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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

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Joint Research Centre
Institute for Health and Consumer Protection
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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.

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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.

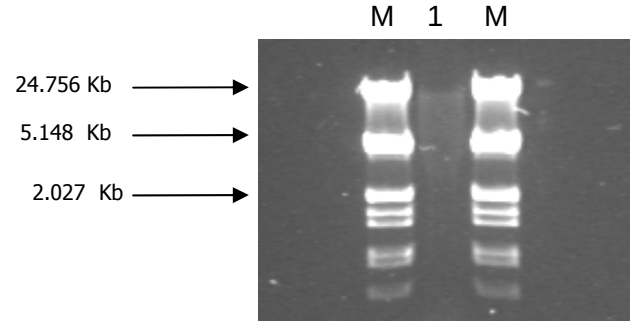
Given the experimental design for a LOD study where it is required to test a large number of replicates in each sample characterised by defined analyte content (GM level) over a decreasing series of concentrations, the Cochran approach was accepted as the most feasible.

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.

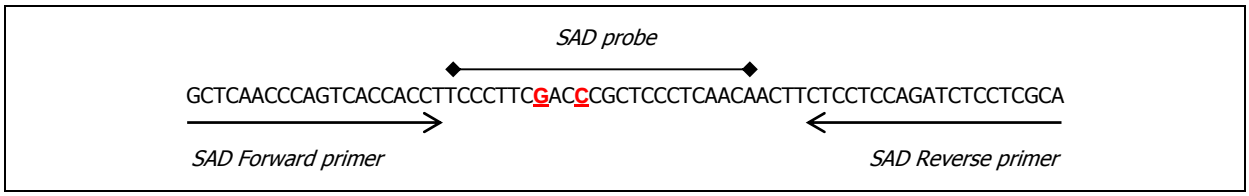


Figure 3. Sequence of the SAD amplicon

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.

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