EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Health, Consumers & Reference Materials
Food & Feed Compliance Unit

Event-specific Method for the Quantification of maize MON 87403 by Real-time PCR

Validated Method

Method development:

Monsanto Company S. A.

1. General information and summary of the methodology

This protocol describes an event-specific real-time quantitative TaqMan® PCR (polymerase chain reaction) procedure for the determination of the relative content of maize event MON 87403 (unique identifier MON-874Ø3-1) genomic DNA to total maize genomic DNA in a sample.

Template DNA extracted by means of suitable methods should be tested for quality and quantity prior to use in PCR assays, in particular in case of complex and difficult matrices. Tests for the presence of PCR inhibitors (e.g. monitor run of diluted series, use of DNA spikes) are also recommended to ensure suitability of the extracted DNA.

For the specific detection of GM event MON 87403, an 88 bp fragment of the region spanning the 3' insert-to-plant junction in maize MON 87403 is amplified using 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 (6-carboxyfluorescein), as reporter dye at its 5' end, and TAMRA (6-carboxytetramethylrhodamine) as quencher dye at its 3' end.

For the relative quantification of maize GM event MON 87403 DNA, a maize taxon-specific reference system amplifies a 79 bp fragment of a maize *high mobility group* (*hmg*) endogenous gene (GeneBank accession number AJ131373.1), using *hmg* gene-specific primers and a *hmg* gene-specific probe labelled with FAM as reporter dye at its 5' end and TAMRA as quencher dye at its 3' end.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Cq" value. For quantification of the amount of MON 87403 genomic DNA in a test sample, Cq values for the MON 87403 and the *hmg* systems are determined for the sample. Standard curves are then used to estimate the relative amount of MON 87403 genomic DNA to total maize genomic DNA.

2. Validation and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from genetically modified and conventional maize seeds. Precision 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 an international collaborative study by the European Union Reference Laboratory for GM Food and Feed (EURL GMFF). The study was undertaken with twelve participating laboratories in June 2016.

A detailed validation report can be found at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx.

2.3 Limit of detection (LOD)

According to the method developer, the relative LOD of the method is at least 0.040 % (related to mass fraction of GM material) in 200 ng of total suitable 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 % (related to mass fraction of GM material) in 200 ng of total suitable maize DNA. The lowest relative GM content of the target sequence included in the collaborative trial was 0.1 % (mass fraction of GM-material).

2.5 Molecular specificity

The method exploits a unique DNA sequence in the region spanning the 3' insert-to-plant junction in maize MON 87403 and is therefore event-specific for the event MON 87403. This was confirmed by bioinformatics analyses.

3. Procedure

3.1 General instructions and precautions

- 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 should have been 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 regularly
- Laboratory benches and equipment should be cleaned periodically, with e.g. 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 room temperature.
- In order to avoid repeated freeze/thaw cycles aliquots should be prepared.

3.2 Real-time PCR for quantitative analysis of maize event MON 87403

3.2.1 General

The real-time PCR set-up for the taxon (*hmg*) and the GMO (event MON 87403) target sequences are carried out in separate vials. Multiplex qPCR (using differential fluorescent labels for the probes) has not been tested or validated by the EURL GMFF.

The method is developed for a total volume of 25 µL per reaction mixture for the GM (event MON 87403) and the taxon (*hmg*) assay with the reagents as listed in Table 2 and Table 3.

3.2.2 Calibration

To establish the calibration curve at least five samples should be prepared and analysed. The range of GM contents in the calibration curve should be equal to or included in the range validated during the international collaborative study, as given in Table 1. In the trial the first point of the calibration curve (S1) was established for a sample containing 10 % (relative to copy number fractions) maize MON 87403 genomic DNA in a total of 200 ng maize genomic DNA (corresponding to 73260 maize genome copies with one haploid genome assumed to correspond to 2.73 pg of maize genomic DNA) [(1)]. Standards S2 to S5 were prepared by fourfold serial dilutions according to Table 1 below.

Table 1. Copy number values of the standard curve samples

Sample code	S1	S2	S3	S4	S5
Total amount of maize DNA in reaction (ng)	200	50	12.5	3.1	0.78
hmg copies	73260	18315	4579	1145	286
MON 87403 copies	7326	1832	458	114	29

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

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

3.2.3 Real-time PCR set-up

1. Thaw, mix and centrifuge the components needed for the run. Keep thawed reagents on ice.
2. In two tubes on ice, add the components in the order mentioned below (except DNA) to prepare the amplification reaction mixes for the MON 87403 maize specific system (Table 2) and the *hmg* reference gene system (Table 3).

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the MON 87403 event-specific assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
87403 QF (10 µM)	450 nM	1.125
87403 QR (10 µM)	450 nM	1.125
87403 QP* (10 µM)	200 nM	0.50
Nuclease free water	-	5.75
DNA (max 200 ng)	-	4.0
Total reaction volume:		25 µL

*TaqMan® probe labelled with 6-FAM at its 5'-end and TAMRA at its 3'-end

Table 3. Amplification reaction mixture in the final volume/concentration per reaction well for the maize *hmg* taxon-specific assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
hmg primer 1 (10 µM)	300 nM	0.75
hmg primer 2 (10 µM)	300 nM	0.75
hmg probe* (10 µM)	160 nM	0.40
Nuclease free water	-	6.6
DNA (max 200 ng)	-	4.0
Total reaction volume:		25 µL

*TaqMan® probe is labeled with 6-FAM at its 5'-end and TAMRA at its 3'-end

3. Mix well and centrifuge briefly.
4. Prepare two 0.5 mL reaction tubes (one for maize event MON 87403 and one for the *hmg* system) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
5. Add to each reaction tube the amount of reaction mix for 3.5 PCR repetitions (73.5 µL for the *hmg* system and 73.5 µL for the MON 87403 maize system). Add to each tube the correct amount of DNA for 3.5 PCR repetitions (14 µL DNA). The additional 0.5 repetition included will ensure adequate volume when loading the samples.

6. Vortex each tube for approx. 10 seconds. This step is mandatory to reduce to a minimum the variability among the repetitions of each sample.
7. Spin down the tubes. Aliquot 25 µL for the MON 87403 system and for the *hmg* reference system in each well.
8. Place an optical cover on the reaction plate and briefly centrifuge the plate at low speed (e.g. approximately 250 x g for 1 minute) to spin down the reaction mixture.
9. Place the reaction plate in the real-time PCR apparatus (possibly apply a compression pad, depending on the model), according to the manufacturer's instructions and your Standard Operating Procedures.
10. Select FAM as reporter dye and TAMRA as quencher dye for the MON 87403 and the *hmg* reference systems. Select ROX as the passive reference dye. Enter the correct reaction volume (25 µL).
11. Run the PCR with the cycling program described in Table 4. Users who plan to use the second derivative maximum analysis method (an option e.g. on Roche LC480 instruments) are advised to program 45 cycles instead of 40, in order to be able to quantify down to Cq 40.

Table 4. Cycling program for MON 87403/*hmg* assays.

Step	**Stage**		**T (°C)**	**Time (s)**	**Acquisition**	**Cycles**
1	UNG*		50	120	No	1
2	Initial denaturation		95	600	No	1
3	Amplification	Denaturation	95	15	No	40**
		Annealing & Extension	60	60	Yes	

*UNG: Uracil-N-glycosylase
** see comment above for users of second derivative maximum analysis method

3.3 Data analysis

After the real-time PCR, analyse the run following the procedure below:

a) Analyse data: automatic baseline and threshold settings have given the best results at the EURL GMFF.

b) Save the settings and export all the data for further calculations.

3.4 Calculation of results

After having defined a threshold value within the logarithmic phase of amplification, the instrument's software calculates the Cq values for each reaction.

The standard curves are generated both for the *hmg* and the MON 87403 specific assays by plotting the Cq 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 number in the unknown samples.

To obtain the percentage value of event MON 87403 DNA in the unknown sample, the MON 87403 copy number is divided by the copy number of the maize endogenous gene *hmg* and multiplied by 100 (GM% = MON 87403/*hmg* x 100).

4. Equipment and Materials

4.1 Equipment

- Real-time PCR instrument for plastic reaction vessels (glass capillaries are not recommended for the described buffer composition) and appropriate analysis software
- 96-well reaction plates
- Optical caps/adhesion covers
- Micropipettes
- Standard bench top centrifuge with rotor or standard microfuge fit for 0.5 mL reaction tubes, centrifuge for 96-Well reaction plates
- Vortex
- Racks for reaction tubes, also cooled
- 0.5, 1.5 mL and 5 or 15 mL DNAse free reaction tubes

4.2 Reagents

- TaqMan® Universal PCR Master Mix. Applied Biosystems Part No 4318157.
- Nuclease free water

4.3 Primers and Probes

Table 5. Primers and probes for the MON 87403 and *hmg* methods

	Name	DNA Sequence (5' to 3')	Length (nt)
MON 87403			
Forward primer	87403 QF	CTT TCT TTT TCT CCA TAT TGA CCA TCA TAC	30
Reverse primer	87403 QR	TAC TCC GGA ATG AGT GCT CTG TAT C	25
Probe	87403 QP	6-FAM- TCA TTG CGA TCC ACA TTT CCC TAC ATG G-TAMRA	28
hmg			
Forward primer	hmg primer 1	TTG GAC TAG AAA TCT CGT GCT GA	23
Reverse primer	hmg primer 2	GCT ACA TAG GGA GCC TTG TCC T	22
Probe	hmg probe	6-FAM- CAA TCC ACA CAA ACG CAC GCG TA-TAMRA	23

FAM: 6-carboxyfluorescein; TAMRA: carboxytetramethylrhodamine;

5. References

1. Plant DNA C-values Database. Royal Botanic Gardens, Kew, http://data.kew.org/cvalues/