



T-nos::spc Construct-Specific Method for the Detection of CDC Triffid Flax (Event FP967) Using Real-Time PCR

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Genetic ID (Europe) AG
Am Mittleren Moos 48
86167 Augsburg Germany
+49.821.747.7630
www.genetic-id.de

Genetic ID NA, Inc.
504 N. 4th. St.
Fairfield, Iowa 52556 USA
+1.641.472.9979
www.genetic-id.com

1. Background

CDC Triffid (FP967) flax/linseed was developed by the Crop Development Centre (CDC) at the University of Saskatchewan. This flax is tolerant to soil residues of sulfonylurea herbicides such as triasulfuron and metsulfuron-methyl. FP967 flax received Canadian regulatory approval in 1996 for feed, and in 1998 for food. FP967 flax received regulatory approval in the United States in 1998 for food and feed. CDC Triffid (Event FP967) is currently the only approved GM flax, however Triffid certified seed has never been sold to produce flax for food, feed or for processing. In 2001 Triffid was officially de-registered by the Canadian Food Inspection Agency and all remaining seed stocks of CDC Triffid were destroyed.

FP967 flax is currently not authorized in the European Union for food or feed use. Due to the zero tolerance regulatory policy in the EU for GM events that have not been authorized, qualitative assays which detect and discriminate potential GM flax from other transgenic crops are sufficient.

2. Assay development

In March 2009 Genetic ID Europe received linseed/flax seed samples from customers for GM analysis. These samples were shown to be positive for T-nos. A positive result for a GM target gene was unexpected for linseed/flax samples and therefore prompted a series of further analyses. Samples were tested for the potential presence of other species such as canola, soy and corn which also contain the T-nos construct. Also, additional target genes were investigated which are present in the GM construct of CDC Triffid. T-nos positive samples shown to be devoid of traces of canola, soy or corn tested positive for the kanamycin resistance gene *nptII* from *Escherichia coli* and the native promoter for nopaline synthase, P-nos from *Agrobacterium tumefaciens*, both of which are present in CDC Triffid (1,2).

Since the target genes nptII and P-nos are also present in certain varieties of GM canola, a CDC Triffid specific test is necessary to definitively discriminate potential traces of GM canola from potentially transgenic flax. Because the spectinomycin/streptomycin resistance gene (spc) from the *E. coli* vector used for creating the CDC Triffid flax is, according to currently approved GM events, unique for CDC Triffid flax (3), T-nos samples were first tested for the presence of spc construct and were demonstrated to be positive. Although this result was highly indicative of the presence of CDC Triffid, it was necessary to provide customers a test which would detect a region overlapping two transgenic genes which would confirm that genetically modified DNA was contained in the sample and also exclude the possibility of a false positive. For these reasons the German surveillance laboratory of Hessen (LHL) chose to utilize the p-nos::nptII overlapping sequence. However, this construct is also present in certain GM canola varieties (4). We chose an overlap which includes the spectinomycin resistance gene (spc). The T-nos::spc construct-specific test we developed is directed to the overlapping region T-nos to spc within the transgenic construct present in CDC Triffid FP967. *Whereas the T-nos::spc construct is present in CDC Triffid, it is not present in any other GM event that is commercialized or has received regulatory approval. Importantly, if an unapproved GM event contains a construct with this overlap then this assay would also detect it thus providing additional important information on the presence of unapproved GM events.*

3. General information for the construct-specific GID flax test

For GM flax (*Linum usitatissimum*) a construct-specific Real-time PCR method for the detection of CDC Triffid Flax (Event FP967) was developed. The procedure includes (i) a DNA extraction procedure, (ii) a Real-time PCR system for a flax specific “house-keeping” target gene (iii) a Real-time PCR system specific for the GM construct utilized in CDC Triffid Flax (Event FP967), and (iv) as reference, a plasmid containing sequences for the house-keeping (species reference) target gene and the GM CDC Triffid flax construct.

The target gene used for the flax species reference is a 77 bp fragment of the “house-keeping” target gene stearyl-acyl carrier protein desaturase 2, EC 1.14.19.2 (SAD). For detection of CDC Triffid Flax (Event FP967) a 95 bp overlap fragment is used. This T-nos::spc overlap bridges the NOS terminator and Spectinomycin resistance gene contained within the T-DNA of the FP967 construct. The transgene integrated in at least two unlinked loci (2).

In order to obtain an amplicon specific for CDC Triffid flax, primers for T-nos and Spc 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 T-nos linked to Spc. The T-nos::spc specific primer set was then designed to cover the overlap region.

For reference, a synthetic plasmid “FlaxGM” was created to contain both the 77 bp SAD and 95 bp T-nos::spc fragment. Both are present at a single copy.

4. DNA extraction and purification

For DNA extraction and purification the silica membrane column based Fast ID Genomic DNA Extraction kit was used. The method was tested and validated according to the definitions of the Joint Research Center of the EU (JRC) for performance requirements of GMO testing methods (5). The DNA purification met the described criteria. The **average DNA yield** as measured by UV spectropscopy was 30ng/μl which is sufficient for PCR analyses. For evaluation of the **fragmentation state**, DNA from each of six samples was subjected to agarose gel electrophoresis. The Fast ID kit resulted in intact genomic DNA. 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.

5. Real-time PCR

The PCR system uses 45 cycles of standard cycling conditions with Taqman® chemistry for a singlex PCR system. This test also passed the criteria for performance requirements for GMO testing methods (5). In quantitative terms, the **amplification efficiency** and **R² coefficient** for the primer sets directed against both targets, SAD and T-nos::spc, were within the defined expectations.

The **specificity of the SAD primer** set was confirmed against all major crop plant species as well as against representative mammal, bird and fish species. DNA was tested in duplicate at 200ng per reaction. The **specificity of T-nos::spc** was evaluated against reference material from ~30 GM events. The absence of amplification plots for each event proved the specificity of this test. In addition, no amplification plots were observed for natural non-GM flax. The specificity of this test for CDC Triffid was confirmed by the official surveillance laboratory of Bavaria/Germany, LGL Bavaria, and by the State Institute for Chemical and Veterinary Analysis in Freiburg/Germany (CVUA) who tested flax samples and obtained amplicons identical in length and sequence to those of FP967 positive control reference material (provided by LGL Bavaria) (6). Based on this finding the method was submitted to JRC for further validation and availability to all ENGL laboratories.

The **limit of detection (LOD)** of T-nos::spc is at least 0.01% GMO. For the plasmid the LOD was determined to be >5 copies per reaction. This is equivalent to ~0.002%GM (2.8×10^5 copies of the flax genome are contained within 200ng DNA with a genome size for flax of $1C=0.70\text{pg}$) (7).

6. Summary

A construct-specific Real-time PCR method for the detection and quantification of CDC Triffid Flax was developed due to the unexpected detection of T-nos in some flax-containing samples. This test detects Event FP967 as confirmed by

German surveillance laboratories (4). The method uses a primer set targeting the construct-specific T-nos to Spc region of CDC Triffid flax. A plasmid “FlaxGM” containing one copy of the amplicon of T-nos::spc and the flax reference SAD serves as reference. The primer sets are specific for species and the CDC Triffid flax construct with a sensitivity which allows the detection of 0.01% GM flax. All Real-time parameters tested (amplification efficiency, linearity, precision and LOD) were within the definition of minimum performance requirements.

7. References

- (1) Petition for Determination of Non-regulated Status for CDC Triffid Flax: <http://www.agbios.com/docroot/decdocs/04-225-005.pdf>
- (2) Decision Document 98-24 of the Canadian Food Inspection Agency: 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: <http://www.agbios.com/docroot/decdocs/01-290-031.pdf>
- (3) <http://www.agbios.com/dbase.php>
- (4) http://www.untersuchungsämter-bw.de/pub/beitrag.asp?subid=3&Thema_ID=17&ID=1206&Pdf=No
- (5) Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing by ENGL, 2008
- (6) http://www.ua-bw.de/uploaddoc/cvuafr/gm_linseed_further_analytical_informations.pdf
- (7) Bennett, M.D. and Leitch, I.J. (2004) Plant DNA C-values database (release 3.0); <http://www.rbgekew.org.uk/cval/homepage.html>