Event-specific Method for the Quantification of Maize Line MON 88017 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:

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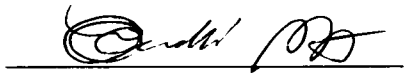

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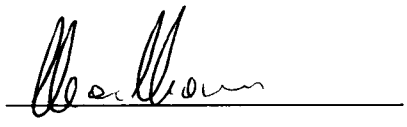

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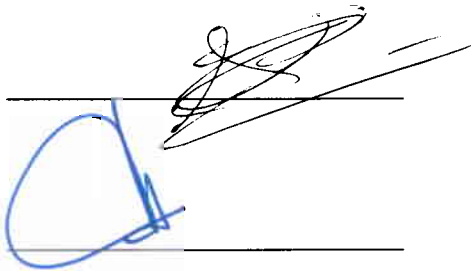

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Content

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 event MON 88017 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 88017 DNA, a 94-bp fragment of the integration region of the construct inserted into the plant genome (located at the 5' flanking DNA region) is amplified using two specific primers. PCR products are measured at each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with the fluorescent dye 6-FAM as a reporter at its 5′ end and with TAMRA as a quencher dye at its 3' end. The 5' nuclease activity of the Taq DNA polymerase results in the specific cleavage of the probe, leading to increased fluorescence, which is then monitored.

For the relative quantification of maize event MON 88017 DNA, a maize-specific reference system amplifies a 79-bp fragment of the maize endogenous *hmg* gene (*high mobility group*), using two specific primers and a *hmg* gene-specific probe labelled with 6-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 88017 DNA in a test sample, MON 88017 and *hmg* Ct values are determined for the sample. Standard curves are then used to estimate the relative amount of maize event MON 88017 DNA to total maize DNA.

2. Validation status and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from maize 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 laboratories in July 2007.

Each participant received twenty blind samples containing MON 88017 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 MON 88017 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 data provided by the applicant, the relative LOD of the method is at least 0.045% in 200 ng of total maize DNA. The relative LOD was not assessed in collaborative study.

2.4 Limit of quantification (LOQ)

According to the data provided by the applicant, the relative LOQ of the method is at least 0.09% in 200 ng of total maize DNA. The lowest relative GM content of the target sequence included in the international collaborative study was 0.09%.

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 event MON 88017 and thus imparts event-specificity to the method.

The specificity of MON 88017 assay (forward/reverse primers and probe) was experimentally tested in real-time PCR by the applicant against DNA extracted from samples containing Roundup Ready® maize MON 88017 (positive control), Roundup Ready® canola (RT200), Roundup Ready® canola (RT73), conventional canola, Roundup Ready® maize (GA21), Roundup Ready® maize (NK603), YieldGard® Corn Borer maize (MON810), YieldGard® Rootworm maize (MON863), Lysine maize (LY038), conventional maize, Roundup Ready® cotton (MON 1445), Bollgard® cotton (MON 531), Bollgard® cotton (MON 757), BollgardII® cotton (MON 15985), Roundup Ready® Flex cotton (MON 88913), conventional cotton, Roundup Ready® soybean (40-3-2), conventional soybean, Roundup Ready® wheat (MON 71800), conventional wheat, lentil, sunflower, buckwheat, rye berries and peanut.

According the applicant, none of the GM lines tested, except the positive control MON 88017, produced amplification signals.
The specificity of the maize reference assay *hmg* was experimentally tested by the applicant against DNA extracted from samples containing Roundup Ready® corn MON 88017, Roundup Ready® canola (RT200), Roundup Ready® canola (RT73), conventional canola, Roundup Ready® maize (GA21), Roundup Ready® maize (NK603), YieldGard® Corn Borer maize (MON810), YieldGard® Rootworm maize (MON863), Lysine maize (LY038), conventional maize, Roundup Ready® cotton (MON 1445), Bollgard® cotton (MON 531), Bollgard® cotton (MON 757), BollgardII® cotton (MON 15985), Roundup Ready® Flex cotton (MON 88913), conventional cotton, Roundup Ready® soybean (40-3-2), conventional soybean, Roundup Ready® wheat (MON 71800), conventional wheat, lentil, sunflower, buckwheat, rye berries and peanut.

Only the positive control maize line MON 88017 and Roundup Ready® maize (GA21), Roundup Ready® maize (NK603), YieldGard® Corn Borer maize (MON810), YieldGard® Rootworm maize (MON863), Lysine maize (LY038) and conventional maize produced amplification signals.

3. Procedure

3.1 General instructions and precautions

- The procedures require sterile conditions working experience.
- Laboratory organisation, e.g. “forward flow direction” during PCR-setup, should follow international guidelines, 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.
- Equipment used should be sterilised prior to use and any residue of DNA should be removed. All material used (e.g. vials, containers, pipette tips, etc.) should be suitable for PCR and molecular biology applications; it should 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 MON 88017 maize

3.2.1 General

The PCR set-up for the taxon specific target sequence (*hmg*) and for the GMO (event MON 88017) 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 MON 88017, in a total volume of 50 µL per reaction mixture with the reagents as listed in Table 1 and, for *hmg*, in a total volume of 25 µL per reaction mixture with the reagents as listed in 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 88017 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 88017 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 88017 specific system.

Component	Final concentration	µL / reaction
TaqMan® Universal PCR Master Mix (2x)	1x	25
MON 88017 AF (10 µM)	150 nM	0.75
MON 88017 AR (10 µM)	150 nM	0.75
MON 88017 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 maize *hmg* reference system.

Component	Final concentration	µL / reaction
Buffer A (10x)	1x	2.5
Hmg F (10 µM)	300 nM	0.75
hmg R (10 µM)	300 nM	0.75
hmg P (5 µM)	160 nM	0.80
$MgCl_2$ (25 mM)	6.5 mM	6.5
dNTPs mix (10 mM)	200 µM	0.5
Nuclease free water	#	8.95
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 88017 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 MON 88017 and 21 x 3 = 63 µL master mix for three PCR repetitions for *hmg*). 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 for MON 88017 and 25 µL for *hmg*. 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 MON 88017 and maize *hmg* 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. MON 88017) 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 88017 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 MON 88017 DNA in the unknown sample, the MON 88017 copy number is divided by the copy number of the maize reference gene (*hmg*) and multiplied by 100 to obtain the percentage value (GM% = MON 88017/*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)
- Analysis software
- 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
- 0.5 M EDTA. Sigma Cat. No. E-7647-01-0
- PCR Nucleotide Mix (10mM dNTPs). Promega Cat. No. C114G
- TaqMan® 1000X Rxn Gold/Buffer A Pack. Applied Biosystems Cat. No. 4304441
- AmplTaq Gold Polymerase. Applied Biosystems Cat. No. N808-0244
- Nuclease-free water. Sigma Cat. No. W-4502
- 1 M Tris-HCl, pH 8.0. Sigma Cat. No. T-3038

4.3 Primers and Probes

Name	Oligonucleotide DNA Sequence (5' to 3')
MON 88017 target sequence	
MON 88017 AF	5' – GAG CAG GAC CTG CAG AAG CT – 3'
MON 88017 AR	5' – TCC GGA GTT GAC CAT CCA – 3'
MON 88017 AP (Probe)	6-FAM-TCC CGC CTT CAG TTT AAA CAG AGT CGG GT-TAMRA
Reference gene hmg target sequence	
Hmg F	5' – TTG GAC TAG AAA TCT CGT GCT GA– 3'
Hmg R	5' – GCT ACA TAG GGA GCC TTG TCC T– 3'
hmg P (Probe)	6-FAM- CAA TCC ACA CAA ACG CAC GCG TA -TAMRA

5. References

1. Arumuganathan K., Earle E. D., 1991. Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter* 9: 208-218.