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

Part 2: Construct-specific real-time PCR method for detection of event FP967 in linseed and linseed products

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

Partie 2: Méthode PCR en temps réel spécifique de la construction pour la détection d'un évènement FP967 dans les graines de lin et les produits à base de graines de lin

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Foreword

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

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

In other circumstances, particularly when there is an urgent market requirement for such documents, a technical committee may decide to publish other types of document:

— an ISO Publicly Available Specification (ISO/PAS) represents an agreement between technical experts in an ISO working group and is accepted for publication if it is approved by more than 50 % of the members of the parent committee casting a vote;

— an ISO Technical Specification (ISO/TS) represents an agreement between the members of a technical committee and is accepted for publication if it is approved by 2/3 of the members of the committee casting a vote.

An ISO/PAS or ISO/TS is reviewed after three years in order to decide whether it will be confirmed for a further three years, revised to become an International Standard, or withdrawn. If the ISO/PAS or ISO/TS is confirmed, it is reviewed again after a further three years, at which time it must either be transformed into an International Standard or be withdrawn.

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.

ISO/TS 21569-2 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 16, *Horizontal methods for molecular biomarker analysis*.

ISO/TS 21569 consists of the following parts, under the general title *Horizontal methods for molecular biomarker analysis — Methods of analysis for the detection of genetically modified organisms and derived products*:

— *Part 2: Construct-specific real-time PCR method for detection of event FP967 in linseed and linseed products*

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

Part 2: Construct-specific real-time PCR method for detection of event FP967 in linseed and linseed products

1 Scope

This method describes a procedure for the detection of a DNA sequence present in a genetically modified linseed (*Linum usitatissimum*) line (event FP967, also named as "CDC Triffid"). For this purpose, extracted DNA is used in a real-time PCR and the genetic modification (GM) is specifically detected by amplification of a 105 bp DNA sequence representing the transition between the nopalin synthase gene terminator (*Tnos*) from *Agrobacterium tumefaciens* and the dihydrofolate reductase gene (*dfrA1*) from a Class 1 integron of *Escherichia coli*.

The method described is applicable for the analysis of DNA extracted from foodstuffs. It may also be suitable for the analysis of DNA extracted from other products such as feedstuffs and seeds. The application of this method requires the extraction of an adequate amount of amplifiable DNA from the relevant matrix for the purpose of analysis.

2 Normative references

ISO 21569, *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Qualitative 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 24276 apply.

4 Principle

DNA is extracted from the test sample applying a suitable method. The DNA analysis consists of two parts:

a) Verification of the amount, quality and amplifiability of the extracted DNA, e.g. by means of a target taxon specific real-time PCR with primers amplifying a 68 bp long fragment from the linseed-specific (*Linum usitatissimum*) stearoyl-acyl carrier protein desaturase 2 gene (SAD) (Reference [1]).

b) Detection of the *Tnos-dfr* construct in a real-time PCR (Reference [1]).

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 of an adequate quality. Unless stated otherwise, solutions should be prepared by dissolving the corresponding reagents in water and autoclaved. For all operations in which gloves are used, it should be ensured that these are powder-free. The use of aerosol-protected pipette tips serves as protection against cross-contamination.

5.1 PCR reagents

5.1.1 Thermostable DNA polymerase (for hot-start PCR).

5.1.2 PCR buffer solution (contains magnesium chloride and deoxyribonucleoside triphosphate dATP, dCTP, dGTP and dUTP).

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

5.1.3 Oligonucleotides (see Table 1).

Table 1 — Oligonucleotides

Name	DNA sequence of the oligonucleotide	Final concentration in the PCR
Tnos-dfr construct as the target sequence (Reference [1]):		
NOST-Spec FW	5'-AgC gCg CAA ACT Agg ATA AA-3'	800 nmol/l
NOST-Spec RV	5'-ACC TTC Cgg CTC gAT gTC TA-3'	800 nmol/l
NOST-Spec Probe	5'-(FAM)-CgC gCg Cgg TgT CAT CTA Tg-(BHQ)-3'[a]	100 nmol/l
[a] FAM: 6-Carboxyfluorescein, BHQ: black hole quencher.		

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

5.1.4 Standard DNA for calibration

A standard DNA solution of a known concentration (ng/µl) is used to calculate the copy numbers of the *Tnos-dfr* target sequence.

When using genomic linseed DNA as the standard DNA, the number of haploid genome equivalents per microlitre, n_{hgEq}, shall be calculated on the basis of the molecular mass of the linseed haploid genome which is approximately 0,7 pg (Reference [2]) and by applying Equation (1):

$$n_{hgEq} = \frac{[DNA] \times 1\,000}{m_{hg}} \quad (1)$$

where

[DNA] is the DNA concentration in nanograms per microlitre;

m_{hg} is the haploid genome mass, in picograms.

In the collaborative trial, a plasmid was used as standard DNA which contains a copy of the 105 bp *Tnos-dfr* fragment and the 68 bp large SAD gene fragment, respectively. Because the exact number of integrations of the *Tnos-dfr* construct in event FP967 in linseed is not known at the time of the specification of this document, the calculated GM-content only represents an estimation which is based on the assumption that the target sequence is present as a single copy per haploid genome.

6 Apparatus

6.1 General

Regarding the apparatus and materials, see ISO 21569. In addition to the usual laboratory equipment the following equipment is required.

6.2 PCR device

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

7 Sampling

All samples shall be identified unambiguously.

8 Procedure

8.1 Test sample preparation

It should be ensured that the test sample used for DNA extraction is representative of the laboratory sample, e.g. by grinding or homogenizing the samples. Take into consideration the measures and operational steps specified in ISO 21571 and ISO 24276.

8.2 Preparation of the DNA extracts

Concerning the preparation of DNA from the test sample, the general instructions and measures described in ISO 21571 should be followed. It is recommended that one of the DNA extraction methods described in ISO 21571:2005, Annex A be chosen.

8.3 DNA extraction

It is recommended that the DNA extraction be performed by means of the CTAB method with a test portion of 1 g of the homogenized sample (see ISO 21571:2005, A.3.1).

Due to problems of purity, an additional purification step (gel filtration, e.g. by means of micro spin columns) may be necessary.

As long as comparability is ensured, other extraction and purification methods (e.g. kit systems) can be applied, using lower test portions, if necessary (Reference [1]).

8.4 PCR setup

The method is described for a total volume of 25 µl per PCR. The reagents given in Table 2 should be used.

Reagents are completely thawed at room temperature and should be briefly centrifuged before use. Each reagent should be carefully mixed immediately before pipetting. A 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. A volume of 5 µl of sample DNA is used.

Table 2 — Addition of reagents

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	see Table 1
Probe	see Table 1
Water	add to obtain 25 µl

[a] In the collaborative study, TaqMan Universal Mastermix (Applied Biosystems) was used as the PCR buffer solution. This information is given for the 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. If necessary, adapt the amounts of the reagents and the temperature-time programme.

Mix the reagent mixture, centrifuge briefly and pipette 20 µl into each reaction vial. For the PCR reagent control, add 5 µl water into the respective reaction set-up. 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 set-ups into the thermal cycler and start the temperature-time programme.

8.5 Temperature–time programme

The temperature-time programme, as outlined in Table 3, has been used in the validation study. It was used in combination with the TaqMan Universal Mastermix. 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	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	

9 Accept/reject criteria

9.1 General

A corresponding real-time PCR device-specific data analysis programme is used for the identification of PCR products. The amplification results may be given in a different manner, depending on the device used. In the absence of detectable PCR products (negative result), e.g. “undetermined”, “no amp”, or the maximum number of possible cycles is given in the report. If the amplification of the DNA target sequence occurred in a sample (positive result), a sigmoid shaped amplification curve can be observed and the cycle number is calculated at which a predetermined fluorescence threshold value was exceeded (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 shall be applied.

9.2 Identification

The target sequence is considered as detected, if

— by using the *Tnos-dfr* specific primers NOST-Spec FW and NOST-Spec RV and the probe NOST-Spec-Probe, a sigmoid shaped amplification curve can be observed and a predetermined fluorescence threshold value was exceeded

— by using a linseed specific real-time PCR (Reference [1]), a sigmoid shaped amplification curve can be observed and a predetermined fluorescence threshold value was exceeded

— in the PCR control set-ups with no added DNA (PCR reagent control, negative extraction control), no sigmoid shaped amplification curve can be observed and a predetermined fluorescence threshold value was not exceeded, and

— in the set-ups for the amplification control (positive DNA target control, PCR inhibition control) the expected C_t values (or C_p values) are achieved.

10 Validation status and performance criteria

10.1 Robustness of the method

The robustness of the method has not been tested with respect to small modifications of factors such as reagent concentrations (e.g. primers, probe) or reaction conditions (e.g. annealing temperature).

NOTE In the collaborative trial, the robustness of the method has been checked with regard to different real-time PCR machines (ABI 7500, ABI 7700, ABI 7900, RotorGene 3000, RotorGene 6000, LightCycler 480).[1)] The real-time PCR machine had no influence on the performance of the method.

10.2 Intralaboratory trial

Experiments with DNA extracted from FP967 seeds were carried out by the European Union Reference Laboratory for Genetically Modified Food and Feed (EURL-GMFF) in order to verify the specificity and sensitivity of the construct-specific method (Reference [1]). The experimental testing of the specificity indicated that the *Tnos-dfr* construct-specific PCR assay does not detect other genetically modified events under the conditions tested. The limit of detection method established in 60 PCR replicates each at 50, 25, 10, 5, 1 and 0,1 copies of the target sequence (theoretically calculated) showed 60 positive reactions with 5 copies and 58 positive reactions with 1 copy.

10.3 Collaborative trial

The method has been validated in a collaborative study (Reference [3]) coordinated by the German Federal Office of Consumer Protection and Food Safety (BVL), in accordance with the IUPAC protocol (Reference [4]) with a total of 11 participants. The participants received 14 DNA samples for the analysis. The samples contained different concentrations of the *Tnos-dfr* target sequence. All samples were marked with random coding numbers.

To prepare the samples, genomic DNA was extracted from GM linseed event FP967 (reference material CDC-FL001-2 from the University California, Riverside/USA[1)]), from a GM-positive linseed product (market samples from CVUA, Freiburg[1)]) as well from non-GM rapeseed (winter rapeseeds, KWS[1)]), non-GM linseeds (LGL, Oberschleißheim[1)]) or non-GM potato flour (ERM-BF421a from IRMM, Geel[1)]) and used as initial DNA solutions. The DNA concentrations were determined photospectrometrically. Copy numbers were calculated on the basis of the genome sizes assuming an integration of one copy of the target sequence per haploid genome. The DNA concentration (in pg/µl) was divided by the published average 1C value for linseed (0,7 pg, Reference [2]), oilseed rape (1,23 pg, Reference [5]) and potato (1,8 pg, Reference [5]), respectively. Non-GM rapeseed DNA was adjusted to approx. 4,8 × 10^4 copies

1) Examples of products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.