Event-specific Method for the Quantification of Maize MON 87460 Using Real-time PCR

Protocol

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Joint Research Centre
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Method development:

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Method validation:

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Content

1. General information and summary of the methodology

This protocol describes an event-specific quantitative real-time PCR (polymerase chain reaction) procedure for the determination of the relative content of maize event MON 87460 DNA to total maize DNA in a sample.

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

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

For the detection of maize event MON 87460 DNA, an 82 bp fragment of the region spanning the 5′ insert-to-plant junction is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with fluorescent dye FAM (6-carboxyfluorescein) as a reporter at its 5′ end and with TAMRA (carboxytetramethylrhodamine) as a quencher dye at its 3′ end.

For the relative quantification of maize event MON 87460, a maize-specific reference system amplifies a 79 bp fragment of the maize *high mobility group* gene (*hmg*), using specific primers and an *hmg* 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 MON 87460 DNA in a test sample, Ct values for the MON 87460 and *hmg* system are determined for the sample. Standard curves are then used to estimate the relative amount of MON 87460 DNA to total maize DNA.

2. Validation and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from homogenised maize grains.
The reproducibility and trueness of the method were tested through an international collaborative ring trial using DNA samples at different GM contents.

2.2 Collaborative trial

The method was validated in a collaborative study coordinated by the EU-RL GMFF. The study was undertaken with twelve participating laboratories in December 2009.

Each participant received twenty blind samples containing maize MON 87460 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 GM level in four unknown samples. Two replicates of each GM level were analysed on the same PCR plate.

A detailed validation report can be found at http://gmo-crl.jrc.ec.europa.eu/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 the 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 of the recombination region between the insert and the plant genome; the sequence is specific to maize event MON 87460 and thus imparts event-specificity to the method.

The specificity of the GMO assay was assessed by the applicant in real-time PCR against genomic DNA extracted from oilseed rape RT73, RT200; maize GA21, NK603, MON810, MON863, MON88017, LY038, MON89034, MON87460; cotton MON531, MON1445, MON15985, MON88913; soybean GTS 40-3-2, MON89788; wheat MON71800 and of conventional maize, oilseed rape, cotton, soybean, wheat, rice, millet, lentil, sunflower, and oat. According to the method developer, none of the materials tested, except the positive control maize event MON 87460, yielded detectable amplification.

The specificity of the maize reference assay *hmg* was assessed by the applicant in real-time PCR against genomic DNA extracted from oilseed rape RT73, RT200; maize GA21, NK603, MON810, MON863, MON88017, LY038, MON89034, MON87460; cotton MON531, MON1445, MON15985, MON88913; soybean GTS 40-3-2, MON89788; wheat MON71800 and of conventional maize, oilseed rape, cotton, soybean, wheat, rice, millet, lentil, sunflower, and oat. According to the method developer, none of the materials tested, except the positive controls represented by all maize events and conventional maize, yielded detectable amplification.

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 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.
- All the equipment 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 hypochlorite 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 87460

3.2.1 General

The PCR set-ups for the taxon-specific target sequence (*hmg*) and for the GMO (event MON 87460) are 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 and 25 µL for the MON 87460 and for the *hmg* systems respectively, 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 sample containing 10% MON 87460 maize DNA in a total of 200 ng of maize DNA (corresponding to approximately 73394 maize genome copies with one genome assumed to correspond to 2.725 pg of haploid maize genomic DNA)[(1)]. The other three standard samples are prepared by serial dilution.

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 using the 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. To prepare the amplification reaction mixtures add the following components (Tables 1 and 2) in two reaction tubes (one for the MON 87460 assay and one for the *hmg* assay) on ice in the order mentioned below (except DNA).

Table 1. Amplification reaction mixture in the final volume/concentration per reaction well for the MON 87460 assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	25.0
MON 87460 forward (20 µM)	600 nM	1.5
MON 87460 reverse (20 µM)	600 nM	1.5
MON 87460 probe (5 µM)	250 nM	2.5
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 assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.50
hmg primer 1 (10 µM)	300 nM	0.75
hmg primer 2 (10 µM)	300 nM	0.75
hmg-probe (5 µM)	160 nM	0.80
Nuclease free water	#	6.20
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 87460 and one for the *hmg* reaction 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 of the MON 87460 system and 21 x 3 = 63 µL master mix for the *hmg* system). Add to each tube the correct amount of DNA (e.g. 4 x 3 = 12 µL DNA for three PCR repetitions for both systems). Vortex each tube for approx. 10 sec. This step is mandatory to reduce to a minimum the variability among the repetitions of each sample.
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 250x *g* for 1 minute at 4 °C) to spin down the reaction mixture.
7. Place the plate in the instrument.
8. Run the PCR with cycling conditions described in Table 3:

Table 3. Cycling program for MON 87460/*hmg* assays

Step	Stage		T°C	Time (sec)	Acquisition	Cycles
1	Decontamination		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 87460) 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. 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 87460 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 DNA copy numbers in the unknown sample.

To obtain the percentage value of event MON 87460 DNA in the unknown sample, the MON 87460 copy number is divided by the copy number of the maize reference gene (*hmg*) and multiplied by 100 (GM% = MON 87460/hmg x 100).

4. Materials

4.1 Equipment

- Real-time PCR instrument for plastic reaction plates (glass capillaries are not recommended for the described buffer composition)
- Plastic reaction plates 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)
- Micro-centrifuge
- Centrifuge for plates
- Micropipettes
- Vortex
- Rack for reaction tubes
- 0.5/1.5/2.0 mL reaction tubes

4.2 Reagents

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

4.3 Primers and Probes

Table 4. Primers and probes sequences

Name	Oligonucleotide DNA Sequence (5′ to 3′)	Length (nt)
MON 87460 target sequence		
MON 87460 forward	5′ – CAC gTT gAA ggA AAA Tgg ATT g – 3′	22
MON 87460 reverse	5′ – TCg CgA TCC TCC TCA AAg AC – 3′	20
MON 87460 probe	6 - FAM 5' – Agg gAg TAT gTA gAT AAA TTT TCA AAg CgT TAg ACg gC – 3' TAMRA	38
Taxon specific *hmg* target sequence		
hmg primer 1	5′ – TTg GAC TAg AAA TCT CgT gCT gA – 3′	23
hmg primer 2	5′ – gCT ACA TAg ggA gCC TTg TCC T – 3′	22
hmg-probe	6-FAM 5′ – CAA TCC ACA CAA ACg CAC gCg TA – 3′ TAMRA	23

FAM: 6-carboxyfluorescein; TAMRA: tetramethylrhodamine

5. References

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