TECHNICAL SPECIFICATION

ISO/TS 21569-7

First edition
2022-12

Horizontal methods for molecular biomarker analysis — Methods of analysis for the detection of genetically modified organisms and derived products—

Part 7:

Real-time PCR based methods for the detection of CaMV and *Agrobacterium* Ti-plasmid derived DNA sequences

Reference number
ISO/TS 21569-7:2022(E)

ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Email: copyright@iso.org
Website: www.iso.org

Published in Switzerland

Contents

Page

Foreword

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

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 34, *Food products,* Subcommittee SC 16, *Horizontal methods for molecular biomarker analysis*.

A list of all parts in the ISO 21569 series can be found on the ISO website.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Horizontal methods for molecular biomarker analysis — Methods of analysis for the detection of genetically modified organisms and derived products —

Part 7: Real-time PCR based methods for the detection of CaMV and *Agrobacterium* Ti-plasmid derived DNA sequences

1 Scope

This document specifies a procedure for the detection of a DNA sequence of the open reading frame five (ORF V) from cauliflower mosaic virus (CaMV) and a procedure for the detection of the DNA sequence of the nopaline synthase (*nos*) gene from tumour-inducing (Ti) plasmids of phytopathogenic *Rhizobium radiobacter* (formerly named *Agrobacterium tumefaciens*). The procedures can be used in the context of screening for genetically modified crop/plants and their derived products to further clarify a positive PCR result for a specific promoter or terminator of CaMV (P-35S, T-35S), or both, and the *nos* gene (P-*nos*, T-*nos*), respectively.

The methods specified in this document will detect and identify naturally occurring CaMV or *Rhizobium radiobacter* (Ti plasmid) DNA, or both, if present in the sample in the absence of a genetically modified plant event containing the specified target sequences.

Both methods are based on the real-time polymerase chain reaction (PCR) and are applicable for the analysis of DNA extracted from foodstuffs and other products such as feedstuffs and seeds/grains. The application of the methods requires the extraction of an adequate amount of amplifiable DNA from the relevant matrix.

With appropriate calibration material, the CaMV ORF V or *nos* copy number, or both, can be estimated and compared, respectively, with the estimated copy number for the promoter (P-35S, P-*nos*) or the terminator (T-35S, T-*nos*) sequences, or both. Thereby, conclusions are possible about the presence of an unknown genetically modified organism (GMO) in addition to any detected CaMV DNA or *Rhizobium radiobacter* Ti plasmid DNA, or both, in a test sample.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 16577, *Molecular biomarker analysis — Vocabulary for molecular biomarker analytical methods in agriculture and food production*

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

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

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

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 16577 apply.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

4 Principle

DNA extracts of test portions are used that showed a positive PCR result in screening tests for specific promoter/terminator sequences derived from CaMV and/or from Ti plasmid of *Rhizobium radiobacter*. The tests consist of two parts, namely:

a) detection of the CaMV *ORF V* and/or the *nos* DNA sequence in a real-time PCR;

b) estimation of the copy numbers on basis of the measured Cq values compared to a standard curve using reference materials, if the CaMV *ORF V* and/or the *nos* gene target sequences are amplified.

For further confirmation, in case of positive results in the *nos* PCR tests, it is recommended to perform a further test for the detection of chromosomal *Rhizobium radiobacter* DNA.[1]

5 Reagents and materials

Chemicals of recognized analytical grade, appropriate for molecular biology shall be used. The water used shall be double-distilled or PCR-grade water (i.e. nuclease and nucleic acid free). For all operations in which gloves are used, it should be ensured that these are powder-free. The use of aerosol-protected pipette tips as protection against cross-contamination is recommended.

5.1 Thermostable DNA polymerase, (for hot-start PCR). PCR buffer solution, which contains magnesium chloride and deoxyribonucleoside triphosphates (dNTPs). Ready-to-use reagent mixtures or mixes of individual components can be used. Reagents and polymerases which lead to equal or better results may also be used.

5.2 Positive control materials. DNA extracted from *Rhizobium radiobacter* strains with Ti plasmid (DSM-5172 or ATCC 33970D 5) and from CaMV isolates (DSMZ PV-0226; DSMZ PV-0227; DSMZ PV-0228; DSMZ PV-0229).

5.3 Oligonucleotides. See Tables 1 and 2.

Equivalent reporter dyes and/or quencher dyes may be used for the probe if they can be shown to yield similar or better results.

Table 1 — Oligonucleotides for detection of *nos*

Name	DNA sequence of the oligonucleotide	Final concentration in the PCR
nos gene sequence from *Rhizobium radiobacter* Ti plasmids as target		
At-nop-f2	5′-CCA gCC RTS TAC TgA TTA TTg TMA C-3′	300 nmol/l
At-nop-r2	5′-TgC gAg TTC RCC gTT gAA g-3′	300 nmol/l
At-nop-s1	5′-(FAM)-CCg TgC ggA CgT TCA CgA CAg-(BHQ1)-3′[a]	150 nmol/l

[a] FAM: 6-Carboxyfluorescein, BHQ1: black hole quencher 1.

Table 2 — Oligonucleotides for detection of CaMV *ORF V*

Name	DNA sequence of the oligonucleotide	Final concentration in the PCR
ORF V sequence from CaMV as target		
CaMV-ORFV-fd2	5′-ATY AAg CCC AgY AAA AgC CC-3′	300 nmol/l
CaMV-ORFV-rd2	5′-CTY CgC TTC TCg gCT TCR TT-3′	300 nmol/l
CaMV-ORFV-P2	5′-(FAM)-CAT ggC ACC AgC CTT CTT ggT CAA C-(BHQ1)-3′[a]	150 nmol/l
[a] FAM: 6-Carboxyfluorescein, BHQ1: black hole quencher 1.		

5.4 Standard DNA for calibration. DNA solution of a known concentration (ng/µl) to estimate the copy number of the target sequence.

When using genomic CaMV or *Rhizobium radiobacter* DNA as the standard DNA, the number of genome equivalents should be calculated on the basis of the molecular mass of the genome according to Formula (1):

$$\gamma_E = \frac{(\delta \times 1\,000)}{\gamma_M} \tag{1}$$

where

γ_E is the number of genome equivalents per microliter (µl);

δ is the DNA concentration in nanograms per microliter (ng/µl);

γ_M is the genome mass in femtograms (fg).

The respective copy number for the target sequence can be calculated based on the genome equivalents.

6 Apparatus

Requirements concerning apparatus and materials shall be in accordance with ISO 21569. In addition to the usual laboratory equipment, the following equipment shall be used.

6.1 Real-time PCR device, which shall be suitable for the excitation of fluorescent molecules and the detection of fluorescence signals generated during PCR.

7 Procedure

7.1 Preparation of test samples

The test portion used for DNA extraction should be representative of the laboratory sample, e.g. by grinding or homogenizing of the samples. Measures and operational steps that should be considered are described in ISO 21571 and ISO 24276.

7.2 Preparation of DNA extracts

For the extraction of DNA from the test portion, the general instructions and requirements specified in ISO 21571 shall be followed.

7.3 PCR setup

7.3.1 The method description applies to a total volume of 25 µl per reaction mixture with the setup given in Tables 3 and 4. The PCR reagent mixture is prepared containing all components except for the

sample DNA. The required amount of PCR reagent mixture depends on the number of reactions to be performed.

Table 3 — Reaction setup for the amplification of the *nos* target DNA sequence

Reagent	Volume
Sample DNA (up to 200 ng) or controls	5 µl
PCR buffer solution[a] (contains $MgCl_2$, dNTPs and hot-start DNA polymerase)	12,5 µl
Primers At-nop-f2 and At-nop-r2	see Table 1
Probe At-nop-s1	see Table 1
Water	add to obtain 25 µl

[a] In the interlaboratory trial, the PerfeCTa qPCR ToughMix (Quanta BioSciences) was used as PCR buffer solution. This information is given for convenience of users of this document and does not constitute an endorsement by ISO of the product names. Equivalent products from other manufacturers may be used if they can be shown to give equivalent or better results. If necessary, adapt the amounts of the reagents and the temperature-time programme.

Table 4 — Reaction setup for amplification of the CaMV ORF V target DNA sequence

Reagent	Volume
Sample DNA (up to 200 ng) or controls	5 µl
PCR buffer solution[a] (contains $MgCl_2$, dNTPs and hot-start DNA polymerase)	12,5 µl
Primers CaMV-ORFV-fd2 and CaMV-ORFV-rd2	see Table 2
Probe CaMV-ORFV-P2	see Table 2
Water	add to obtain 25 µl

[a] In the interlaboratory trial, the PerfeCTa qPCR ToughMix (Quanta BioSciences) was used as PCR buffer solution. This information is given for convenience of users of this document and does not constitute an endorsement by ISO of the product names. Equivalent products from other manufacturers may be used if they can be shown to give equivalent or better results. If necessary, adapt the amounts of the reagents and the temperature-time programme.

7.3.2 Mix the PCR reagent mixture, centrifuge briefly and pipette 20 µl into each reaction vial.

7.3.3 For the amplification reagent control, add 5 µl of water into the respective reaction setup.

7.3.4 Pipette either 5 µl of sample DNA or 5 µl of the respective control solution (extraction blank control, positive DNA target control).

7.3.5 If necessary, prepare a PCR inhibition control as specified in ISO 24276.

7.3.6 Carefully seal the reaction setup, transfer them into the real-time PCR device and start the temperature-time program (Table 5).

7.4 Temperature-time programme

The temperature-time programme as outlined in Table 5 was used in the validation study. The use of different reaction conditions and real-time PCR devices may require specific optimization. The time for initial denaturation depends on the master mix used.

Table 5 — Temperature-time programme

Step	Parameter		Temperature	Time	Cycles
1	Initial denaturation		95 °C	2 min	1
2	Amplification	Denaturation	95 °C	15 s	45
		Annealing and Elongation	60 °C	90 s	

8 Accept/reject criteria

8.1 General

The evaluation of PCR amplification results is performed with the respective device-specific data analysis programme. If the amplification of the target sequence was successful in a sample (positive result), the cycle number is calculated at which a specified fluorescence threshold was exceeded for the first time (Cq value).

8.2 Identification of *nos*

The *nos* gene target sequence is considered as detected, if:

— a sigmoid-shaped amplification curve and typical increase in the measured fluorescence is detected using the specific primers At-nop-f2 and At-nop-r2 and the probe At-nop-s1;

— no amplification and increase in fluorescence have occurred in the PCR control reactions with no DNA added (PCR reagent control, extraction blank control).

In the case of a negative PCR result, the expected Cq values are achieved in the amplification controls (positive DNA target control, PCR inhibition control).

The Cq value obtained for *nos* with the test sample DNA should be compared with the Cq values obtained in positive PCR tests for the genetic elements P-*nos* or T-*nos*. If the values are approximately the same, it is plausible to interpret that the Cq values for P-*nos* or T-*nos* or both can be explained solely by a Ti plasmid from wild-type *Rhizobium radiobacter* detected via the *nos* gene. If the Cq values for P-*nos* or T-*nos* or both are significantly smaller, it can be suspected that the sample contains DNA from an unknown GMO. In any case, the absence of genetically modified (GM) linseed FP967 shall be clarified (see 9.1). To clarify positive results for *nos*, an examination for the presence of chromosomal *Rhizobium radiobacter* DNA is useful.[1]

8.3 Identification of CaMV ORF V

The target sequence is considered to be detected in the samples if, in the real-time PCR assay:

— a sigmoid-shaped amplification curve and typical increase in the measured fluorescence is detected using the specific primers CaMV-ORFV-fd2 and CaMV-ORFV-rd2 and the probe CaMV-ORFV-P2;

— no amplification and increase in fluorescence have occurred in the PCR control reactions with no DNA added (PCR reagent control, extraction blank control).

In the case of a negative PCR result, the expected Cq values shall be achieved in the amplification control approaches (positive DNA target control, PCR inhibition control).

The Cq value measured for the sample should be compared with the Cq values for the genetic elements P-35S or T-35S. If the values are approximately the same, it is plausible to interpret that the measured Cq values for P-35S and/or T-35S can be explained by the detected CaMV or virus DNA alone. If the Cq values for P-35S and/or T-35S are significantly smaller, it can be suspected that the sample additionally contains DNA of an unknown GMO.

9 Validation status and performance criteria

9.1 Specificity

The amplicon sequences were validated *in silico* against the National Center for Biotechnology Information (NCBI) nucleotide sequence and patent sequence databases using the program BLASTN[2]. No similarity was found with any other published sequence except with the *nos* sequences of related *Rhizobium radiobacter* strains and the ORF V sequences of CaMV isolates (see Table 6). Of the total 113 CaMV deposited in the NCBI sequence database which were used for comparison, 94 sequences (83 %) showed complete identity with the primer and probe sequences (database assessed 2015-07-28). Fourteen sequences (12 %) showed one mismatch to the primer and probe sequences. For five of these sequences, the mismatch is located at the 5′-terminal nucleotide of the probe and is therefore unlikely to affect the 5′-nuclease activity of the polymerase and detection. For two sequences, no match at two nucleotide positions was found.

The primer, probe and amplicon sequences were additionally tested for similarities to sequences of GMOs and of genomes of more than 100 plants (including *Brassica rapa*, *Glycine max*, *Oryza sativa*, *Solanum lycopersicum* and *Zea mays*) compiled in the database of the European Reference Laboratory for Genetically Modified Food and Feed (EURL-GMFF) using ePCR programs.[3][4] No similarities were found to any sequences except for those targeted by the oligonucleotides.

Genomic DNAs from different *Rhizobium radiobacter* and CaMV strains were examined as parts of interlaboratory trial validations. In addition, other bacterial, plant and DNAs from diverse GMOs were tested to provide experimental data on specificity. Only the relevant bacterial strains containing a Ti plasmid or CaMV strains showed positive reactions in these tests (see Table 6).

The *nos* detection method also detects DNA from the flax event FP967, which contains a complete *nos* gene including the P-*nos* and T-*nos* sequences. DNAs of other GM plants were negative in all tests.

Table 6 — Specificity of the *nos* and the CaMV ORF V detection methods

PCR test result	Reference material	Theoretical identity
nos positive	*Rhizobium radiobacter* (*Agrobacterium tumefaciens*) strains: C58 (DSM-5172); CFBP-1903; CFBP-2719; GM plants: FP967 linseed	*Rhizobium radiobacter* (*Agrobacterium tumefaciens*) strain: C58 (AE007871.2) Ti plasmids: X74123, JX901132.1, JX901135.1, JX901137.1, JX901131.1, X77327.1, AB016260.1, AJ237588.1, V00087.1, AB291242.1
CaMV ORF V positive	CaMV isolates: DSMZ PV-0226 (isolate from cabbage, Denmark); DSMZ PV-0227 (isolate from the John Innes Institute, England); DSMZ PV-0228 (isolate of the BBA, Germany); DSMZ PV-0229 (isolate of the BBA, Germany)	CaMV isolates: 94 isolates in NCBI GenBank)
nos and CaMV ORF V negative	*Rhizobium radiobacter* (*Agrobacterium tumefaciens*) strains (tested only for *nos*): CFBP-2713; CFBP-2413; CFBP-2682 Other bacterial strains (tested only for *nos*): *Staphylococcus pasteuri* (DSM-10656), *Bordetella trematum* (DSM-11334), *Caulobacter vibrioides* (DSM-4738), *Aeromonas media* (DSM-4881), *Erwinia pyrifoliae* (DSM-12163), *Providencia rettgeri* (DSM-4542), *Rauoltella terrigena* (DSM-2687), *Escherichia coli* (DSM-301), *Pseudomonas putida* (DSM-7162) GM soybean: A2704-12 (AOCS 0707-B), A5547-127 (AOCS 0707-C), GTS 40-3-2 (IRMM-410S), MON89788 (AOCS 0906-B), DP-356043 (ERM-BF425d), DP-305423 (ERM-BF426d), MON87701 (AOCS 0809-A), FG72 (AOCS 0610-A), DAS 68416-4 (ERM-BF432d), CV127 (AOCS0911-C), MON87705 (AOCS 0210-A), MON87708	

Table 6 *(continued)*

PCR test result	Reference material	Theoretical identity
	(AOCS 0311-A), DAS-44406-6 (ERM-BF436b), MON87769 (AOCS 0809-B) GM maize: Bt11 (ERM-BF412f), TC1507 (ERM-BF418d), DAS-59122-7 (ERM-BF424d), T25 (AOCS 0306-H), Bt176 (ERM-BF411f), NK603 (ERM-BF415f), MON863 (ERM-BF416d), MON88017 (AOCS 0406-D), MON89034 (AOCS 0906-E), DP-98140-6 (ERM-BF427d), MIR604 (ERM-BF423d), MIR162 (AOCS 1208-A), 3272 (ERM-BF420c), GA21 (ERM-BF414f), MON810 (ERM-BF413gk), DAS-40278-9 (ERM-BF433c), MON87460 (AOCS 0709-A) GM rapeseed: Topas 19/2 (AOCS 0711-D), T45 (AOCS 0208-A), Ms1 (AOCS 0711-A), Rf1 (AOCS 0711-B), Rf2 (AOCS 0711-C), Rf3 (AOCS 0306-G), Ms8 (AOCS 0306-F), GT73/RT73 (AOCS 0304-B), 73496 (ERM-BF434b), MON88302 (AOCS 1011-A) GM-cotton: 281-24-236 x 3006-210-23 (ERM-BF422b), LLCotton25 (AOCS 0306-E), GHB119 (ERM-BF428c), T304-40 (ERM-BF429c), MON1445 (AOCS 0804-B), MON531 (AOCS 0804-C), MON15985 (AOCS 0804-D), GHB614 (AOCS 1108-A), MON88913 (AOCS 0906-D) GM rice: LLRice62 (AOCS 0306-I) GM potato: EH92-527-1 (ERM-BF421b), AM04-1020 (ERM-BF430b), AV43-6-G7 (ERM-BF431e) GM sugar beet: H7-1 (ERM-BF419b) Plant species: *Beta vulgaris, Bertholletia excels, Sesamum indicum, Olea europaea, Nicotiana tabacum, Solanum tuberosum, Solanum melongena, Solanum lycopersicum, Helianthus annuus, Daucus carota, Linum usitatissimum, Medicago falcata x Medicago sativa, Trifolium pratense, Lupinus spec., Lens culinaris, Vigna radiata, Phaseolus vulgaris, Glycine max, Cicer arietinum, Arachis hypogaea, Cucurbita pepo, Prunus dulcis, Sinapis, Brassica napus, Zea mays, Sorghum bicolor, Setaria italic, Panicum miliaceum, Oryza sativa, Triticum spelta, Triticum aestivum, Secale cereale, Hordeum vulgare, Avena sativa*.	

9.2 Sensitivity

In a preliminary experiment, the absolute detection limits ($LOD_{95\ \%}$) for the *nos* and CaMV detection method were determined by modelling the detection probability.[5] For this purpose, three different laboratories examined dilution series of plasmids containing the target sequences. An $LOD_{95\ \%}$ of approximately 3 copies was calculated from the results for both methods. In the interlaboratory trials, detection limits of at least 3,3 copies for the *nos*-PCR and of 4 copies for the CaMV ORF V-PCR were determined (see Table 11).

9.3 Robustness

The robustness of the *nos* and the CaMV ORF V detection method was tested with respect to changes in the following factors: real-time PCR devices (ABI 7500, ABI 7500 Fast, Roche LC96, Mx3005P, Roche LC480, BioRad CFX96) and PCR master mix kits (Basic Mastermix, Eurofins GeneScan; QuanTi

Tect Probe PCR Mix, Qiagen; PerfeCTa qPCR ToughMix, Quanta Biosciences)[1]. These deviations of the experimental conditions did not cause any difficulties in detecting the target sequences. In the interlaboratory trials, no specific influence on the performance or difficulties were observed. Hence, both methods can be considered robust.

9.4 Interlaboratory trials

9.4.1 General

The reliability of the method was tested in interlaboratory trials coordinated by the Federal Office of Consumer Protection and Food Safety (BVL) with 19 participants in accordance with ISO 5725-2[6]. Each participant received 12 (*nos*) or 16 (CaMV ORF V) vials containing DNA solutions as test samples. Samples were labelled with randomized codes. In order to stabilize the DNA solutions, 15 ng/µl of genomic maize DNA was added as background DNA.

For the *nos* interlaboratory trial, six samples contained extracted DNA from *Rhizobium radiobacter* strains harbouring Ti plasmids. Four samples contained DNA from *Rhizobium radiobacter* strains lacking Ti plasmid and two other samples contained maize MIR604 genomic DNA (see Table 7).

All copy number estimations of the target sequences for extracted DNAs were performed by digital PCR. The DNA concentrations were determined fluorometrically. The concentrations of the MIR604 and MON810 DNAs (100 copies/µl) were provided by the manufacturer.

Table 7 — *nos* interlaboratory trial test samples

Sample	Reference material for DNA extraction (origin)	Nominal concentrations of the target sequences (copies/µl)		
		nos	**T-*nos***	**Maize DNA**[a]
1 to 3	ATCC 33970D-5 (*Rhizobium radiobacter* strain C58)	5	5	5770
4 to 6	DSM-30208 (*Rhizobium radiobacter* strain H-100)	5	5	5770
7 to 8	DSM-30206 (*Rhizobium radiobacter* strain F/1)	—	—	5770
to 10	DSM-30205 (*Rhizobium radiobacter* strain B 6)	—	—	5770
to 12	GM maize MIR604 (ERM BF423d)	—	5	5870

[a] Calculated copy number for maize reference gene-specific PCR (due to the addition of 15 ng/µl maize DNA as background DNA).

For the CaMV ORF V interlaboratory trial, eight samples contained extracted DNA from radish leaf material (*Raphanus sativa*) infected with different CaMV reference strains. Six samples contained DNA from non-infected radish leaf material (negative control material), and two samples contained maize MON810 genomic DNA (see Table 8).

1) This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products. Equivalent products from other manufacturers may be used if they can be shown to give equivalent or better results.

Table 8 — CaMV ORF V interlaboratory trial test samples

Sample	Reference material for DNA extraction (origin)	Nominal concentrations of the target sequences (copies/µl)		
		CaMV ORF V	P-35S	Maize DNA[a]
1 to 2	DSMZ PV-0226 CaMV (cabbage, Denmark)	5	5	5770
3 to 4	DSMZ PV-0227 CaMV (John Innes Institute)	5	5	5770
5 to 6	DSMZ PV-0228 CaMV (M.Schröder BBA)	5	5	5770
7 to 8	DSMZ PV-0229 CaMV (H.J. Vetten BBA)	5	5	5770
9 to 14	DSMZ NC-0206 negative control (*Raphanus sativa)*	—	—	5770
15 to 16	GM maize MON810 (ERM BF413f)	—	5	5820

[a] Calculated copy number for maize reference gene-specific PCR (due to the addition of 15 ng/µl maize DNA as background DNA).

All sample DNAs were analysed as single determinations and additionally using in-house established PCR methods for T-*nos*, P-35S and a maize reference gene.

To perform the PCR, participants were provided with the oligonucleotides and a master mix suitable for real-time PCR. As positive controls and to validate the detection limits and PCR efficiencies, participants were also provided with DNA solutions of control plasmids (pGSE266; pGSE660) with target sequence concentrations of nominally 500 copies/µl and a buffer solution (0.1x TE with 20 µg/ml salmon sperm DNA).

9.4.2 False-positive rate/false-negative rate

Table 9 provides an overview on the results of the interlaboratory trials with information on the false-positive and false-negative rates. Results from 19 laboratories were available.

Table 9 — Evaluation of the results of the interlaboratory trials

Parameter	*nos*	CaMV ORF V
Number of participating laboratories	19	19
Number of laboratories that submitted PCR results	19	19
Number of laboratories with PCR results included in the evaluation	19	19
Samples per laboratory	12	16
Accepted PCR results (total)	227	304
PCR results for samples containing the target sequence	113	152
PCR results for samples that did not contain the target sequence	114	152
False positive PCR results (rate in per cent)	0 (0 %)	1 (0,7 %)
False-negative PCR results (rate in per cent)	0 (0 %)	0 (0 %)

For the *nos*-positive samples 1 to 6, one laboratory did not provide a result for sample 6, thus a total of 113 PCR results were received. No false-positive or false-negative results were submitted.

For the CaMV positive and negative samples, 152 PCR results each could be used for the evaluation. One false-positive result and no false-negative results were submitted.

The mean Cq value for the *nos*-positive samples was 34 (Cq range from 30,7 to 36,8). T-*nos* PCR results were also reported for the samples from 18 participants. For all *nos*-positive samples, positive PCR results were also reported for T-*nos*. The mean Cq value for T-*nos* was 34,2 (Cq range of 30,9 to 39,8). For *nos*-negative samples 7 to 12, weak T-*nos* amplification signals were obtained in three cases (Cq mean 37,9). Positive results (Cq mean 24,4) were given for all sample DNAs tested with regard to the maize reference gene-specific PCR. For the two MIR604 GM maize DNA samples, T-*nos* positive PCR results (Cq mean of 33,1) and for *nos* exclusively negative results were reported by all laboratories.

The mean Cq value for the CaMV-positive samples was 33,2 (Cq range of 30,0 to 36,6). A Cq value of 39,9 was reported for the false-positive result. Samples from 18 participants were also tested for P-35S and corresponding PCR results were reported. Only in one case, no positive P-35S PCR result was reported for CaMV-positive samples 1 to 8. The mean Cq value for P-35S was 33,9 (Cq range of 26,9 to 41,8), which was in the similar Cq value range as the CaMV ORF V-PCR. For CaMV-negative samples 9 to 16, weak P-35S amplification signals were obtained in six cases (Cq mean 38,8). Positive PCR results (Cq mean 24,4) for maize reference gene-specific PCR were reported for all sample DNAs tested. For the two MON810 GM maize DNA samples, P-35S positive PCR results (Cq mean of 34,2) were reported by all laboratories and only negative results were reported for CaMV ORF V.

9.4.3 PCR efficiency and detection limit

Serial dilution series had to be prepared by the participants of the interlaboratory trials with the provided control plasmid DNA solutions and buffers. Four concentration levels with nominally 500, 100, 20 and 4 copies/µl of the target sequences had to be prepared and measured as triplicate determinations. Based on the Cq values for the standard curves, the values for slopes, correlation coefficients and PCR efficiencies were calculated for each laboratory (see Table 10).

Table 10 — Evaluation of the standard curves for the determination of PCR efficiency

Laboratory	PCR device	Slope (s)		Correlation coefficient (R^a)		PCR efficiency (%)	
		nos	CaMV ORF V	*nos*	CaMV ORF V	*nos*	CaMV ORF V
A	ABI 7900HT	−3,44	−2,91	0,99	1,00	95	121
B	ABI QS6	−3,44	−3,24	1,00	1,00	95	104
C	ABI 7900HT	−3,50	−3,12	1,00	1,00	93	109
D	ABI 7500	−3,24	−3,25	0,96	0,99	104	103
E	ABI 7500 Fast	−3,53	−3,18	0,99	0,99	92	106
F	Roche LC96	−3,68	−3,25	0,99	0,99	87	103
G	BioRad CFX96	−3,64	−3,31	0,99	1,00	88	101
H	ABI 7900HT	−3,59	−3,32	1,00	1,00	90	100
I	BioRad CFX96	−3,38	−3,14	0,99	0,99	97	108
K	Mx3005P	−3,49	−3,07	0,99	0,99	94	112
L	BioRad CFX96	−3,52	−3,11	1,00	1,00	92	110
M	Mx3005P	−3,37	−3,42	1,00	1,00	98	96
N	ABI QS5	−3,42	−3,43	1,00	0,99	96	96
O	ABI 7500 Fast	−2,96	−3,47	0,99	1,00	118	94
P	ABI 7500	−3,47	−3,24	1,00	0,99	94	104
Q	ABI 7500	−3,14	−3,17	1,00	0,99	108	107
R	Mx3005P	−3,46	−3,23	0,99	0,98	95	104
S	ABI 7900HT	−3,79	−3,35	0,99	0,96	84	99
T	Mx3005P	−3,52	−3,31	1,00	0,99	92	100

[a] This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products. Equivalent products from other manufacturers may be used if they can be shown to give equivalent or better results.

In addition, six concentration levels in the range of the detection limit of the PCR methods (nominal copy numbers of 2 / 1 / 0,4 / 0,2 / 0,1 and 0,02 per µl) were examined. Six PCR replicates each with 5 µl DNA solution per PCR should be determined by each participant.

The evaluation of all PCR results submitted was carried out according to the mathematical-statistical procedure for calculating the limit of detection ($LOD_{95\ \%}$) of the laboratory standard deviations and other performance characteristics relating to the probability of detection (POD).[5] A summary of the results is provided in Table 11. One laboratory was not included in the statistical evaluation of the *nos*