



EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit



Event-specific Method for the Quantification of Maize MON 87427 Using Real-time PCR

Validated Method

9 June 2015

Method development:

Monsanto Company

Quality assurance

The EURL GMFF is ISO 17025:2005 accredited [certificate number: ACCREDIA 1172 (Flexible Scope for DNA extraction and qualitative/quantitative PCR) - Accredited tests are available at http://www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7].

The original version of the document containing evidence of internal checks and authorisation for publication is archived within the EURL GMFF quality system.

The EURL GMFF is also ISO 17043:2010 accredited (proficiency test provider) and applies the corresponding procedures and processes for the management of ring trials during the method validation.

The EURL GMFF conducts its activities under the certification ISO 9001:2008 of the Institute for Health and Consumer Protection (IHCP) provided by SGS.

Address of contact laboratory:

European Commission, Joint Research Centre (JRC)
Institute for Health and Consumer Protection (IHCP)
Molecular Biology and Genomics Unit (MBG)
European Union Reference Laboratory for GM Food and Feed
Via E. Fermi 2749, 21027 Ispra (VA) – Italy
Functional mailbox: eurl-gmff@jrc.ec.europa.eu

Content

1. GENERAL INFORMATION AND SUMMARY OF THE METHODOLOGY	4
2. VALIDATION AND PERFORMANCE CHARACTERISTICS	4
2.1 GENERAL	4
2.2 COLLABORATIVE TRIAL.....	4
2.3 LIMIT OF DETECTION (LOD)	5
2.4 LIMIT OF QUANTIFICATION (LOQ).....	5
2.5 MOLECULAR SPECIFICITY	5
3. PROCEDURE.....	6
3.1 GENERAL INSTRUCTIONS AND PRECAUTIONS.....	6
3.2 REAL-TIME PCR FOR QUANTITATIVE ANALYSIS OF MAIZE EVENT MON 87427	7
3.2.1 General	7
3.2.3 Real-time PCR set-up	7
3.3 DATA ANALYSIS	9
3.4 CALCULATION OF RESULTS.....	9
4. EQUIPMENT AND MATERIALS	10
4.1 EQUIPMENT	10
4.2 REAGENTS.....	10
4.3 PRIMERS AND PROBES.....	10
5. REFERENCES.....	11

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 87427 DNA to total maize 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. 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 maize event MON 87427, a 95-bp fragment of the region spanning the 5' insert-to-plant junction in maize MON 87427 event 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 a reporter at its 5' end, and the dye TAMRA (6-carboxytetramethylrhodamine) as a quencher at its 3' end.

For the relative quantification of maize event MON 87427 DNA, a maize taxon-specific system amplifies a 79-bp fragment of a *high mobility group (hmg)* maize endogenous gene (Accession number, GeneBank: 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 a quencher at its 3' end.

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 87427 DNA in a test sample, Ct values for the MON 87427 and the *hmg* systems are determined for the sample. Standard curves are then used to estimate the relative amount of MON 87427 DNA to total maize DNA.

2. Validation and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from mixtures of 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 December 2012-January 2013.

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 0.04% (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 5' insert-to-plant junction in maize MON 87427 and is therefore event-specific for the event MON 87427.

The specificity of the event-specific assay was assessed by the applicant in duplicate real-time PCR reactions, according to the method described (Tables 1, 2, 3 and 4), using genomic DNA extracted from 100% oilseed rape RT73; maize NK603, MON 810, MON 863, MON 88017, MON 89034, MON 87460, MON 87427 (1% GM); cotton MON 531, MON 15985, MON 1445, MON 88913; soybean 40-3-2, MON 89788, MON 87701, MON 87769 and conventional oilseed rape, maize, cotton, soybean, wheat, millet, lentil, sunflower, peanut (shelled), and quinoa.

According to the method developer the MON 87427 method did not react with any sample except the positive control.

This was further verified *in silico* by the EURL GMFF by means of bioinformatics analysis, on the basis of the sequence data provided by the applicant.

The specificity of the maize taxon-specific assay *hmg* was assessed by the method developer in duplicate real-time PCR reactions, according to the method described (Tables 1, 2, 3 and 4), with genomic DNA extracted from 100% oilseed rape RT73; maize NK603, MON 810, MON 863, MON 88017, MON 89034, MON 87460, MON 87427 (1%); cotton MON 531, MON 15985, MON 1445, MON 88913; soybean 40-3-2, MON 89788, MON 87701, MON 87769 and conventional oilseed rape, maize, cotton, soybean, wheat, millet, lentil, sunflower, peanut (shelled) and quinoa.

According to the method developer the maize-specific reference system did not react with any sample except the positive control maize lines.

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 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 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 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 87427

3.2.1 General

The qPCR set-up for the taxon (*hmg*) and the GMO (event MON 87427) 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 with the reagents as listed in Table 2 and Table 3.

3.2.2 Calibration

The calibration curves have to be established on at least five samples. The first point of the calibration curve (S1) should be established for a sample containing 10% maize MON 87427 DNA in a total of 200 ng of maize 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 are to be prepared by serial dilutions (dilution factor 3.80) 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	53	14	3.6	0.96
<i>hmg</i> copies	73260	19413	5128	1318	351
MON 87427 copies	7326	1941	513	132	35

* Total nanograms are rounded to the integral value

A calibration curve is to be produced by plotting the Ct 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 software.

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 spin down the components needed for the run. Keep thawed reagents on ice.
2. To prepare the amplification reaction mixtures add the components listed in Table 2 and 3 in two reaction tubes (one for the MON 87427 assay and one for the *hmg* assay) on ice in the order mentioned below (except DNA).

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the MON 87427 assay.

Component	Final concentration	µL/reaction
TaqMan [®] Universal PCR Master Mix (2x)	1x	12.5
MON 87427 primer 1 (10 µM)	450 nM	1.125
MON 87427 primer 2 (10 µM)	450 nM	1.125
MON 87427 probe (5 µM)	200 nM	1.0
Nuclease free water	-	5.25
DNA (max 200 ng)	-	4
Total reaction volume:		25 µL

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

Component	Final concentration	µL/reaction
TaqMan [®] Universal PCR Master Mix (2x)	1x	12.5
<i>hmg</i> primer 1 (10 µM)	300 nM	0.75
<i>hmg</i> primer 2 (10 µM)	300 nM	0.75
<i>hmg</i> probe (5 µM)	160 nM	0.80
Nuclease free water	-	6.2
DNA (max 200 ng)	-	4
Total reaction volume:		25 µL

3. Vortex for approx. 5 seconds and spin down.
4. Prepare two reaction tubes (one for the maize MON 87427 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 correct amount of reaction mix for 3.5 PCR repetitions (73.5 µL for the *hmg* system and for the MON 87427 maize system). Add to each tube the correct amount of DNA for 3.5 PCR repetitions (14 µL DNA). The volume for the additional 0.5 repetition will ensure adequate volume when loading the samples. Vortex each tube for approx. 5 seconds. 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 250 x *g* for 1 minute at 4 °C) to spin down the reaction mixture.
7. Place the plate into the instrument.

8. Run the PCR with the cycling program described in Table 4.

Table 4. Cycling program for MON 87427/ *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	45
		Annealing & Extension	60	60	Yes	

*UNG: Uracil-N-glycosylase

3.3 Data analysis

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

- a) Set the threshold: display the amplification curves of one assay (e.g. MON 87427) 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 (only needed for some analysis software). 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 exponential 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), b) and c) on the amplification plots of the other system (e.g. *hmg*).
- e) 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 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 87427 specific assays 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 number in the unknown samples.

To obtain the percentage value of event MON 87427 DNA in the unknown sample, the MON 87427 copy number is divided by the copy number of the maize endogenous gene *hmg* and multiplied by 100 (GM% = MON 87427/*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)
- Plastic reaction vessels suitable for real-time PCR instrument (enabling undisturbed fluorescence detection)
- Software for analysis of the runs (mostly integrated in the software of the real-time PCR instrument)
- Microcentrifuge
- Micropipettes
- Centrifuge for PCR plates
- Vortex
- Rack for reaction tubes
- 0.2/1.5/2.0 mL reaction tubes

4.2 Reagents

- TaqMan[®] Universal PCR Master Mix. Applied Biosystems Part No 4304437.

4.3 Primers and Probes

Table 5. Primers and probes for the MON 87427 and *hmg* methods

		DNA Sequence (5' to 3')	Length (nt)
<i>MON 87427</i>			
Forward primer	MON 87427 primer 1	5' – ACg gAA ACg gTC ggg TCA AAT g – 3'	22
Reverse primer	MON 87427 primer 2	5' – CCA TgT AgA TTT CCC ggT TTT CTC – 3'	24
Probe	MON 87427 probe	6-FAM 5' – TCg ggA CAA TAT ggA gAA AAA gAA AgA g – 3' TAMRA	28
<i>hmg</i>			
Forward primer	<i>hmg</i> primer 1	5' – TTg GAC TAg AAA TCT CgT gCT gA – 3'	23
Reverse primer	<i>hmg</i> primer 2	5' – gCT ACA TAg ggA gCC TTg TCC T – 3'	22
Probe	<i>hmg</i> probe	6-FAM 5' – CAA TCC ACA CAA ACg CAC gCg TA – 3' TAMRA	23

FAM: 6-carboxyfluorescein; TAMRA: carboxytetramethylrhodamine;

5. References

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