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

## Part 5: Real-time PCR based screening method for the detection of the FMV promoter (P-FMV) DNA sequence

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## National foreword

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

## Part 5: Real-time PCR based screening method for the detection of the FMV promoter (P-FMV) DNA sequence

*Méthodes horizontales d'analyse moléculaire de biomarqueurs — Méthodes d'analyse pour la détection des organismes génétiquement modifiés et des produits dérivés —*

*Partie 5: Méthode de dépistage PCR en temps réel pour la détection de la séquence ADN du promoteur FMV (P-FMV)*

Reference number
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# Foreword

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The committee responsible for this document is ISO/TC 34, *Food products*, Subcommittee SC 16, *Horizontal methods for molecular biomarker analysis*.

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

TECHNICAL SPECIFICATION ISO/TS 21569-5:2016(E)

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

# Part 5: Real-time PCR based screening method for the detection of the FMV promoter (P-FMV) DNA sequence

## 1 Scope

This document specifies a procedure for the detection of a DNA sequence used in genetically modified (GM) plants by means of a real-time PCR (polymerase chain reaction). The method detects a 78 base pairs long segment of the *Figwort mosaic virus* 34S promoter DNA sequence. This segment in some GM plants is indicated as FMV promoter (P-FMV) and in other GM plants as FMV enhancer (E-FMV).

The method was developed and validated for the analysis of DNA extracted from foodstuffs. It may be suitable also for analysis of other products such as feedstuffs and seeds. The procedure requires the extraction of an adequate quantity and quality of amplifiable DNA from the test sample.

The DNA sequence amplified by the P-FMV element-specific method can be detected in samples which contain DNA of the naturally occurring *Figwort mosaic virus*. For this reason, it is necessary to confirm a positive screening result. Further analyses are required using construct-specific or event specific methods.

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

ISO 21570, *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Quantitative nucleic acid based methods*

ISO 21571:2005, *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 terminological databases for use in standardization at the following addresses:

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

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

# 4 Principle

DNA is extracted from the test portion applying a suitable method (see ISO 21571). The DNA analysis consists of two parts:

a) verification of the amount, quality and amplifiability of the extracted DNA, e.g. by a taxon-specific PCR assay (according to ISO 21569 and ISO 21570), see also Reference [1];

b) detection of the P-FMV DNA sequence in a real-time PCR, see Reference [2].

# 5 Reagents and materials

## 5.1 General

For the purpose of this document, only chemicals and water of recognized analytical grade, appropriate for molecular biology shall be used. Unless stated otherwise, solutions should be prepared by dissolving the corresponding reagents in water and be autoclaved. For all operations for which gloves are used it should be ensured that these are powder-free. The use of aerosol protected pipette tips (protection against cross contamination) is recommended.

## 5.2 PCR reagents

**5.2.1 Thermostable DNA polymerase** (for hot-start PCR).

**5.2.2 PCR buffer solution** (containing magnesium chloride and deoxyribonucleoside triphosphates dNTPs.

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

**5.2.3 Oligonucleotides** (see Table 1)[1)].

**Table 1 — Oligonucleotides**

| Name | DNA sequence of the oligonucleotide | Final concentration in PCR |
|---|---|---|
| P-FMV as the target sequence (GeneBank accession number X06166[2],[3]): | | |
| pFMV-F | 5'-CAA AAT AAC GTG GAA AAG AGC T–3' | 340 nmol/l |
| pFMV-R | 5'-TCT TTT GTG GTC GTC ACT GC–3' | 340 nmol/l |
| Probe pFMV | 5'-(FAM)-CTG ACA GCC CAC TCA CTA ATG C-(BHQ1)-3'[a] | 120 nmol/l |

[a] FAM: 6-Carboxyfluorescein, BHQ-1: Black Hole Quencher® 1 (non-fluorescent chromophore). This information is given for convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products from other manufacturers may be used if they can be shown to give equivalent or better results.

# 6 Apparatus

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

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

1) In the interlaboratory trial performed for P-FMV, participants were provided with dried aliquots (per 50 reactions) of primer/probe -mixes (to be stored in dark until the start of the interlaboratory trial). Per aliquot 375 µl PCR grade water was added and allowed to settle.

# 7 Procedure

## 7.1 Preparation of test sample

It should be ensured that the test sample used for DNA extraction is representative of the laboratory sample, e.g. by grinding or homogenizing of the laboratory sample. Measures and operational steps to be taken into consideration should be according to ISO 21571 and ISO 24276.

## 7.2 Preparation of DNA extracts

Concerning the preparation of DNA from the test portion the general instructions and measures described in ISO 21571 shall be followed. It is recommended to choose one of the DNA extraction methods described in ISO 21571:2005, Annex A.

## 7.3 PCR setup

The method described applies for a total volume of 25 µl per PCR. The reaction setup is given in Table 2.

Reagents are completely thawed at room temperature. Each reagent should be carefully mixed and briefly centrifuged immediately before pipetting. A PCR reagent mixture is prepared which contains all components except for the sample DNA. The required amount of the PCR reagent mixture depends on the number of reactions to be performed, including at least one additional reaction as a pipetting reserve. Add 5 µl of sample DNA to each reaction.

**Table 2 — Reaction setup for the amplification**

| | |
|---|---|
| Total reaction volume | 25 µl |
| Sample DNA (up to 200 ng) or controls | 5 µl |
| PCR buffer solution[a] (including $MgCl_2$, dNTPs and hot-start DNA polymerase) | 12,5 µl |
| Primer pFMV-F and pFMV-R | see Table 1 |
| Probe pFMV | see Table 1 |
| Water | to 25 µl |

[a] In the collaborative trial the QuantiTect Multiplex PCR NoROX Kit (Qiagen GmbH, Hilden/Germany) was used. This information is given for convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products from other manufacturers may be used if they yield similar or better results. If necessary, adapt the amounts of the reagents and the temperature-time programme.

Mix the PCR reagent mixture, centrifuge briefly and pipette 20 µl into each reaction vial. For the amplification reagent control, add 5 µl of water into the respective reaction setup. Pipette either 5 µl of sample DNA or 5 µl of the respective control solution (extraction blank control, positive DNA target control). If necessary, prepare a PCR inhibition control as described in ISO 24276.

Transfer the reaction setups into the thermal cycler and start the temperature-time programme.

## 7.4 Temperature-time programme

The temperature-time programme as outlined in Table 3 has been used in the validation study. The use of different reaction conditions and real-time PCR cyclers may require specific optimization. The time for initial denaturation depends on the master mix used.

**Table 3 — Temperature-time programme**

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

# 8 Accept/reject criteria

## 8.1 General

A corresponding real-time PCR device-specific data analysis programme is used for the identification of PCR products. The amplification results may be expressed in a different manner, depending on the device used. In the absence of detectable PCR products (e.g. negative controls) the result can be expressed as "undetermined", "no amp", or the maximum number of reaction cycles performed. If the amplification of the DNA target sequence in a sample (e.g. positive controls) occurred, a sigmoid shaped amplification curve should be observed. The cycle number at the crossing point of the amplification curve and the fluorescence threshold is calculated ($C_t$ value or $C_p$ value).

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

A positive result can be also obtained if the sample contains DNA derived from the *Figwort mosaic virus*, which can naturally infect plants. To proof the presence of a GM plant derived product additional tests using construct-specific or event specific methods are required for confirmation.

## 8.2 Identification

The target sequence is considered as detected, if

— by using the P-FMV specific primers pFMV-F and pFMV-R and the probe pFMV, a sigmoid shaped amplification curve is observed and a $C_t$ value or $C_p$ value is calculated,

— in the PCR control reactions with no added DNA (PCR reagent control, extraction blank control), no amplification has occurred, and

— in the reactions for the amplification controls (positive DNA target control, PCR inhibition control) the expected $C_t$ values (or $C_p$ values) are achieved.

# 9 Validation status and performance criteria

## 9.1 General

Validation followed a two part process:

a) in-house validation followed by a pilot interlaboratory study;

b) a collaborative trial validation.

## 9.2 Robustness

The robustness of the method was tested with respect to small changes in the conditions of the following factors: real-time PCR-instruments[2] (ABI7500, Rotor-Gene Q, Stratagene MX 3005P); PCR-master mix kits (QuantiTect Multiplex PCR NoROX Kit, SureMaster Probe Kit); volumes, 19 µl or 21 µl master mix plus 5 µl sample DNA); annealing temperature (59 °C and 61 °C); and primer or probe concentration (both lowered by 30 % respectively). For each tested factor three PCR replicates were analysed, each with 20 copies of the target sequence. In these tests the method showed positive results under the changed conditions in all PCR reactions as expected and thus the method can be considered robust.

## 9.3 Collaborative trial

The reliability of the method was verified in a collaborative trial with 16 participants, organized by the CVUA Freiburg in accordance with the IUPAC protocol.[4] The participants received 18 DNA samples for the analysis. The samples contained the P-FMV target sequence at various concentrations, or did not contain the target sequence. All samples were labelled with random coding numbers. Each participant received 3 vials (triplicate-blind) containing the following DNA solutions:

— non GM rapeseed;

— non GM soy;

— GT73 rapeseed, adjusted to a calculated concentration of 4 copies/µl;

— GT73 rapeseed, adjusted to a calculated concentration of 10 copies/µl;

— MON89788 soy, adjusted to a calculated concentration of 4 copies/µl;

— MON89788 soy, adjusted to a calculated concentration of 10 copies/µl.

For conducting the PCR experiments participants received the PCR master mix and the oligonucleotides (primers and probes).

To prepare the samples, genomic DNA was extracted from ground seeds of GT73 canola (AOCS 0304-B from AOAC Boulder/USA) and of MON89788 soy (AOCS 0906-B).[3] The DNA was adjusted with 0,2x TE buffer to a nominal concentration of 10 copies/µl and 4 copies/µl of the target sequence in a background of 20 ng/µl of rapeseed or soy DNA.

The DNA concentrations were determined by fluorescence detection using the PicoGreen assay.[5] The copy numbers were established on the basis of the genome equivalents. For soy a haploid genome mass of 1,15 pg and for rapeseed of 1,23 pg was taken.[6] The integration of one copy of the P-FMV target sequence in rapeseed GT73 and soy MON89788 genome was assumed,[7],[8] and the GM material was considered homozygous.

To prepare samples not containing the target sequence, DNA was extracted from non GM rapeseed or soy (market samples) and adjusted to a concentration of approximately 20 ng/µl.

The DNA samples and a plasmid DNA standard, were tested for the presence of FMV DNA using the method described in Annex A.[9] Negative results were obtained in each case.

The collaborative trial was designed for calculating the false positive and false negative rates. Each DNA sample was tested by the participants in a single PCR test with 5 µl of the respective DNA solution, using the procedure and the conditions given in Tables 2 and 3. The results of the collaborative trial are listed in Table 4, more details on precision are provided in Table 5.

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2) This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the products named. Equivalent products may be used if they can be shown to lead to the same results.

3) This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the products named. Equivalent products may be used if they can be shown to lead to the same results.

**Table 4 — Results of the collaborative trial**

| | |
|---|---|
| Number of laboratories | 16 |
| Number of laboratories submitting results | 16 |
| Number of samples per laboratory | 18 |
| Number of accepted results | 288 |
| Number of accepted samples containing the P-FMV target sequence | 192 |
| Number of accepted samples not containing the P-FMV target sequence | 96 |
| False-positive results | 0 |
| False-positive results [%] | 0 |
| False-negative results | 2 |
| False-negative results [%] | 1,0 |

In order to calculate the corresponding copy numbers from the $C_t$ values determined from the DNA samples and to establish the LOD, each participant additionally received a plasmid DNA solution (adjusted to 800 copies/µl of the target sequence in 20 ng/µl sonicated salmon-sperm DNA). The plasmid DNA concentration was measured by digital PCR (QX100 Droplet Digital PCR System, Bio-Rad GmbH, Munich/Germany[4)]). To produce serial dilutions, the participants received as dilution buffer a 0,2x TE buffer containing 20 ng/µl sonicated salmon sperm DNA. The plasmid DNA standards (dilutions with 800, 200, 40, 10 copies/µl) were measured twofold in the same PCR run with the DNA samples. Table 5 summarizes the results achieved with the DNA samples.

A quantification of the target sequence copy number might be possible above 50 copies per PCR with sufficient precision. The verification of the method for copy number quantification, however, was not subject of the collaborative trial.

**Table 5 — Precision results obtained in the collaborative trial**

| Samples (nominal target copy number) | No of laboratories | Positive tests/ total tests | Copy numbers calculated | | | $RSD_R$[c] % |
|---|---|---|---|---|---|---|
| | | | Mean | $S_r$[a] | $S_R$[b] | |
| GT73 rapeseed (50 copies) | 16 | 48/48 | 76,6 | 17,1 | 23,4 | 30,5 |
| GT73 rapeseed (20 copies) | 16 | 46/48 | 33,6 | 7,8 | 11,0 | 32,8 |
| MON89788 soy (50 copies) | 16 | 48/48 | 39,5 | 10,1 | 11,7 | 29,7 |
| MON89788 soy (20 copies) | 16 | 48/48 | 13,5 | 5,8 | 6,3 | 46,6 |
| Non GM rapeseed | 16 | 0/48 | – | – | – | – |
| Non GM soy | 16 | 0/48 | – | – | – | – |

[a] Repeatability standard deviation.

[b] Reproducibility standard deviation.

[c] Relative reproducibility standard deviation.

## 9.4 Sensitivity

The method developer showed that the absolute limit of detection (LOD) for the method is around 5 DNA copies to 10 DNA copies.

In the collaborative trial, the plasmid DNA was further diluted for the determination of the LOD. Serial dilutions were produced by the 16 laboratories in the range of 0,02 copies/µl to 4 copies/µl using 0,2x TE buffer containing 20 ng/µl sonicated salmon sperm DNA. Each participant measured 10 replicates per concentration level. A positive result was still achieved for 5 copies per PCR in 160 out of 160 tests (Table 6).

4) This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the products named. Equivalent products may be used if they can be shown to lead to the same results.

In addition, the probability of detection (POD) of the P-FMV method was examined according to a new mathematical-statistical model.[11] The qualitative data of all PCR test results at the different dilution levels were taken to estimate the laboratory standard deviations $\sigma_L$ representing the relative between-laboratory variability at POD = 0.95. For the P-FMV method, a value of 0,23 was obtained.

**Table 6 — Collaborative trial results for the limit of detection (LOD)**

| P-FMV copy number per PCR (nominal) | Number of positive results $C_t < 45$) out of 160 results |
|---|---|
| 20 | 160 |
| 10 | 160 |
| 5 | 160 |
| 1 | 96 |
| 1 diluted 1:10 | 24 |

## 9.5 Specificity

The P-FMV target sequence is part of the promoter region of gene VI in the genome of *Figwort mosaic virus*.[2] The amplified segment is part of a sequence which is used as promoter or enhancer for gene expression in several GM plants (Table 7). In MON1445, MON89034, GT73 and H7-1 the inserted element is indicated as FMV promoter (P-FMV), in MON89788, MON87705, MON88913 and MON88302 the inserted element is indicated as FMV enhancer (E-FMV).

The performances specified in Table 7 were established with DNA from different GM plants. Theoretically expected data were established by queries in public databases.[12],[13]

In addition, P-FMV was detected with DNA from figwort mosaic virus and several plant transformation vectors (pMON99036, pMON93914) show complete identity to the target sequence according to sequence data. This was established by comparing the target sequence to the sequence data in the GenBank nucleotide sequence collection ("non redundant" database with all GenBank, RefSeq, EMBL, DDBJ and PDB sequences) and the database for patented nucleotide sequences by using the programme BLASTN.

In Annex A, a method for detection of another sequence of the *Figwort mosaic virus* genome is described.[9] In case of a positive signal for the P-FMV target sequence this method may allow testing for a contamination by the native FMV.

**Table 7 — Specificity of the P-FMV detection method**

| GM plant | Theoretically expected | Experimental confirmation |
|---|---|---|
| **Alfalfa** | | |
| J101 | + | n.d. |
| J163 | + | n.d. |
| **Cotton** | | |
| MON 1445 | + | + |
| MON 88913 | + | + |
| LL25 | - | - |
| MON 1076 | + | n.d. |
| MON 1698 | + | n.d. |
| **Maize** | | |
| MON 89034 | + | + |
| 3272 | - | - |
| 5912 | - | - |
| Bt176 | - | - |

**Table 7** *(continued)*

| GM plant | Theoretically expected | Experimental confirmation |
|---|---|---|
| Bt11 | - | - |
| GA21 | - | - |
| TC1507 | - | - |
| MIR604 | - | - |
| MON 810 | - | - |
| MON 863 | - | - |
| NK603 | - | - |
| T25 | - | - |
| CBH-351 | - | - |
| MON 88017 | - | - |
| **Papaya** | | |
| 55-1 | - | - |
| 63-1 | - | - |
| **Potato** | | |
| HLMT15-3 | + | n.d. |
| HLMT15-15 | + | n.d. |
| HLMT15-46 | + | n.d. |
| **Rapeseed** | | |
| GT73 | + | + |
| MON 88302 | + | + |
| MS8 | - | - |
| RF3 | - | - |
| Falcon GS40/90 | - | - |
| OXY-235 | - | - |
| GT200 | + | n.d. |
| ZSR500/502/503 | + | n.d. |
| **Rice** | | |
| LL601 | - | - |
| LL62 | - | - |
| Bt63 | - | - |
| **Sugar beet** | | |
| H7-1 | + | + |
| GTSB77 | + | n.d. |
| **Soybean** | | |
| A2704-12 | - | - |
| GTS 40-3-2 | - | - |
| 305423 | - | - |
| 356043 | - | - |

## 10 Test report

The test report should be carried out as specified in ISO 24276 and other applicable standards (e.g. ISO/IEC 17025.

# Annex A
(informative)

# Detection of the *Figwort mosaic virus* (FMV) open reading frame VII

The target sequence is a 113 base pair long DNA sequence from the open reading frame VII region of the *Figwort mosaic virus* (FMV ORF VII).[10]

The validation of the method described in this annex was not part of the collaborative trial. For detailed information about sensitivity, specificity and robustness of the method see Reference [9].

**Table A.1 — Oligonucleotides**

| Name | DNA sequence of the oligonucleotides | Final concentration in the PCR |
|---|---|---|
| FMV ORF VII as target sequence[9],[10] | | |
| FMVorf7-F4 | 5'-TAT GCT AGG CTT AAC GGG CTT C-3' | 400 nmol/l |
| FMVorf7-R4 | 5'-GGA TCC GAG ATT TTC TTG AAG TT-3' | 400 nmol/l |
| Probe FMVorf7-FAM | 5'-(FAM)-TCA TCT CCA AGC GAG AAT TTC AGC TGT TTC-(BHQ1)-3'[a] | 200 nmol/l |
| [a] FAM: 6-carboxyfluorescein, BHQ-1: Black Hole Quencher® 1 (non-fluorescent chromophore). 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 yield similar or better results. If necessary, adapt the amounts of the reagents and the temperature-time programme. | | |

**Table A.2 — Reaction setup for the amplification**

| | |
|---|---|
| Total reaction volume | 25 µl |
| Probe-DNA (up to 200 ng) or control DNA | 5 µl |
| PCR-buffer solution[a] (containing $MgCl_2$, dNTP and hot-start DNA Polymerase) | 12,5 µl |
| Primer FMVorf7-F4 and FMVorf7-R4 | see Table A.1 |
| Probe FMVorf7-FAM | see Table A.1 |
| Water | to 25 µl |
| [a] QuantiFast Probe PCR Kit (Qiagen GmbH, Hilden/Germany). 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 yield similar or better results. If necessary, adapt the amounts of the reagents and the temperature-time programme. | |

**Table A.3 — Temperature-time programme**

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

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