TECHNICAL SPECIFICATION

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Second edition
2021-07

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

Analyse moléculaire de biomarqueurs — Partie 2: Méthode PCR en temps réel construit-spécifique pour la détection d'un événement FP 967 dans les graines de lin et les produits à base de graines de lin

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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*.

This second edition cancels and replaces the first edition (ISO/TS 21569-2:2012), which has been technically revised.

The main changes compared to the previous edition are as follows:

— the single target copy integration into the genome has been updated;

— an explanation of *dfr* A/Spectinomycin resistance cassette juxtaposition has been added;

— minor typographical improvements have been made.

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.

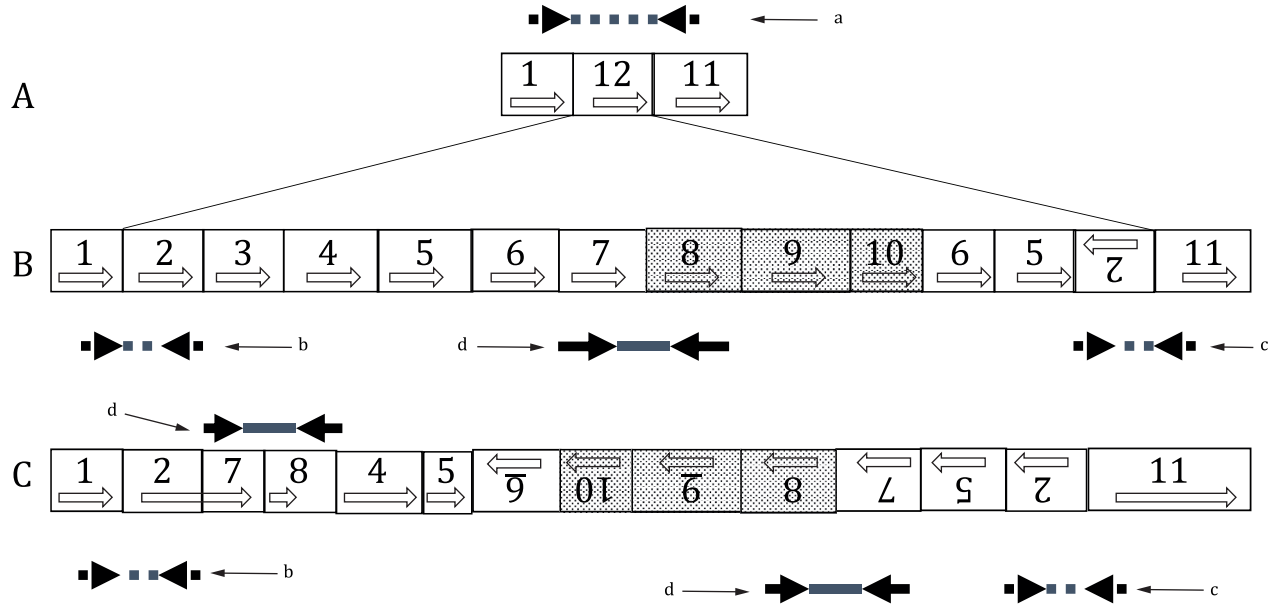
Introduction

Flaxseed (*Linum usitatissimum* L.) FP967 (CDC Triffid Flax) is the only GMO linseed flax listed in the International Service for the Acquisition of Agro-biotech Applications (ISAAA)[1]. FP967 was regenerated from a single Norlin Flax hypocotyl (regenerant number 12115) transformed with an agrobacterium/Ti plasmid system containing the NPT-11 gene encoding kanamycin resistance and a modified *Arabidopsis* acetolate synthase gene with reduced enzyme affinity for chlorosulfuron[2][3][4][5][6][7]. The *in planta* T-DNA construct includes a repeat and re-arrangement of the T-DNA forming an inverted-repeat structure of the right border, as confirmed by next generation sequencing and PCR cloning. The FP967 GM construct is stable within the recombinant plant genome and demonstrates functional resistance to the sufonylurea herbicides chlorsufuron, metsulfuron, and triasulfuron[8].

Published event-specific assays for FP967 have been described[8][9]. One generates two products from the recombinant and one product from the non-recombinant[8]. The other generates a single product but requires an internal control PCR test for linseed-specific (*Linum usitatissimum*) stearoyl-acyl carrier protein desaturase 2 gene (SAD)[9]. Event-specific assays are most useful for proprietary and breeding uses when exact identity or copy number of a transgene is required.

The FP967 PCR assay described in this document is construct-specific[10]. It generates a 105 bp product spanning the junction between the T-nos and dfrA1 elements of the transgene construct. Construct-specific assays are usually used as generic GM screening tools able to cross-detect different GM events carrying the same gene fusion. Because FP967 is the only flaxseed construct to carry a spectinomycin selectable marker and the only listed GM flax event, the described assay is conclusive for genetically modified identification among approved GMOs. It has been widely accepted and deployed and has been effective identifying and eliminating unwanted adventitious presence from unrelated breeding lines and commercial stocks. It is also more sensitive than reported for the available event-specific test because there are two copies of the target in the recombinant (see Figure 1). Adding event-specific testing options to the testing portfolio would require considerable effort (especially experimental comparison and validation to recommend one of the available event-specific assays) with no ultimate benefit to the final purpose.

Next generation sequencing and PCR cloning of the T-DNA of FP967 revealed a repeat and rearrangement of an internal T-DNA fragment forming an inverted-repeat structure of the right border of the T-DNA in the flax genome. Although, there is only a single copy of the FP967 T-DNA, the order and arrangement of the NOS gene, the *Arabidopsis* acetolactate synthase (NP_001189794.1), pBR322 (J01749.1), neomycin phosphotransferase II (AY909580.1), and the *Escherichia coli* spectinomycin resistance/dihydrofolate reductase (SpecR/DHFR) region are no longer consistent with the original plasmids used to transform FP967[8]. This rearrangement was not anticipated in the development of the construct specific assay. Figure 1 provides a graphic depicting the genomic position of the insert, the anticipated recombinant structure and the deduced recombinant structure based on DNA sequencing. It also shows the location of the event and construct-specific PCR assays on each of these.



Key

A	insertion site of flax genome	5	pBR322	12	FP 967 insertion site
B	anticipated recombinant T-DNA	6	left inside homology	a	Non-recombinant PCR.
C	deduced recombinant T-DNA	7	nopaline synthase	b	Left side event specific PCR.
1	flaxseed genomic region 1	8	spectinomycin resistance gene	c	Right side event specific PCR.
2	right border	9	chimeric neomycin phosphotransferase	d	Construct specific PCR.
3	undetermined sequence	10	*Arabidopsis* acetolactate synthase		
4	left border	11	flaxseed genomic region 2		

NOTE As a result of the rearrangement of the T-DNA in the recombinant two copies of the target amplicon were formed. This increases the sensitivity of the construct specific assay.

Figure 1 — FP967 insertion into the flax genome

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 document specifies 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 nopaline 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 can 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

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 — Terms and definitions*

ISO 21569, *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 terminological databases for use in standardization at the following addresses:

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

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

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)[10];

b) detection of the *Tnos-dfr* construct in a real-time PCR[10].

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 triphosphates: 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[10]:		
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[11] and by applying Formula (1):

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

where

C_{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 that contained a single copy of the 105 bp *Tnos-dfr* fragment and the 68 bp large SAD gene fragment, respectively. There is a single copy the *Tnos-dfr* construct in event FP967 in linseed[7]. The calculated GM-content is based on the single copy presence of the target sequence 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. Samples should be representative of the lot.

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, for example, 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 be chosen. DNA extraction from flaxseed for GMO analysis has been described and evaluated[12].

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).

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[10][12].

8.4 PCR setup

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

Reagents should be completely thawed at room temperature and 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), for example, “undetermined”, “no amplification”, 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. The cycle number at the crossing point of the amplification curve and the fluorescence threshold can be calculated [cycle threshold (C_t) or cycle quantification (C_q)].

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