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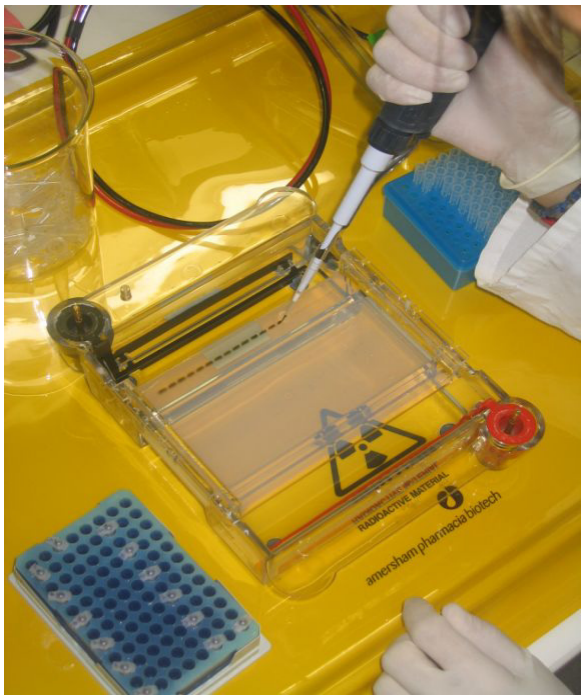
Report on the Verification of the Performance of a Construct-specific Assay for the Detection of Flax CDC Triffid Event FP967 Using Real-time PCR

Validation Report and Protocol

C. Delobel, N. Foti, C. Savini, A. Patak, M. Van den Bulcke, M. Ermolli, S. Folloni G. Pinski, M. Mazzara, G. Van den Eede



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European Commission
Joint Research Centre
Institute for Health and Consumer Protection

Contact information
Address: Molecular Biology and Genomics Unit
E-mail: JRC-BGMO@ec.europa.eu
Tel.: +39 0332 789379
Fax: +39 0332 786159

http://ihcp.jrc.ec.europa.eu/
http://www.jrc.ec.europa.eu/

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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

15 October 2009

Joint Research Centre
Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit

Executive Summary

Further to the detection by the German authorities of the unauthorised flax CDC Triffid event FP967 (Unique Identifier CDC-FLØØ1-2) in materials imported from Canada, a notification was sent through the Rapid Alert System for Food and Feed (RASFF) in September 2009.

On 21st August 2009, the Community Reference Laboratory for Genetically Modified Food and Feed received from the German authorities a construct-specific method for the detection of flax CDC Triffid event FP967, developed by Genetic ID, Augsburg (Germany). The method developer declared this method as specific for event FP967 as it targets a transition sequence spanning the *nopaline synthase* (*nos*) terminator gene and the *spectinomycin/streptomycin* resistance gene, construction being found only in the flax FP967 event.

On 11th September 2009, the CRL-GMFF received from the German authorities the FP967 positive control in the form of DNA extracted from seeds. Seeds were provided to the German authorities by the University of California, Riverside, USA. The CRL-GMFF carried out experiments on the control sample received in order to verify the specificity and the Limit of Detection (LOD) of the construct-specific method.

The CRL-GMFF observed that the NOST-Spec (nos terminator – spectinomycin resistance gene) construct-specific method generates a PCR amplification product of 105 bp, whose sequence is homologous to a transition sequence spanning the *nopaline synthase* (nos) terminator gene and the *dihydrofolate reductase* gene. The experimental testing of the specificity indicates that the NOST-Spec construct-specific assay does not detect genetically modified events under the conditions reported. The limit of detection (LOD) established is between 1 and 5 haploid genome copies of FP967.

Drafted by:
C. Delobel

Report Verification Team:
1) M. Querci

2) M. Ermolli

Scientific and technical approval:
M. Mazzara

Compliance with CRL Quality System:
S. Cordeil

Authorisation to publish:
G. Van den Eede

Address of contact laboratory:
European Commission, Joint Research Centre
Institute for Health and Consumer Protection (IHCP)
Molecular Biology and Genomics Unit – Community Reference Laboratory for GM Food and Feed
Via Fermi 2749, 21027 Ispra (VA) – Italy

Content

1. Introduction

German authorities reported the detection of the unauthorised flax called CDC Triffid event FP967 (Unique Identifier CDC-FLØØ1-2) in materials imported from Canada. A notification was sent through the Rapid Alert System for Food and Feed (RASFF) in September 2009.

Event FP967 is approved for food, feed and cultivation in the US and Canada and is declared to contain at least the following genetic elements: *nopaline synthase* promoter gene (*pnos*) linked to the *neomycin phosphotransferase II* gene (*nptII*) as well as the *nopaline synthase* terminator gene (*tnos*) linked to the *spectinomycin/streptomycin* resistance gene (*spc*) [1].

On 21st August 2009, the Community Reference Laboratory for Genetically Modified Food and Feed (CRL-GMFF) received from the German authorities a real-time PCR method for construct-specific detection of flax event FP967, developed by Genetic ID, Augsburg (Germany) [2].

The real-time PCR method targets a transition sequence spanning the *tnos* and the *spc* genes. According to the method developer this construct is only found in event FP967 which allows specific detection of that GMO.

On 11th September 2009, the CRL-GMFF received from the German authorities the FP967 positive control as DNA extracted from seeds. Seeds were provided to the German authorities by the University of California, Riverside, USA.

The CRL-GMFF carried out experiments on the control sample received in order to verify the specificity and sensitivity of the construct-specific method received for the qualitative detection of event FP967.

The present report describes the outcome of the tests performed.

2. Experimental design, materials and methods

2.1. DNA concentration and integrity

The concentration of the FP967 DNA sample received was determined by fluorescence detection, after extensive homogenisation, using PicoGreen dsDNA Quantitation Kit (Molecular Probes). The DNA concentration was determined on the basis of a five-point standard curve ranging from 0 ng/mL to 500 ng/mL using a Modulus (Turner Biosystems) as fluorescence detector. The DNA integrity was verified by agarose-gel electrophoresis. For subsequent analysis the size of the 1-C value of the linseed genome was estimated to be 0.7 pg [3].

2.2. Sequencing of the NOST-Spec construct-specific amplicon

Direct sequencing was performed on the amplicon generated by the two NOSt-Spec primers [2]. Five microliters of the FP967 DNA sample received by the CRL-GMFF were used as a template DNA for the PCR amplification. The amplification product was purified from a 1% agarose gel, following the instruction provided by the Agarose GelExtract Mini kit (5PRIME).

The purified PCR product was sequenced using both primers of the NOST-Spec assay. Cycle sequencing reactions were performed in duplicate for both strands, using the BigDye® Terminator v

1.1 cycle sequencing kit (Applied Biosystems) and analysed by the ABI 3730 DNA analyzer (Applied Biosystems); each replicate was processed with a different purification method, using CENTRI-SEP columns (Princeton Separation) and ethanol).

2.3. Specificity

2.3.1. Bioinformatics analysis

Bioinformatics analyses were conducted by homology searches, BLASTN 2.2.15 [4], with the sequences of the primers/probe of the NOST-Spec construct-specific assay developed by Genetic ID [2] against i) the GMO database maintained by the Joint Research Centre (Central DNA Core Sequence Information System - CCSIS), ii) the Genbank nt, Vector, Univec and patent databases.

2.3.2. Experimental testing

The NOST-Spec method was tested against genomic DNA from a selection of genetically modified (GM) events for which the prediction of total or partial homologies with one or both NOST-Spec primers arose from the bioinformatics analysis: soybean event GTS 40-3-2, maize events MIR162, Bt11, GA21, MIR604, MON863, NK603, MON87460, 3272, MON89034, MON88017, oilseed rape events Rf1, Rf2, Rf3, Ms1, MS8, cotton events LL25, MON 1445, MON 531, MON 15985, and the potato event EH92-527-1. The same GM events were also tested with their respective target-taxon reference systems, i.e. *adh* for maize, *lectin* for soybean, *cruA* for oilseed rape, *acp* for cotton and *ugp* for potato, according to the methods submitted to the CRL-GMFF under Regulation (EC) No 1829/2003.

2.4. Limit of Detection

The optimal sample size (number of replicates n per assayed GM level) was estimated to determine the limit of detection (LOD), defined as the GM level (p) detected at least 95% of the time, thus ensuring ≤5% false negative results. The number n was estimated to generate a 0.95 confidence interval whose upper bound does not exceed 5%.

For an accurate estimate of the 0.95 (1-α) confidence interval (depending on the degrees of freedom used to compute p), the F-distribution was used based on the relationship between such distribution and the binomial distribution [5]. The method is derived from Bliss (1967) [6] and re-proposed by Zar (1999) [7]. According to this method, in a sample of n data, X of which showing the character of interest, confidence limits (L1: lower limit, L2: upper limit) of a proportion p are computed as follows:

$$L_1 = \frac{X}{X + (n - X + 1) \cdot F_{\alpha/2,\nu1,\nu2}}$$

$$L_2 = \frac{(X + 1) \cdot F_{\alpha/2,\nu1,\nu2}}{n - X + (X + 1) \cdot F_{\alpha/2,\nu1,\nu2}}$$

where the degrees of freedom $\nu 1$ and $\nu 2$ are:

$$\nu 1 = 2 \cdot (n - X + 1)$$
$$\nu 2 = 2 \cdot X$$

and the degrees of freedom $'\nu 1$ and $'\nu 2$ are:

$$'\nu 1 = \nu 2 + 2$$
$$'\nu 2 = \nu 1 - 2$$

Based on this method, with X = 1 and α = 0.05, L2 = 0.05 results from n = 100.

As suggested by various statisticians (e.g. Cochran, 1977 [8]), the simplest approach to estimate the confidence interval of a sample proportion p, is the use of the normal distribution (z) and its standard deviation p · (1-p):

$$L_1 = p - z_{\alpha/2} \cdot \sqrt{\frac{p \cdot (1-p)}{n-1}}$$

$$L_2 = p + z_{\alpha/2} \cdot \sqrt{\frac{p \cdot (1-p)}{n-1}}$$

Based on this simplified approach, with X = 1 and α = 0.05, L2 = 0.05 results from n = 60, thus resulting in an experimental absolute LOD set at 59 positive tests (n - X) over 60 replicates.

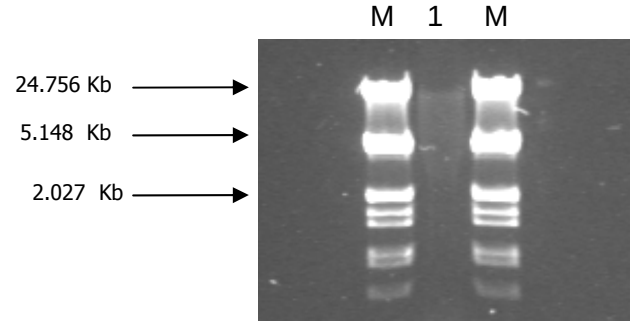
Given the experimental design for a LOD study where it is required to test a large number of replicates in each sample characterised by defined analyte content (GM level) over a decreasing series of concentrations, the Cochran approach was accepted as the most feasible.

The FP967 DNA sample received by the CRL-GMFF was diluted to a concentration of 100 haploid genome copies in one microliter. Subsequently a serial dilution was prepared from the first diluted sample to obtain concentrations of 5, 2.5, 1, 0.5, 0.1 and 0.01 copies/μL. Ten μL of each concentration were analysed by real-time PCR using the NOST-Spec construct-specific assay provided [2]. Sixty replicates were tested for each concentration.

3. Results

3.1. DNA concentration and integrity

The concentration of the FP967 DNA sample received by the CRL-GMFF was estimated equal to 7 ng/μL. The sample was visualied on a 0.8% agarose gel electrophoresis run at 70 V for 1 h to evaluate DNA integrity (Figure 1).



M= *Lambda DNA/EcoRI+HindIII (0.1 mg DNA/mL 0.05 mg)*
Lane 1= Flax FP967, 35 ng

Figure 1. Visualisation of the FP967 DNA sample by gel electrophoresis

The flax FP967 DNA shows a high molecular weight band close to 24 Kb together with smaller fragments in the form of "smear" from about 24 Kb down to about 2 Kb. The integrity of the genomic DNA is therefore considered suitable for the purpose of PCR amplification with the method described.

3.2. Sequencing of the NOST-Spec construct-specific amplicon

From the NOST-Spec Forward primer sequencing reactions, a 71-nucleotide sequence was obtained. On this sequence, 9 of the probe nucleotides as well as the whole sequence of the NOST-Spec reverse primer could be identified.

From the NOST-Spec Reverse primer sequencing reactions, a 74-nucleotide sequence was obtained. On this sequence, the full sequence of both the probe and the NOST-Spec forward primer could be identified.

Figure 2 shows the consensus sequence of the amplification product generated by the NOST-Spec primers.

Figure 2. Sequence of the FP967 amplicon

The amplification product generated by the NOST-Spec construct-specific assay is 105 bp, while it is 95 bp in the method developed by Genetic ID [2]. Parallel experiments conducted by the Belgian National Reference Laboratory (CRA-W) on two independent samples confirmed the sequence as reported in Figure 2.

The CRL-GMFF also received from the same Belgian National Reference Laboratory (CRA-W) the sequencing results of the amplicon produced by the target-taxon reference system SAD [2]. The amplification product generated by the SAD real-time PCR method is 68 bp. The sequencing results were obtained from two independent samples. Figure 3 shows the sequence of the *stearoyl-acyl carrier protein desaturase* gene (SAD) obtained by the CRA-W.

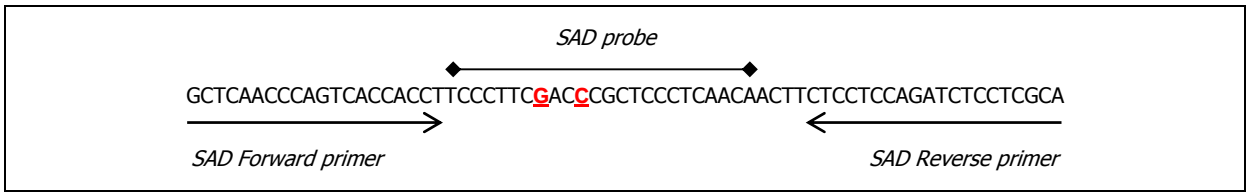


Figure 3. Sequence of the SAD amplicon

The sequence shows a mismatch of two nucleotides between the sequence of the SAD probe and that described by CRA-W laboratory. By performing a homology search of both sequences (i.e. from CRA-W and from Genetic ID), it results that the two variants of the sequence match with a sequence of the *stearoyl-acyl carrier protein desaturase* gene, namely Genbank nt accession number AJ006958 (CRA-W sequence) and accession number X70962 (Genetic ID sequence).

3.3. Specificity

The construct-specific method developed by Genetic ID is described as targeting a transition sequence spanning the *tnos* and the *spc* gene. However, the method developer considers that this combination of genetic elements is only found in the flax event FP967 and thus allows the specific detection of that event. The specificity of the method was evaluated by the CRL-GMFF both *in silico* and experimentally.

3.3.1. Bioinformatics analysis

The NOST-Spec forward primer and the NOST-Spec probe showed a perfect homology with the sequence of the *nopaline synthase* terminator gene (*tnos*). This leads to many matches of the combination (forward primer + probe) with all GM events containing the *tnos* sequence.

The NOST-Spec reverse primer showed a complete match with a sequence of the *dihydrofolate reductase* gene (*dhfr*) but no homology with the *Spectinomycin/streptomycin* resistance gene.

The blast search of the combination of the two primers and the probe against the GMO database (CCSIS) maintained at the JRC revealed a perfect homology with a vector used for the transformation of one particular GM maize event, leading to a potential amplification product of about 2.8 Kb. However the region where the reverse primer matches is not in the T-DNA and thus it should not be present in the GM event.

The above *in silico* analysis was confirmed by the bioinformatics analysis performed on the sequence obtained from the FP967 DNA control sample (see section 3.2.), nucleotides 1-55 perfectly match with the sequence of the *tnos* and nucleotides 54-105 perfectly match with the sequence of the *dhfr*.

3.3.2. Experimental assessment

Based on the results of the bioinformatics analysis, the CRL-GMFF tested the specificity of the NOST-Spec method on the following GM events: soybean event GTS 40-3-2, maize events MIR162, Bt11, GA21, MIR 604, MON863, NK603, MON87460, 3272, MON89034, MON88017, oilseed rape events Rf1,

Rf2, Rf3, Ms1, MS8, cotton events LL25, MON 1445, MON 531, MON 15985, and the potato event EH92-527-1. Results of the specificity test are shown in Table 1.

Table 1. Results of the specificity test of the NOST-Spec assay

Event name	Species	GM %	Target DNA quantity in PCR reactions	NOST-Spec assay (Ct value / sd)	Taxon-specific reference system (Ct number (sd) / Ref system)
GTS 40-3-2	soybean	100	50 ng	-	24.93 (0.06) / lectin
MIR162	Maize	100	50 ng	-	27.72 (0.05) / adh
Bt11	Maize	100	50 ng	-	29.93 (0.12) / adh
GA21	Maize	10	50 ng	-	29.72 (0.10) / adh
MIR 604	Maize	10	50 ng	-	28.68 (0.09) / adh
MON863	Maize	100	50 ng	-	28.19 (0.04) / adh
NK603	Maize	100	50 ng	-	30.28 (0.07) / adh
MON87460	Maize	100	50 ng	-	28.13 (0.05) / adh
3272	Maize	100	50 ng	-	27.77 (0.02) / adh
MON89034	Maize	100	50 ng	-	28.09 (0.53) / adh
MON88017	Maize	100	50 ng	-	29.57 (0.12) / adh
Rf1	Oilseed rape	100	25 ng	-	24.75 (0.14) / CruA
Rf2	Oilseed rape	100	25 ng	-	24.70 (0.16) / CruA
Rf3	Oilseed rape	100	25 ng	-	23.16 (0.21) / CruA
Ms1	Oilseed rape	10	25 ng	-	20.46 (0.10) / CruA
MS8	Oilseed rape	100	25 ng	-	24.06 (0.26) / CruA
LL25	Cotton	100	50 ng	-	26.13 (0.04) / Acp
MON 1445	Cotton	100	50 ng	-	26.81 (0.01) / Acp
MON 531	Cotton	100	50 ng	-	26.70 (0.03) / Acp
MON 15985	Cotton	100	50 ng	-	26.48 (0.06) / Acp
EH92-527-1	Potato	100	50 ng	-	16.39 (0.03) / UGP
FP967	Linseed	100	10 copies	30.05 (0.21)	31.10 (1.41) / SAD
FP967	Linseed	100	40 copies	28.76 (0.82)	29.06 (0.27 / SAD
FP967	Linseed	100	400 copies	25.49 (0.07)	25.97 (0.05) / SAD
NTC				-	

NTC: No template control
sd: standard deviation

The data of the specificity test indicate that, under the conditions described for the NOST-Spec assay [2], the construct-specific method does not detect GM events for which the bioinformatics analysis indicated homologies.

3.4. Limit of detection (LOD)

The sensitivity of the NOST-Spec detection method was evaluated through the determination of the Limit of Detection (LOD) tested on the FP967 DNA sample received by the CRL-GMFF. Table 2 reports the results of the experiment carried out.

The information available on genetic analysis of flax event FP967 [1] suggest that the original recombinant insert (T-DNA) was integrated in at least two unlinked loci, with a possible partially linked third locus. However, at the moment of the compilation of this report, there is no evidence that the NOST-Spec method can detect more than one inserted copy per genome. Therefore, in assessing the LOD of the method, the CRL-GMFF assumed the presence of one amplifiable copy per haploid genome.

Table 2. Result of the LOD of the NOST-Spec assay

PCR plate number	Sample	FP967 copy number	Average Ct value / Standard deviation	Positive / total PCR reactions
# 1	FP967 control sample	50	31.66 / 0.14	60/60
# 1	FP967 positive control	100	27.30 / 0.07	3/3
# 1	NTC	---	---	---
# 2	FP967 control sample	25	32.67 / 0.21	60/60
# 2	FP967 positive control	50	31.63 / 0.11	3/3
# 2	NTC	---	---	---
# 3	FP967 control sample	10	34.09 / 0.30	60/60
# 3	FP967 positive control	50	31.85 / 0.05	3/3
# 3	NTC	---	---	---
# 4	FP967 control sample	5	35.05 / 0.44	60/60
# 4	FP967 positive control	50	31.68 / 0.13	3/3
# 4	NTC	---	---	---
# 5	FP967 control sample	1	37.59 / 0.89 *	58/60
# 5	FP967 positive control	50	31.76 / 0.11	3/3
# 5	NTC	---	---	---
# 6	FP967 control sample	0.1	39.31 / 1.59 *	16/60
# 6	FP967 positive control	10	35.50 / 0.09	3/3
# 6	NTC	---	---	---

NTC: No template control
**: calculated on measured data*

Considering the results presented in Table 1, the LOD of the NOST-Spec construct-specific assay is between 1 and 5 haploid genome copies of FP967, based on the 1-C value of the flax genome.

4. Conclusions

Further to the detection in materials imported from Canada of the unauthorised flax event FP967 (Unique Identifier CDC-FLØØ1-2) and RASFF notification thereof, the Community Reference Laboratory for Genetically Modified Food and Feed (CRL-GMFF) received from the German authorities a positive control sample as DNA extracted from FP967 seeds as well as a construct-specific detection method developed by Genetic ID.

The CRL-GMFF performed bioinformatics analysis and experimental testing of the NOST-Spec construct-specific method to determine its specificity and sensitivity (Limit of Detection).

The CRL-GMFF observed that the NOST-Spec construct-specific method generates a PCR amplification product of 105 bp; the amplicon shows homology to a sequence spanning the *nopaline synthase* terminator gene and the *dihydrofolate reductase* gene. The experimental testing of the specificity indicates that the NOST-Spec construct-specific assay does not detect genetically modified events under the conditions reported. The Limit of Detection (LOD) established is between 1 and 5 haploid genome copies of FP967.

5. References

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6. Acknowledgment

The Community Reference Laboratory for Genetically Modified Food and Feed would like to acknowledge the contribution of Eric Janssen, Yordan Muhovski and Gilbert Berben from the Walloon Agricultural Research Centre (CRA-W) - Department Quality of Agricultural Products (Belgium), for having provided the sequences of the NOST-Spec construct-specific and of the SAD amplicons.

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The method below was developed by Genetic ID NA, Inc. as a “NOST-Spec Construct-specific method for the detection of the CDC Triffid Flax (Event FP967) Using Real-time PCR”.

The method was transmitted to the Community Reference Laboratory for GM Food and Feed (CRL-GMFF) of the European Commission Joint Research Centre by the German Authorities.

The information given in this document is made publicly available as received with no further modifications. The CRL-GMFF cannot be held responsible for the truthfulness of the information provided in this document.

The entire risk as to the use, quality, analytical results and performance of the method is with the final user of the method.

NOST-Spec Construct-specific Method for the Detection of CDC Triffid Flax (Event FP967) Using Real-time PCR

Genetic ID NA, Inc.

Version 3
30. October 2009

1. General Information

For GM flax (*Linum usitatissimum)* a construct-specific Real-time PCR method is proposed for the detection and quantification of CDC Triffid Flax (event FP967). The procedure includes (1) a DNA extraction procedure, (2) a Real-time system for a flax specific “house-keeping” target gene (3) a Real-time system specific for the GM construct utilized in CDC Triffid Flax (event FP967), and (4) as reference a plasmid containing a 77 bp fragment of the house-keeping target gene SAD and a 95 bp fragment of the GM CDC Triffid flax construct.

DNA Extraction

For DNA extraction and purification the silica membrane column based Fast ID Genomic DNA Extraction kit was compared to the Machery&Nagel Plant II kit. Subsequent validation was performed using the Fast ID kit only.

Real-time PCR system

The PCR system uses standard cycling conditions with Taqman® chemistry for a singlex PCR system. The oligonucleotide probes use FAM as fluorescent reporter dye and Blackhole 1 as quencher. An ABI7500 instrument was used.

Target Genes

For flax species reference a 77 bp fragment specific for the “house-keeping” target gene stearoyl-acyl carrier protein desaturase 2, EC 1.14.19.2 (SAD) is used. For detection of CDC Triffid Flax (event FP967) a 95 bp overlap fragment is used. This NOST-Spec overlap bridges the NOS terminator and spectinomycin resistance gene contained within the T-DNA of the FP967 construct (Petition for Determination of Nonregulated Status for CDC Triffid Flax of the US Department of Animal and Plant Health Inspection Service). The spectinomycin resistance gene is unique for this GM flax as it is not used in currently approved GM events of any species (http://www.agbios.com/dbase.php).

The transgene integrated in at least two unlinked loci (Decision Document 98-24: Determination of the Safety of the Crop Development Centre's 'CDC Triffid', a Flax (*Linum usitatissimum* L.) Variety Tolerant to Soil Residues of Triasulfuron and Metsulfuron-methyl) of the Canadian Food Inspection Agency).

Sequences

In order to obtain an amplicon specific for CDC Triffid flax, primers for NOST and spectinomycin resistance gene were used to amplify the DNA region between these two genes in GM flax. A BLAST search of the resultant 1.3kbp overlap sequence confirmed the presence of NOST linked to spectinomycin resistance gene. The NOST-Spec specific primer set was then designed to cover the overlap region. The SAD, NOST and spectinomycin resistance gene sequences were derived from http://www.ncbi.nlm.nih.gov/nuccore/X70962, http://www.ncbi.nlm.nih.gov/nuccore/DQ666282, http://www.ncbi.nlm.nih.gov/nuccore/206148847

Reference

A synthetic plasmid reference “FlaxGM” was created to contain both the 77 bp SAD and 95 bp NOST-Spec fragment. Both fragments are present at a single copy. This plasmid is used for the construction of the Ct/log concentration standard curve for both the flax reference SAD and the GM target gene NOST-Spec. Target gene values of samples are extrapolated from the standard curve and the GM content is calculated by dividing the NOST-Spec value by the SAD value for the sample (normalization).

2. DNA Extraction and Purification

Sample matrix was ground flax seeds. The Fast ID Genomic DNA Extraction kit and Machery&Nagel Plant II kit were compared for their ability to extract pure DNA suitable for real time PCR application. Sample size in both cases was 100mg of ground flax seeds. (*Note:* For this validation a sample size of 100 mg

was chosen following the Machery&Nagel Plant II kit instructions. However, for routine GMO analysis of flax a sample size of at least 200 mg is recommended).
DNA extraction and purification was performed according to the manufacturer's instruction for the Machery&Nagel Plant II kit. The DNA extraction and purification using the Fast ID kit followed the protocol with the optional chloroform extraction. Briefly, the procedure of the Fast ID kit involves:

- Lysis of 100mg sample with 1ml of Genomic Lyse buffer. This buffer was premixed with 20μl RNAse (1mg/ml) and, per ml of Genomic Lyse, with 10μl Proteinase K (10mg/ml).
- Incubation for 10-30min at 65°C followed by a brief centrifugation
- Extraction with 1ml of chloroform
- Addition of 1ml of Genomic Bind buffer and passage through the DNA binding column
- Consecutive washing steps with Genomic Wash solution and 75% ethanol
- Elution of the purified DNA with 150μl 1xTE

Some difficulties were observed with handling of the samples when following the Machery&Nagel Plant II kit instructions such as clogging of the columns and observation of precipitates. DNA yield was greater using the Fast ID Genomic DNA Extraction kit, therefore this kit was used for all subsequent validation experiments.

a. DNA concentration

A ground flax sample was extracted six times on two consecutive days for the Fast ID kit and on one day for the Plant II kit and the concentration and purity evaluated. The concentration of DNA was measured with UV spectroscopy. The average yield was 30ng/μl (standard deviation 6.19, 21% CV) for the Fast ID kit and 7.5ng/μl (standard deviation 2.39, 32% CV) for the Plant II kit (see Table 1). The average yield was 4.5μg and 1.1μg per 100mg sample for the Fast ID kit or Plant II kit, respectively.

Day	Sample #	Concentration μg/ml
Fast ID Genomic DNA Extraction kit		
1	#1	19.50
1	#2	35.80
1	#3	30.35
1	#4	23.20
1	#5	38.60
1	#6	35.05
2	#7	37.15
2	#8	27.30
2	#9	22.45
2	#10	33.25
2	#11	28.55
2	#12	27.90
Machery&Nagel Plant II kit		
1	#1	6.30
1	#2	9.70
1	#3	6.55
1	#4	6.00
1	#5	5.30
1	#6	11.30

Table 1: DNA concentration of 12 sample extraction repetitions for the Fast ID kit and 6 sample extraction repetitions for the Plant II kit.

b. DNA fragmentation state

For evaluation of the fragmentation state, DNA from each of the six samples from the third day extraction of the Fast ID kit and the Plant II kit was subjected to agarose gel electrophoresis. Electrophoresis was performed on a 2% NuSieve 3:! Agarose gel cast with Gelstar dye for marking double stranded DNA. Molecular marker was the 50-2,000bp Marker from Sigma P9577). The Fast ID and the Plant II kit both resulted in intact genomic DNA. Data for samples extracted with the Fast ID kit are shown in Figure 1.

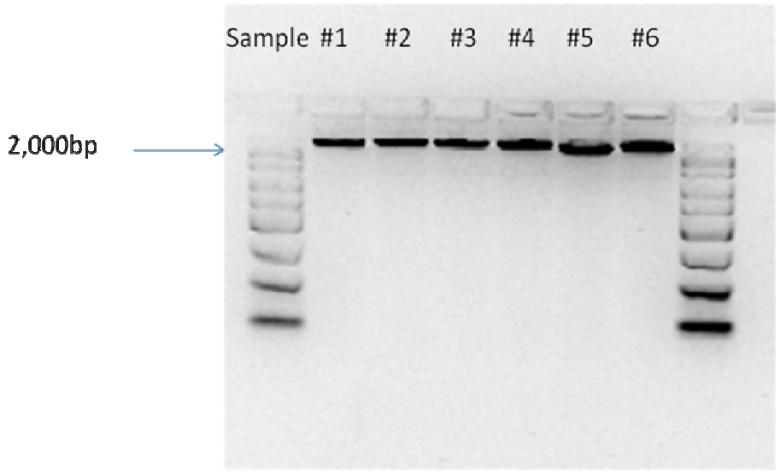

Figure 1: Agarose gel electrophoresis of six genomic DNA samples extracted from ground flax sees extracted and purified with Fast ID Genomic DNA Extraction kit.

c. Purity of DNA extracts

Purity and absence of inhibition of the DNA was evaluated by serial 1:2 dilutions of DNA extracted from six replicates of the Fast ID kit. The data indicate the absence of inhibition (Table 2a, 2b).

	Undiluted (20μg/ml)	**Diluted**			
DNA Extract	**1:1**	**1:2**	**1:4**	**1:16**	**1:32**
1	22.87	23.89	25.00	25.93	26.87
2	22.63	23.64	24.66	25.76	26.52
3	23.98	24.95	25.85	26.85	27.66
4	22.15	23.15	24.09	25.08	26.00
5	24.03	25.05	26.07	26.99	28.02
6	21.17	22.18	23.10	24.11	25.01

Table 2a: Ct values of undiluted and serially twofold diluted DNA sample extracts after amplification of the flax housekeeping gene SAD.

DNA Extract	R^2	Slope	Ct extrapolated	Ct measured	ΔCt
1	0.999	-3.335	22.90	22.87	0.03
2	0.997	-3.285	22.66	22.63	0.03
3	0.999	-3.205	24.01	23.98	0.03
4	1.000	-3.201	22.17	22.15	0.02
5	1.000	-3.294	24.05	24.03	0.02
6	1.000	-3.188	21.19	21.17	0.02

Table 2b: Comparison or extrapolated Ct values versus measured Ct values of DNA extracts after amplification of the SAD gene. Slopes and R^2 values are from the Ct/log-concentration standard curves. ΔCt values are (Ct extrapolated – Ct measured).

ΔCt values are <0.5 cycles which indicates the absence of inhibiting, PCR suppressing compounds in the purified DNA. Linear regression values are for all samples >0.99 and the slopes are between -3.6 and -3.1.

3. Real-time PCR

a. Real Time PCR set-up

In a 96 well plate the following components were added to obtain a total volume of 25μl per reaction (Table 3) and covered with optical sealing tape:

Component	Final Concentration	μl / reaction
Taqman Universal PCR Master Mix (2x)	1x	12.50
FW Primer	800nM	1.00
RV Primer	800nM	1.00
Probe	100nM	0.125
Purified Water	NA	0.375

Template DNA (max 200ng)	NA	10.00

Table 3: Reaction mixture for the amplification of SAD and NOST-Spec target genes.

Sequences of primers are as follows (Table 4):

Name	**Oligonucleotide DNA Sequence (5' to 3')**
SAD	
FW Primer	GCT CAA CCC AGT CAC CAC CT
RV Primer	TGC GAG GAG ATC TGG AGG AG
Probe	6-FAM-5'- TGT TGA GGG AGC GTG TTG AAG GGA -3'-BHQ
NOST-Spec	
FW Primer	AGC GCG CAA ACT AGG ATA AA
RV Primer	ACC TTC CGG CTC GAT GTC TA
Probe	6-FAM-5'- CGC GCG CGG TGT CAT CTA TG -3'-BHQ

Table 4: Sequences of primers and probes

For each sample extract, two reactions are performed per target gene: two reactions for the NOST-Spec and two reactions for the SAD. The FlaxGM plasmid for reference was used at 10^5, 10^4, 10^3 and 10^2 copy numbers per reaction for each primer set. Standard Real-time PCR cycling conditions were used as described in table 5:

Step	**Stage**	**Temperature, °C**	**Time, sec**	**Data Acquisition**	**Cycles**
1	UNG	50°C	120	No	1
2	Denaturation	95°C	600	No	1
3	Amplification	95°C	15	No	45
		60°C	60	Yes	

Table 5: Cycling program for amplification of SAD and NOST-Spec target genes.

b. Data Analysis

Data are analyzed for each target gene separately. The threshold is set within the exponential phase of the PCR for all amplification plots where no “fork effect” between repetitions of the same sample is observed. The baseline is set 3 cycles before the cycle at which the first of the amplification curves crosses the threshold. Data are exported for further calculations. The duplicate values per sample duplicate and primer are averaged. For normalization per sample duplicate the averaged GM target gene value (NOST-Spec) is divided by the averaged reference gene value (SAD). In order to obtain the %GM per sample duplicate the value obtained must be divided by two because the plasmid contains one copy of the GM target gene NOST-Spec whereas CDC Triffid event FP967 contains two unlinked insertions of the T-DNA (see Introduction). Finally, the %GM is calculated as the average of sample duplicate %GM values.

c. Specificity of SAD

The specificity of the SAD primer set was tested against all major crop plant species as well as against representative mammal, bird and fish species. DNA’s were tested in duplicate at 200ng per reaction. No amplification was observed for wheat, barley, rice, and canola. For soy and corn, amplification plots were observed at a level of ~0.0005ng flax DNA. Controls were as expected: amplification for the positive control (flax DNA) and absence of amplification for the negative control (non-template control).

d. Amplification Efficiency of SAD

The amplification efficiency in two experiments was 0.96 and 1.01. The first of the experiments was run with the FlaxGM plasmid as reference for

the range from 10,000 to 10 copies per reaction of target gene. The second experiment was run with flax DNA in the range 200ng to 0.1ng DNA per reaction.

e. R^2 Coefficient of SAD

The R^2 coefficient in two experiments was 0.982 and 0.999. The first of the experiments was run with the FlaxGM plasmid as reference for the range from 10,000 to 10 copies of target gene. The second experiment was run with flax DNA in the range 200ng to 0.1ng DNA per reaction.

f. Limit of Detection (LOD) of SAD

In order to determine the LOD 200ng flax DNA per reaction were used. In two experiments 24 of 24 reactions resulted in amplification at Ct~32.

g. Specificity of NOST-Spec

The following GM events were tested in duplicate at 200ng per reaction and no amplification was observed: Corn DBT418, Bt176, Bt11, GA21, DLL25, CBH-351, T14, Mon810, NK603, TC1507, Mon863, DAS-59122, Mon88017, MIR604, Soy Mon40-3-2, Mon89788, Canola MonGT73, HCN92, Oxy-235, Laureate23-198, MS8xRF3, MS1xRF1, MS1xRF2, T45, Cotton Mon1445, Mon531, BXN, Potato RBMT21-129, Alfalfa J101,J163, Zucchini ZW20. In addition, no amplification plots were observed in natural non-GM flax. Controls were as expected: amplification for the positive control (GM flax) and absence of amplification for the negative control (non-template control).

h. Amplification Efficiency of NOST-Spec

The amplification efficiency was 0.92 in two experiments for the range from 10,000 to 10 copies per reaction of target gene.

i. R^2 Coefficient of NOST-Spec

The R^2 coefficient was 0.983 in two experiments for the range from 10,000 to 10 copies of target gene.

j. Precision of NOST-Spec

For determination of the precision of the flax GM test utilizing NOST-Spec and SAD for flax reference gene, the DNA of two available samples was extracted and purified with the Fast ID kit and subjected to Real-time PCR. The samples were run in two experiments in triplicates. Target gene values for SAD and NOST-Spec were obtained from the Ct/log concentration standard curve, the NOST-Spec value normalized by the SAD value and divided by two in order to obtain a %GM value for flax (Table 6). For each sample 6 data points were averaged and the relative standard deviation %CV calculated. Sample 1 resulted in an average of 0.62 %GM (%CV 2.9%) and sample 2 in an average of 0.008% GM (%CV 22%).

Replica	%GM Sample 1	%GM Sample 2
1	0.637	0.007
2	0.593	0.006
3	0.633	0.006
4	0.640	0.010
5	0.619	0.009
6	0.611	0.009

Table 6: %GM values for two samples run in replicas in two Real-time experiments.

In order to define the Limit of Quantification (LOQ) and dynamic range samples encompassing a wider range of %GM is desirable. The unavailability of such material held up further studies at this time.

k. Limit of Detection (LOD) of NOST-Spec

In order to determine the LOD, the plasmid was diluted to 40, 20, 10 and 5 copies per reaction. Each dilution was run in 46 repetitions. The non-template control was negative. Each of the dilutions yielded 46 of 46 reactions. Therefore the LOD of the plasmid is estimated to be >5 copies per reaction. This is equivalent to ~0.002%GM: $2.8x10^5$ copies of the flax genome are contained within 200ng DNA with a genome size of 1C=0.70pg for flax (Bennett, M.D. and Leitch, I.J. (2004) Plant DNA C-values database (release 3.0); http://www.rbgkew.org.uk/cval/homepage.html). Therefore the LOD for this flax assay is at least 0.01% GM.

4. Summary

A construct-specific Real-time PCR method is proposed for the detection and quantification of CDC Triffid Flax (event FP967). This method uses a primer set targeting the construct specific NOST to spectinomycin resistance gene region of the GM flax. SAD was used as flax house-keeping reference gene. A plasmid “FlaxGM” containing one copy of the amplicon of NOST-Spec and SAD serves as reference. The primer sets are specific for species and CDC Triffid flax at least to extend as it has been tested with this validation. All Real-time parameters tested (amplification efficiency, linearity, precision and LOD) were within the definition of minimum performance requirements (Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing by ENGL, 2008). Upon availability, additional work is needed with a wider range of flax GM samples.

European Commission

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Abstract

Further to the detection by the German authorities of the unauthorised flax CDC Triffid event FP967 (Unique Identifier CDC-FLØØ1-2) in materials imported from Canada, a notification was sent through the Rapid Alert System for Food and Feed (RASFF) in September 2009.

On 21st August 2009, the Community Reference Laboratory for Genetically Modified Food and Feed received from the German authorities a construct-specific method for the detection of flax CDC Triffid event FP967, developed by Genetic ID, Augsburg (Germany). The method developer declared this method as specific for event FP967 as it targets a transition sequence spanning the *nopaline synthase* (*nos*) terminator gene and the *spectinomycin/streptomycin* resistance gene, construction being found only in the flax FP967 event.

On 11th September 2009, the CRL-GMFF received from the German authorities the FP967 positive control in the form of DNA extracted from seeds. Seeds were provided to the German authorities by the University of California, Riverside, USA. The CRL-GMFF carried out experiments on the control sample received in order to verify the specificity and the Limit of Detection (LOD) of the construct-specific method.

The CRL-GMFF observed that the NOST-Spec (nos terminator – spectinomycin resistance gene) construct-specific method generates a PCR amplification product of 105 bp, whose sequence is homologous to a transition sequence spanning the *nopaline synthase* (nos) terminator gene and the *dihydrofolate reductase* gene. The experimental testing of the specificity indicates that the NOST-Spec construct-specific assay does not detect genetically modified events under the conditions reported. The limit of detection (LOD) established is between 1 and 5 haploid genome copies of FP967.

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