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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.

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

Genetic ID NA, Inc.

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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.

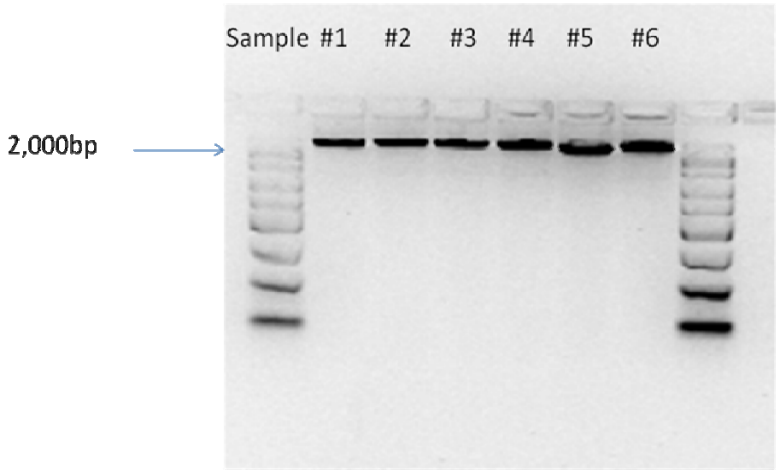

Figure 1: Agarose gel electrophoresis of six genomic DNA samples extracted from ground flax sees extracted and purified with Fast ID Genomic DNA Extraction kit.

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.