Event-specific Method for the Quantification of Maize Line MON 89034 Using Real-time PCR

Protocol

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

Method development:

Monsanto Company

Collaborative trial:

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

Drafted by
C. Savini

Report Verification Team
1) M. Ermolli

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 2749, 21027 Ispra (VA) - Italy

Content

1. General information and summary of the method

This protocol describes an event-specific real-time quantitative TaqMan® PCR procedure for the determination of the relative content of maize event MON 89034 DNA to total maize DNA in a sample.

The PCR assay was optimised for use in real-time PCR instruments for plastic reaction vessels.

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 maize event MON 89034 DNA, a 77-bp fragment of the integration region of the construct inserted into the plant genome (located at the 3' insert-to-plant junction) 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) at its 3' end.

For the relative quantification of maize event MON 89034 DNA, a maize specific reference system amplifies a 79-bp fragment of the maize endogenous *hmg* gene (high mobility group), using a two specific primers and an *hmg* 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 MON 89034 DNA in a test sample, MON 89034 and *hmg* Ct values are determined for the sample. Standard curves are then used to estimate the relative amount of maize event MON 89034 DNA to total maize DNA.

2. Validation status and performance characteristics

2.1 General

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

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 an international collaborative study by the Joint Research Centre (JRC) of the European Commission. The study was undertaken with twelve participating laboratories in November 2007.

Each participant received twenty blind samples containing maize MON 89034 genomic DNA at five GM contents, ranging from 0.09% 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 maize MON 89034 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 0.04% in 200 ng of total maize 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 0.085% in 200 ng of total maize DNA. The lowest relative GM content of the target sequence included in collaborative trial was 0.09 %.

2.5 Molecular specificity

According to the method developer, the method exploits a unique DNA sequence in the region of recombination between the insert and the plant genome. The sequence is specific to maize event MON 89034 and thus imparts event-specificity to the method.

The specificity of event-specific assay was experimentally tested in real-time PCR by the applicant against DNA extracted from plant materials containing the specific targets of Roundup Ready® canola (RT200), Roundup Ready® canola (RT73), conventional canola, Roundup Ready® maize (GA21), Roundup Ready® maize (NK603), YieldGard® corn borer maize (MON 810), YieldGard® Rootworm/Roundup Ready® maize (MON 88017) , YieldGard® Rootworm maize (MON 863), lysine maize (LY038), MON 89034 maize, conventional maize, Roundup Ready® cotton (MON 1445), Bollgard® cotton (MON 531), Bollgard® cotton (MON 757), BollgardII® cotton (MON 15985), MON 88913 cotton, conventional cotton, Roundup Ready® soybean 40-3-2, MON 89788 soybean, conventional soybean, Roundup Ready® wheat (MON71800), conventional wheat, lentil, quinoa, sunflower nuts, buckwheat, pinenuts, rye berries, millet, peanut (shelled).
None of the GM-lines tested, except the positive control maize line MON 89034, produced detectable amplification signals.

The specificity of the maize reference assay *hmg* was experimentally tested by the applicant against DNA extracted from plant materials containing Roundup Ready® canola (RT200), Roundup Ready® canola (RT73), conventional canola, Roundup Ready® maize (GA21), Roundup Ready® maize (NK603), YieldGard® corn borer maize (MON 810), YieldGard® Rootworm/Roundup Ready® maize (MON 88017), YieldGard® rootworm maize (MON863), lysine maize (LY038), MON 89034 maize, conventional maize, Roundup Ready® cotton (MON 1445), Bollgard® cotton (MON 531), Bollgard® cotton (MON 757), BollgardII® cotton (MON 15985), MON 88913 cotton, conventional cotton, Roundup Ready® soybean 40-3-2, MON 89788 soybean, conventional soybean, Roundup Ready® wheat (MON71800), conventional wheat, lentil, sunflower, buckwheat, rye berries, peanut.
None of the samples tested, except the control maize lines GA21, NK603, MON 810, MON 863, NON 88017, LY038, MON 89034 and conventional maize, produced detectable amplification signals.

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 24276:2006.
- 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.
- 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 maize event MON 89034

3.2.1 General

The PCR set-up for the taxon specific target sequence (*hmg*) and for the GMO (event MON 89034) 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 for the MON 89034 reaction and of 25 µL for the *hmg* 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% MON 89034 maize DNA in non-GM maize DNA for a total of 200 ng of DNA (corresponding to approximately 73394 maize genome copies with one genome assumed to correspond to 2.725 pg of haploid maize 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.

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 MON 89034 system and one for the *hmg* 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 MON 89034 specific system.

Component	Final concentration	µL/reaction
TaqMan® 2x PCR Master Mix	1x	25
MON 89034 primer 1 (10 µM)	450 nM	2.25
MON 89034 primer 2 (10 µM)	450 nM	2.25
MON 89034 probe (5 µM)	100 nM	1.00
Nuclease free water	#	15.5
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 maize *hmg* reference system.

Component	Final concentration	µL/reaction
Nuclease free water	#	8.95
TaqMan® buffer A (10x)	1x	2.5
$MgCl_2$ (25 mM)	6.5 mM	6.5
dNTP mix (10 mM each)	200 µM each	0.5
AmpliTaq Gold polymerase (5 U/µl)	1.25 U	0.25
hmg primer 1 (10 µM)	300 nM	0.75
hmg primer 2 (10 µM)	300 nM	0.75
hmg probe (5 µM)	160 nM	0.8
Template DNA (max 200 ng)	#	4.0
Total reaction volume:		25

3. Mix gently and centrifuge briefly.
4. Prepare two reaction tubes (one for the MON 89034 event and one for the *hmg* 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 for the MON 89034 reactions and 21 x 3 = 63 µL for *hmg* reactions). 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 (or 25 depending on the reaction mixture) 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. Cycling program for maize MON 89034 and *hmg* systems

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. MON 89034) 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. *hmg* 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 *hmg* and the MON 89034 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 MON 89034 maize DNA in the unknown sample, the copy number of the GMO is divided by the copy number of the maize reference gene (*hmg*) and multiplied by 100 to obtain the percentage value (GM% = MON 89034/*hmg* x 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® 2X PCR Master Mix, Applied Biosystems Part No 4304437
- TaqMan® 1000X Rxn Gold/Buffer A Pack (10x) Applied Biosystems Part No 4304441

4.3 Primers and Probes

Name	Oligonucleotide DNA Sequence (5' to 3')
MON 89034 target sequence	
MON 89034 primer 1	5' – TTC TCC ATA TTG ACC ATC ATA CTC ATT– 3'
MON 89034 primer 2	5' – CGG TAT CTA TAA TAC CGT GGT TTT TAA A– 3'
MON 89034 (Probe)	6-FAM 5' – ATC CCC GGA AAT TAT GTT – 3' MGBNFQ
Reference gene hmg target sequence	
hmg primer 1	5' – TTG GAC TAG AAA TCT CGT GCT GA– 3'
hmg primer 2	5' – GCT ACA TAG GGA GCC TTG TCC T – 3'
hmg (Probe)	6-FAM 5' – CAA TCC ACA CAA ACG CAC GCG TA – 3' TAMRA