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AMENDMENT 1
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Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Quantitative nucleic acid based methods

AMENDMENT 1

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

AMENDEMENT 1

Reference number
ISO 21570:2005/Amd.1:2013(E)

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Foreword

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Amendment 1 to ISO 21570:2005 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 16, *Horizontal methods for molecular biomarker analysis*.

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Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Quantitative 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 21570:2005. The footnote numbers given are for use solely within this amendment.

Page 1, Clause 2

Update entries 1 to 3 as follows and delete footnote 1).

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

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

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

Page 2, 7.1, last paragraph; Page 8, A.1.5.1; Page 15, B.1.5.1; Page 23, C.1.5.1; Page 31, C.2.5.1; Page 38, C.3.5.1; Page 45, C.4.5.1; Page 53, C.5.5.1; Page 60, C.6.5.1; Page 68, C.7.5.1; Page 75, C.8.5.1; Page 83, C.9.5.1; Page 90, D.1.5.1; Page 96, D.2.5.1

Delete “ISO 24276:—”, insert “ISO 24276:2006”.

Page 4, Clauses 8–10

Replace the existing text with the following.

8 Interpretation

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

a) Fit for quantification of the target sequence provided:

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

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

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

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

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

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

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

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

9 Expression of results

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

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

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

10 Test report

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

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

b) the practical LOQ;

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

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

e) the reference material used;

f) the results expressed according to Clause 9;

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

h) the definition of the measurement uncertainty used.

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

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Table 1 — Expression of results

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

Page 11, Annex A

Add A.2 and A.3.

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

A.2.1 Principle

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

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

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

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

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

— ISO 5725;[51]–[56]

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

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

A.2.2 Scope

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

A.2.3 Validation status and performance criteria

A.2.3.1 Robustness of the method

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

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

A.2.3.2 Intralaboratory trial

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

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

A.2.3.3 Collaborative trial

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

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

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

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

A.2.3.4 Molecular selectivity

A.2.3.4.1 General

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

Table A.6 — Oligonucleotide primers and probe sequences

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

A.2.3.4.2 Experimental

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

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

A.2.3.4.3 Theoretical

The theoretical specificity of the *SPS* gene primers and probe was assessed through a similarity search using the BLASTN 2.0MP-WashU program (Reference [64], search date: 2010-01-09). The 81 bp sequence

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used as query is part of the NCBI accession number U33175 (nucleotides 1055–1135). The results of the blast search confirmed the complete identity of the query sequence with rice *SPS* gene sequence, and no similarity with other genes and species.

A.2.4 Principle and summary

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

A.2.5 Terms and definitions

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

A.2.6 Sample type and amounts

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

The participants received the following samples.

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

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

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

A.2.8 Estimation of measurement uncertainty

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

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A.2.9 Interferences

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

A.2.10 Physical and environmental conditions

The procedures require experience of working under sterile conditions.

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

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

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

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

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

Pipettes should be calibrated regularly, if necessary.

A.2.11 Apparatus and equipment

Usual laboratory equipment and in particular the following.

A.2.11.1 **Microcentrifuge**.

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

A.2.11.3 **Micropipettes**.

A.2.11.4 **Vortex mixer**.

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

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

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

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

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

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

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

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

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

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

A.2.12 Reagents and materials

A.2.12.1 General

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

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A.2.12.2 DNA extraction

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

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

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

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

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

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

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

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

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

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

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

A.2.12.2.12 **Hydrochloric acid**, ρ(HCl) = 370 g/l.

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

A.2.12.3 Quantitative real-time PCR

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

A.2.13 Sample collection, transport, preservation and storage

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

A.2.14 Preparation of test sample

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

A.2.15 Instrument calibration

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

A.2.16 Analysis steps

A.2.16.1 General

A.2.16.1.1 Preparation of the DNA for standard curve construction

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

For specific requirements, see ISO 21571.

A.2.16.1.2 PCR reagents

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A.2.16.1.3 PCR master mix

See A.2.12.3.

A.2.16.1.4 Primers and probe

See Table A.6.

A.2.16.2 Procedure

A.2.16.2.1 General

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

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

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

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

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

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

A.2.16.2.2 PCR controls

See A.2.6.

A.2.16.2.3 Preparation of standards

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

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

A.2.16.2.4 Temperature–time programme (PCR)

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

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

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

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A.2.16.2.5 Accept or reject criteria

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

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

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

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

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

A.2.16.2.6 Identification

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

A.2.17 Sample identification

See A.2.6.

A.2.18 Interpretation and calculations of the results

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

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

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

A.2.19 Record keeping

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

A.2.20 Reporting

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

A.2.21 Safety measures

No specific requirements. See ISO 24276.

A.2.22 Pollution prevention and waste disposal

No specific requirements. See ISO 24276.

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

A.3.1 Principle

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

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

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

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

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

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

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

A.3.2 Scope

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

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

A.3.3 Validation status and performance criteria

A.3.3.1 Robustness of the method

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

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

A.3.3.2 Intralaboratory trial

The tomato *LAT52* gene has been validated for use as endogenous reference gene in GM tomato identification and quantification (Reference [62]). The *LAT52* PCR system was also tested by three

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operators in GMDL-SJTU using the tomato genomic DNA, providing satisfactory results; in particular, in quantitative PCR, the bias was below 25 % over the dynamic range (i.e. 0,05 ng to 1,00 ng).

A.3.3.3 Collaborative trial

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

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

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

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

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

A.3.3.4 Molecular selectivity

A.3.3.4.1 General

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

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Table A.9 — Oligonucleotide primers and probe sequences,

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

A.3.3.4.2 Experimental

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

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

A.3.3.4.3 Theoretical

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

A.3.4 Principle and summary

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

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

A.3.5 Terms and definitions

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

A.3.6 Sample type and amounts

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

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

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

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

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A.3.8 Estimation of measurement uncertainty

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

A.3.9 Interferences

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

A.3.10 Physical and environmental conditions

The procedures should be performed under sterile working conditions.

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

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

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

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

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

Pipettes should be checked for precision and calibrated frequently.

A.3.11 Apparatus and equipment

Usual laboratory equipment and in particular the following.

A.3.11.1 **Microcentrifuge**.

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

A.3.11.3 **Micropipettes**.

A.3.11.4 **Vortex mixer**.

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

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

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

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

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

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

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

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

A.3.12 Reagents and materials

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

A.3.12.1 **Real-time PCR master mixture**.

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A.3.12.2 Oligonucleotides (primers and probe).

A.3.13 Sample collection, transport, preservation and storage

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

A.3.14 Test sample preparation

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

A.3.15 Instrument calibration

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

A.3.16 Analysis steps

A.3.16.1 General

A.3.16.1.1 Preparation of the DNA for standard curves construction

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

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

A.3.16.1.2 PCR reagents

A.3.16.1.3 Real-time PCR master mixture

See A.3.12.

A.3.16.1.4 Primers

See Table A.9.

A.3.16.2 Procedure

A.3.16.2.1 General

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

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

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

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

Place the plate into the instrument.

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

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A.3.16.2.2 PCR controls

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

A.3.16.2.3 Preparation of standards

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

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

A.3.16.2.4 Temperature–time programme (PCR)

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

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

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

A.3.16.2.5 Accept or reject criteria

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

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

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

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

A.3.16.2.6 Identification

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

A.3.17 Sample identification

See A.3.6.

A.3.18 Interpretation and calculations of the results

After the quantitative real-time PCR run is completed, the threshold line is set in the area where the amplification profiles are parallel (exponential phase of PCR — logarithmic mode) and where there is no “fork effect” between repetitions of the same sample. Determine the cycle number at which the

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threshold line crosses the first amplification curve and set the baseline three cycles before that value. Export all the data for further calculations.

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

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

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

A.3.19 Record keeping

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

A.3.20 Reporting

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

A.3.21 Safety measures

No specific requirements. Refer to ISO 24276.

A.3.22 Pollution prevention and waste disposal

No specific requirements. Refer to ISO 24276.

Page 103, Bibliography

Add the following references.

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