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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

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Foreword

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

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

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.

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_{rice}/m_{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_{tomato}/m_{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.