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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

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Foreword

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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).

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This document was prepared by Technical Committee ~~16~~ISO/TC 34, *Food* ~~Products~~*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.

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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~~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.

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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.

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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*

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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 ~~Molecular biomarker analysis — Vocabulary for molecular biomarker analytical methods in agriculture and food production~~ apply.

ISO and IEC maintain ~~terminological~~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.

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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]

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5 Reagents and materials

Chemicals of recognized analytical grade, appropriate for molecular biology shall be used~~, as a rule~~. 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 PCR reagents**~~

~~5.1.1~~ 5.1 Thermostable DNA polymerase, (for hot-start PCR). PCR buffer solution

~~Contains~~, 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).

~~5.1.2~~ 5.3 Oligonucleotides

~~(~~. See Tables 1 and 2~~)~~.

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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'-5′-CCA gCC RTS TAC TgA TTA TTg TMA C-3'3′	300 nmol/l
At-nop-r2	5'-5′-TgC gAg TTC RCC gTT gAA g-3'3′	300 nmol/l
At-nop-s1	5'-(5′-(FAM)-CCg TgC ggA CgT TCA CgA CAg-(BHQ1)-3'[a]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'-5′-ATY AAg CCC AgY AAA AgC CC-3'3′	300 nmol/l
CaMV-ORFV-rd2	5'-5′-CTY CgC TTC TCg gCT TCR TT-3'3′	300 nmol/l
CaMV-ORFV-P2	5'-(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.		

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

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

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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).:

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

~~Number of genome equivalents per µl = DNA concentration [ng/µl]*1000 / genome mass [fg] (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.

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6 Apparatus

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

6.1 Real-time PCR device

~~Shall~~, 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

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7.3.1 The method description applies to a total volume of 25 µl per reaction mixture with the setup given in ~~Table~~ Tables 3 and ~~Table~~ 4~~, respectively~~. 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.

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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 ~~program~~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

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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 ~~program~~programme.

7.3.~~1~~2 Mix the PCR reagent mixture, centrifuge briefly and pipette 20 µl into each reaction vial.

7.3.~~2~~3 For the amplification reagent control, add 5 µl of water into the respective reaction setup.

7.3.~~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.~~4~~5 If necessary, prepare a PCR inhibition control as specified in ISO 24276.

7.3.~~5~~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 ~~program~~programme

The temperature-time ~~program~~programme as outlined in Table 5 ~~has been~~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.

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Table 5 — Temperature-time ~~program~~programme

<table>
<tr><th>Step</th><th colspan="2">Parameter</th><th>Temperature</th><th>Time</th><th>Cycles</th></tr>
<tr><td>1</td><td colspan="2">Initial denaturation</td><td>95 °C</td><td>2 min</td><td>1</td></tr>
<tr><td rowspan="2">2</td><td rowspan="2">Amplification</td><td>Denaturation</td><td>95 °C</td><td>15 s</td><td rowspan="2">45</td></tr>
<tr><td>Annealing and Elongation</td><td>60 °C</td><td>90 s</td></tr>
</table>

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8 Accept/~~Reject~~reject criteria

8.1 General

The evaluation of PCR amplification results is performed with the respective device-specific data analysis ~~program~~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).

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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).

~~NOTE:~~ 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 ~~must~~shall be clarified (see ~~section~~ 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 ~~must~~shall be achieved in the amplification control approaches (positive DNA target control, PCR inhibition control).

~~NOTE:~~ 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.

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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~~, respectively (~~ (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~~]. 14~~). Fourteen sequences (12 %) showed one mismatch to the primer and probe sequences. For five of these sequences, the mismatch is located at the ~~5'-~~5′-terminal nucleotide of the probe and is therefore unlikely to affect the ~~5'-~~5′-nuclease activity of the polymerase and detection~~, respectively.~~. 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~~, respectively.~~. In addition, other bacterial, plant and DNAs from diverse

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