

Collaborative trial validation of a construct-specific real-time PCR method for detection of genetically modified linseed event ‘CDC Triffid’ FP967

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Abstract A real-time PCR-based method for construct-specific detection of the genetically modified (GM) linseed event ‘CDC Triffid’ FP967 originating from Canada has been validated in a collaborative trial. The construct-specific method amplifies a 105 bp long fragment of the transgenic insertion present in FP967 spanning the junction of the terminator region of the nopalyn synthase gene from *Agrobacterium tumefaciens* (*Tnos*) to a sequence region coding for the dehydrofolate reductase gene (*dfr*) from a class I integron from *Escherichia coli*. This region is characteristic for the construct used to develop FP967. A total of 11 laboratories participated in the collaborative study. For PCR analysis, each laboratory received 14 DNA samples comprising 7 double-blind DNA samples. The samples consisted of two low GM-levels of FP967 DNA (10 or 50 copies per PCR), of DNA from two different GM-positive linseed products and of DNA from GM-negative linseed, potato and rapeseed materials, respectively. All but one of the FP967-positive DNA samples were detected correctly. No false-positive results were reported. The results demonstrate that the linseed event FP967 is

detectable even at low copy number concentrations. The limit of detection (LOD) determined with plasmid DNA was shown to be at 5 copies of the *Tnos*–*dfr* sequence. The data provided show that the method can be applied successfully in different laboratories and is fit-for-purpose to test for the presence of the EU-unauthorised linseed event ‘CDC Triffid’ FP967.

Keywords Genetically modified linseed · Detection method · Real-time PCR · Collaborative trial · CDC Triffid · FP967

Introduction

In 2009, several linseed products originating from Canada were found to contain genetically modified (GM) DNA which was attributed to the GM linseed (*Linum usitatissimum*) event FP967 (‘CDC-Triffid’). This GM linseed has tolerance to soil residues of sulfonylurea-based herbicides and was developed by the Crop Development Centre (CDC) at the University of Saskatchewan in Canada in the 1990s [1]. It received regulatory feed and environmental safety authorisation in 1996, and food safety authorisation in 1998 in Canada, but was never released for commercial production according to communications of the Canadian Grain Commission (CGC) [2].

Official findings of genetic modifications in linseed products on the European market have been notified to the Rapid Alert System for Food and Feed (RASFF) for information of the regional food and feed control authorities in the EU member states [3]. According to the current legislation, no food and feed products derived from GM linseed are authorised in the EU. In order to prevent further imports of unauthorised GM linseed from Canada, the

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European Commission adopted a sampling and testing protocol for Canadian flaxseed exported to the EU published by the CGC [4]. This protocol is based on the EU Commission Recommendation 787/2004/EC [5] and should be also used by Member States for the control on imported products, in order to avoid discrepancy of results, with no need for adopting specific European Commission emergency measures. For detection of the genetic modification in linseed products, the protocol refers to a real-time PCR method which targets a construct of two genetic elements that are specifically present in event FP967 [6, 7]. The method developer declared that the method targets the transition sequence spanning the terminator region of the *Agrobacterium tumefaciens* nopaline synthase gene (*Tnos*) and a spectinomycin/streptomycin resistance gene and thus named the method 'NOST-Spec' [7]. However, bioinformatic and nucleotide sequence analysis revealed that the amplified region spans the transition of *Tnos* and a sequence of the dehydrofolate reductase gene (*dfr*) from a class I integron isolated from *Escherichia coli* [8]. Therefore, the method is named as *Tnos-dfr* in the following text.

The 'NOST-Spec' construct-specific real-time PCR method has been extensively validated for its sensitivity and specificity at the methods developer's laboratory [7] and in a verification study done by the European Union Reference Laboratory for GM Food and Feed (EURL-GMFF) [8] but its performance characteristics are not established in a multi-laboratory study until now.

The Federal Office of Consumer Protection and Food Safety and its working group 'Development of methods for identifying foodstuffs produced by means of genetic engineering techniques' has the task to validate food and feed analytical methods in ring trials in order to assess the performance data concerning the inter-laboratory repeatability and reproducibility and to confirm that these methods are fit-for-purpose and transferable to multiple laboratories. The results of a recently conducted collaborative validation study of the construct-specific *Tnos-dfr* real-time PCR method for detection of linseed event FP967 are presented in this work.

Materials and methods

Sample materials

FP967 (CDC Triffid) reference material (whole seeds, provided by Dr. Alan McHughen, University of California, Riverside, USA) and non-GM linseed (whole seeds) were provided by the Bavarian Health and Food Safety Authority (LGL Oberschleißheim, Germany). A market sample from a consumer store (genomic DNA, FP967 positive) was provided by the State Institute for Chemical and Veterinary Analysis of Food (CVUA Freiburg,

Germany). Negative control material for non-GM potato (milled, dried powder produced from whole tubers of non-modified starch potato cultivar KURAS, ERM-BF421a) was obtained from the Institute for Reference Materials and Measurements (IRMM, Geel/Belgium). Negative control material for non-GM rapeseed (whole seeds, variety KW1519) was kindly provided by KWS Saat AG (Einbeck, Germany). Plasmid FP967/CDC Triffid was kindly provided by Genetic ID AG (Augsburg, Germany).

Sample preparation

Grinding of whole rapeseeds was done using a blender (Thermomix TM31, Vorwerk, Wuppertal, Germany). For grinding of linseeds, a mixer mill (Type MM400, Retsch, Haan, Germany) was used. Test portions of 100–400 mg were used for DNA extraction using either the CTAB method [9] for flour from potato tubers or the Fast ID Genomic DNA Extraction Kit (Genetic ID, Fairfield, USA) for flour from rapeseed and linseed. DNA was quantified by UV spectrophotometry (BioPhotometer 6131, Eppendorf, Hamburg, Germany).

Samples containing DNA extracted from FP967 seeds were adjusted to 10 or 50 target sequence copies per 5 μ L. DNA from GM-positive linseed market samples was extracted using the Wizard kit system (Promega, Mannheim, Germany) and additionally purified in a Microspin S300 HR column (GE Healthcare, München, Germany). The DNAs were finally adjusted to Ct 32 and Ct 30 when using 5 μ L per PCR, respectively. Copy numbers as given in Table 1 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 ng) was divided by the published average 1C value for linseed (0.7 pg), oilseed rape (1.23 pg) and potato (1.8 pg), respectively [10]. Non-GM rapeseed DNA was adjusted to $\sim 4.8 \times 10^4$ copies per 5 μ L; non-GM potato and linseed DNA were adjusted to $\sim 5 \times 10^4$ genome copies per 5 μ L. The different DNA solutions were finally subdivided to 14 coded DNA samples (double-blind) for each participant of the collaborative trial (Table 1).

As standard DNA, the FP967/CDC Triffid plasmid (Genetic ID AG, Augsburg, Germany) containing one copy of the 105-bp fragment of the *Tnos-dfr* region was used. The plasmid DNA was diluted to a concentration of 2,500 copies per 5 μ L. Copy numbers were calculated on basis of the concentration (1×10^5 copies/ μ L after reconstitution of the lyophilisate in 100 μ L H₂O) indicated by the provider of the plasmid DNA.

Collaborative trial

The collaborative trial was organised by the Federal Office of Consumer Protection and Food Safety (Berlin,

Table 1 Description of DNA test samples and standard DNA used in the collaborative trial study

Sample DNA	Calculated target sequence copy number [per PCR]	Test sample type
FP967 (whole seeds)	50 ^a	Blind duplicate
FP967 (whole seeds)	10 ^a	
GM-positive linseeds (market sample Ct = 30)	n.d.	
GM-positive linseeds (market sample Ct = 32)	n.d.	
Non-GM linseeds (whole seeds)	0	
Non-GM rapeseeds (whole seeds)	0	
Non-GM potato (flour)	0	
Plasmid FP967/CDC Triffid	2,500	

^a The number GM target sequence copies (cp) per PCR was calculated on basis of the genome sizes and the number of integrations per haploid genome (see materials and methods)

^b DNA stock solution used as starting calibration standard and for preparation of five dilutions

n.d. not determined

Table 2 Primers and probes used for real-time PCR

Method (amplicon length)	Name	Sequence (5'–3')	Final concentration in PCR [nmol/L]	Reference
<i>Tnos-dfr</i> (105 bp)	NOST-Spec FW	AgC gCg CAA ACT Agg ATA AA	800	[7, 8]
	NOST-Spec RV	ACC TTC Cgg CTC gAT GTC TA	800	
	NOST-Spec Probe	6-FAM—CgC gCg Cgg TgT CAT CTA Tg—BHQ	100	

Germany) and its working group “Development of methods for identifying foodstuffs produced by means of genetic engineering techniques”. Eleven laboratories experienced in GMO testing from Germany, Austria and Switzerland participated in the study. DNA test samples and standard DNA provided to the participants are described in Table 1. Sample coding was done in a randomised manner. The standard DNA had to be used by the participants as starting calibration DNA standard and for serial dilutions with TE buffer [2 mM Tris–HCl and 0.2 mM ethylenediaminetetraacetic acid (EDTA), adjusted to pH 8.0]. In addition, each laboratory received appropriate amounts of lyophilised oligonucleotide primers and probe (Table 2) and a real-time PCR reagent kit (TaqMan[®] Universal PCR Mastermix No AmpErase[®] UNG of Applied Biosystems, Darmstadt, Germany). The coded DNA test samples were shipped on dry ice and had to be kept cool in the laboratories until the analysis.

Real-time PCR

Real-time PCR for detection of the *Tnos-dfr* construct present in event FP967 was performed according to the so-called “NOST-Spec” method with minor modifications [7, 8]. Primers and probes (purchased from BioTez, Berlin, Germany) are described in Table 2. Reactions were carried

out in 1× TaqMan[®] Universal PCR Mastermix (Applied Biosystems, Darmstadt, Germany) using the corresponding primer and probe concentrations [7]. Instead of 10 µL as described in [7], sample DNA or standard DNA was added in a volume of 5 µL to obtain the final PCR volume of 25 µL. For amplification, the thermal cycling programme used was an initial activation step of 50 °C for 2 min, followed by a denaturation step at 95 °C for 10 min and 45 cycles at 95 °C for 15 s and 60 °C for 60 s. Different real-time PCR instruments were used by the participants (ABI 7500, ABI 7700, ABI 7900, RotorGene 3000, RotorGene 6000, LightCycler 480).

For target-taxon-specific detection and copy number quantification, the DNA samples were analysed using real-time PCR systems specific for endogenous reference genes (UGPase for potato [11]; SAD for linseed [7]; *cruA* for rapeseed [12]).

Data analysis

Calculations of slope and correlation coefficients of the standard curve were done with an Excel spreadsheet provided to all participants. Thereafter, the copy number in the unknown sample DNA was estimated by interpolation from the standard curve. Submitted results were subjected to statistical tests to identify any outliers [13].

Results and discussion

The collaborative trial for validation of the *Tnos-dfr* real-time PCR method (also known as ‘NOST-Spec’ method [7]) was carried out in 2009 and was designed according to internationally accepted guidelines [14, 15]. Considering the modular approach for method validation, the collaborative study did not include the DNA extraction step [16]. A set of 14 coded DNA samples, a plasmid DNA for generating a standard curve and all required reagents were sent to all 11 participating laboratories. The detailed description of the DNA samples is given in Table 1. For the plasmid standard DNA, it was required that laboratories prepared five dilutions to obtain DNA solutions with estimated concentrations of 2,500, 500, 150, 50 and 10 copies of the *Tnos-dfr* target sequence per reaction. A further dilution of the plasmid DNA was prepared to generate a sensitivity sample with a concentration of 5 copies at the limit of detection (LOD). The calibrants had to be analysed as duplicates in parallel to the unknown DNA sample (single PCR determinations) in one real-time PCR run.

The participants submitted the filled Excel spreadsheet with corresponding Ct-values measured for the 14 unknown DNA samples and the DNA standards. Except for one sample, all FP967-positive DNAs were detected when using the *Tnos-dfr* real-time PCR (Table 3). For the FP967-negative DNA samples, all except one laboratory reported that no amplification occurred. The respective laboratory measured a Ct value of 42.8 for one non-GM linseed DNA sample. In the respective quantification report, zero copies were calculated and this result was thus also assessed as negative. Accordingly, the resulting false-positive rate is 0% (Table 3). For the DNA samples prepared from the FP967 seeds material and diluted to 10 or 50 copies of the target sequence, a mean Ct-value of 34.2 ± 1.4 and of 32.1 ± 1.2 was measured, respectively. DNA samples extracted from FP967-positive market products showed mean Ct values of 28.8 ± 1.3 and of 31.3 ± 1.1 , respectively. For the 5 copy plasmid DNA samples, a mean Ct of 35.6 ± 1.9 as calculated from 21 PCR-positive determinations was calculated.

Table 3 Collaborative trial study results

Number of laboratories	11
Number of laboratories submitting results	11
Number of samples per laboratory	14
Number of accepted results	137*
Number of samples containing the <i>T-nos-dfr</i> construct	71
Number of samples not containing the <i>T-nos-dfr</i> construct	66
False-positive results	0 (0%)
False-negative results	1 (1.4%)

* One laboratory reported an insufficient volume for one test sample

Table 4 Quantitative results obtained in the collaborative trial for the *Tnos-dfr* construct-specific detection method

Sample DNA (target copies per PCR)	Ratio of positive results/determinations	Mean copy number detected ^a	Relative standard deviation RSD _R (%)
FP967 (10 cp)	22/22	11	47
FP967 (50 cp)	21/22	40	24
Market sample Ct = 30 (n.d.)	22/22	314	19
Market sample Ct = 32 (n.d.)	22/22	66	29
Non-GM linseed	0/22	0	–
Non-GM rapeseed	0/22	0	–
Non-GM potato	0/22	0	–

^a Mean values of the *Tnos-dfr* copies calculated on basis of single determinations using the standard curve generated by 9 laboratories. Data of 2 laboratories were eliminated after statistical outlier tests

n.d. not determined

Six different real-time PCR cyclers models were used by the different laboratories. No specific difficulties or unusual observations were reported or identified in the evaluation of the results, indicating the methods robustness to different real-time PCR cyclers.

Quantitative results and precision data for the analyses of the *Tnos-dfr* positive samples obtained by the interpolations from the standard curve generated with the FP967 plasmid DNA are shown in Table 4. Before the calculation of precision data, different statistical tests were used to identify outliers. The Cochran tests identified no significant outliers, but using the serial Grubbs tests at a confidence level of 95% the data of two laboratories with inconsistently high copy numbers were outlying the acceptance limits. All data were also tested for normal distribution by calculating Q–Q plots. As expected, each data set is normal distributed when the two highest values identified by the Grubbs test were removed. Therefore, the calculations of the mean copy numbers and the relative standard deviations under reproducibility conditions (RSD_R) were calculated with data from only 9 laboratories (Table 4). The RSD_R values calculated for the 10 and 50 copy FP967 DNA samples conform to the performance requirements for real-time PCR-based GMO testing methods defined in the guidance document published by the European Network of GMO Laboratories (ENGL) [17]. The results of the study show that the *Tnos-dfr* real-time PCR assay is capable of detecting the transgenic FP967 construct at a concentration of 5 copies of the target sequence, which is consistent with the LOD of 1–5 copies reported in the EURL-GMFF method verification [8].

In conclusion, the data of this collaborative trial study verify the sensitivity and specificity results of the in-house validation and the method verification reports [7, 8]. The study shows that the *T-nos-dfr* construct-specific method is

transferable to other laboratories and is fit-for-purpose to test for the presence of the EU-unauthorised linseed event FP967. For the extraction of DNA from linseed products yielding genomic DNA of sufficient quality and quantity for PCR, either a commercial kit product indicated by the Canadian Grain Commission and the EURL-GMFF [4, 18] or the standard CTAB method recommended in a guidance document of the German § 64 working group should be applied [9, 19].

It is noted that the presence of linseed event FP967 has been reported not only for products imported from Canada but from other non-EU countries [3]. Up to now, the Canadian Grain Commission (CGC) has published sampling and testing protocols for linseed shipments to the EU and to Japan, but not to other countries [4, 20]. Thus, it appears that Canadian linseed bulk vessel exports shipped elsewhere may not have passed the CGC testing scheme protocol for excluding the presence of event FP967. Therefore, it may occur that linseed cargos of Canadian origin entering the EU market from a third country are tested positive for event FP967. The construct-specific method for detection of FP967 in linseed products from abroad may thus be used by EU control laboratories until proven non-GM linseed seedstocks have completely replaced FP967 admixed varieties in Canada.

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