Event-specific Method for the Quantification of Soybean Line 40-3-2 Using Real-time PCR

Protocol

20 January 2009

Joint Research Centre
Institute for Health and Consumer Protection
Biotechnology & GMOs Unit

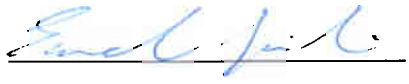
Method development:

Monsanto Company

Method validated through collaborative trial by:

Community Reference Laboratory for GM Food and Feed (CRL-GMFF)
Biotechnology & GMOs Unit

Drafted by
E. Grazioli



Report Verification Team
1) W. Moens

2) M. Querci

Scientific and technical approval
M. Mazzara

Compliance to CRL Quality System
S. Cordeil

Authorisation to publish
G. Van den Eede

Address of contact laboratory:
European Commission, Joint Research Centre
Institute for Health and Consumer Protection (IHCP)
Biotechnology and GMOs Unit – Community Reference Laboratory for GM Food and Feed
Via Fermi 1, 21020 Ispra (VA) - Italy

Content

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 soybean event 40-3-2 DNA to total soybean DNA in a sample.

The PCR assay was 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 use in PCR assay. Tests for the presence of PCR inhibitors (e.g. monitor run of diluted series, use of DNA spikes) are recommended.

For the specific detection of soybean event 40-3-2 DNA, an 84-bp fragment of the recombination region between the insert and the plant genome (located at the 5' plant DNA region) 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 the fluorescent dye FAM as a reporter at its 5′ end and with the non-fluorescent quencher MGBNFQ (minor groove binding non-fluorescent quencher [Applied Biosystems]) at its 3' end.

For the relative quantification of soybean event 40-3-2 DNA, a soybean-specific reference system amplifies a 74-bp fragment of the soybean endogenous *lectin* gene (*lec*), using a pair of specific primers and a *lec* 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 quantification of the amount of event Roundup Ready 40-3-2 DNA in a test sample, Roundup Ready 40-3-2 and *lec* Ct values are determined for the sample. Standard curves are then used to estimate the relative amount of soybean event 40-3-2 DNA to total soybean DNA.

2. Validation status and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from soybean seeds and grains containing mixtures of genetically modified and conventional soybean.

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

2.2 Collaborative trial

The method was validated in a collaborative study by the Joint Research Centre (JRC) of the European Commission. The study was undertaken with fourteen participating laboratories in October 2006.

Each participant received twenty blind samples containing Roundup Ready 40-3-2 genomic DNA at five GM contents, ranging from 0.10 % to 8.0 %.

Each test sample was analysed by PCR in three repetitions. The study was designed as a blind quadruplicate collaborative trial; each laboratory received each level of GM Roundup Ready 40-3-2 in four unknown samples. Four replicates of each GM level were analysed on the same PCR plate.

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

2.3 Limit of detection (LOD)

According to the method developer, the relative LOD of the method is at least 0.045% in 200 ng of total soybean DNA. The relative LOD was not assessed in a collaborative study.

2.4 Limit of quantification (LOQ)

According to the method developer, the relative LOQ of the method is at least 0.09% in 200 ng of total soybean DNA. The lowest relative GM content of the target sequence included in collaborative trial was 0.10 %.

2.5 Molecular specificity

The method exploits a unique DNA sequence in the region of recombination between the insert and the plant genome. The sequence is specific to soybean event 40-3-2 and thus imparts event-specificity to the method.

The specificity of event-specific assay was experimentally tested in real-time PCRs against DNA extracted from plant materials containing the specific targets of Roundup Ready® soybean 40-3-2, Roundup Ready® Canola (RT200), Roundup Ready® Canola (RT73), Conventional Canola, Roundup Ready® Corn (GA21), Roundup Ready® Corn (NK603), YieldGard® Corn Borer Corn (MON810), YieldGard® Rootworm/Roundup Ready® Corn (MON88017) , YieldGard® Rootworm corn (MON863), Lysine Maize (LY038), Conventional Corn, Roundup Ready® Cotton (MON1445), Bollgard® Cotton(MON531), Bollgard® Cotton (MON757), BollgardII® Cotton (MON15985), Conventional Cotton, Conventional Soybean, Roundup Ready® Wheat (MON71800), Conventional Wheat, Lentil, Quinoa, Sunflower, Buckwheat, Pinenuts, Rye berries, Millet, Barley, Basmati rice, Peanut.
None of the GM-lines tested, except the positive control soybean line 40-3-2, yielded detectable amplicons.

The specificity of the soybean reference assay *lec* was experimentally tested by the applicant against DNA extracted from plant materials containing Roundup Ready® soybean 40-3-2, Roundup Ready® Canola (RT200), Roundup Ready® Canola (RT73), Conventional Canola, Roundup Ready® Corn (GA21), Roundup Ready® Corn (NK603), YieldGard® Corn Borer Corn (MON810), YieldGard® Rootworm/Roundup Ready® Corn (MON88017) , YieldGard® Rootworm corn (MON863), Lysine Maize (LY038), Conventional Corn, Roundup Ready® Cotton (MON1445), Bollgard® Cotton (MON531), Bollgard® Cotton (MON757), BollgardII® Cotton (MON15985), Conventional Cotton, Conventional Soybean, Roundup Ready® Wheat (MON71800), Conventional Wheat, Lentil, Quinoa, Sunflower, Buckwheat, Pinenuts, Rye berries, Millet, Barley, Basmati rice, Peanut. None of the samples tested, except the positive control soybean line 40-3-2 and from conventional soybean, yielded detectable amplicons.

3. Procedure

3.1 General instructions and precautions

- The procedures require experience of working under sterile conditions.
- Laboratory organisation, e.g. “forward flow direction” during PCR-setup, should follow the guidelines given by relevant authorities, e.g. ISO, CEN, Codex Alimentarius Commission.
- PCR-reagents should be stored and handled in a separate room 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 should be sterilised 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 unable to adsorb protein or DNA.
- In order to avoid contamination, filter pipette tips protected against aerosol should be used.
- Powder-free gloves should be used and changed frequently.
- Laboratory benches and equipment should be cleaned periodically with 10% sodium hypochloride solution (bleach).
- Pipettes should be checked regularly for precision and calibrated, if necessary.
- All handling steps - unless specified otherwise - should 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 soybean event 40-3-2

3.2.1 General

The PCR set-up for the taxon specific target sequence (*Lec*) and for the GMO (event 40-3-2) 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 200 ng of template DNA per reaction well is recommended.
The method is developed for a total volume of 50 µl per reaction mixture with the reagents as listed in Table 1 and Table 2.

3.2.2 Calibration

The calibration curves consist of five samples. The first point of the calibration curves is a 10% 40-3-2 in non-GM soybean DNA for a total of 200 ng of DNA (corresponding to 176991 soybean genome copies with one genome assumed to correspond to 1.13 pg of haploid soybean genomic DNA) [(1)].

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

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

The ratio of transgene copy number and reference gene copy number multiplied by 100 gives the % GM contents of the samples.

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 the 40-3-2 system and one for the *lec* 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 40-3-2 specific system.

Component	Final concentration	µl/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	25
40-3-2 AF (10 µM)	150 nM	0.75
40-3-2 AR (10 µM)	150 nM	0.75
40-3-2 AP (5 µM)	50 nM	0.50
Nuclease free water	#	19
Template DNA (max 200 ng)	#	4.0
Total reaction volume:		50

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the soybean *Lec* reference system.

Component	Final concentration	µl/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	25
lec F (10 µM)	150 nM	0.75
lec R (10 µM)	150 nM	0.75
lec P (10 µM)	50 nM	0.25
Nuclease free water	#	19.25
Template DNA (max 200 ng)	#	4.0
Total reaction volume:		50

3. Mix gently and centrifuge briefly.
4. Prepare two reaction tubes (one for the Roundup Ready 40-3-2 and one for the *Lec* master mixes) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
5. Add to each reaction tube the correct amount of master mix (e.g. 46 x 3 = 138 µl master mix for three PCR repetitions). Add to each tube the correct amount of DNA (e.g. 4 x 3 = 12 µl DNA for three PCR repetitions). Vortex each tubes for approx. 10 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 microcentrifuge. Aliquot 50 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 and Table 4:

Table 3. Cycling program for 40-3-2 system

Step	Stage		T°C	Time (sec)	Acquisition	Cycles
1	UNG		50 °C	120	No	1
2	Initial denaturation		95 °C	600	No	1
3	Amplification	Denaturation	95 °C	15	No	45
		Annealing & Extension	55 °C	60	Yes	

Table 4. Cycling program for soybean *Lec* system

Step	Stage		T°C	Time (sec)	Acquisition	Cycles
1	UNG		50 °C	120	No	1
2	Initial denaturation		95 °C	600	No	1
3	Amplification	Denaturation	95 °C	15	No	45
		Annealing & Extension	60 °C	60	Yes	

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. Roundup Ready 40-3-2) 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. *Lec* system).

e) Save the settings and export all the data to a text 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 instrument's software calculates the Ct-values for each reaction.

The standard curves are generated both for the *lec* and the 40-3-2 specific systems 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.

For the determination of the amount of event 40-3-2 DNA in the unknown sample, the 40-3-2 copy number is divided by the copy number of the soybean reference gene (*lec*) and multiplied by 100 to obtain the percentage value (GM% = 40-3-2/*lec* * 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 run analysis (mostly integrated in the software of the real-time PCR instrument)
- Microcentrifuge
- Micropipettes
- Vortex
- Rack for reaction tubes
- 1.5/2.0 ml reaction tubes

4.2 Reagents

- TaqMan® Universal PCR Master Mix (2X). Applied Biosystems Part No 4304437

4.3 Primers and Probes

Name	Oligonucleotide DNA Sequence (5' to 3')
40-3-2 target sequence	
40-3-2 AF	5' – TTC ATT CAA AAT AAG ATC ATA CAT ACA GGT T – 3'
40-3-2 AR	5' – GGC ATT TGT AGG AGC CAC CTT – 3'
40-3-2 AP (Probe)	6-FAM 5' – CCT TTT CCA TTT GGG – 3' MGBNFQ
Reference gene Lec target sequence	
lec F	5' – CCA GCT TCG CCG CTT CCT TC – 3'
lec R	5' – GAA GGC AAG CCC ATC TGC AAG CC – 3'
lec P (Probe)	6-FAM 5' – CTT CAC CTT CTA TGC CCC TGA CAC – 3' TAMRA