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

Event-specific method for the quantitation of sugar beet line H7-1 using real-time PCR

Protocol

Method development:
KWS SAAT AG and Monsanto Company

Method validation:
Joint Research Centre – European Commission
Biotechnology & GMOs Unit

Contents

Document Approval		
Name / *Function*	**Date**	**Signature**
Marco Mazzara *Sector Head*	14 5 08	
Stephane Cordeil *Quality Manager*	19.05.08	
Guy Van den Eede *B&GMOs Unit Head*	19.05.08	

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 event H7-1 DNA to total sugarbeet DNA in a sample.

The PCR assay has been optimised for use in real-time PCR instruments for plastic reaction vessels. Glass capillaries are not recommended for the buffer composition described in this method.

Template DNA extracted by means of suitable methods should be tested for quality and quantity prior to the use in PCR assay. Tests for the presence of PCR inhibitors (e.g. monitor run, use of DNA spikes) are recommended.

For specific detection of event H7-1 genomic DNA, a 108-bp fragment of the recombination region of parts of the construct inserted into the plant genome is amplified using two specific primers. 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 quantitation of event H7-1 DNA, a sugar beet reference system amplifies a 121-bp fragment of the glutamine synthetase (GS) gene, a sugarbeet endogenous gene, using a pair of GS gene-specific primers and a GS gene-specific probe labelled with FAM and TAMRA as described above.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Ct" value. For quantitation of the amount of event H7-1 DNA in a test sample, event H7-1 and GS Ct values are determined for the sample. Standard curves are then used to calculate the relative content of event H7-1 DNA to total maize DNA.

2. Validation status and performance characteristics

2.1 General

The method has been optimised for DNA extracted from seeds, containing mixtures of genetically modified H7-1 and conventional sugar beet.

The reproducibility and trueness of the method was 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 thirteen laboratories.

Each participant received twenty unknown samples containing H7-1 sugar beet genomic DNA at five concentration levels, between 0.1 % and 5.0 %.

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 H7-1 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

According to the method developer, the absolute LOD of the method is 10 copies. 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 ≤ 0.045%. The lowest relative concentration of the target sequence included in collaborative trial was 0.10%.

2.5 Molecular specificity

The method utilizes a unique DNA sequence of the recombination region of parts of the construct inserted into the plant genome. The sequence is specific to H7-1 and thus imparts event-specificity to the detection method.

The specificity was assessed by Blastsearch 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 resulting from the event-specific amplification of the transition region of the sugar beet genomic DNA into the specific event.
No 100% match with other plant GMO sequences was found.

The specificity was experimentally tested against DNA extracted from plant materials containing the specific targets (at least 500 genomic copies/reaction) of Bt176 Corn, NK603 Corn, Mon810 Corn, Bt11 Corn, GA21 RR-Corn, T25 Corn, Mon863 Corn, RR-Soybean, RR Rapeseed, and against DNA from non-GM sources, i.e. rice, wheat, spinach, potato, chard, beetroot, autumn beet and sugarbeet.

None of the materials yielded detectable amplification.

Experimental tests conducted by the applicant have shown that the GS reference system cannot discriminate between sugar beet (*Beta vulgaris*) and autumn beet (*Brassica rapa*). Out of the six autumn beet varieties, chosen to represent the genetic variability of *Brassica rapa*, two reacted with the sugar beet reference system. However, in such cases, Ct figures of autumn beet, in subsequent tests carried out by the applicant upon request of the CRL, differ significantly from those obtained from samples of sugar beet when the same amount of DNA was analyzed, corresponding to a *Brassica rapa* reactivity from two thousand to at least thirty-two thousand times less than that of *Beta vulgaris*.

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. "forward 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 H7-1 sugarbeet

3.2.1 General

The PCR set-up for the taxon specific target sequence (GS) and for the GMO (H7-1) target sequence should be carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.

The use of maximum 125 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% H7-1 in non-GM sugar beet DNA for a total of 125 ng of DNA per reaction (corresponding to 100,000 sugar beet genome copies with one genome assumed to correlate to 1.25 pg of haploid sugarbeet genomic DNA) (Arumuganathan & Earle, 1991). A series of 1:5 dilutions (starting from the first point) down to 1 ng of total sugarbeet DNA per reaction 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 H7-1 system and one for the GS 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 GS specific system.

Component	Final concentration	µl/reaction
PCR buffer I 10x	1x	2.5
Rox Reference Dye (25µM)	1 µM	1
$MgCl_2$ (25mM)	5 mM	5
dATPs (10mM)	0.2 mM	0.5
dCTPs (10mM)	0.2 mM	0.5
dGTPs (10mM)	0.2 mM	0.5
dUTPs (20mM)	0.4 mM	0.5
GluA3-F primer (100µM)	150 nM	0.0375
GluA3-R primer (100µM)	150 nM	0.0375
GluD1 probe (100µM)	100 nM	0.025
Ampli Taq Gold (5U/µl)	0.04 U/µl	0.2
Nuclease free water	#	9.20
Template DNA (see 3.2.1 and 3.2.2)		
Total reaction volume:		25

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for H7-1 specific system.

Component	Final concentration	µl/reaction
PCR buffer I 10x	1x	2.5
Rox Reference Dye (25µM)	1 µM	1
$MgCl_2$ (25mM)	7 mM	7
dATPs (10mM)	0.2 mM	0.5
dCTPs (10mM)	0.2 mM	0.5
dGTPs (10mM)	0.2 mM	0.5
dUTPs (20mM)	0.4 mM	0.5
H7PLT1 primer (100µM)	400 nM	0.1
ZRH7-R2 primer (100µM)	400 nM	0.1
ZRH7 probe (100µM)	100 nM	0.025
Ampli Taq Gold (5U/µl)	0.04 U/µl	0.2
Nuclease free water	#	7.075
Template DNA (see 3.2.1 and 3.2.2)		
Total reaction volume:		25

3. Mix gently and centrifuge briefly.
4. Prepare two reaction tubes (one for the H7-1 and one for the GS 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. H7-1) 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. GS 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 GS and H7-1 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 H7-1 DNA in the unknown sample, the H7-1 copy number is divided by the copy number of the sugarbeet reference gene (GS) and multiplied by 100 to obtain the percentage value (GM% = H7-1/GS * 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

Reagents

(equivalents may be substituted)

- PCR buffer I 10x (Applied Biosystems Part No. N8080240)
- $MgCl_2$ (Applied Biosystems Part No. N8080240)
- Rox (Invitrogen PartNo. 12223-012)
- dATP (Amersham-Pharmacia Part No.27-2040-01)
- dCTP (Amersham-Pharmacia Part No.27-2040-02)
- dGTP (Amersham-Pharmacia Part No.27-2040-03)
- dUTP (Amersham-Pharmacia Part No.27-2040-01)
- ATGold (Applied Biosystems Part No. N8080240)
- TE-Buffer pH=8.0 (10/1 mM) (Applichem Part No. A2575,1000)

Primers and Probes

Name	Oligonucleotide DNA Sequence (5' to 3')
H7-1 target sequence	
H7PLT1	5'- TGG GAT CTG GGT GGC TCT AAC T -3'
ZRH7-R2	5'- AAT GCT GCT AAA TCC TGA G -3'
ZRH7 (Probe)	FAM-5'- AAG GCG GGA AAC GAC AAT CT -3'-TAMRA
Reference gene GS target sequence	
GluA3-F	5'- GAC CTC CAT ATT ACT GAA AGG AAG -3'
GluA3-R	5'- GAG TAA TTG CTC CAT CCT GTT CA -3'
GluD1 (Probe)	FAM-5'- CTA CGA AGT TTA AAG TAT GTG CCG CTC -3'-TAMRA

5. References

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