 25 mmol/l	1,5 mmol/l	1,5
dNTP solution, 2,5 mmol/l (each)	0,2 mmol/l (each)	2
FMV 34S Primer F 5 µmol/l	0,2 µmol/l	1
FMV 34S Primer R, 5 µmol/l	0,2 µmol/l	1
Taq DNA Polymerase, 5 IU/µl	1 IU	0,2

[a] If the PCR buffer solution already contains $MgCl_2$, the final concentration of $MgCl_2$ in the reaction mixture is adjusted to 1,5 mmol/l.

B.7.16.3.2 PCR controls

Each PCR should contain the positive control, negative control, and blank control (water), as described in ISO 24276.

B.7.16.3.3 Preparation of standards

A DNA solution with a certain concentration (ng/µl) and copy number of the FMV 34S target sequence can be prepared as standard.

B.7.16.3.4 Temperature–time programme

The temperature–time programme given in Table B.22 has been optimized for use in MJ Research PTC-225,[1)] Applied Biosystems 2720[1)] and Eppendorf Mastercycler Gradient[1)] thermal cyclers. Other thermal cyclers may be used, but it is necessary to verify the thermal cycling conditions for the instrument used. The qualitative PCR temperature–time programme refers to Table B.22.

Table B.22 — Qualitative PCR temperature–time programme

Activation and initial denaturation	3 min/94 °C
Amplification	30 s/94 °C 30 s/54 °C 40 s/72 °C
No. cycles	40
Final elongation	3 min/72 °C

B.7.16.3.5 Accept or reject criteria

Method performance requirements used to evaluate the results of the collaborative study are as follows.

A fragment of 196 bp should be detected in the RT73 rapeseed positive control (sample P), and no target fragment detected in negative control (sample N and M) and blank control. The detection of a fragment with a size of 196 bp indicates that the sample DNA solution contains amplifiable *FMV 34S* promoter.

A false-positive result can occur since the amplified sequence is derived from figwort mosaic virus, which naturally infects plants. Positive results may indicate the presence of a GM plant-derived product, but should not be interpreted as a proof for the presence of GM plant-derived products without additional confirmation.

In order to distinguish between a viral infection and GM material, methods for the detection of figwort mosaic virus and/or further screening methods for GMO detection should be used.

B.7.16.3.6 Identification

In the method described here, identification is based only on PCR product size estimated by the DNA size standards. A fragment of 196 bp should be the specific product, other bands existing in the agarose electrophoresis mean unspecific products.

B.7.17 Sample identification

All samples should be identified unambiguously.

B.7.18 Interpretation and calculations of the results

The expected amplicon of the *FMV 34S* promoter is 196 bp in size.

The detection of fragment with a size of 196 bp indicates that the sample DNA solution contains amplifiable *FMV 34S* promoter with a size similar to that derived from RT73 rapeseed, and the result should be expressed as “For sample X, *FMV 34S* promoter sequence was detected”.

If no expected DNA fragment with 196 bp in size was obtained, the result should be expressed as “For sample X, *FMV 34S* promoter sequence was not detected”. The LOD of the analyses shall be given.

B.8 Real-time PCR based screening method for the detection of the *bar* gene of *Streptomyces hygroscopicus*

B.8.1 Principle

The method describes a procedure for the detection of a DNA sequence from the phosphinothricin acetyl transferase gene (*bar*) from *Streptomyces hygroscopicus*. The *bar* gene is frequently found in genetically modified plants (e.g. rice, rapeseed, maize and cotton) since plants carrying this gene are insensitive towards phosphinothricin-containing herbicides. The *bar* screening method described is based on a real-time PCR and can be used for screening of DNA extracted from genetically modified plants containing the *bar* gene sequence.

The DNA sequence amplified by the *bar* screening method and originating from *Streptomyces hygroscopicus* can be detected in samples which contain DNA of these naturally occurring bacteria. For this reason, it is necessary to confirm any positive *bar* screening result. To do so, the respective sample DNA should be subjected to a follow-up analysis.

B.8.2 Validation status and performance criteria

B.8.2.1 Robustness

The robustness of the method was tested by applying the following modifications in the reaction set-up:

- reduction of the primer concentration from 140 nmol/l to 100 nmol/l;
- reduction of the probe concentration from 100 nmol/l to 75 nmol/l.

All reactions were done in three replicates using the same amount of template DNA.

The reduction of the probe concentration did not influence the cycle threshold value.

The reduction of the primer concentration resulted in an increase of the cycle threshold value by 0,4 on average. This aberration can be neglected for a qualitative method.

In the collaborative trial, the robustness of the method was checked with regard to different real-time PCR devices and master mixes. Neither the real-time PCR devices nor the master mixes used had an influence on the performance of the method.

B.8.2.2 Intralaboratory trial

In intralaboratory trials, the method provided satisfactory and consistent results. The method was tested with a dilution series of 5 % mass fraction (DNA copies/DNA copies) LL62 rice DNA and five PCR replicates at each step. The relative confidence intervals (P = 95 %) for the measured copy numbers at 2 500, 500, 100, 50, 25, 10 and 5 copies were 5,6 %, 12,2 %, 27,2 %, 18,2 %, 22,4 %, 84,5 % and 45,2 %, respectively. The method was also tested for its performance with different real-time PCR-instruments (see B.8.10.1), different PCR master mixes (see B.8.15.4.1), and with DNAs extracted from different plants (see B.8.2.4.3). The results of these tests also showed that the method provides satisfactory and consistent results.

B.8.2.3 Collaborative trial

The performance of the method has been assessed in a collaborative study coordinated by the German Federal Office of Consumer Protection and Food Safety (BVL, References [58][59]). The study was performed in accordance with the IUPAC protocol (Reference [48]). In all, 15 laboratories participated in the study. For the analysis, the participants received 12 DNA samples with different concentrations of copies of the *bar* gene sequence as well as 6 DNA samples not containing any *bar* gene sequences. All samples were marked with random coding numbers.

To prepare the samples, genomic DNA from the leaves of LL62 rice or MS8 rapeseed plants and genomic DNA extracted from non-genetically modified rice grains or rapeseed seeds (*bar* negative) were used as initial solutions. All genomic DNA solutions were certified reference materials (purchased from Bayer CropScience, Gent, Belgium). The DNA concentrations were determined photometrically, and the copy numbers calculated from this on the basis of genome equivalents, according to the equation given in the Note to B.8.15.4.3. For rice, the haploid genome mass taken as a basis was 0,5 pg and for rapeseed, 1,33 pg (Reference [55]). Regarding the genomic LL62 rice or MS8 rapeseed reference DNA used, single integration of the *bar* target sequence into the rice or rapeseed genome, respectively (References [60][61]) was assumed, as well as for homozygous LL62 rice and hemizygous MS8 rapeseed, according to the manufacturer's information.

As far as the unknown samples were concerned, each participant received three vials each of which contained sub-samples of the following DNA solutions (mass fractions of genetically modified DNA, adjusted on the basis of the previously calculated copy numbers of the initial DNA solutions): 0,1 % LL62 rice DNA (20 ng/µl); 0,02 % LL62 rice DNA (20 ng/µl); 0,1 % MS8 rapeseed DNA (40 ng/µl); 0,02 % MS8 rapeseed DNA (40 ng/µl); non-genetically modified (non-GM) rice (20 ng/µl); non-genetically modified (non-GM) rapeseed (40 ng/µl).

Furthermore, for the purposes of calculating the copy numbers of the *bar* gene in the samples, all participants received a standard DNA with 5 % mass fraction (DNA copies/DNA copies) LL62 rice DNA (designated as S-2500, with a concentration of 5 ng/µl) which had been prepared using the same initial DNA solutions and in the same manner as was the case with the samples. On the basis of this LL62 rice standard DNA, the participants in the collaborative study had to prepare a dilution series with 0,2× TE in order to obtain DNA solutions for five calibration points (2 500, 500, 150, 50 and 10 copies of the *bar* target sequence) and one LL62 rice DNA solution as the sensitivity control with five copies of the *bar* sequence.

Each sample was analysed by the participants using a single assay with 5 µl of the respective DNA solution and the *bar* PCR system, under the conditions described in Tables B.25 to B.28. The DNA solutions for the calibration and the LL62 rice DNA solution with five copies were measured in duplicate. The measurements were performed with different real-time PCR devices (see B.8.10.1).

The results of the collaborative study in the form of an overview and as a qualitative evaluation for the determination of the false-positive and false-negative rates are shown in Table B.23.

Table B.23 — Results of the collaborative study

Parameter (collaborative study 2008)	**Value**
No. laboratories	15
No. laboratories having presented results	15
No. samples per laboratory	18
No. accepted results	270
No. samples containing the bar target sequence	180
No. samples containing no bar target sequence	90
False-positive results	0 (0 %)
False-negative results	0 (0 %)

In order to calculate the respective copy numbers from the determined cycle threshold (C_t) values of the samples, the 5 DNA calibration solutions were measured together with the samples in the same run of the PCR analysis. The calibration curve was drawn by plotting the C_t values versus the logarithm of the copy numbers of the *bar* target sequence which had been prescribed for the calibration solutions. The respective copy numbers for the samples as well as for the LL62 rice DNA solution with five copies were calculated from the calibration curves by interpolation. In Table B.24, a summary of the results thus determined is given.

Table B.24 — Evaluation of the collaborative study (quantitative)

GMO content in samples (GM copies/genome equivalents) % mass fraction	**No. positive results/ total results**	**Calculated copy numbers *bar* gene**		**% *bar* gene (relative to copy number of species)**[c]
		Mean[a]	$C_{V,R}$,[b] %	Mean[a]
0,1 % LL62 rice	45/45	218	24	0,11
0,02 % LL62 rice	45/45	46	25	0,02
0,1 % MS8 rapeseed	45/45	86	17	0,11
0,02 % MS8 rapeseed	45/45	21	31	0,03
Non-GM rice	0/45	0	—	—
Non-GM rapeseed	0/45	0	—	—

[a] Average value of the calculated copy numbers from all single assays.

[b] Coefficient of variation under reproducibility conditions.

[c] Based on the assumption that 150,000 rapeseed genome copies (200 ng rapeseed DNA) or 200,000 rice genome copies (100 ng rice DNA), respectively, were used per reaction.

B.8.2.4 Molecular selectivity

B.8.2.4.1 General

The method has been designed to target the phosphinothricin acetyl transferase gene (*bar*) of *Streptomyces hygroscopicus* described in the GenBank® database (Reference [83], accession No. X17220).

The DNA sequence amplified by the *bar* screening method can be detected in samples originating from genetically modified plants containing the *bar* gene from *Streptomyces hygroscopicus,* but also in samples which contain DNA of naturally occurring *Streptomyces hygroscopicus* bacteria.

For this reason, it is necessary to confirm any positive *bar* screening result. To do so, the respective sample DNA should be subjected to a follow-up analysis.

B.8.2.4.2 Theoretical

The theoretical specificities of the primers and the probe were assessed by a BLASTN search in the GenBank®/EMBL/DDBJ databases using the amplicon sequence (Reference [83], accession number FN550386). The result of the BLAST confirmed an 100 % identity only with the expected target sequences (search date 2009-11-15).

B.8.2.4.3 Experimental

In samples containing DNA extracted from non-genetically modified rice or rapeseed, the *bar* target sequence was not detected (Table B.24). When experimentally determining the specificity, no cross-reaction of the *bar* gene detection method with DNA extracted from the following genetically modified plants was observed:

- GM rice: Bt63;
- GM rapeseed: Liberator pHoe6/Ac (ACS-BN009-3), GT73 (MON-00073-7), Falcon GS40/90 phoe6/AC (ACS-BN010-4), TOPAS19/2 (HCN92) (ACS-BN007-1), OXY 235 (ACS-BN011-5), T45 (HCN 28) (ACS-BN008-2), LPAAT/Trierucin (pRESS), Laurat pCGN3828 (CGN-89465-2);
- GM maize: GA21 (MON-00021-9), Bt11 (SYN-BT 011-1), MON 809, MON810 (MON-00810-6), MON863 (MON-00863-5), MON 88017 (MON-88017-3), NK603 (MON-00603-6), DAS1507 (DAS-01507-1), DAS59122 (DAS-59122-7), MIR604 (SYN-IR604-5), 3272 (SYN-E3272-5), T14 (ACS-ZM002-1), T25 (ACS-ZM003-2);
- GM soya: GTS 40-3-2 (MON-04032-6), A2704-12 (ACS-GM005-3), A5547-127 (ACS-GM006-4), 305423 (DP-305423-1), 356043 (DP-356043-5), MON89788 (MON-89788-1);
- GM potato: EH92-527-1 (BPS-25271-9);
- GM sugar beet: GTSB77 (SY-GTSB77-8), H7-1 (KM-000H71-4);
- GM cotton: MON1445 (MON-01445-2), MON531 (MON-00531-6), MON15985 (MON-15985-7), 3006-210-23×281-24-236 (DAS-21023-5 × DAS-24236-5).

For the following genetically modified plants, it was experimentally shown that the *bar* gene detection method is suitable as a screening method:

- GM rice: LL62 (ACS-OS002-5), LL601 (BCS-OS003-7);
- GM rapeseed: MS1 (ACS-BN004-7), MS1xRF1 (ACS-BN004-7×ACS-BN001-4), MS8 (ACS-BN005-8);
- GM maize: Bt176 (SYN-EV176-9), CBH-351 (ACS-ZM004-3), DBT418 (DKB-89614-9);
- GM cotton: LL25 (ACS-GH001-3).

All DNAs used in experimental specificity tests were controlled for the ability to be amplified and for inhibitors with taxon specific methods before use (data not shown).

B.8.3 Principle and summary

A 60 bp DNA fragment from the phosphinothricin acetyl transferase gene (*bar*) of *Streptomyces hygroscopicus* is amplified and detected by real-time PCR. Accumulated PCR products are measured over each cycle (real-time) by means of a target sequence-specific oligonucleotide probe which is labelled with two fluorescent dyes (FAM as reporter dye and TAMRA as quencher) and binds in between the two primers in the DNA sequence range (so-called "TaqMan®1) chemistry") (Reference [54]).

B.8.4 Terms and definitions

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

B.8.5 Sample type and amounts

Ensure that the test sample is representative of the laboratory sample, e.g. by grinding or homogenization. Measures and operational steps to be taken into consideration are described in ISO 21571.

B.8.6 Limit of detection

The method was tested with samples containing low copy numbers of the *bar* gene (Table B.24). In all samples containing the *bar* target sequence, this sequence was detectable. Also the sensitivity control with five copies of LL62 rice DNA resulted in an amplification of the *bar* target sequence in all laboratories (30 single assays, data from the results obtained with the standard DNA dilution used for calibration are taken, see B.8.2.3). Based on these results, the LOD (relative to the matrix) is less than or equal to 0,02 % mass fraction (DNA samples with this amount of the *bar* copies to the genome copies of the respective species) or as an absolute value is less than or equal to five copies.

B.8.7 Estimation of measurement uncertainty

The coefficient of variation under reproducibility conditions ($C_{V,R}$) was 25 % or 31 %, respectively, at the level of 0,02 % mass fraction of LL62 rice or MS8 rapeseed. In the case of the samples with a mass fraction of the *bar* target sequence of 0,1 % on the basis of LL62 rice or MS8 rapeseed, the coefficient of variation under reproducibility conditions ($C_{V,R}$) was 24 % or 17 %. Thus, the precision data meet the requirements of ISO 24276 for the quantification of GMO target sequences. According to that method, the $C_{V,R}$ should be 25 % max. with quantitative procedures and 33 % max. between the LOD and the limit of quantification.

B.8.8 Interferences

The amount, quality, and ability to amplify the nucleic acid template influences the analytical result obtained (see ISO 21571). Therefore the nucleic acid used for the analysis should be checked, e.g. by means of a target taxon-specific PCR method.

B.8.9 Physical and environmental conditions

See ISO 24276 for details.

B.8.10 Apparatus and equipment for real-time PCR

B.8.10.1 Thermal cycler equipped with

- an **energy source** suitable for the excitation of fluorescent molecules;
- an **optical detection system** suitable for the detection of the fluorescence signals generated during PCR.

Within the framework of the collaborative study, devices of the ABI 7500[1)] type (Applied Biosystems, Darmstadt) were primarily used (8 laboratories), furthermore ABI 7700[1)] (2×), ABI 7900[1)] (2×), BioRad iCycler[1)] (1×), Mx3005p[1)] (1×) and LightCycler[1)] (1×) were used.

B.8.10.2 Reaction vessels and **caps** or **closures** which can be repeatedly heated to 100 °C and cooled to 4 °C without damage and which do not influence the fluorescence signal generated during the amplification process.

B.8.11 Reagents and materials

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

B.8.12 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.

B.8.13 Test sample preparation

See ISO 21571.

B.8.14 Instrument calibration

Instruments (e.g. thermocyclers) should be calibrated as per ISO/IEC 17025.[41]

B.8.15 Analysis steps

B.8.15.1 General

DNA is extracted from the test sample applying a suitable procedure. The DNA analysis consists of:

- verification of the amount, quality and amplifiability of the extracted DNA, e.g. by means of a PCR specific for the target taxon (see ISO 21569 and ISO 21570[43]);
- detection of the *bar* gene sequence in a real-time PCR.

B.8.15.2 Preparation of the DNA extracts

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

B.8.15.3 PCR reagents

B.8.15.3.1 Thermostable DNA polymerase (for hot-start PCR)

B.8.15.3.2 PCR buffer solution (contains $MgCl_2$ and deoxyribonucleoside triphosphates dATP, dCTP, dGTP and dUTP).

Ready-to-use reagent mixtures or individual components can be used as the PCR buffer solution.

B.8.15.3.3 Oligonucleotides

Table B.25 — Oligonucleotides

Name	DNA sequence of the oligonucleotide	Final concentration in the PCR	
	bar gene as the target sequence	Plastic reaction vials	Glass capillaries
RapB-F1	5’–ACA AgC ACg gTC AAC TTC C–3’	140 nmol/l	340 nmol/l
RapB-R1	5’–gAg gTC gTC CgT CCA CTC–3’	140 nmol/l	340 nmol/l
RapB-S1	5’–(FAM)–TAC CgA gCC gCA ggA ACC–(TAMRA)–3’[a]	100 nmol/l	120 nmol/l

[a] FAM: 6-carboxyfluorescein, TAMRA: 6-carboxytetramethylrhodamine. Equivalent reporter and/or quencher dyes can be used for the probe if they can be shown to yield similar or better results.

B.8.15.4 Procedure

B.8.15.4.1 PCR set-up

The description of the procedure is applicable to an overall volume of 25 µl per PCR set-up, with the reagents stated in Table B.26.

Table B.26 — Reaction set-up for the amplification of the *bar* gene sequence per reaction vial

Overall volume		25 µl
Sample DNA (up to 200 ng) or controls		5 µl
PCR buffer solution (including $MgCl_2$, dNTPs and DNA polymerase)[a]		12,5 µl
Primer	RapB-F1 and RapB-R1	see Table B.25
Probe	RapB-S1	see Table B.25
Water		To 25 µl

[a] In the collaborative study, TaqMan®1) universal master mix (Applied Biosystems, Darmstadt) was applied as the PCR buffer solution when using real-time PCR devices by the company Applied Biosystems; QuantiTect Probe1) PCR kit (Qiagen GmbH, Hilden) was applied when using other real-time PCR devices with plastic vials; QuantiTect Multiplex PCR No-Rox1) master mix (Qiagen GmbH, Hilden) was applied when using real-time PCR devices with glass capillaries. Equivalent products by other manufacturers may be used if they can be shown to yield similar or better results.

B.8.15.4.2 PCR controls

As a positive control, genomic DNA reference materials derived from leaves of the GM event MS8 and of the GM event LLRice62 (AOCS, Urbana, USA) may be used.

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

B.8.15.4.3 Preparation of standards

DNA solution with a known concentration (ng/µl) and copy number of the *bar* gene sequence calculated from this concentration.

NOTE When using genomic plant DNA as the standard DNA, the number of genome equivalents per microlitre, c_{GE}, can be calculated, as a first step, on the basis of the molecular mass of the respective haploid genome (Reference [49]) of the plant species, applying the following equation:

$$c_{GE} = \frac{\rho_{DNA} \times 1\,000}{m_{HG}}$$

where

ρ_{DNA} is the mass concentration of DNA, in nanogram per microlitre;

m_{HG} is the mass of the haploid genome, in picogram.

B.8.15.4.4 Temperature–time programme

If applying the PCR described here, the temperature–time programme listed in Table B.27 has proven suitable for plastic reaction vials and that in Table B.28 for glass capillaries.

Table B.27 — Temperature–time programme for plastic reaction vials

Step	Parameter		Temperature °C	Time	Fluorescence measurement	Cycles
1	Initial denaturation		95	10 min	no	1
2	Amplification	Denaturation	95	15 s	no	45
		Annealing and elongation	60	60 s	yes	

Table B.28 — Temperature–time programme for glass capillaries

Step	Parameter		Temperature °C	Time	Fluorescence measurement	Cycles
1	Initial denaturation		95	15 min	no	1
2	Amplification	Denaturation	95	10 s	no	45
		Annealing and elongation	60	15 s	yes	

B.8.15.4.5 Accept or reject criteria

The evaluation is performed applying the relevant device-specific data analysis program. The indication of the amplification result differs partly, depending on the real-time PCR device used. If no PCR products are detectable (negative result), the indication in the result report is, for example, “undetermined”, “no amp” or the maximum set number of cycles. If an amplification of the DNA target sequence took place in a sample (positive result), the number of cycles is calculated at which a prescribed fluorescence threshold value has been exceeded (C_t value or C_p value).

If, due to atypical fluorescence measurement data, the automatic evaluation does not provide a reasonable result, it may be necessary to manually set the baseline and the threshold value prior to evaluating the data. In doing so, follow the device-specific advice given in the technical manual for the application of the evaluation software.

B.8.15.4.6 Identification

The target sequence is considered as detected when:

- by using the *bar* gene-specific primers RapB-F1 and RapB-R1 and the probe RapB-S1, an increase in the measured fluorescence can be determined which is due to amplification;
- in the PCR control set-ups with no added DNA (PCR reagent control, negative extraction control), no increase in the fluorescence can be determined which is due to amplification;
- in the set-ups for the amplification control (positive DNA target control, PCR inhibition control) the expected C_t values are achieved.

B.8.16 Sample identification

All samples should be identified unambiguously.

B.8.17 Calculations

The results of the collaborative study support the conclusion that this method is suitable for screening for components of GMOs and the quantitative determination of the *bar* target sequence. Results obtained by quantifying the copy number of the *bar* gene, however, can only be used for determining the content of genetically modified material if information regarding to the number of *bar* gene integrations and the degree of zygosity of the plant species detectable in the sample are available.

B.9 Detection of certain DNA sequences frequently used in genetically modified organisms and originating from the cauliflower mosaic virus (*CaMV 35S* promoter, *P35S*) as well as from *Agrobacterium tumefaciens* (*T-nos*) in foodstuffs — Screening method

B.9.1 Principle

This clause describes a procedure for the simultaneous detection of DNA sequences from the *35S* promoter of the cauliflower mosaic virus (*P35S*) and from the terminator of the nopaline synthase gene (*T-nos*) of *Agrobacterium tumefaciens*. Since both *P35S* and *T-nos* are used as regulatory elements in many genetically modified plants, this method is suitable for screening for DNA of genetically modified organisms (GMOs) in foodstuffs. Basically, it is applicable to the analysis not only of all foodstuffs, but also of other products (e.g. feeding stuffs, seeds). The application of the method requires that an amount of amplifiable DNA can be extracted from the respective matrix which is sufficient for analysis.

Since both the *P35S* and the *T-nos* DNA sequences can also be detected in samples which contain DNA from the cauliflower mosaic virus or from *Agrobacterium tumefaciens*, but no genetically modified DNA sequences, a follow-up analysis should be carried out to confirm any positive results.

The screening method described is based on the amplification and detection of two DNA target sequences (here, *P35S* and *T-nos*) in one reaction (so-called duplex real-time PCR).

B.9.2 Validation status and performance criteria

B.9.2.1 Robustness of the method

The robustness of the method has not been tested to small modifications.

NOTE In the collaborative trial, the robustness of the method was checked with regard to different real-time PCR devices [ABI 7500,[1] ABI 7900,[1] Stratagene MX3005[1]]. The real-time PCR devices used had no influence on the performance of the method.

B.9.2.2 Intralaboratory trial

Detailed information about results of in-house validation is given in Reference [62].

B.9.2.3 Collaborative trial

B.9.2.3.1 General

The method has been validated in a collaborative study by the working group “Development of methods for identifying foodstuffs produced by means of genetic engineering techniques” of the German Federal Office of Consumer Protection and Food Safety (BVL). In all, 10 laboratories participated in the collaborative study (Reference [62]). Some 10 different DNA solutions or DNA mixtures, respectively, were used (see Tables B.30 and B.31). The DNA used was extracted from reference materials (ERM BF series) (IRMM, Geel, Belgium) with the certified mass fractions of 4,29 % GA21 (414F) or 0,1 % GA21 (414B), 5 % MON810 (413F) or 0,1 % MON810 (413B) as well as 5 % Bt11 (412F), 1 % Bt11 (412D) and 0,1 % Bt11 (412B); furthermore, DNA extracted from a maize flour sample previously tested with a negative result was used. The mass fractions 0,02 %, 0,05 % or 2,5 %, respectively, stated in Tables B.30 and B.31, column 1, were obtained by mixing these DNA solutions and should, therefore, be assumed to be approximate values regarding the indication “% mass fraction”.

These DNA solutions were sent to the participating laboratories in a coded form. In addition, for the purposes of quantifying the *P35S* and the *T-nos* copy numbers, each laboratory received a dilution series of the standard DNA which had been prepared with DNA from Bt11-certified reference material (ERM BF-412F, 5 % mass fraction Bt11) in accordance with Table B.29. Furthermore, each laboratory was provided with the oligonucleotides (Table B.32) as well as the buffer solution for the real-time PCR [TaqMan®[1] universal PCR master mix, Applied Biosystems, Darmstadt].

Each DNA sample had to be analysed with five repetitions so that each participant had to report 50 results.

Eight laboratories used the real-time PCR device ABI 7500,1) one laboratory used the real-time PCR device ABI 7900,1) and one the real-time PCR device Stratagene MX3005.1)

B.9.2.3.2 Standard DNA for calibration

A DNA solution containing both the *P35S* and the *T-nos* sequences, e.g. extracted from Bt11-certified reference material as well as a dilution series prepared from this solution and comprising five dilution levels, was used as standard DNA for calibration (see Table B.29).

Within the framework of the collaborative study the standard DNA was prepared as follows: DNA extraction from Bt11-certified reference material (ERM BF-412F, 5 % mass fraction Bt11) by means of a DNeasy Plant Mini Kit (Qiagen, Hilden). The copy number of plant species DNA per 5 µl, C_{DNA}, is given by

$$c_{\mathrm{DNA}} = \frac{\rho_{\mathrm{DNA}} \times 5 \times 1\,000}{m_{\mathrm{HG}}}$$

where

ρ_{DNA} is the mass concentration of DNA, in nanogram per microlitre;

m_{HG} is the mass of the haploid genome, in picogram.

The DNA mass concentration was determined spectrometrically. For maize, a haploid genome mass of 2,73 pg was taken as a basis (Reference [63]) and a double integration of the P35S and the *T-nos* target sequences into the maize genome as well as heterozygote material regarding the GMO assumed (Reference [62]).

From a 5 % mass fraction Bt11 DNA solution (stock solution), the dilutions (with 0,2× TE) listed in Table B.29 were prepared.

Table B.29 — Preparation of a dilution series

Level	Preparation	Copy number maize DNA per 5 µl	Copy number P35S per 5 µl	Copy number *T-nos* per 5 µl
S 1	Respective dilution of DNA extract	50 000	2 500	2 500
S 2	1 Vol. S1 + 4 Vol. 0,2× TE	10 000	500	500
S 3	1 Vol. S2 + 1 Vol. 0,2× TE	5 000	250	250
S 4	1 Vol. S3 + 4 Vol. 0,2× TE	1 000	50	50
S 5	1 Vol. S4 + 4 Vol. 0,2× TE	200	10	10

B.9.2.3.3 Slope of the calibration function, efficiency

In some laboratories, the dilution series resulted in a slight inhibition, particularly with the *T-nos* detection system, with the result that the level S1 standard could not be used for calculating the regression curve. By trend, the P35S detection system exhibited a slightly better efficiency.

The lowest efficiencies of 66 % for the *T-nos* system and 83 % for the P35S system, respectively, were obtained in the same laboratory. However, the data of all laboratories were taken into consideration in the evaluation, outliers were not eliminated.

B.9.2.3.4 Sensitivity and precision

Tables B.30 and B.31 summarize the proportions of positive results as well as the precision data for the individual samples. According to this, a proportion of 0,02 % mass fraction Bt11 was clearly detectable with both systems (50/50 or 49/50 reactions, respectively). This applies likewise to mass fractions of 0,05 %

GA21 or 0.05 % MON810, including the sample mixtures with high proportions of the competitive target sequence and the competition by one of the two PCR systems (*T-nos* or P35S, respectively) which may occur.

As far as the *T-nos* system was concerned, however, the precision data ($C_{V,R}$), except for level 1 % mass fraction Bt11, were not sufficient to meet the requirements of ISO 24276 regarding quantification (a $C_{V,R}$ of 25 % to 61 %). According to ISO 24276, $C_{V,R}$ should be 25 % maximum with quantitative procedures and 33 % max. between the LOD and the limit of quantification.

On the other hand, for the P35S system the limit of quantification criterion was approximately met with all samples of mass fraction 1,0 % or above as well as with sample 0,1 % mass fraction Bt11 (a $C_{V,R}$ of 13 % to 38 %).

B.9.2.3.5 Specificity

Particularly when applying the *P35S* system, positive signals in very low quantities were observed in the samples previously established to be “P35S negative” (in one case C_t was 36, otherwise C_t was ≥38), corresponding to less than 10 copies in all cases. One possible reason for this consists in minimal contaminations of the “0 % mass fraction” maize flour by materials containing *P35S* and *T-nos*.

Prior to preparing the negative controls, it became apparent that all tested “0 % mass fraction” reference materials, e.g. “0 % mass fraction MON810” or “0 % mass fraction GA21” contained traces of the P35S or *T-nos* sequences. The materials were negative only with regard to the certified GMO, but not with regard to contaminations by other GMOs. Therefore, the laboratories used their own maize flour mixtures which had been found to be negative in previous analyses. Nevertheless, with these mixtures it was also observed that very weakly positive results could not be ruled out completely in some individual PCR set-ups. Follow-up analyses by means of “singleplex” PCR confirmed that the “0 % mass fraction GM maize”, “0,05 % mass fraction GA21” and “2,5 % mass fraction GA21” samples were slightly contaminated by *P35S*-containing components, the “0 % mass fraction GM maize” sample contained *T-nos* components as well.

These results indicate that the observed sporadic weakly positive results in the controls are false-positive results due not to the procedure, but to the material.

B.9.2.3.6 Recovery and trueness

The laboratories received DNA extracts which had previously been set to approximately 50 000 copies of the maize reference gene per set-up (Table B.29). The percentage proportions of the P35S or the *T-nos* sequence, respectively, relative to the maize reference gene, are given in the “mean” and “standard deviation” columns of Tables B.30 and B.31. Regarding the 0,1 % mass fraction and 1,0 % mass fraction Bt11, proportions of 0,11 % or 0,94 % respectively, were measured applying the P35S system; when using the *T-nos* system, the proportions obtained were 0,07 % or 0,81 %, respectively.

A precise quantification of MON810 or GA21 was not possible within the framework of the collaborative study applying the chosen test scheme, as DNA from Bt11 was used as the quantification standard. Factors such as different integration frequency of the P35S or the *T-nos* sequences as compared with the Bt11 sequence or different zygosity or ploidy in the reference material used to prepare the DNA standards may yield diverging results. The values for these samples, therefore, appear in brackets in Tables B.30. and B.31.

B.9.2.3.7 Summarizing evaluation

The data support the conclusion that this method can be used for a semiquantitative screening for components of GMOs.

B.9.2.4 Molecular selectivity

B.9.2.4.1 General

B.9.2.4.1.1 *35S* promoter of the cauliflower mosaic virus (*P35S*)

The method has been designed to target a sequence described in the GenBank® database (Reference [83], accession number FN550389).

Table B.30 — Results of the collaborative study regarding the P35S detection system

P35S detection system		**Average efficiency**: 93 % (83 % to 99 %)				
Reference material GM maize (setpoint value) % mass fraction	**Proportion of positive results/overall results** [a]	C_t values		Copy numbers P35S		
		Mean[b]	Standard deviation[c]	Mean	Standard deviation	$C_{V,R}$[d] %
0,02 % Bt11 (P35S pos)	50/50	36,4	1,0	13	4,8	38
0,1 % Bt11 (P35S pos)	50/50	34,1	0,5	56	15	27
1,0 % Bt11 (P35S pos)	50/50	30,8	0,6	470	72	15
2,5 % MON810 (P35S pos)	50/50	29,4	0,6	(1,170)	(116)	10
0,05 % MON810 (P35S pos)	50/50	34,4	0,8	(29)	9,4	32
2,5 % GA21 (P35S neg)	7/50[e]	— (C_t min = 36)	—	—	—	—
0,05 % GA21 (P35S neg)	10/50[e]	— (C_t min = 38)	—	—	—	—
0,05 % MON810 + 2,5 % GA21 (P35S pos)	50/50	34,5	5,0	(43)	(18)	42
2,5 % MON810 + 0,05 % GA21 (P35S pos)	50/50	29,4	0,6	(1,192)	(152)	13
0 % GM maize	10/50[e]	— (C_t min = 38)	—	—	—	—
Footnotes a to e and the Note to Table B.31 apply here also.						

Table B.31 — Results of the collaborative study regarding the *T-nos* detection

T-nos **detection**		**Average efficiency**: 102 % (66 % to120 %)				
		C_t values		Copy numbers T-nos		
Reference material GM maize (setpoint value) % mass fraction	**Proportion of positive results/ overall results**[a]	Mean[b]	Standard deviation[c]	Mean	Standard deviation	$C_{V,R}$[d] (%)
0,02 % Bt11 (*T-nos* pos)	49/50	37,6	1,4	5,7	3,5	61
0,1 % Bt11 (*T-nos* pos)	50/50	34,7	0,9	37	13	35
1,0 % Bt11 (*T-nos* pos)	50/50	31,2	0,7	404	101	25
2,5 % MON810 (*T-nos* neg)	0/50	—	—	—	—	—
0,05 % MON810 (*T-nos* neg)	0/50	—	—	—	—	—
2,5 % GA21 (*T-nos* pos)	50/50	27,9	4,2	(4,500)	(1,722)	38
0,05 % GA21 (*T-nos* pos)	50/50	33,6	0,9	(81)	(23)	28
0,05 % MON810 + 2,5 % GA21 (*T-nos* pos)	50/50	27,8	4,1	(4,650)	(1,850)	40
2,5 % MON810 + 0,05 % GA21 (*T-nos* pos)	50/50	33,9	1,0	(69)	(27)	40
0 % GM maize	5/50[e]	— (C_t min = 39)	—	—	—	—

[a] Results with C_t values > 40 were regarded as negative results.

[b] Average value.

[c] Standard deviation.

[d] coefficient of variation under reproducibility conditions.

[e] weak amplifications were observed in some laboratories in individual PCR set-ups (e.g. 1 or 2 out of 5 reactions).

Values in brackets: Data are given for information only; evaluation of trueness not possible since quantification was performed on the basis of the Bt11 standard series.

A list of genetically modified plants containing the *CaMV 35S* promoter is provided in References [64][68]. All DNAs used in these experimental specificity tests were controlled for the ability to be amplified and for inhibitors with taxon specific methods before use (data not shown).

A false-positive result can occur since the amplified sequence is derived from cauliflower mosaic virus infecting cauliflower and other members of the Brassicaceae (Cruciferae) family, as well as Resedaceae and Solanaceae (References [65][66]). Positive results with samples derived from 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 (Reference [67]).

See ISO 21570:2005,[43] Annex B.1.

B.9.2.4.1.2 Terminator of the nopaline synthase gene (*T-nos*) of *Agrobacterium tumefaciens*

The method has been designed to target the *Agrobacterium tumefaciens* nopaline synthase terminator sequence described in the GenBank® database (Reference [83], accession number FN550390).

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

B.9.2.4.2 Theoretical

The theoretical specificities of the primers and the probes were assessed by a BLASTN search in the GenBank®/EMBL/DDBJ databases (Reference [83], search date: 2009-11-15). The result of the BLAST confirmed an 100 % identity only with the expected target sequences.

B.9.2.4.3 Experimental

B.9.2.4.3.1 *35S* promoter of the cauliflower mosaic virus (*P35S*)

See ISO 21570:2005,[43] Annex B.1.

Updated information about genetically modified plants detectable or not detectable by the P35S-method is given in Reference [68], see also Reference [53].

B.9.2.4.3.2 Terminator of the nopaline synthase gene (*T-nos*) of *Agrobacterium tumefaciens*

Testing for the specificity of the *T-nos* real-time PCR in a singleplex-reaction no amplification has been observed using DNA from non-GM crop-plants and derived processed food matrices in performance tests. No amplification was observed with the DNA isolated from the events Bt176 (SYN-EV176-9); MON810 (MON-00810-6); TC1507 (DAS-01507-1); and T25 (ACS-ZM003-2).

For the following genetically modified plants, it was experimentally shown that the *T-nos* detection method is suitable as a screening method:

- GM rice: Bt63;
- GM rapeseed: OXY 235 (ACS-BN011-5), MS1 (ACS-BN004-7), MS1×RF1 (ACS-BN004-7×ACS-BN001-4), MS8 (ACS-BN005-8);
- GM maize: GA21 (MON-00021-9), Bt11 (SYN-BT 011-1), MON863 (MON-00863-5), NK603 (MON-00603-6), CBH-351 (ACS-ZM004-3):
- GM soya: MON40-3-2 (MON-04032-6);
- GM papaya: SunUp 55-1;
- GM tomato: Zeneca.

Updated information about genetically modified plants detectable or not detectable by the *T-nos*-method is given in Reference [68], see also Reference [53].

B.9.3 Principle and summary

Detection of the *P35S* and the *T-nos* sequences takes place in a duplex real-time PCR. The primers used amplify a fragment with a length of 82 bp from the P35S sequence as well as a fragment with a length of 84 bp from the *T-nos* sequence. The PCR products are detected during the real-time PCR by means of specific oligonucleotide probes. Each probe is labelled by two fluorescent dyes (FAM or Yakima yellow as reporter dye and BHQ1 as non-fluorescent quencher) and binds in between the two primers in the DNA sequence range (so-called TaqMan®1) chemistry, Reference [54]).

When applying the P35S and/or the *T-nos* screening method, a follow-up analysis should be performed for further confirmation of positive results.

B.9.4 Terms and definitions

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

B.9.5 Sample type and amounts

Ensure that the test sample is representative of the laboratory sample, e.g. by grinding or homogenization. Measures and operational steps to be taken into consideration are described in ISO 21571.

B.9.6 Limit of detection and range of use

The method was tested with samples containing low copy numbers of the 35S-promotor-sequence and the *T-nos* terminator-sequence (Tables B.30 and B.31).

In all samples containing the P35S-target sequence at a level of 10 copies, the sequence was detectable, thus representing the assessed LOD of the method for the P35S-sequence. Similar results were obtained in the range of 25 to 1 500 copies. For this evaluation the data from the results obtained with the standard DNA dilution series used for calibration are taken.

In 49 out of 50 samples containing the *T-nos* sequence at a level of 10 copies, the sequence was detectable, thus representing the assessed LOD of the method for the *T-nos* sequence. The sequence was detectable in all samples containing the *T-nos* sequence in the range of 25 to 1 500 copies. For this evaluation, the data from the results obtained with the standard DNA dilution series used for calibration are taken.

B.9.7 Estimation of measurement uncertainty

The measurement uncertainty was assessed in a collaborative study. The results are given in B.9.2.3.4.

B.9.8 Interferences

The amount, quality, and ability to amplify the nucleic acid template influences the analytical result obtained, see ISO 21571. Therefore the nucleic acid used for the analysis requires checking, e.g. by means of a target taxon-specific PCR method.

B.9.9 Physical and environmental conditions

See ISO 24276 for details.

B.9.10 Apparatus and equipment

Regarding the apparatus and materials, reference is made to Clauses 5 and 6 and to ISO 21570.[43] Usual molecular biological laboratory equipment and in particular the following.

B.9.10.1 Apparatus and equipment for DNA extraction

B.9.10.1.1 Centrifuge able to centrifuge 1,5 ml and 2 ml reaction vials at 14 500 × *g*.

B.9.10.2 Apparatus and equipment for real-time PCR

B.9.10.2.1 Thermal cycler equipped with:

- an **energy source** suitable for the excitation of fluorescent molecules;
- an **optical detection system** suitable for the detection of the fluorescence signals generated during PCR.

B.9.10.2.2 Reaction vessels and **caps** or **closures** which can be repeatedly heated to 100 °C and cooled to 4 °C without damage and which do not influence the fluorescence signal generated during the amplification process.

B.9.11 Reagents and materials

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

B.9.12 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.

B.9.13 Test sample preparation

See ISO 21571.

B.9.14 Instrument calibration

Instruments (e.g. thermocyclers) should be calibrated as per ISO/IEC 17025.[41]

B.9.15 Analysis steps

B.9.15.1 General

Prior to carrying out the method, DNA is extracted from the test sample applying a suitable procedure (ISO 21571). The analysis consists of:

a) checking the amount, quality, and the ability to amplify the extracted DNA e.g. by means of a PCR specific of the target taxon (ISO 21570[43]);

b) detection of the P35S and the *T-nos* sequences in a duplex real-time PCR.

When applying the P35S and/or the *T-nos* screening method, a follow-up analysis should be performed for further confirmation of positive results.

B.9.15.2 Preparation of the DNA extracts

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

B.9.15.3 PCR reagents

B.9.15.3.1 General. Ready-to-use reagent mixtures or individual components can be used.

B.9.15.3.2 Thermostable DNA polymerase (for hot-start PCR)

B.9.15.3.3 PCR buffer solution (contains $MgCl_2$ and deoxyribonucleoside triphosphates dATP, dCTP, dGTP and dUTP).

B.9.15.3.4 Oligonucleotides. See Table B.32.

Table B.32 — Oligonucleotides

Name	DNA sequence of the oligonucleotide
P35S as the target sequence (ISO 21570[43])	
35S-F	5'-gCC TCT gCC gAC AgT ggT–3'
35S-R	5'–AAg ACg Tgg TTg gAA CgT CTT C–3'
35S-TMP FAM	5'–(FAM)–CAA AgA Tgg ACC CCC ACC CAC g–(BHQ1)–3' [a]
T-nos as the target sequence (References [72][73])	
180-F	5'–CAT gTA ATg CAT gAC gTT ATT TAT g–3'
180-R	5'–TTg TTT TCT ATC gCg TAT TAA ATg T–3'
TM-180	5'–(YY)–ATg ggT TTT TAT gAT TAg AgT CCC gCA A–(BHQ1) –3' [a]

[a] FAM: 6-carboxyfluorescein; YY: Yakima yellow; BHQ1: black hole quencher 1. Equivalent reporter and/or quencher dyes can be used for the probes if they can be shown to yield similar or better results.

B.9.15.4 Procedure

B.9.15.4.1 PCR set-up

The description of the procedure is applicable to an overall volume of 25 µl per PCR set-up, with the reagents stated in Table B.33.

Table B.33 — Reaction set-up for the duplex real-time PCR

Reagent	Final concentration	Volume per reaction µl
PCR buffer solution[a] (including MgCl2, dNTPs and DNA polymerase)	1×	12,5
Primer 35S-F, c = 2 µmol/l[b]	100 nmol/l	1,25
Primer 35S-R, c = 2 µmol/l[b]	100 nmol/l	1,25
Probe 35S-TMP FAM, c = 2 µmol/l[b]	100 nmol/l	1,25
Primer 180-F, c = 20 µmol/l[b]	1 000 nmol/l	1,25
Primer 180-R, c = 20 µmol/l[b]	1 000 nmol/l	1,25
Probe TM-180 YY, c = 4 µmol/l[b]	200 nmol/l	1,25
DNA extract (sample or DNA standard)	up to 200 ng[c]	5
Overall reaction volume	–/–	25

[a] In the collaborative study, TaqMan®1) universal PCR master mix (Applied Biosystems, Darmstadt, G) was used as the PCR buffer solution. Equivalent products of other manufacturers may be used if they can be shown to yield similar or better results. If the PCR buffer solution contains a system to avoid carry-over contaminations on the basis of the enzyme uracil-*N*-glycosylase (UNG), an additional UNG activation step is required for the temperature–time programme.

[b] Other working concentrations may be used.

[c] In the collaborative study DNA solutions were used which contained approximately 50 000 genome copies of the maize DNA per set-up.

B.9.15.4.2 PCR controls

Appropriate controls should be included as described in ISO 24276.

B.9.15.4.3 Preparation of standards

The preparation of standards is described in B.9.2.3.2.

B.9.15.4.4 Temperature–time programme

If applying the PCR described here, the temperature–time programme listed in Table B.34 has proven suitable for plastic reaction vials.

Table B.34 — Temperature–time programme

Step	Parameter		Temperature °C	Time	Fluorescence measurement	Cycles
1	UNG activation (optional)		50	2 min	no	1
2	Initial denaturation		95	10 min	no	1
3	Amplification	Denaturation	95	15 s	no	45
		Annealing and elongation	60	60 s	yes	

B.9.15.4.5 Accept or reject criteria

The evaluation is performed by applying the relevant device-specific data analysis program. The indication of the amplification result differs partly, depending on the real-time PCR device used. If no PCR products are detectable (negative result), the indication in the result report is, for example, “undetermined”, “no amp” or the maximum set number of cycles. If amplification of the DNA target sequence took place in a sample (positive result), the number of cycles is calculated at which a prescribed fluorescence threshold value has been exceeded (C_t value or C_p value).

If, due to atypical fluorescence measurement data, the automatic evaluation does not provide a reasonable result, it may be necessary to manually set the baseline and the threshold value prior to evaluating the data. In doing so, follow the device-specific advice given in the technical manual for the application of the evaluation software.

B.9.15.4.6 Identification

The target sequences are considered as detected when:

- by using the P35S-specific primers 35S-F and 35S-R and the probe 35S-TMP, an increase in the measured fluorescence can be determined which is due to amplification;
- by using the *T-nos*-specific primers 180-F and 180-R and the probe TM-180, an increase in the measured fluorescence can be determined which is due to amplification;
- in the PCR control set-ups with no added DNA (PCR reagent control, negative extraction control), no increase in the fluorescence can be determined which is due to amplification;
- in the set-ups for the amplification control (positive DNA target control, PCR inhibition control) the expected C_t values are achieved.

B.9.16 Sample identification

All samples should be identified unambiguously.

B.9.17 Calculations

The results of the collaborative study support the conclusion that this method is suitable for a semiquantitative screening for components of GMOs.

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Insert C.6 to C.8 after the existing text.

C.6 Construct-specific method for the qualitative detection of genetically modified DNA sequences in papaya ring spot resistant papaya [SunUp, Rainbow)]

C.6.1 Principle

This method describes construct-specific procedures for the qualitative detection of genetically modified DNA sequences in papaya ring spot virus (PRSV) resistant papaya (*Carica papaya*). Information on the genetic construct introduced into the papaya genome is available in Reference [70].

The application of this method requires a sufficient amount of amplifiable DNA to be extractable from the relevant matrix for the purpose of analysis. The method is described as PCR verified by restriction and furthermore as real-time PCR. The method is based on the detection of DNA sequences of the 35S promoter of the cauliflower mosaic virus (CaMV) and of the junction between the CMV/PRSV CP DNA sequences (chimeric cucumber mosaic virus coat protein/papaya ring spot virus coat protein) (Reference [71]).

C.6.2 Validation status and performance criteria

C.6.2.1 Robustness of the method

In the collaborative study, the robustness of the method has been checked. The primers have been checked in PCR and real-time PCR. Furthermore, the method has been tested with different PCR instruments, i.e. PCR block cycler and real-time PCR block cycler. By these tests, the robustness of the method has been shown (see the collaborative study data).

C.6.2.2 Intralaboratory trial

Before the collaborative study was performed, intralaboratory trials demonstrated that the CTAB extraction as well as kit extraction (Reference [71]) were successful and that the method is robust with respect to different PCR instruments and PCR applications (see C.6.2.1, C.6.2.4.3 and C.6.6).

C.6.2.3 Collaborative trial

The method has been validated in a collaborative trial by the working group “Development of methods for identifying foodstuffs produced by means of genetic engineering techniques” of the German Federal Office of Consumer Protection and Food Safety (BVL) in 2005-11. The collaborative trial involved 10 laboratories (Reference [72]).

In the collaborative trial, samples were tested comprising a range of 10 % to 100 % mass fraction of genetically modified papaya fruit pieces [SunUp) papaya event 55-1 (unique identifier: CUH-CP551-8) or 63-1 (unique identifier: CUH-CP631-7)]. The samples were sent to the participating laboratories in a coded form. In the collaborative study a DNA extraction by CTAB was performed with a test portion size of 2 g (Reference [72]). After DNA extraction, a species-specific PCR was performed in order to check the amplifiability of the extracted DNA. The specific detection of the genetically modified papaya DNA sequences was performed once by PCR followed by restriction enzyme analysis. Furthermore, the method was tested by real-time PCR. The results of the collaborative study are listed in Table C.18.

Table C.18 — Results of the collaborative study

Parameter	Conventional PCR	Real-time PCR
No. laboratories	10	10
No. laboratories submitting results	10	10
No. samples per laboratory	6	6
No. accepted results	60	60
No. samples containing genetically modified PRSV-resistant papaya	39	34
No. samples containing not genetically modified papaya	21	26
False-positive results	2	0
False-negative results	2	2

In total, two laboratories submitted incorrect evaluations. One of them had been provided with three identical samples with a mass fraction of 10 % of GM papaya, one of the three samples having yielded false-negative results in conventional and real-time PCR. The other laboratory had, among the coded samples, been provided with a sample consisting of 10 % mass fraction of GM papaya which also yielded false-negative results in conventional and real-time PCR. Of the three samples which were non-GM papaya, this laboratory obtained false-positive results for two samples in conventional PCR, whereas these samples were recognized correctly by real-time PCR (Reference [72]).

C.6.2.4 Molecular selectivity

C.6.2.4.1 General

The method has been designed to target DNA sequences specific for the genetically modified papaya line 55-1 [unique identifier: CUH-CP551-8, papaya SunUp] and 63-1 [unique identifier: CUH-CP631-7, papaya Rainbow^TM1)^]. The method is described in Reference [72]. Information on the genetic construct introduced into the papaya genome is available in Reference [70].

C.6.2.4.2 Theoretical

Sequences of the primer sunup-af1, sunup-ar1, 35S-F and the probe 35S-T are based on genetically modified papaya Rainbow1) gene sequence {GenBank® (Reference [83], accession No. FJ467933, search date 2008-12-22}. The sequences of the primers sunup-af1, 35S-F and the probe 35S-T are based on the 35 promoter sequence. The selectivity in the PCR detection system is based on the sequence of the primer sunup-ar1 which is specific for papaya transgenic cultivar Rainbow1) and yielded no further match with a GenBank® entry (Reference [83], BLAST date 2009-01-28). No sequence homology with DNA sequences of non-genetically modified papaya varieties and other crop plants has been found in databank searches.

C.6.2.4.3 Experimental

In the intralaboratory study, various GM plants (see Table C.19) and 100 % mass fraction GM papaya [SunUp papaya event 55-1 (unique identifier: CUH-CP551-8) or 63-1 (unique identifier: CUH-CP631-7)] were tested with usually 500 genome copies each with two replications. Only the GM papaya showed the specific PCR product with a length of 152 bp.

Table C.19 — GM plants tested

Bt11 maize (SYN-BT 011-1)	Bxn$^{\text{TM}}$1) cotton (10211, BXN-10211-9; 10215, BXN-10215-4; 10222, BXN-10222-2)
MON810 maize (MON-00810-6)	Bollgard II cotton (MON15985-7)
T25 maize (ACS-ZM003-2)	GTS40-3-2 soya bean (MON-04032-6)
GA21 maize (MON-00021-9)	FlavrSavr tomato (CGN-89564-2)
CBH351 maize (ACS-ZM004-3)	NewLeaf potato (Bt6, NMK-89812-3; Bt10, NMK-89175-5; Bt12, NMK-89601-8; Bt16, NMK-89167-6; Bt17, NMK-89593-9; Bt18, NMK-89906-7; Bt23, NMK-89675-1)
GT73 canola (MON-00073-7)	LL sugar beet (ACS-BV001-3)
T45 canola (ACS-BN008-2)	—
MS1/RF1 canola (ACS-BN004-7×ACS-BN001-4)	—

C.6.3 Principle and summary

A 152 bp DNA fragment, starting from DNA sequences of the 35S promoter of the cauliflower mosaic virus (CaMV) to DNA sequences of the junction between the viral coat proteins is amplified by PCR. This amplification is specific for the genetically modified papaya CUH-CP551-8 and CUH-CP631-7.

The method is described first as PCR followed by verification by restriction enzyme analysis. The method is also described as real-time PCR, i.e. the verification is performed by a specific oligonucleotide probe which is labelled by two fluorescent dyes (“TaqMan1) probe”).

C.6.4 Terms and definitions

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

C.6.5 Sample type and amounts

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

Ensure that the test sample is representative of the laboratory sample, e.g. by grinding or homogenization. Measures and operational steps to be taken into consideration are described in ISO 21571. In the collaborative study, samples for DNA extraction were taken from homogenized papaya fruit pieces.

C.6.6 Limit of detection and range of use

In the collaborative study, samples were tested comprising a range of 10 % to 100 % mass fraction of GM papaya. It was possible to detect a mass fraction of 10 % of genetically modified papaya in papaya (see Table C.20).

To determine the practical sensitivity in the sample to be analysed, a genome size of 372 Mbp for papaya is assumed.

The determination of the copy number which, with a probability of 95 %, will lead to a positive result in the PCR was performed in the intralaboratory study. A PCR carried out 10 times (internationally agreed procedure, e.g. EURACHEM Guide[84]) with the primer pair sunup-af1 and sunup-ar1 yielded a positive result in all reactions when using five genome copies.

Table C.20 — No. samples yielding false-negative or false-positive results

No. samples	Content of GM papaya % mass fraction	Conventional PCR		Real-time PCR	
		False-positive results	False-negative results	False-positive results	False-negative results
20	0	2	0	0	0
20	10	0	2	0	2
20	100	0	0	0	0

C.6.7 Estimation of measurement uncertainty

The measurement uncertainty should be assessed in intralaboratory studies.

C.6.8 Interferences

The amount, quality, and ability to amplify the nucleic acid template influences the analytical result obtained (see ISO 21571). Therefore, the nucleic acid used for the analysis should be checked, e.g. by means of a target taxon-specific PCR method.

C.6.9 Physical and environmental conditions

See ISO 24276 for details.

C.6.10 Apparatus and equipment

Usual molecular biological laboratory equipment and in particular the following.

C.6.10.1 Apparatus and equipment for PCR followed by restriction enzyme analysis

C.6.10.1.1 Thermal cycler.

C.6.10.1.2 Thermostat or **water bath**.

C.6.10.2 Apparatus and equipment for real-time PCR

C.6.10.2.1 Thermal cycler equipped with an energy source suitable for the excitation of fluorescent molecules and an **optical detection system** suitable for the detection of the fluorescence signals generated during PCR

C.6.10.2.2 Reaction tubes and **caps** or **closures** which can be repeatedly heated to 100 °C and cooled to 4 °C without damage and which do not influence the fluorescence signal generated during the amplification process.

C.6.11 Reagents and materials

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

C.6.12 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.

C.6.13 Test sample preparation

See ISO 21571.

C.6.14 Instrument calibration

See ISO/IEC 17025.[41]

C.6.15 Analysis steps

C.6.15.1 General

DNA is extracted from the test sample applying a suitable procedure. DNA analysis consists of the following parts.

a) Verification of the amount, quality and amplifiability of the extracted DNA, e.g. by means of a PCR specific for the target taxon.

NOTE DNA sequences for conventional and real-time PCR are provided in Table C.21. Details are provided in References [72][73].

b) Detection of the genetic modification present in PRSV-resistant papaya. Here, a target sequence specific for this genetic modification is amplified by PCR. This can be done by conventional PCR or real-time PCR. The PCR products of a conventional PCR are separated by agarose gel electrophoresis and analysed, by means of suitable DNA size markers, for the PCR product sizes to be expected.

c) The amplified DNA sequence is verified by restriction enzyme digestion if a conventional PCR has been performed. In the case of the real-time PCR, the verification step is included in b) by using a fluorescent-labelled probe.

C.6.15.2 Preparation of the DNA extracts

For details concerning DNA extraction, see ISO 21571. In the collaborative study, the CTAB extraction has been successfully used for the papaya fruit pieces. Test portion size was 2 g in the collaborative study.

C.6.15.3 DNA quantification

For details concerning quantification of the DNA extracts, see ISO 21571.

C.6.15.4 DNA integrity evaluation

Quality and amplifiability of the extracted DNA are tested, e.g. by a PCR specific for the target taxon.

C.6.16 PCR reagents

C.6.16.1 General

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

Ready-to-use PCR buffer mixtures or individual components can be used.

C.6.16.2 PCR

The reaction solutions required for the PCR are generally stored in aliquots at −20 °C.

C.6.16.2.1 Thermostable DNA polymerase (for hot-start PCR), 5 IU/µl.

C.6.16.2.2 PCR buffer stock.

C.6.16.2.3 Magnesium chloride solution, c = 25 mmol/l, optional (if PCR buffer stock is without magnesium chloride).

C.6.16.2.4 Deoxyribonucleoside triphosphate (dNTP) solution, containing dATP, dCTP, dGTP, and dTTP, at c = 2,5 mmol/l each.

C.6.16.2.5 Primers and **probes**. See Table C.21

Table C.21 — Oligonucleotides

Name	DNA sequence of the oligonucleotides
DNA sequences of the genetically modified papaya as target sequence for conventional PCR	
sunup-af1	5'- TTC ATT Tgg AgA ggA CAg ggT AC –3'
sunup-ar1	5'- TCA TTC TTg gAC TgA CgA CgT –3'
DNA sequences of the genetically modified papaya as target sequence for real-time PCR	
35S-F	5'– gAC gTA Agg gAT gAC gCA CAA –3'
sunup-ar1	5'- TCA TTC TTg gAC TgA CgA CgT –3'
35S-T[a]	5'– FAM – CCC ACT ATC CTT CgC AAg ACC CTT CC – TAMRA –3'

[a] FAM: 6-carboxyfluorescein, TAMRA: 6-carboxytetramethylrhodamine. Equivalent reporter and/or quencher dyes can be used for the probe if they can be shown to yield similar or better results.

C.6.16.3 Restriction enzymes *Kpn* I, *NIa* III, *Tsp* 509 I

C.6.17 Procedure

C.6.17.1 PCR master mix set-up

For the volume of 25 µl per PCR, the reagents are given in Tables C.22 and C.23. The PCR can also be performed with a smaller or larger volume if the solutions of the PCR mix are adapted accordingly. A volume of 5 µl of DNA extract is used.

In the collaborative study, the final concentrations of the reagents given in Tables C.22 and C.23 proved to be suitable.

After carefully thawing the reagents, they should be centrifuged immediately prior to use. While preparing the PCR batch, the reagents are kept in an ice bath, if required.

Each reagent should be thoroughly mixed prior to pipetting. A PCR mix is prepared containing all PCR components with the exception of the DNA extract. The amount of PCR mix required depends on the number of reactions to be carried out including at least one additional reaction.

Mix the PCR mix, centrifuge briefly, and pipette into the PCR reaction vessels 20 µl per vessel.

Pipette either 5 µl of DNA extract, 5 µl of the negative extraction control, 5 µl for the PCR reagent control, 5 µl of water into the control reaction (PCR without DNA template) or 5 µl of the positive extraction control into the corresponding PCR tubes.

Centrifuge the reaction batches briefly and place into the real-time device. Start the temperature–time programme in accordance with the manufacturer's instructions.

Table C.22 — Addition of reagents (conventional PCR)

Reagent	Final concentration	Volume per reaction µl
Sample DNA	10 ng to 50 ng	5
Water	—	12,3
10× PCR buffer (without $MgCl_2$)[a]	1×	2,5
$MgCl_2$ solution, 25 mmol/l	1,5 mmol/l	1,5
dNTP solution, 10 mmol/l	0,2 mmol/l	0,5
Primer sunup-af1, 10 µmol/l	0,6 µmol/l	1,5
Primer sunup-ar1, 10 µmol/l	0,6 µmol/l	1,5
Thermostable DNA polymerase (for hot-start PCR), 5 IU/µl	1 IU	0,2

[a] If the PCR buffer solution already contains magnesium chloride, the final concentration of magnesium chloride in the reaction mixture is adjusted to 1,5 mmol/l.

Table C.23 — Concentration of reagents (block cycler real-time PCR)

Reagent	Final concentration
Sample DNA	10 ng to 50 ng
Water	—
2x PCR buffer, e.g. QuantiTect Probe$^{TM1)}$ PCR kit (Qiagen)	1×
Primer 35S-F, 10 µmol/l	0,4 µmol/l
Primer sunup-ar1, 10 µmol/l	0,4 µmol/l
Probe 35S-T	0,4 µmol/l

C.6.17.2 PCR controls

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

C.6.17.3 Preparation of standards

Commercially available DNA size standards can be used for estimation of the PCR product sizes.

C.6.17.4 Temperature–time programmes

Temperature–time programmes are given in Tables C.24 and C.25. The denaturation time given in Tables C.24 and C.25 take into account the use of AmpliTaq Gold®1) DNA polymerase. When using a different polymerase, the activation and initial denaturation should be adapted accordingly.

Table C.24 — Temperature–time programme for conventional PCR

Parameter	Duration and temperature
Activation and initial denaturation	10 min at 95 °C
Amplification	25 s at 94 °C
	30 s at 62 °C
	40 s at 72 °C
No. cycles	40
Final elongation	7 min at 72 °C

Table C.25 — Temperature–time programme for real-time PCR with block cycler

Parameter	Duration and temperature
Activation and initial denaturation	15 min at 95 °C
Amplification	15 s at 94 °C
	60 s at 60 °C
No. cycles	45
Final elongation	30 s at 40 °C

C.6.17.5 Accept or reject criteria

C.6.17.5.1 For conventional PCR

The products of conventional PCR are detected by gel electrophoresis (e.g. according to ISO 21571:2005, B.2). The PCR products are separated by molecular weight in an agarose gel electrophoresis. Use 10 µl of the PCR products for the analysis by agarose gel electrophoresis and document accordingly. The PCR product produced by this conventional PCR has a length of 152 bp. The conventional PCR fulfils the acceptance criteria if

— the controls gave the expected results;

— the PCR products have the specific length of 152 bp.

The confirmation of the PCR products is performed according to C.6.17.6.1.

C.6.17.5.2 For real-time PCR

The evaluation is performed applying the relevant device-specific data analysis program. The format of the amplification result may vary depending on the real-time PCR device used. Examples of no detectable PCR products (negative result) are: “undetermined”, “no amp” or the maximum set number of cycles. If an amplification of the DNA target sequence took place in a sample (positive result), the number of cycles is calculated at which a prescribed fluorescence threshold value has been exceeded (C_t value or C_p value).

If, due to atypical fluorescence measurement data, the automatic evaluation does not provide a reasonable result, it may be necessary to manually set the baseline and the threshold value prior to evaluating the data. In doing so, follow the device-specific advice given in the technical manual for the application of the evaluation software.

C.6.17.6 Identification

C.6.17.6.1 Identification of the PCR products of conventional PCR by restriction enzyme analysis

A PCR product of the specific target DNA sequence is confirmed by performing a restriction enzyme analysis on the PCR products. At least one of the restriction enzymes given in Table C.26 is selected and used in accordance with Tables C.26 and C.27.

Table C.26 — Restriction enzymes, incubation conditions and number and size of restriction fragments

Restriction enzyme	Incubation conditions	Number/size of restriction fragments
Kpn I	37 °C, 4 h	24 bp and 128 bp
Nla III	37 °C, 4 h/65 °C, 20 min	94 bp and 58 bp
Tsp 509 I	65 °C, 4 h	77 bp and 75 bp

Table C.27 — Addition of reagents for restriction analysis

Reagent	Volume
PCR product	5 µl
Restriction enzyme (at least 10 IU)	*x* µl
Reaction buffer (10×)	2 µl
Water	to 20 µl

For the analysis of the restriction fragments, a gel electrophoresis can be performed in accordance with ISO 21571:2005, B.2.

The target sequence is considered as detected in conventional PCR if

— the results of the taxon-specific PCR give a positive result;

NOTE The result of the taxon-specific PCR is needed to exclude false-negative results.

— a PCR product with a size of 152 bp is obtained with the primer pair sunup-af1/sunup-ar1;

— the 152 bp fragment is restricted by restriction enzyme *Kpn* I into two fragments of 24 bp and 128 bp, respectively, or by restriction enzyme *Nla* III into two fragments of 94 bp and 58 bp, respectively, or by restriction enzyme *Tsp* 509 I into two fragments of 77 bp and 75 bp, respectively;

NOTE Due to the separation properties of the agarose used, the 24 bp DNA fragment is sometimes not observable in the gel electrophoresis in all cases after digestion with *Kpn* I. The separation of the two DNA fragments of 77 bp and 75 bp, respectively, cannot necessarily be observed after restrictions with the enzyme *Tsp* 509 I.

— the PCR control set-ups with no added DNA (PCR reagent control, negative extraction control), are negative;

— in the set-ups for the amplification control (positive DNA target control, PCR inhibition control), a PCR product of 152 bp can be detected, which, after restriction analysis with the specified enzymes, shows the corresponding profile.

C.6.17.6.2 Identification of the PCR products of real-time PCR

When using the real-time PCR the target sequence is to be considered as detected if

— the results of the taxon-specific PCR give a positive result;

NOTE The result of the taxon-specific PCR is needed to exclude false-negative results.

— by using the primers 35S-F and sunup-ar1, and the probe 35S-T, an increase in the measured fluorescence can be determined which is due to amplification;

— in the PCR control set-ups with no added DNA (PCR reagent control, negative extraction control), no increase in the fluorescence can be determined which is due to amplification;

— in the set-ups for the amplification control (positive DNA target control, PCR inhibition control), the expected C_t values are achieved.

C.6.18 Sample identification

All samples should be identified unambiguously.

C.6.19 Calculations

The results of the collaborative study support the conclusion that this method is suitable for a semiquantitative screening for components of GMOs.

C.7 Construct-specific method for the detection of modified DNA sequences from genetically modified rice line TT51-1 (cultivar Bt63)

C.7.1 Principle

This is a method for the detection of DNA from genetically modified insect-resistant rice line TT51-1 (cultivar Bt63) (Reference [74]) in raw or processed materials (rice grains, rice noodles) by amplification of a 83 bp single copy sequence representing the junction region between the synthetic *CryIA(c)* and the *Nos* terminator (Reference [75]) by real-time PCR.

This method cannot be used to distinguish between different rice varieties containing the same construct. For further specification (e.g. detection of the event), a follow-up analysis should be carried out.

C.7.2 Validation status and performance criteria

C.7.2.1 Robustness

The robustness of the method was tested by applying the following modifications in the reaction set-up:

— reduction of the primer concentration from 300 nmol/l to 200 nmol/l;

— reduction of the probe concentration from 100 nmol/l to 75 nmol/l.

All reactions were done in three replicates using the same amount of template DNA.

The reduction of the primer concentration did not influence the cycle threshold (C_t) value.

The reduction of the probe concentration resulted in an increase of the C_t value by 0,8 × C_t on average. This increase can be considered of limited significance for a qualitative method.

C.7.2.2 Intralaboratory trial

The performance of the method was assessed by analysing four parallel DNA extractions of a mixture of milled Bt63 rice in conventional rice at levels of 5 %, 0,5 %, and 0,1 % mass fraction. The test revealed C_t-values of 30,0 ± 0,3 ($C_{V,r}$ = 0,9 %) for the 5 % fortification level; 34,1 ± 0,5 ($C_{V,r}$ = 1,5 %) for the 0,5 % fortification level; and 36,2 ± 0,5 ($C_{V,r}$ = 1,5 %) for the 0,1 % fortification level (Reference [75]).

C.7.2.3 Collaborative study

The method has been validated in a collaborative study under coordination of the German Federal Office of Consumer Protection and Food safety (BVL) by the working group “Development of methods for identifying foodstuffs produced by means of genetic engineering techniques” (Reference [77]). The number of participants as well the number of samples followed the criteria according to ISO 5725-2[39] and the IUPAC protocol (Reference [48]).

For the analysis, the participants received six rice flour samples, six rice noodle samples, and three DNA samples. The samples contained different concentrations of the *cry1A(c)-T-nos* sequence or no sequences of the construct (negative samples), respectively. All samples were marked with random coding numbers.

To prepare the rice samples, whole rice grains or rice noodles were used. Based on parts by mass, finely ground non-GM rice grains and “Bt63” rice grains (JRC, Ispra) were used to prepare a 0.1 % mass fraction Bt63 mixture; subsequently, a 0,05 % mass fraction Bt63 mixture was prepared by further homogenous mixing with the non-GM rice grain flour. The GM rice noodle samples used were two different Bt63 positive samples from official monitoring laboratories which had already been reported through the European Rapid Alert System. The negative samples used were non-GM rice noodles.

The rice noodles were ground to form homogenous flours. As far as the unknown samples were concerned, each participant received two vials containing sub-samples of 1 g each of the following rice flours:

a) 0,1 % mass fraction Bt63 rice;

b) 0,05 % mass fraction Bt63 rice;

c) non-GM rice;

d) Bt63 positive rice noodles (sample a);

e) Bt63 positive rice noodles (sample b);

f) Bt63 negative rice noodles.

For DNA extraction, the CTAB method as outlined in ISO 21571:2005, A.3 was employed using a test portion of 1 g.

In order to determine the LOD, DNA samples consisting of a solution of a previously linearized plasmid comprising a fragment with a length of 717 bp of the *cry1A(c)-T-nos* sequence were prepared. The concentrations of the sample DNAs were adjusted to 4 copies/µl and 1 copy/µl, based on the size of the plasmid and applying the PicoGreen[1)] method; the plasmid DNA solutions were stabilized with 10 ng/µl of maize genomic DNA. Each of the participants received a coded vial containing a plasmid DNA solution with 0 copies/µl or 1 copy/µl and 4 copies/µl, respectively, of the *cry1A(c)-T-nos* sequence. Furthermore, for performing positive controls, two vials each of which contained 1 g of Bt63 positive rice noodle flours and one vial containing Bt63 DNA were provided.

The data from the collaborative study are listed in Table C.28 and C.29.

Table C.28 — Results of the collaborative study — Evaluation of the results for the rice samples

Parameter (collaborative study of 2008)	Value
No. laboratories	17
No. laboratories submitting results	17
No. samples per laboratory	12
No. accepted results	191[a]
No. samples containing ´Bt63-rice´	129
No. samples containing non-GM rice	62
False-positive results	1 (1,6 %)
False-negative results	0 (0 %)

[a] One laboratory reported leakage of four samples during the overnight incubation and therefore these test samples could not be analysed; for a total of five test samples no rice DNA was detectable in three different laboratories. One laboratory reported for five test samples (two rice grain and three rice noodle samples) unusually low gos9 C_t-values ($C_t \leq 14$) and therefore results for these test samples were excluded in the evaluation.

Table C.29 — Results of the collaborative study — Evaluation of the results for the plasmid DNA samples

Parameter (collaborative study of 2008)	Value
No. laboratories	17
No. laboratories submitting results	17
No. Bt63 plasmid DNA samples per laboratory	3
No. determinations per plasmid DNA sample	2
No. positive results/overall number of determinations with 0 copies of Plasmid DNA	0/34
No. positive results/overall number of determinations with 5 copies of Plasmid DNA	32/34
No. positive results/overall number of determinations with 20 copies of Plasmid DNA	34/34

C.7.2.4 Molecular selectivity

C.7.2.4.1 General

This subclause fulfils the requirements outlined in Clause 7.

The method is described in Reference [75]. Information on the genetic construct introduced into the rice genome is available in Reference [76].

C.7.2.4.2 Theoretical

No sequence homology with DNA sequences of non-genetically modified rice varieties and other crop plants has been found in databank searches {GenBank® database (Reference [83]), BlastN® 2.2.21, date: 2009-07-21}. Moreover the primer set was designed to amplify a DNA sequence specific for an artificial junction region not expected to occur in nature.

C.7.2.4.3 Experimental

No amplification has been observed using DNA from non-GM soya beans, rape seed, maize, and rice.

No amplification has been observed also for the following genetically modified lines (Reference [75]):

Cotton lines: MON531 (MON-ØØ531-6), MON15985 (MON-15985-7), MON15985×MON1445 (MON-15985-7 × MON-Ø1445-2), MON531×MON1445 (MON-ØØ531-6 × MONØ1445-2), 3006-210-23×281-24-236 (DAS-21Ø23-5 × DAS-24236-5)

Maize lines: Bt11 (SYN-BTØ11-1), Bt176 (SYN-EV176-9), GA21 (MON-ØØØ21-9), T25 (ACS-ZMØØ3-2), MON863 (MON-ØØ863-5), MON810 (MON-ØØ81Ø-6), TC1507 (DAS-Ø15Ø7-1), 59122 (DAS-59122-7), MON89Ø34 (DAS-89Ø34-3), MIR6Ø4 (SYN-IR6Ø4-5), MON88017 (MON-88Ø17-3), LY038 (REN-ØØØ38-3), 3272 (SYN-E3272-5)

Potato line: EH92-527-1 (BPS-25271-9)

Rapeseed lines: RF1 (ACS-BNØØ1-4), RF2 (ACS-BNØØ2-5, RF3 (ACS-BNØØ3-6), MS1 (ACS-BNØØ4-7), MS8 (ACS-BNØØ5-8), Gt 73 (MON-ØØØ73-7), GS40 / 90pHoe6 / Ac (ACS-BN010-4)

Rice line: LLRice62 (ACS-OSØØ2-5), LLRice 601 (BCS-OSØØ3-7)

Soybean line: GTS 40-3-2 (MON-Ø4Ø32-6)

All DNAs used in experimental specificity tests were controlled for the ability to be amplified and for inhibitors with taxon specific methods before use (data not shown).

C.7.3 Principle and summary

An 83 bp DNA fragment, spanning between the synthetic *cryIA(c)*-gene to the *nos* terminator over a 15 bp spacer sequence is amplified and detected by real-time PCR. The real-time PCR system is based on a specific hydrolysis probe PCR which is labelled with 6-carboxyfluorescein (FAM) as reporter molecule and 6-carboxytetramethylrhodamine (TAMRA) as quencher molecule (Reference [54]).

C.7.4 Terms and definitions

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

C.7.5 Sample type and amounts

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

C.7.6 Limit of detection

Based on the assumption that there is only one copy of the genetic construct per haploid genome and that one haploid rice genome copy has a molecular mass of 0,47 pg, the LOD is less than or equal to five copies, also in presence of conventional rice DNA (used as target taxon) and maize DNA (used as an example for non-target taxon) (References [79][77]).

The LOD (relative to the matrix) is less than or equal to 0,05 % mass fraction (DNA samples with this amount of the *cry1A(c)-T-nos* target copies to rice genome copies) (Reference [78]).

C.7.7 Estimation of measurement uncertainty

The measurement uncertainty was assessed in a collaborative study. The results are given in C.7.2.3.

C.7.8 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.

C.7.9 Physical and environmental conditions

See ISO 24276 for details.

C.7.10 Apparatus and equipment

Regarding the apparatus and materials, see ISO 21569. Employ usual molecular biological laboratory equipment and in particular the following.

C.7.10.1 Apparatus and equipment for DNA extraction

C.7.10.1.1 Thermostat or **water bath**, preferably with shaking function.

C.7.10.1.2 Centrifuge, able to centrifuge 1,5 ml and 2 ml reaction vials at 14 500 × g.

C.7.10.2 Apparatus and equipment for real-time PCR

C.7.10.2.1 Thermal cycler, equipped with

- an **energy source** suitable for the excitation of fluorescent molecules;
- an **optical detection system** suitable for the detection of the fluorescence signals generated during PCR.

C.7.10.2.2 Reaction vessels and caps or closures which can be repeatedly heated to 100 °C and cooled to 4 °C without damage and which do not influence the fluorescence signal generated during the amplification process.

C.7.11 Reagents and materials

Unless otherwise stated, only reagents that conform to the specifications of ISO 24276 and only molecular biology grade water shall be used.

C.7.11.1 Reagents for the DNA extraction with CTAB

See ISO 21571.

C.7.11.2 Reagents for the real-time PCR

C.7.11.2.1 Thermostable DNA polymerase (for hot-start PCR).

C.7.11.2.2 PCR buffer solution (contains $MgCl_2$ and deoxyribonucleoside triphosphates dATP, dCTP, dGTP and dUTP).

Ready-to-use reagent mixtures or individual components can be used as the PCR buffer solution. In the collaborative study the PCR buffer from TaqMan1) universal master mix (Applied Biosystems, Darmstadt) was used.2)

C.7.11.2.3 Oligonucleotides. See Table C.30.

Table C.30 — Oligonucleotides

Name	DNA sequence of the oligonucleotide	Final concentration in the PCR
	cry1A(c)-T-nos construct as the target sequence	
T51F	5'–gAC TgC Tgg AgT gAT TAT CgA CAg A–3'	300 nmol/l
T51R	5'–AgC TCg gTA CCT CgA CTT ATT CAg–3'	300 nmol/l
T51p	5'–(FAM)–TCg AgT TCA TTC CAg TTA CTg CAA CAC TCg Ag–(TAMRA)–3'[a]	100 nmol/l

[a] FAM: 6-carboxyfluorescein, TAMRA: 6-carboxytetramethylrhodamine. Equivalent reporter dyes and/or quencher dyes can be used.

C.7.12 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.

C.7.13 Test sample preparation

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 the ISO 21571.

C.7.14 Instrument calibration

Instruments (e.g. thermocyclers) should be calibrated as per ISO/IEC 17025.[41]

C.7.15 Analysis steps

C.7.15.1 General

DNA is extracted from the test sample applying a suitable procedure. The DNA analysis consists of:

a) verification of the amount, quality and amplifiability of the extracted DNA, e.g. by means of a PCR specific for a target taxon sequence of rice;

b) detection of the *cry1A(c)-T-nos* construct in a real-time PCR.

C.7.15.2 Preparation of the DNA extracts

C.7.15.2.1 DNA extraction

Concerning the extraction of DNA from the test sample, the general instructions and measures described in ISO 21571, should be followed.

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2) Other reagentscan be used if they yield similar or better results.

CTAB was used for the extraction of DNA as outlined in ISO 21571:2005, A.3 with the following modification. The method was validated using 1 g of finely ground test sample material for DNA extraction. The DNA extraction method was modified by adding 10 µl of proteinase K solution according to ISO 21571:2005, A.3.1.5.8 to the CTAB extraction buffer turning the optional proteinase K step into a mandatory step and an incubation overnight at 65 °C under continuous mixing.

Should the material swell in such a way that the particles are no longer freely movable in a suspension, additional CTAB extraction buffer is used to reduce sample viscosity. This should be done in single steps of 1 ml CTAB extraction buffer each. This is often required when extracting DNA from rice noodles.

C.7.15.2.2 DNA quantification

For specific requirements, see ISO 21571:2005, Annex B.

C.7.15.2.3 DNA integrity evaluation

The DNA integrity is determined indirectly by applying a target taxon specific real-time PCR resulting in a PCR product of similar length (Reference [75]).

C.7.15.3 PCR reagents

See C.7.11.2.

C.7.15.4 Procedure

C.7.15.4.1 PCR set-up

The description of the procedure is applicable to an overall volume of 25 µl per PCR set-up, using the reagents stated in Table C.31.

Prior to being applied, the carefully thawed reagents should be briefly centrifuged. Ensure that each reagent is carefully mixed immediately before pipetting. A PCR reaction mixture containing all PCR components except the DNA extract is prepared. The required amount of PCR reaction mixture depends on the number of reactions to be performed, including at least one additional reaction as the pipetting reserve. Use 5 µl of DNA extract.

Table C.31 — Reaction set-up for the amplifications

Overall volume		25 µl
Sample DNA (up to 200 ng) or controls		5 µl
PCR buffer solution[a] (including $MgCl_2$, dNTPs and DNA polymerase)		12,5 µl
Primer	T51F + T51R	See Table C.30
Probe	T51p	See Table C.30
Water		Difference to 25 µl

[a] In the collaborative study, TaqMan[1)] universal master mix (Applied Biosystems, Darmstadt) was applied as the PCR buffer solution. Equivalent products of other manufacturers may be used if they yield similar or better results.

Mix the reaction set-up, centrifuge briefly and pipette 20 µl into each reaction vial.

For the PCR reagent control, pipette 5 µl of water into the respective set-up.

Pipette either 5 µl of the DNA extract, 5 µl of the negative extraction control or 5 µl of the positive extraction control into each of the remaining set-ups.

Set up a PCR inhibition control, if necessary.

Transfer the reaction set-ups into the thermal cycler and start the temperature–time programme.

C.7.15.4.2 PCR controls

As positive control material, genomic DNA of rice line TT51-1 ("cultivar Bt63") or a commercially available plasmid containing the target sequence can be used.

C.7.15.4.3 Temperature–time programme

If applying the PCR described here, the temperature–time programme stated in Table C.32 has proven suitable.

Table C.32 — Temperature–time programme

Step	Parameter		Temperature °C	Time	Fluorescence measurement	Cycles
1	Initial denaturation		95	10 min	no	1
2	Amplification	Denaturation	95	20 s	no	45
		Annealing and elongation	60	60 s	yes	

C.7.15.4.4 Accept or reject criteria

The evaluation is performed applying the relevant device-specific data analysis program. The indication of the amplification result differs partly, depending on the real-time PCR device used. In the case of no detectable PCR products (negative result), the indication in the result report is, for example, "undetermined", "no amp" or the maximum set number of cycles. If an amplification of the DNA target sequence took place in a sample (positive result), the number of cycles is calculated at which a prescribed fluorescence threshold value has been exceeded (C_t value or C_p value).

If, due to atypical fluorescence measurement data, the automatic evaluation does not provide a reasonable result, it may be necessary to manually set the baseline and the threshold value prior to evaluating the data. In doing so, follow the device-specific advice given in the technical manual for the application of the evaluation software.

To detect small quantities of admixtures of genetically modified rice lines containing the construct *cry1A(c)-T-nos*, at least 5 000 haploid genome copies are required in the target taxon specific system. This copy number corresponds to lower C_t values in the rice-specific PCR.

C.7.16 Sample identification

All samples should be identified unambiguously.

C.8 Construct-specific method for the detection of the *ctp2-cp4-epsps* sequence for screening for components of genetically modified organisms in foodstuffs

C.8.1 Purpose, relevance and scientific basis

This method describes a construct-specific procedure for screening of DNA extracted from genetically modified plants containing the *ctp2-cp4-epsps* gene sequence.

The transition from CTP2 (chloroplast transit peptide signal sequence from *Arabidopsis thaliana*) to the herbicide tolerance gene *cp4-epsps* (5-enolpyruvylshikimate-3-phosphate synthase gene from *Agrobacterium tumefaciens* strain CP4) is frequently found in genetically modified plants (Reference [53]).

Basically, the method described is applicable not only to the analysis of all foodstuffs, but also of other products (e.g. feeding stuffs, seeds). The application of the method requires that an amount of amplifiable DNA can be extracted from the respective matrix which is sufficient for analysis. The method described is based on a real-time PCR.

For further specification of the detection (e.g. detection of the event), a follow-up analysis should be carried out.

C.8.2 Validation status and performance criteria

C.8.2.1 Robustness

The robustness of the method has not been tested to small modifications.

In the collaborative trial, the robustness of the method has been checked with regard to different real-time PCR devices (see C.8.10.1) and master mixes (see Table C.36, footnote b). Neither the real-time PCR devices nor the master mixes used had influence on the performance of the method.

C.8.2.2 Intralaboratory trial

In intralaboratory trials, the method provided satisfactory and consistent results. The method was tested with dilution series of DNA extracted from 4,9 % mass fraction NK 603 maize and from 100 % mass fraction GT73 rapeseed certified reference materials, respectively. Five PCR replicates were tested at each dilution step. In the tests with GT73 DNA the relative confidence intervals (P = 95 %) for the measured copy numbers at 2 000, 400, 100, 25, 10 and 5 copies were 7,4 %, 12,1 %, 10,5 %, 13,7 %, 24,7 % and 41,0 %, respectively. In the tests with NK603 DNA, the relative confidence intervals (P = 95 %) for the measured copy numbers at 2 500, 500, 250, 50, 10 and 5 copies were 5,6 %, 13,9 %, 3,3 %, 39,4 %, 52,6 % and 58,8 %, respectively. The method was also tested for its performance with DNAs extracted from different plants (see C.8.2.4.3). The results of these tests also showed that the method provides satisfactory and consistent results.

C.8.2.3 Collaborative trial

C.8.2.3.1 General

The performance of the method has been assessed in a collaborative study (Reference [59]) coordinated by the Federal Office of Consumer Protection and Food Safety and performed with 11 participants. For the analysis, the participants received 12 DNA samples with different concentrations of the *ctp2-cp4-epsps* gene sequence as well as 6 DNA samples supposed not to contain this sequence. All samples were marked with random coding numbers.

For DNA extraction, the CTAB method as outlined in ISO 21571:2005, A.3, was used. To prepare the DNA samples, genomic DNA extracted from 0,1 % mass fraction NK603-certified reference material (ERM BF 415b by the IRMM, Geel), from non-GM maize flour or GT73 rapeseed and non-GM rapeseed-certified reference materials (0304-A and 0304-B by AOCS, USA) were used. The non-GM maize flour and rapeseed flour had not shown any amplification in the PCR test carried out before that regarding the *ctp2-cp4-epsps* sequence. The 0,02 % mass fraction NK603 DNA or the 0,02 % mass fraction GT73 DNA, respectively, was prepared by mixing the 0,1 % mass fraction NK603 or the 0,1 % mass fraction GT73 DNA solution, respectively, with non-GM maize or rapeseed DNA at a ratio of 1 → 5.

Aliquots of the following DNA solutions were provided as unknown samples:

a) 0,1 % mass fraction NK603 DNA, approximately 27 ng/µl;

b) 0,02 % mass fraction NK603 DNA, approximately 27 ng/µl;

c) non-genetically modified (non-GM) maize DNA, approximately 27 ng/µl;

d) 0,1 % mass fraction GT73 rapeseed DNA, approximately 13 ng/µl;

e) 0,02 % mass fraction GT73 rapeseed DNA, approximately 13 ng/µl;

f) non-genetically modified (non-GM) rapeseed DNA, approximately 13 ng/µl.

C.8.2.3.2 Qualitative evaluation of the results

The data from the collaborative study are listed in Table C.33.

Table C.33 — Results of the collaborative study (qualitative)

Parameter (interlaboratory trial of 2008)	Value
No. laboratories	11
No. laboratories having presented results	11
No. samples per laboratory	18
No. accepted results	198
No. samples containing the *ctp2-cp4-epsps* sequence	132
No. samples containing no *ctp2-cp4-epsps* sequence[a]	66
False-positive results	13 (19,7 %)[a]
False-negative results	0 (0 %)

[a] See also C.8.2.4.3.

C.8.2.3.3 Semiquantitative evaluation of the results

To calculate the *ctp2-cp4-epsps* copy numbers in the samples, all participants received a standard DNA, which had been extracted from 5 % mass fraction NK603-certified reference material (ERM-BF415f, IRMM). The DNA concentration had been determined spectrometrically, and the copy numbers had been calculated from this on the basis of genome equivalents, taking the proportion given in the certificate as a basis (4,91 % mass fraction in ERM-BF415f). From this NK603 standard DNA, the participants of the collaborative study had to prepare a dilution series with 0,2× TE in order to obtain DNA solutions for 5 calibration points (2 000, 500, 150, 50 and 10 copies of the *ctp2-cp4-epsps* target sequence) and another DNA solution as the sensitivity control with 5 copies of the *ctp2-cp4-epsps* target sequence. In order to calculate the corresponding copy numbers from the sample cycle threshold (C_t) values determined, the 5 DNA calibration solutions were measured together with the samples in the same analytical PCR run. The C_t values were plotted against the logarithm of the copy numbers to give the calibration curve. In Table C.34, a summary of the results thus determined is given.

Table C.34 — Evaluation of the collaborative study (semiquantitative)

GM material content (GM copies/genome equivalents) % mass fraction	No. positive results/total results	Calculated copy numbers *ctp2-cp4-epsps* sequence	
		Mean[a]	$C_{V,R}$,[b] %
0,1 % NK603 maize	33/33	50	35
0,02 % NK603 maize	33/33	11	41
0,1 % GT73 rapeseed	33/33	36	32
0,02 % GT73 rapeseed	33/33	9	50
non-GM maize[c]	12/33	0	—
non-GM rapeseed	1/33	0	—

[a] Mean value of the calculated copy numbers from all single assays.

[b] Coefficient of variation under reproducibility conditions.

[c] See also C.8.2.4.3.

The results obtained by two laboratories which used glass capillaries were similar to those obtained in the collaborative study applying devices for plastic reaction vials as far as the sensitivity, average values and standard deviation of the copy numbers, as well as the specificity, were concerned (data not shown).

C.8.2.3.4 Sensitivity and precision

Table C.34 summarizes the proportions of positive results as well as the precision data for the individual samples. The method was tested with samples containing low copy numbers of the *ctp2-cp4-epsps* sequence. In all samples containing the *ctp2-cp4-epsps* sequence, this sequence was detectable. Also the sensitivity control with five copies of NK603 maize DNA resulted in an amplification of the *ctp2-cp4-epsps* target sequence in all 22 determinations. Based on these results, the LOD (relative to the matrix) can, therefore, be stated as a mass fraction of at least 0,02 % (DNA samples with this relative relation of the *ctp2-cp4-epsps* copies to the genome copies of the respective species) or as an absolute value of ≤5 copies as shown in the tests with NK603 maize DNA.

The coefficient of variation under reproducibility conditions ($C_{V,R}$) was 35 % or 32 %, respectively, at the levels of 0,1 % NK603 maize and 0,1 % GT73 rapeseed. Thus, the precision data slightly exceed the $C_{V,R}$ values required by ISO 24276 for the quantification.

Potential outliers were not eliminated prior to calculating the precision data. Checking of the suitability of the method for quantification was not the major objective of the collaborative study.

C.8.2.4 Molecular selectivity

C.8.2.4.1 General

The method is described in References [59][80]. Information on the genetic construct introduced into the NK603 maize genome is also available in Reference [81].

C.8.2.4.2 Theoretical

The theoretical specificities of the primers and the probe were assessed by a BLASTN 2.2.1 search in the GenBank®/EMBL/DDBJ databases using the amplicon sequence (Reference [83], accession number FN550387). No sequence homology with DNA sequences of non-genetically modified plants and other crop plants was found (search date 2009-11-15). Moreover, the primer set was designed to amplify a DNA sequence specific for an artificial junction region not expected to occur in nature.

C.8.2.4.3 Experimental

When experimentally determining the specificity prior to the collaborative study, no cross-reactions of the *ctp2-cp4-epsps* detection method with DNA from the following genetically modified plants was observed:

- GM rice: LL62 (ACS-OS002-5), LL601 (BCS-OS003-7);
- GM rapeseed: Liberator pHoe6/Ac (ACS-BN009-3), Falcon GS40/90 pHoe6/Ac (ACS-BN010-4), Laurat (pCGN3828) (CGN-89465-2), TOPAS19/2 (ACS-BN007-1), MS1×RF1 (ACS-BN004-7×ACS-BN001-4), MS8 (ACS-BN005-8), T45 (HCN 28) (ACS-BN008-2);
- GM maize: 3272 (SYN-E3272-5), DAS59122 (DAS-59122-7), Bt176 (SYN-EV176-9), MON810 (MON-00810-6), T14 (ACS-ZM002-1), T25 ACS-ZM003-2), DAS1507 (DAS-01507-1), GA21 (MON-00021-9);
- GM soya: 305423 (DP-305423-1), 356043 (DP-356043-5), MON40-3-2 (MON-04032-6), A 2704-12 (ACS-GM005-3), A5547-127 (ACS-GM006-4);
- GM potatoes: EH92-527-1 (BPS-25271-9).

For the following genetically modified plants it was experimentally shown that the *ctp2-cp4-epsps* detection method is suitable as a screening method:

- GM rapeseed: GT73 (MON-00073-7);
- GM maize: MON809, MON88017 (MON-88017-3), NK603 (MON-00603-6);
- GM sugar beet: GTSB77, H7-1 (KM-000H71-4);

— GM soya: MON89788 (MON-89788-1).

All DNAs used in experimental specificity tests were controlled for the ability to be amplified and for inhibitors with taxon specific methods before use (data not shown).

A continuously updated list (screening table) of genetically modified plant events detectable (or non-detectable) using this method is available (see Reference [68]).

Concerning the non-GM maize DNA sample which, based on previous determinations, had been assumed to be "negative", an amplification was reported by the laboratories for 12 of the total of 33 determinations (a C_t of 37 or more, average C_t of 38,5). This corresponds to less than five copies of the *ctp2-cp4-epsps* target sequence. A possible reason for this are minimal contaminations of the maize flour used, and previously tested to be negative, by materials containing *ctp2-cp4-epsps* during the preparation and aliquotation of the samples.

In order to avoid false-positive results when using the *ctp2-cp4-epsps* method, fluorescence signals at a C_t value of ≥37,0 which correspond to low copy numbers of the target sequence (≤5 copies), should not be interpreted as positive analysis results.

Regarding the non-GM rapeseed DNA samples extracted from non-genetically modified rapeseed reference material (AOCS, 0304-A), a very low amplification (C_t of 39,4) was detectable in only one of the 33 determinations.

C.8.3 Principle and summary

An 88 bp DNA fragment, spanning the junction between the CTP2 (chloroplast transit peptide signal sequence from *Arabidopsis thaliana*) sequence and the herbicide tolerance gene *cp4-epsps* (5-enolpyruvylshikimate-3-phosphate synthase gene from *Agrobacterium tumefaciens* strain CP4) is amplified by real-time PCR and detected by means of a specific oligonucleotide probe which is labelled by two fluorescent dyes ("TaqMan[1] probes") (Reference [54]).

C.8.4 Terms and definitions

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

C.8.5 Sample type and amounts

Ensure that the test sample is representative of the laboratory sample, e.g. by grinding or homogenization. Measures and operational steps to be taken into consideration are described in ISO 21571. For the collaborative trial, a set of DNA samples were used (see C.8.2.3.1).

C.8.6 Limit of detection

The method was tested with samples containing low copy numbers of the *ctp2-cp4-epsps* target sequence (Table C.34). In all samples containing the *ctp2-cp4-epsps* sequence, this target was detectable. Also the sensitivity control with five copies of NK603 maize DNA resulted in an amplification of the *ctp2-cp4-epsps* target sequence in all laboratories (22 single assays, data from the results obtained with the standard DNA dilution used for calibration are taken, see C.8.2.3.4). Based on these results, the LOD (relative to the matrix) is less than or equal to 0,02 % mass fraction when using NK603 DNA (sample DNA with this amount of the *ctp2-cp4-epsps* copies to the genome copies of the respective species) or as an absolute value is less than or equal to 5 copies.

C.8.7 Estimation of measurement uncertainty

The measurement uncertainty was assessed in a collaborative study. The results are given in C.8.2.3.2.