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INSTITUTE FOR HEALTH AND CONSUMER PROTECTION
COMMUNITY REFERENCE LABORATORY FOR GM FOOD AND FEED

Event-specific Method for the Quantification of Amylopectin Potato Event EH92-527-1 Using Real-time PCR

Protocol

Method development:
BASF Plant Science GmbH

Method validation:
Joint Research Centre – European Commission
Biotechnology & GMOs Unit
Community Reference Laboratory for GM Food and Feed

Contents

Document Approval		
Name / *Function*	**Date**	**Signature**
Marco Mazzara *Sector Head*	**14/09/2006**	**Signed**
Stephane Cordeil *Quality Manager*	**14/09/2006**	**Signed**
Guy Van den Eede *B&GMOs Unit Head*	**14/09/2006**	**Signed**

Address of contact laboratory:

European Commission, Joint Research Centre
Institute for Health and Consumer Protection (IHCP)
Biotechnology and GMOs Unit – Community Reference Laboratory
Via Fermi 1, 21020 Ispra (VA) - Italy

1. General information and summary of the methodology

This protocol describes an event-specific real-time quantitative TaqMan® PCR procedure for the determination of the relative content of Amylopectin event EH92-527-1 DNA to total potato DNA in a sample in a simplex format.

The PCR assay has been optimised for use in an ABI Prism® Sequence Detection System (Applied Biosystems).

The quantitative event-specific detection method for EH92-527-1 potato DNA is used in conjunction with a DNA extraction method which yields DNA of sufficient purity.

Based on the sequence information about the integration site (right border) of the transgenic potato line EH92-527-1 and the transformed plasmid pHoxwG, the PCR system was established on the transition between the plant genome and the nos promoter. Two primers and one probe were chosen to amplify and detect an event-specific 134-bp fragment.
PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with two fluorescent dyes: FAM as a reporter dye at its 5′ end and TAMRA as a quencher dye at its 3′ end.

For relative quantification of event EH92-527-1 DNA, a potato-specific reference system that targets the UDP-glucose pyrophosphorylase (UGPase) gene from *Solanum tuberosum* amplifies a 88-bp fragment that is located in the intron 2 of the UDP-glucose pyrophosphorylase (UGPase) gene (1).

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the “Ct” value. For quantification of the amount of event EH92-527-1 DNA in a test sample, event EH92-527-1 and UDP-glucose Ct values are determined for the sample. Standard curves are then used to calculate the relative content of event EH92-527-1 DNA to total potato DNA.

2. Validation status and performance characteristics

2.1 General

The reproducibility and trueness of the method were tested through collaborative trial using samples at different GMO contents.

2.2 Collaborative trial

The method was validated in a collaborative trial by the Joint Research Centre (JRC) of the European Commission. The study was undertaken with 12 laboratories.

Each participant received twenty unknown samples containing EH92-527-1 potato genomic DNA at five concentration levels, between 0.10 % and 5.5 %.

Each test sample was analyzed by PCR in three repetitions. The study was designed as a blind quadruplicate collaborative trial; each laboratory received each level of GM EH92-527-1 in four unknown samples. Two replicates of each GM level were analyzed on the same PCR plate.

A detailed validation report can be found under http://gmo-crl.jrc.it/statusofdoss.htm

2.3 Limit of detection

The LOD was calculated by the applicant based on six replicates and found to correspond to a theoretical 0.625 genome copies/reaction. The relative LOD was not assessed in a collaborative trial. The lowest relative concentration of the target sequence included in collaborative trial was 0.10%.

2.4 Limit of quantitation

According to the method developer, the relative LOQ of the method is 0.09%. The lowest relative concentration of the target sequence included in collaborative trial was 0.10%.

2.5 Molecular specificity

2.5.1 Blast search for EH92-527-1 system

The BLAST search were done at the National Center for Biotechnology Information (NCBI) with the "Standard nucleotide-nucleotide BLAST [blastn]" (www.ncbi.nlm.nih.gov/blast/Blast.cgi) on the amplicon sequence of the event-specific system.
No 100% match with other plant GMO sequences was found.

The specificity was experimentally tested against DNA extracted from different plant materials (10000 genomic copies/reaction): NewLeaf potato, a number of non GM potato lines ("Prevalent", "Hinderburg", "La Ratte", "Pink fir Apple"), non GM potato "Prevalent" (cell culture), pHoxwG (transformation plasmid), maize Bt176, soy RuR, rapeseed Seed Link, non GM rice, non GM wheat and a number of transgenic cell culture

potato lines derived from transformation with a construct related to pHoxWG (AM99-1089, AM00-1005, AM02-1003, AM02-1017).
None of the materials yielded detectable amplification. Only the event EH92-527-1 gave a positive signal.

2.5.1 Blast search for the Potato-specific (UGPase) reference system

The BLAST search were done at the National Center for Biotechnology Information (NCBI) with the "Standard nucleotide-nucleotide BLAST [blastn]" (www.ncbi.nlm.nih.gov/blast/Blast.cgi) on the amplicon sequence of the species-specific system.
Except for *Solanum tuberosum* UDP-glucose pyrophosphorylase (UGPase) gene, matches only for *Solanum demissum.*

The specificity was experimentally tested against DNA extracted from different GM and non-GM plant materials (10000 genomic copies/reaction): soybean rapeseed, maize, cotton, wheat, rice, tomato sweet pepper, egg-plant, tobacco. Detectable amplification was found in tomato, sweet pepper, egg-plant, tobacco. No amplification was detected in non-*Solanaceae* species.

3. Procedures

3.1 General instructions and precautions

- All handling of reagents and controls should occur in an ISO 9001:2000 or ISO 17025 environment or equivalent.
- The procedures require experience of working under sterile conditions.
- Laboratory organization, e.g. "flow direction" during PCR-setup, should follow the guidelines given by relevant authorities like e.g. ISO, CEN, Codex alimentarius commission.
- PCR-reagents shall be stored and handled in a separate room and freezer and in equipment where no nucleic acids (with exception of PCR primers or probes) or DNA degrading or modifying enzymes have been handled previously. All handling of PCR reagents and controls requires dedicated equipment – especially pipettes.
- All the equipment used must be sterilized prior to use and any residue of DNA has to be removed. All material used (e.g. vials, containers, pipette tips, etc.)

must be suitable for PCR and molecular biology applications. They must be DNase-free, DNA-free, sterile and shall not adsorb protein or DNA.

- In order to avoid contamination, filter pipette tips protected against aerosol should be used.
- Use only powder-free gloves and change them frequently.
- Clean lab-benches and equipment periodically with 10% sodium hypochloride solution (bleach).
- Pipettes should be checked regularly for precision and calibrated, if necessary.
- All handling steps - unless specified otherwise - shall be carried out at 0 - 4°C.
- In order to avoid repeated freeze/thaw cycles, aliquots should be prepared.

3.2 Real-time PCR for quantitative analysis of EH92-527-1 potato

3.2.1 General

The PCR set-up for the taxon specific target sequence (UGPase) and for the GMO (EH92-527-1) target sequence should be carried out in separate vials.

The use of maximum of 200 ng of template DNA per reaction well is recommended.

The method is developed for a total volume of 25 µl per reaction mixture with the reagents as listed in Table 1 and Table 2.

3.2.2 Calibration

Separate calibration curves with each primer/probe system are generated in the same analytical amplification run.

The calibration curves consist of four samples. The first point of the calibration curves is a 10% EH92-527-1 in non-GM potato DNA for a total of 200 ng of DNA (corresponding to 111111 potato genome copies with one genome assumed to correlate to 1.8 pg of haploid potato genomic DNA) (2).
A series of 1:5 dilutions down to 1.6 ng of total potato DNA/sample (S4) starting from S1 may be used.

A calibration curve is produced by plotting Ct-values against the logarithm of the target copy number for the calibration points. This can be done e.g. by use of spreadsheet software, e.g. Microsoft Excel, or directly by options available with the sequence detection system software.

The copy numbers measured for the unknown sample DNA is obtained by interpolation from the standard curves.

3.2.3 Real-time PCR set-up

1. Thaw, mix gently and centrifuge the required amount of components needed for the run. **Keep thawed reagents at 1-4°C on ice**.
2. In two reaction tubes (one for EH92-527-1 system and one for the UGPase system) on ice, add the following components (Tables 1 and 2) in the order mentioned below (except DNA) to prepare the master mixes.

Table 1. Amplification reaction mixture in the final volume/concentration per reaction well for the reference UGPase specific system.

Component	Final concentration	µl/reaction
PCR buffer II 10 x	1 x	2.5
Rox Reference Dye (50 x)	1 x	0.5
Tween-20 1%	0.01%	0.25
Glycerol 20%	0.8%	1
dATPs (10 mM)	200 µM	0.5
dCTPs (10 mM)	200 µM	0.5
dGTPs (10 mM)	200 µM	0.5
dUTPs (20 mM)	400 µM	0.5
$MgCl_2$ (100 mM)	5.5 mM	1.38
UGP-af7 (10 µM)	400 nM	1
UGP-ar8 (10 µM)	400 nM	1
UGP-sf1 (10 µM)	200 nM	0.5
Ampli Taq Gold (5U/µl)	1 U/rxn	0.2
Nuclease free water	#	9.67
Template DNA (see 3.2.1 and 3.2.2)		(5)
Total reaction volume:		25

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for EH92-527-1 specific system.

Component	Final concentration	µl/reaction
PCR buffer II 10 x	1 x	2.5
Rox Reference Dye (50 x)	1 x	0.5
Tween-20 1%	0.01%	0.25
Glycerol 20%	0.8%	1
dATPs (10 mM)	200 µM	0.5
dCTPs (10 mM)	200 µM	0.5
dGTPs (10 mM)	200 µM	0.5
dUTPs (20 mM)	400 µM	0.5
$MgCl_2$ (100 mM)	4 mM	1
Event527-bf1 (Primer1) (10 µM)	300 nM	0.75
St527-R1 (Primer 2) (10 µM)	300 nM	0.75
St527-S2 (Probe) (10 µM)	160 nM	0.4
Ampli Taq Gold (5U/µl)	1 U/rxn	0.2
Nuclease free water	#	10.65
Template DNA (see 3.2.1 and 3.2.2)		(5)
Total reaction volume:		25

3. Mix gently and centrifuge briefly.
4. Prepare two reaction tubes (one for the EH92-527-1 and one for the UGPase master mix) for each DNA sample to be tested (reference curve samples, unknown samples and control samples).
5. Add to each reaction tube the correct amount of master mix (e.g. 20 x 3 = 60 µl master mix for three PCR repetitions). Add to each tube the correct amount of DNA (e.g. 5 x 3 = 15 µl DNA for three PCR repetitions). Low-speed vortex each tubes at least three times for approx 30 sec. This step is mandatory to reduce the variability among the repetitions of each sample to a minimum.
6. Spin down the tubes in a micro-centrifuge. Aliquot 25 µl in each well. Seal the reaction plate with optical cover or optical caps. Centrifuge the plate at low speed (e.g.

approximately 250 x *g* for 1 minute at 4 °C to room temperature) to spin down the reaction mixture.

7. Place the plate into the instrument.

8. Run the PCR with cycling conditions described in Table 3:

Table 3. Reaction conditions.

Step	Stage		T°C	Time (sec)	Acquisition	Cycles
1	Initial denaturation		95 °C	600”	No	1x
2a	Amplification	Denaturation	95 °C	15”	No	45x
2b		Annealing & Extension	60 °C	60”	Measure	

3.3 Data analysis

Subsequent to the real-time PCR, analyse the run following the procedure below:

a) Set the threshold: display the amplification curves of one system (e.g EH92-527-1) in logarithmic mode. Locate the threshold line in the area where the amplification profiles are parallel (exponential phase of PCR) and where there is no “fork effect” between repetitions of the same sample. Press the update button to ensure changes affect Ct values. Switch to the linear view mode by clicking on the Y axis of the amplification plot, and check that the threshold previously set falls within the geometric phase of the curves.

b) Set the baseline: determine the cycle number at which the threshold line crosses the first amplification curve and set the baseline three cycles before that value (e.g. earliest Ct = 25, set the baseline crossing at Ct = 25 – 3 = 22).

c) Save the settings

d) Repeat the procedure described in a) and b) on the amplification plots of the other system (e.g. UGPase system).

e) Save the settings and export all the data into an Excel file for further calculations.

3.4 Calculation of results

After having defined a threshold value within the logarithmic phase of amplification as described above, the instruments software calculated the Ct-values for each reaction.

The standard curves are generated both for the UGPase and EH92-527-1 specific system by plotting the Ct-values measured for the calibration points against the logarithm of the DNA copy numbers, and by fitting a linear regression line into these data.

Thereafter, the standard curves are used to estimate the copy numbers in the unknown sample DNA by interpolation from the standard curves.

For the determination of the amount of EH92-527-1 DNA in the unknown sample, the EH92-527-1 copy number is divided by the copy number of the potato reference gene (UGPase) and multiplied by 100 to obtain the percentage value (GM% = EH92-527-1/UGPase * 100).

4. Materials

4.1 Equipment

- Real-time PCR instrument for plastic reaction vessels (glass capillaries are not recommended for the described buffer composition)
- Plastic reaction vessels suitable for real-time PCR instrument (enabling undisturbed fluorescence detection)
- Software for evaluating data after standard curve method (mostly integrated in the software of the real-time PCR instrument)
- Microcentrifuge
- Micropipettes
- Vortex
- Rack for reaction tubes
- 1.5/2.0 ml tubes

4.2 Reagents

(equivalents may be substituted)

- PCR buffer II 10x, $MgCl_2$, AmpliTaq Gold polymerase (Applied Biosystems Part No. N8080241)

- Rox (Invitrogen Part No 12223-012)
- Tween20 for molecular biology (SIGMA Part No P9416-50 ML)
- Glycerol for molecular biology (minimum 99%) (SIGMA Part No G5516-100 ML)
- dATP (Amersham-Pharmacia Part No 27-2050-02)
- dCTP (Amersham-Pharmacia Part No 27-2060-02)
- dGTP (Amersham-Pharmacia Part No 27-2070-02)
- dUTP (Amersham-Pharmacia Part No 27-2040-01)
- TE-Buffer pH=8.0 (10/1 mM) (Applichem Part No A2575,1000)
- Primers and probes (Metabion)

4.3 Primers and Probes

Name	Oligonucleotide DNA Sequence (5' to 3')
EH92-527-1 target sequence	
Event527-bf1	5'-GTG TCA AAA CAC AAT TTA CAG CA -3'
St527-R1	5'-TCC CTT AAT TCT CCG CTC ATG A -3'
St527-S2	6-FAM-AGA TTG TCG TTT CCC GCC TTC AGT T–TAMRA-3'
Reference gene UGPase target sequence	
UGP-af7	5'-GGA CAT GTG AAG AGA CGG AGC -3'
UGP-ar8	5'-CCT ACC TCT ACC CCT CCG C -3'
UGP-sf1 probe	6-FAM-CTA CCA CCA TTA CCT CGC ACC TCC TCA–TAMRA

5. References

(1) Borokov AY, McClean PE, Secor GA (1997), Gene 186 (2):293-7, "Organization and transcription of the gene encoding potato UDP-glucose pyrophosphorylase".

(2) Arumuganathan, K., Earle, E.D. (1991). Nuclear content of some important plant species. Plant Mol Biol Reporter 9, 208-218.