TECHNICAL SPECIFICATION

ISO/TS 21569-2

First edition
2012-09-01

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

Reference number
ISO/TS 21569-2:2012(E)

COPYRIGHT PROTECTED DOCUMENT

ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
Web 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.

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):

$$n_{\mathrm{hgEq}} = \frac{[\mathrm{DNA}] \times 1\,000}{m_{\mathrm{hg}}} \tag{1}$$

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.

per 5 µl; non-GM potato and linseed DNA were adjusted to approx. 5,0 × 10^4 genome copies per 5 µl. The different DNA solutions were finally subdivided to 14 coded DNA samples (double-blind) for each participant of the collaborative trial. Each participant received 2 vials (double-blind) containing the following DNA solutions:

— 100 % FP967 DNA (adjusted to a calculated concentration of 10 copies per 5 µl DNA solution)

— 100 % FP967 DNA (adjusted to a calculated concentration of 50 copies per 5 µl DNA solution)

— GM-positive linseed DNA from market samples (adjusted to C_t = 30 with 5 µl of DNA solution)

— GM-positive linseed samples from market samples (adjusted to C_t = 32 with 5 µl of DNA solution)

— non-GM rapeseed DNA (adjusted to a calculated concentration of 48 660 copies per 5 µl DNA solution)

— non-GM potato DNA (adjusted to a calculated concentration of 50 000 copies per 5 µl DNA solution)

— non-GM linseed DNA (adjusted to a calculated concentration of 50 000 copies per 5 µl DNA solution)

In addition, all participants received a DNA solution with plasmid DNA (FP967/CDC Triffid plasmid (Genetic ID AG, Augsburg, Germany[1)]) for calculation of the copy numbers of the *Tnos-dfr* construct in the samples (initial calculated plasmid DNA concentration of 500 copies per µl after reconstitution of the lyophilisate in 100 µl nuclease-free water). On the basis of this standard DNA solution, a dilution series in 0,2 × TE was prepared by the participants in order to obtain DNA solutions for 5 calibration points (2 500, 500, 150, 50 and 10 copies of the target sequence) as well as a DNA solution for use as sensitivity control with 5 copies. Each sample was analysed by the participants in a single determination with 5 µl of the DNA solutions with the *Tnos-dfr* real-time PCR method under the conditions described in Tables 1 to Table 3. The DNA solutions for calibration as well as the plasmid DNA solution with 5 copies were measured in two PCR replicates. The measurement was carried out using different real-time PCR devices (see 10.1). The results of the collaborative trial study are listed in Table 4 and Table 5.

Table 4 — Results of the collaborative trial

Year of collaborative trial	2009
Number of laboratories	11
Number of laboratories submitting results	11
Number of samples per laboratory	14
Number of accepted results	137[a]
Number of accepted samples containing the *Tnos-dfr* target sequence	71
Number of accepted samples which did not contain the *Tnos-dfr* target sequence	66
False positive results	0 (0 %)
False negative results	1 (1,4 %)
[a] One laboratory reported an insufficient volume of one sample; for two laboratories, the results of the samples containing the *Tnos-dfr* target sequence were eliminated as outliers.	

In order to calculate the corresponding copy numbers from the C_t values determined from the samples, 5 DNA calibration solutions together with the samples were measured in the same PCR analysis run. The calibration curve was created by plotting the C_t value against the logarithm of the copy numbers of the target sequence provided for the calibration solutions. The respective copy numbers for the samples, as well as for the plasmid DNA solution with 5 copies, were calculated by interpolation from the calibration curve (Reference [6]). In Table 5, the summary of the results is presented. Before the calculation of the mean copy numbers and of precision data (Reference [6]), different statistical tests were used to identify outliers. The data of two laboratories with inconsistently high copy numbers were outlying the acceptance limits (Reference [3]). Therefore, the calculations of the mean copy numbers and the coefficients of variation under reproducibility conditions, $C_{V,R}$, were calculated with data from only nine laboratories.

Table 5 — Quantitative results obtained in the collaborative trials

Sample DNA	Number of positive results/total results	Mean copy number detected[a]	$C_{V,R}$[b] (%)
100 % FP967 DNA (10 copies)	22/22	11	47
100 % FP967 DNA (50 copies)	21/22	40	24
DNA extracted from market sample (C_t = 30)	22/22	314	19
DNA extracted from market sample (C_t = 32)	22/22	66	29
non-GM rapeseed DNA	0/22	0	—
non-GM potato DNA	0/22	0	—
non-GM linseed DNA	0/22	0	—

[a] Mean copy number calculated from all results (after outlier elimination)

[b] Coefficient of variation under reproducibility conditions (after outlier elimination)

10.4 Sensitivity

In Table 5, the collaborative trial results for the DNA samples with low copy numbers of the *Tnos-dfr* target sequence are shown. The plasmid DNA set on a concentration of 5 copies per 5 µl resulted in an amplification (C_t average = 35,6 ± 1,9) in all laboratories. In one laboratory, amplification was detected in only one of both determinations. At 10 copies of the target sequence, an amplification signal was obtained in all laboratories (C_t average = 34,2 ± 1,4).

The experimental verification of the *Tnos-dfr* construct-specific method by the EURL-GMFF revealed that the detection limit is 1 to 5 copies (Reference [1]).

10.5 Specificity

The specificity of the primer and the probe was validated *in silico* using sequence alignments of data searches in GenBank/EMBL/DDBJ (search date: 2011-06-16). For this purpose, by use of the programme BLASTN and the sequence of the PCR product from the event FP967 (Reference [2]), a search for matches was performed in both the GenBank nucleotide sequence collection ("non-redundant" database with all GenBank, RefSeq, EMBL, DDBJ and PDB sequences) and the database for patented nucleotide sequences. The result of the search shows no complete identity with other sequences in the databases except for those targeted by the oligonucleotides. Identity of the amplicon sequence occurs only for an approximate 60 bp fragment as part of a larger number of vectors which also contain the terminator region of the nopaline synthase gene. The sequence of this fragment contains, however, no binding site for the reverse primer NOST-Spec RV. The database search with the sequence of this primer revealed complete identity with sequence entries for the *dfrA1* gene from the class 1 integron of *Escherichia coli,* but no match with the spectinomycin/streptomycin resistance gene (Reference [2]).

In an experimental determination of specificity using 50 - 200 ng DNA per reaction, no amplification with DNA from the following other genetically modified (GM) plants was detected, except with DNA from the linseed event FP967 (References [1][3]):

— **GM rapeseed:** Rf1 (ACS-BNØØ1-4), Rf2 (ACS-BNØØ2-5), Rf3 (ACS-BNØØ3-6), MS1 (ACS-BNØØ4-7), MS8 (ACS-BNØØ5-8), GT73 (MON-ØØØ73-3), Oxy235 (ACS-BNØ11-5), T45(HCN92) (ACS-BNØØ8-2), Laurate 23-198 (CGN-89465-2)

— **GM maize:** MIR162 (SYN-IR162-4), Bt11 (SYN-BTØ11-1), GA21 (MON-ØØØ21-9), MIR604 (SYN-IR604-5), MON863 (MON-ØØ863-5), NK603 (MON-ØØ6Ø3-6), MON87460 (MON-8746Ø-4), 3272 (SYN-E3272-5), MON89034 (MON-89Ø34-3), MON88017 (MON-88Ø17-3), DBT418 (DKB-89614-9), B16 (DLL25) (DKB-8979Ø-5), CBH351 (ACS-ZMØØ4-3), T14 (ACS-ZMØØ2-1), MON810 (MON-ØØ81Ø-6), TC1507 (DAS-Ø15Ø7-1), DAS-59122-7 (DAS-59122-7)

— **GM soy:** MON40-3-2 (MON-Ø4Ø32-6), MON89788 (MON-89788-1)

— **GM potato:** EH92-527-1 (BPS-25271-9), RBMT21-129 (NMK-89684-1)

— **GM cotton:** MON1445 (MON- Ø1445-2), MON531 (MON- Ø Ø531-6), MON15985 (MON-15985-7)

— **GM alfalfa:** J101 (MON-ØØ1Ø1-8), J163 (MON- ØØ163-7)

— **GM courgette:** ZW20 (SEM-ØZW2Ø-7)

NOTE The specificity of the real-time PCR method for the linseed specific reference gene SAD has also been experimentally checked by method developers for six plant species frequently found in foodstuffs (wheat, barley, rice, rapeseed, maize, soy) using 200 ng DNA respectively (Reference [1]). With the exception of linseed DNA, a weak amplification was detected in DNA from soy and maize (the signal corresponds to a calculated amount of 0,5 pg linseed DNA). The verification report by the EURL-GMFF indicated, however, that between the SAD probe sequence and the sequence determined in linseed, two differences in the nucleotide positions 8 and 11 exist in the "SAD probe" (Reference [1]).

11 Test report

The test report should be carried out as specified in ISO 24276 and other applicable standards (e.g. ISO/IEC 17025[7]).

Bibliography

[1] Delobel C., Foti N., Savini C., Patak A., Van den Bulcke M., Ermolli M. et al.*Report on the verification of the performance of a construct-specific assay for the detection of flax CDC Triffid event FP967 using real-time PCR – Validation Report and Protocol*. 2009, ISBN: 978-92-79-14875-0

[2] Bennett M.D., & Leitch I.J.Plant DNA C-values Database (release 5.0, December 2010); http://data.kew.org/cvalues/ (11.05. 2012)

[3] Grohmann L., Busch U., Pecoraro S., Hess N., Pietsch K., Mankertz J. Collaborative trial validation of a construct-specific real-time PCR method for detection of genetically modified linseed event "CDC Triffid" FP967. *Eur. Food Res. Technol.* 2011, 232 pp. 557–561

[4] Horwitz W. Protocol for the design, conduct and interpretation of method performance studies. *Pure Appl. Chem.* 1995, 67 pp. 331–343

[5] Arumuganathan K., & Earle E.D. Nuclear DNA content of some important plant species. *Plant Mol. Biol. Rep.* 1991, 9 (3) pp. 208–218

[6] Waiblinger H.-U., Graf N., Broll H., Grohmann L., Pietsch K. Evaluation of real-time PCR results at the limit of detection. *J. Verbr. Lebensm.* 2011, 6 pp. 411–417

[7] ISO/IEC 17025, *General requirements for the competence of testing and calibration laboratories*

ICS 67.050

Price based on 9 pages