

JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

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

Validation report
Corrected version 1

European Union Reference Laboratory for
Genetically Modified Food and Feed

2016



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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 Validation Report

9 June 2015

Corrected version 1 - 18/08/2016 (see page 2)

European Union Reference Laboratory for GM Food and Feed

Executive Summary

In line with its mandate^a the European Union Reference Laboratory for GM Food and Feed (EURL GMFF), in collaboration with the European Network of GMO Laboratories (ENGL), validated an event-specific real-time polymerase chain reaction (qPCR) method for detecting and quantifying the maize event MON 87427 (unique identifier MON-87427-7). The validation study was conducted according to the EURL GMFF validation procedure [<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>] and the relevant internationally accepted guidelines⁽¹⁻⁵⁾.

In accordance with current EU legislation^b, Monsanto Company provided the detection method and the positive and negative control samples (genomic DNA from maize seeds harbouring the MON 87427 event as positive control DNA, genomic DNA from conventional maize seeds as negative control DNA). The EURL GMFF verified the method performance data provided by the applicant, where necessary experimentally, prepared the validation samples (calibration samples and blind samples at different GM percentage [copies GM/total maize genome copies]), organised an international collaborative study and analysed the results.

The EURL GMFF in-house verification and the collaborative study confirmed that the method meets the method performance requirements as established by the EURL GMFF and the ENGL, in line with the provisions of Annex III-3.C.2 to Regulation (EU) No 503/2013 and it fulfils the analytical requirements of Regulation (EU) No 619/2011^c. This validation report is published at <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>.

^a Regulation (EC) No 1829/2003 of 22 September 2003 "on genetically modified food and feed

^b Regulation (EC) No 503/2013 of 3 April 2013 "on applications for authorisation of genetically modified food and feed in accordance with Regulation (EC) No 1829/2003 of the European Parliament and of the Council and amending Commission Regulations (EC) No 641/2004 and (EC) No 1981/2006".

^c Regulation (EU) No 619/2011 of 24 June 2011 laying down the methods of sampling and analysis for the official control of feed as regards presence of genetically modified material for which an authorisation procedure is pending or the authorisation of which has expired.

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.

Modification from the previous version:

Page 14 table 7

Run E	-3.43	96	1.00	-3.39	97	1.00
Run F	-3.34	99	1.00	-3.30	101	1.00

Page 15 table 8

Target GM-levels %	Measured GM level %	Bias % of the target GM-level	Precision (RSDr %)
0.061	0.061	-0.08	16

Page 15 table 9

Target GM-levels %	Measured GM level %	Bias % of the target GM-level	Precision (RSDr %)
0.061	0.048	-21	24

Page 20 §5

... at step 3 of the validation process (experimental testing of samples and methods), the EURL GMFF determined the RSDr% value at the level of 0.1% in mass fraction of GM-material (corresponding to 0.061% expressed in terms of copy number ratio). The experiments were carried out under repeatability conditions on fifteen replicates. The RSDr resulted to range between **16%** and **24%** (Table 8 and 9) depending on the qPCR platform applied, hence also below 25%;

Page 20 table 13

Source	RSDr %	GM %
EURL GMFF tests	16-24%	0.1%

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1. Timeline

In line with Regulation (EC) No 1829/2003, Monsanto Company provided the EURL GMFF with an event-specific method for detection and quantification of maize event MON 87427 (unique identifier MON-87427-7) together with genomic DNA as positive and negative control samples (April 2012).

In response to an earlier submission of the method, the EURL GMFF started its step-wise validation procedure (step 1: dossier reception) before the formal approval by EFSA of the official dossier (January 2013).

The scientific dossier assessment (step 2) focused on the reported method performance characteristics assessed against the ENGL method acceptance criteria^d (see http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf for details on method acceptance criteria and method performance requirements) and it was positively concluded in September 2012.

In step 3 of the procedure (experimental testing), the EURL GMFF verified the purity of the control samples provided and conducted an in-house testing of samples and method.

The positive and negative control DNA, submitted in accordance with Art 5(3)(j) and Article 17(3)(j) of Regulation (EC) No 1829/2003, were found of good quality.

The method performance characteristics were verified in-house by quantifying blinded samples of GM levels within the range 0.1%-8.0% on copy number basis. The experiments were performed under repeatability conditions and demonstrated that the efficiency, linearity, trueness and precision of the method were within the limits established by the ENGL.

In addition, and in line with the requirements of Regulation (EU) No 619/2011, the EURL GMFF also verified *i*) the zygosity ratio of the submitted positive control sample in order to determine the conversion factor between copy numbers and mass fractions, and *ii*) the method precision (relative repeatability standard deviation, RSDr) at the 0.1% level related to mass fraction of GM material. Step 3 was completed in December 2012 with the conclusion that the method could be submitted to collaborative study (step 4).

The collaborative study (step 4) took place in December 2012-January 2013. It demonstrated that the method is well suited for analysing DNA of GM maize MON 87427, appropriately extracted from food or feed, down to a GM content level of 0.1% m/m.

The preparation of the report (step 5) was aligned with the timelines communicated by EFSA for its risk assessment.

^d EURL/ENGL guidance doc "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>)

2. Step 1 (dossier acceptance) and step 2 (scientific dossier assessment and bioinformatics analysis)

Documentation and data supplied by the applicant were evaluated by the EURL GMFF for completeness (step 1) and compliance with the ENGL method acceptance criteria (step 2).

The specificity of the event-specific assay was verified by the applicant and confirmed by the EURL GMFF by means of bioinformatics analysis, on the basis of the sequence data provided by the applicant.

The detection method spans the junction between the transgenic insert and the 5' genomic region. The forward primer "MON 87427 primer 1" binding site was found in the genomic border adjacent to the insertion. The reverse primer "MON 87427 primer 2" binds in the insert, in a region that corresponds to T-DNA border region. The probe "MON 87427 probe" binds in the junction between the T-DNA border region and the 5' genomic region.

The amplicon size is expected to be 95 bp, consistent to what reported by the applicant.

The sequence of the amplicon was analysed by BLAST (NCBI) against local copies of the "nt" and "patents" databases, and no significant similarity was found with any other published sequence except the related patent sequences. In addition, the primers were tested against the sequences of the other GMO events present in the Central Core Sequence Information System of the JRC, as well as the whole genomes of more than 80 plants (including *Brassica rapa*, *Glycine max*, *Oryza sativa*, *Solanum lycopersicum* and *Zea mays*) using the e-PCR prediction tool (NCBI), and no potential amplicon was identified.

The applicant clarified that a 41 base pair rearranged DNA at the site of insertion is consistent with molecular rearrangements that occur from the double-stranded break repair as part of the *Agrobacterium*-mediated transformation process; 31 out of the 41 bp are a direct repeat made up of a sequence motif present in the left border of the T-DNA. It was found that the probe could recognise both repeats at the annealing temperature of 60°C. Additional analysis showed, however, that the second binding site of the probe should not create any problem during the PCR exponential phase, as later confirmed by the experimental phase of validation.

The parameters of the calibration curves (slope, R^2 coefficient) were determined by the applicant by quantifying three test samples at different GM levels (see Table 1).

Table 1. Summary of the average slope and R^2 values obtained by the applicant

MON 87427		<i>hmg</i>	
Slope	R^2	Slope	R^2
-3.53	1.00	-3.48	1.00

According to the ENGL method acceptance criteria, the average value of the slope of the standard curve shall range from -3.1 to -3.6 and the R^2 coefficient shall be ≥ 0.98 .

Table 1 indicates that the slope and R^2 coefficient of the standard curves for the GM-system (MON 87427) and the maize-specific *high mobility group (hmg)* system, as established by the applicant, were within the ENGL acceptance criteria.

Also precision and trueness of the method were established by the applicant and 15 values for each of 3 GM levels (expressed as mass fraction of GM-material) were provided. Table 2 reports precision and trueness values for the three GM-levels as provided by the applicant. Both parameters were within the ENGL acceptance criteria (trueness $\pm 25\%$, RSDr $\leq 25\%$ across the entire dynamic range).

Table 2. Mean %, precision and trueness values provided by the applicant

Expected GMO%	Test results		
	0.085	1.0	10.0
Measured mean GMO %	0.096	1.0	10
Precision (RSDr %)	20	11	7.9
Trueness (bias %)	13	1.7	4.5

3. Step 3 (experimental testing of the samples and method)

3.1 DNA extraction

Genomic DNA was isolated from ground maize seeds and grains, previously submitted for detection of maize event MON 88017, using a "CTAB-based" protocol coupled with PEG purification.

This protocol has already been validated in-house by the EURL GMFF. The protocol for DNA extraction and a report on testing were published in 2008 at http://gmo-crl.jrc.ec.europa.eu/summaries/MON88017_DNAExtr_report.pdf.

In agreement with the ENGL position, endorsing the modularity principle (see also Annex III to Reg. (EU) No 503/2013), and given the similarity in the matrix, the EURL GMFF considers the above mentioned DNA extraction protocol applicable in the context of the validation of the method for maize event MON 87427.

3.2 Method protocol for the PCR analysis

The PCR method provided by the applicant and subsequently validated by the EURL GMFF is an event-specific, quantitative, real-time TaqMan[®] PCR procedure for the determination of the relative content of GM event MON 87427 DNA to total maize DNA. The procedure is a simplex system, in which a maize specific assay targeting the endogenous gene *high mobility group (hmg)*, and the GM target assay for MON 87427 are performed in separate wells. A detailed validated Method Protocol is published by the EURL GMFF at <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>.

For the detection of GM event MON 87427, a 95-bp fragment of the region spanning the 5' plant-to-insert junction in maize MON 87427 is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with 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 GM event MON 87427, a maize taxon-specific system amplifies a 79-bp fragment of a maize *high mobility group (hmg)* endogenous gene, 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.

Standard curves are generated for both the MON 87427 and the *hmg* systems by plotting the Ct values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a regression line into these data. Thereafter, the standard curves are used to estimate the copy numbers in the test sample DNA by interpolation from the standard curves.

For relative quantification of event MON 87427 DNA in a test sample, the MON 87427 copy number is divided by the copy number of the maize reference gene (*hmg*) and multiplied by 100 to obtain the percentage value ($GM\% = \text{MON 87427}/hmg \times 100$).

The absolute copy numbers of the calibration curve samples are calculated by dividing the sample DNA mass (nanograms) by the published average 1C value for the maize genome (2.73 pg)⁽⁶⁾. The copy number values used in the quantification, the GMO contents of the calibration samples, and the total DNA quantity used in the PCR reactions are listed in Table 3.

Table 3. Copy number values of the standard curve samples

Sample code	S1	S2	S3	S4	S5
Total amount of maize DNA in the reaction (ng) *	200	53	14	3.6	0.96
Target taxon <i>hmg</i> copies	73260	19279	5073	1335	351
Target MON 87427 copies	7326	1928	507	134	35

* Total nanograms are rounded to the integral value

3.3 EURL GMFF experimental testing (step 3)

3.3.1 Determination of the zygosity ratio in the positive control sample

Annex II of Regulation (EU) No 619/2011 requires that “when results are primarily expressed as GM- DNA copy numbers in relation to target taxon-specific DNA copy numbers calculated in terms of haploid genomes, they shall be translated into mass fraction in accordance with the information provided in each validation report of the EURL GMFF.” This requires knowledge of the zygosity of the event. In order to satisfy this requirement, the EURL GMFF assessed the zygosity (GM-target to reference target ratio) in the positive control sample submitted by the applicant.

The copy number of the MON 87427 and of the *hmg* targets in the positive control sample were determined by digital PCR (dPCR) performed on the BioMark HD System using the 12.765 digital arrays (Fluidigm).

Reaction mixes were prepared in a final volume of 9 µL and contained 1X TaqMan® Universal PCR Master Mix) (Applied Biosystems, Cat. number 4304437), 1X GE sample loading reagent (Fluidigm PN 85000746), primers and probes at concentrations indicated in the corresponding Validated Method (MON 87427 primer 1 and MON 87427 primer 2 at 450 nM each, MON 87427 probe at 200 nM; *hmg* primer 1 and *hmg* primer 2 at 300 nM each, *hmg* probe at 160 nM), 1 µL of DNA at a concentration of 2 ng/µL and 1.5 ng/µL, used respectively in experiment number 1 and in experiments number 2 and 3 to test the zygosity in five replicates; the concentrations were chosen to avoid panel saturation (optimal between 200<positive partitions<700).

Loading of the digital chip was performed according to the manufacturer's instructions by using the IFC controller (Fluidigm). Approximately 4.6 µL of the reaction mixes were loaded into each well and distributed into the 765 partitions constituting one panel. The experiment was repeated three times for a total number of fifteen data sets for both targets. 'No template controls' were included. Amplification conditions were as reported in Annex 1 or in the Validated Method document (<http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>). Data analysis and copy number calculations were performed using the BioMark digital PCR Analysis software. The range of Ct retention was from 20 to 45.

Calculations of means and variances were carried out according to the procedure outlined for random variables in the Annex 4 of the ENGL guidance document 'Verification of analytical methods for GMO testing when implementing inter-laboratory validated methods'^e.

3.3.2 In-house verification of the method performance against ENGL method acceptance criteria

The method performance characteristics were verified by quantifying on a copy number basis five blinded test samples containing a range of GM levels (0.061%-8.0%). The experiments were performed on an ABI 7500 and ABI 7900 real-time platform under repeatability conditions and followed the protocol described in the material and method section. Test samples with GM levels 0.20%, 0.9%, 3.0% and 8.0% were tested in two real-time PCR runs with two replicates for each GM-level on each plate (total of four replicates per GM-level). The test sample with GM-level 0.061% was tested in 15 replicates in an additional run. Average values of the slope and of the R² coefficient of the standard curves and method trueness and precision over the dynamic range were evaluated against the ENGL method acceptance criteria.

In order to assess the method compliance to Regulation (EU) No 619/2011, the EURL GMFF also determined the zygosity of the GM-insert in the positive control sample and estimated, based on 15 replicates, the method precision (RSDr %) at 0.1% GM level in mass fraction.

3.4 International collaborative trial (step 4)

The international collaborative trial involved twelve randomly selected laboratories, all being "national reference laboratories, assisting the CRL for testing and validation of methods for detection", as listed in annex to Regulation (EC) No 1981/2006 who had expressed their interest in participation. The study was carried out in accordance with the following internationally accepted guidelines:

^e Verification of analytical methods for GMO testing when implementing inter-laboratory validated methods. European Network of GMO Laboratories (ENGL), 2011.
<http://gmo-crl.jrc.ec.europa.eu/doc/ENGL%20MV%20WG%20Report%20July%202011.pdf>

- The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" (Horwitz, 1995) ⁽¹⁾
- ISO 5725 "Accuracy (trueness and precision) of measurement methods and results", Part 1 and Part 2 (ISO, 1994); ISO 5725-1:1994/Cor 1 (ISO 1998) and ISO 5725-2:1994/Cor 1 (ISO, 2002) ⁽²⁻⁵⁾

The objective of the international collaborative study was to verify in experienced laboratories the trueness and precision of the PCR analytical method provided by the applicant and described under 3.2, above and in the "Validated Method" (Annex 1).

3.4.1 List of participating laboratories

The twelve participants in the MON 87427 validation study were randomly selected from 27 national reference laboratories (NRL) that offered to participate.

Clear guidance was given to the selected laboratories for strictly following the standard operational procedures that were provided for the execution of the protocol. The participating laboratories are listed in Table 4.

Table 4. Laboratories participating in the validation of the detection method for maize event MON 87427

Laboratory	Country
Agricultural institute of Slovenia	SI
Crop Research Institute	CZ
Environment Agency Austria	AT
INRAN seed testing station	IT
Italian National Institute for Health	IT
Laboratory Agroalimentary of the Spanish Ministry of Agriculture	ES
Landeslabor Schleswig-Holstein	DE
National Centre for Food	ES
National Institute of Biology	SI
RIKILT Institute of Food Safety	NL
State Institute of Chemical and Veterinarian Analysis - Freiburg	DE
Walloon Agricultural Research Centre	BE

3.4.2 Real-time PCR equipment used in the study

Laboratories involved in the collaborative study used the following real-time PCR equipment: five laboratories used the ABI 7500, three used the ABI 7900, one used ABI 7700, one used the Stratagene Mx 3005P, one the Bio-Rad CFX96 and one the Roche LC480.

This variability of equipment, with its known potential influence on PCR results, reflects the real-life situation in the control laboratories and provides additional assurance that the method is robust and usable under real conditions.

3.4.3 Materials used in the international collaborative study

For the validation of the quantitative event-specific method, test samples were provided by the EURL GMFF to the participating laboratories. They were derived from:

- i) genomic DNA extracted by the applicant from heterozygous maize seeds harbouring the event MON 87427, and
- ii) genomic DNA extracted by the applicant from conventional maize seeds genetically similar to those harbouring the MON 87427 event.

The control samples were prepared by the EURL GMFF from the genomic DNA provided by the applicant in accordance to Regulation (EC) No 1829/2003, Art 2.11^f.

The control samples were used by the EURL GMFF to prepare standards (of known GMO content) and test samples (of undisclosed GM content = blinded samples) by mixing MON 87427 maize DNA and non-GM maize DNA.

The calibration sample S1 was prepared by mixing the appropriate amount of MON 87427 DNA with control non-GM maize DNA to obtain a 10% (copy no/copy no) GM-sample. Calibration samples S2-S5 were prepared by 3.8-fold serial dilutions from the S1 sample.

The twelve NRLs participating in the validation study received the following materials:

- ✓ Five calibration samples with known concentrations of GM-event (140 µL of DNA solution each) labelled from S1 to S5 (Table 3).
- ✓ Twenty blinded test DNA samples (70 µL of DNA solution, each at 45 ng/µL) labelled from U1 to U20, representing five GM levels, each in four replicates (Table 5)

^f Control sample defined as the GMO or its genetic material (positive sample) and the parental organism or its genetic material that has been used for the purpose of the genetic modification (negative sample).

Table 5. MON 87427 GM contents

MON 87427 GM%
GM copy number/maize genome copy number x 100
8.0
3.0
0.90
0.20
0.061

✓ Reaction reagents:

- TaqMan[®] Universal PCR Master Mix (2x), one vial: 8 mL
- distilled sterile water, one vial: 5 mL

✓ Primers and probes (1 tube each) as follows:

hmg taxon-specific assay

- *hmg* primer 1 (10 µM): 240 µL
- *hmg* primer 2 (10 µM): 240 µL
- *hmg* probe (5 µM): 260 µL

MON 87427 assay

- MON 87427 primer 1 (10 µM): 360 µL
- MON 87427 primer 2 (10 µM): 360 µL
- MON 87427 probe (5 µM): 320 µL

3.4.4 Design of the collaborative study

Participating laboratories received a detailed validation protocol that included the exact design of the PCR plates, ensuring that on each PCR plate the samples were analysed for the MON 87427 event-specific system and for the *hmg* taxon-specific system. In total, two plates were run by each participating laboratory.

The laboratories prepared the PCR master-mixes for the maize event MON 87427 and the *hmg* assay in accordance with the description provided in the validation protocol. Calibration and test samples were loaded on the PCR plates as per pre-determined plate layout.

The amplification reaction followed the cycling program specified in the protocol. Participants determined the GM% in the test samples according to the instructions and also reported the raw data to the EURL GMFF on an Excel sheet that was designed, validated and distributed by the EURL GMFF. All data are stored by the EURL GMFF on a dedicated and protected server.

The EURL GMFF analysed the data against the parameters and the limits set by the ENGL, i.e. trueness, precision, amplification efficiency and linearity.

3.4.5 Deviations reported from the protocol

Nine laboratories reported no deviations from the method protocol.

One laboratory repeated one PCR run due to a pipetting error.

Another laboratory repeated the half-plate concerning the *hmg* reference system due to a major technical problem.

One laboratory used automatic baseline and threshold settings.

4. Results

4.1 EURL GMFF experimental testing

4.1.1 Zygosity ratio in the positive control sample

The results of the digital PCR analysis conducted by the EURL GMFF on the MON 87427 and *hmg* targets to determine the zygosity ratio in the positive control samples are shown in Table 6.

Table 6. Zygosity ratio of the MON 87427 and *hmg* targets in the positive control sample

Mean ratio (MON 87427/ <i>hmg</i>)	0.61
Standard deviation	0.048
RSD _r (%)	7.9
Standard error of the mean	0.012
Upper 95% CI of the mean	0.641
Lower 95% CI of the mean	0.587

The mean ratio (MON 87427/*hmg*) equals 0.61%. The 95% confidence interval (CI) spans around 0.61% and therefore the mean ratio is not significantly different from the expected ratio for a maize heterozygous control sample whose GM parental contribution is of female origin and assuming a single-copy endogenous gene target, for an $\alpha = 0.05$.

Hence:

$$0.061 \text{ GM\% in DNA copy number ratio} = 0.1 \text{ GM \% in mass fraction}$$

4.1.2 In-house verification of method performance against ENGL method acceptance criteria

Test samples with GM levels from 0.20% to 8.0% were tested in two real-time PCR runs with two replicates for each GM-level on each plate (total of four replicates per GM-level). The sample at 0.061% GM-level was tested in 15 replicates in one run.

Tests were conducted on ABI 7500 and ABI 7900 as a test of robustness.

The standard curve parameters and the results of efficiency, linearity, trueness and precision obtained in the two real-time PCR runs with the test samples are shown in Tables 7, 8 and 9.

According to the ENGL method acceptance criteria, the average value of the slope of the standard curve shall range from -3.1 to -3.6 and the R^2 coefficient shall be ≥ 0.98 . Table 7 documents that the slopes of the standard curves and the R^2 coefficients were within the limits established by the ENGL. The EURL GMFF in-house results confirm the data provided by the applicant.

Table 7. Standard curve parameters of the real-time PCR tests carried out on ABI7900 and ABI 7500

	MON 87427 system			hmg system		
	Slope	PCR efficiency*	R^2	Slope	PCR efficiency*	R^2
Run A	-3.31	100	1.00	-3.44	95	1.00
Run B	-3.33	100	1.00	-3.43	96	1.00
Run C	-3.25	103	1.00	-3.25	103	1.00
Run D	-3.31	100	1.00	-3.21	105	1.00
Run E	-3.43	96	1.00	-3.39	97	1.00
Run F	-3.34	99	1.00	-3.30	101	1.00

* PCR efficiency (%) is calculated using the formula $\text{Efficiency} = (10^{(-1/\text{slope})} - 1) \times 100$

Runs A-B were used to quantify GM-levels from 0.20 to 8.0% on ABI 7500; Runs C-D were used to quantify GM-levels from 0.20 to 8.0% on ABI 7900; Run E was used to quantify GM-level 0.1% on ABI 7500; Run F was used to quantify GM-level 0.1% on ABI 7900.

According to the ENGL method acceptance criteria the method trueness (measured as bias in % of the target GM level) should be within $\pm 25\%$ of the accepted reference value over the entire dynamic range and the precision, expressed as RSDr % (relative standard deviation of repeatability), should be $\leq 25\%$, also over the entire dynamic range.

Tables 8 and 9 show that trueness and precision of quantification were within the limits established by the ENGL for both PCR machines used.

Table 8. Values of trueness and precision as established by the EURL GMFF in its in-house verification using an ABI 7500

Target GM-levels %	Measured GM level %	Bias % of the target GM-level	Precision (RSDr %)
8.0	8.6	6.9	6.4
3.0	3.5	16	2.6
0.9	0.84	-6.7	8.1
0.20	0.19	-4.3	11
0.061	0.061	-0.08	16

Table 9. Values of trueness and precision as established by the EURL GMFF in its in-house verification using an ABI 7900

Target GM-levels %	Measured GM level %	Bias % of the target GM-level	Precision (RSDr %)
8.0	7.6	-4.9	5.7
3.0	2.9	-2.0	7.0
0.9	0.72	-20	7.1
0.20	0.15	-23	12
0.061	0.048	-21	24

4.2 Results of the international collaborative study

4.2.1 PCR efficiency and linearity

The PCR efficiency (%) and R² values (expressing the linearity of the regression) for the standard curve, reported by participating laboratories are displayed in Table 10. The PCR efficiency (%) was calculated from the standard curve slopes using the formula:

$$\text{Efficiency} = (10 * (-1/\text{slope})) - 1) \times 100$$

Table 10. Values of slope, PCR efficiency and R^2 obtained during the international collaborative trial

Lab	Plate	MON 87427			<i>hmg</i>		
		Slope	PCR Efficiency (%)	R^2	Slope	PCR Efficiency (%)	R^2
1	A	-3.39	97	1.00	-3.40	97	1.00
	B	-3.51	93	0.99	-3.32	100	1.00
2	A	-3.46	95	1.00	-3.51	93	1.00
	B	-3.58	90	1.00	-3.56	91	1.00
3	A	-3.35	99	1.00	-3.45	95	1.00
	B	-3.39	97	1.00	-3.54	91	1.00
4	A	-3.56	91	1.00	-3.50	93	1.00
	B	-3.60	89	1.00	-3.48	94	1.00
5	A	-3.27	102	1.00	-3.42	96	1.00
	B	-3.23	104	1.00	-3.41	97	1.00
6	A	-3.35	99	0.99	-3.52	92	1.00
	B	-3.56	91	1.00	-3.50	93	1.00
7	A	-3.34	99	1.00	-3.42	96	1.00
	B	-3.25	103	1.00	-3.30	101	1.00
8	A	-3.38	97	1.00	-3.39	97	1.00
	B	-3.44	95	1.00	-3.41	97	1.00
9	A	-3.34	99	1.00	-3.41	96	1.00
	B	-3.40	97	1.00	-3.39	97	1.00
10	A	-3.37	98	1.00	-3.39	97	1.00
	B	-3.33	100	1.00	-3.41	97	1.00
11	A	-3.53	92	0.99	-3.36	98	1.00
	B	-3.52	92	0.99	-3.30	101	0.99
12	A	-3.45	95	1.00	-3.43	96	1.00
	B	-3.44	95	1.00	-3.45	95	1.00
Mean		-3.42	96	1.00	-3.43	96	1.00

Table 10 indicates that the efficiency of amplification for the MON 87427 system ranges from 89 to 104 and the linearity from 0.99 to 1.00; the amplification efficiency for the maize-specific system ranges from 91% to 101% and the linearity is about 1.00. The mean PCR efficiency was 96% for both the MON 87427 and the *hmg* assays. The average R^2 of the methods was 1.00 for both the MON 87427 and *hmg* assays. Both PCR efficiency and linearity values were within the ENGL acceptance criteria.

These results confirm the appropriate performance characteristics of the methods tested in terms of efficiency and linearity.

4.2.2 GMO quantification

Table 11 reports the values of quantification for the four replicates of each GM level as reported by each of the twelve participating laboratories.

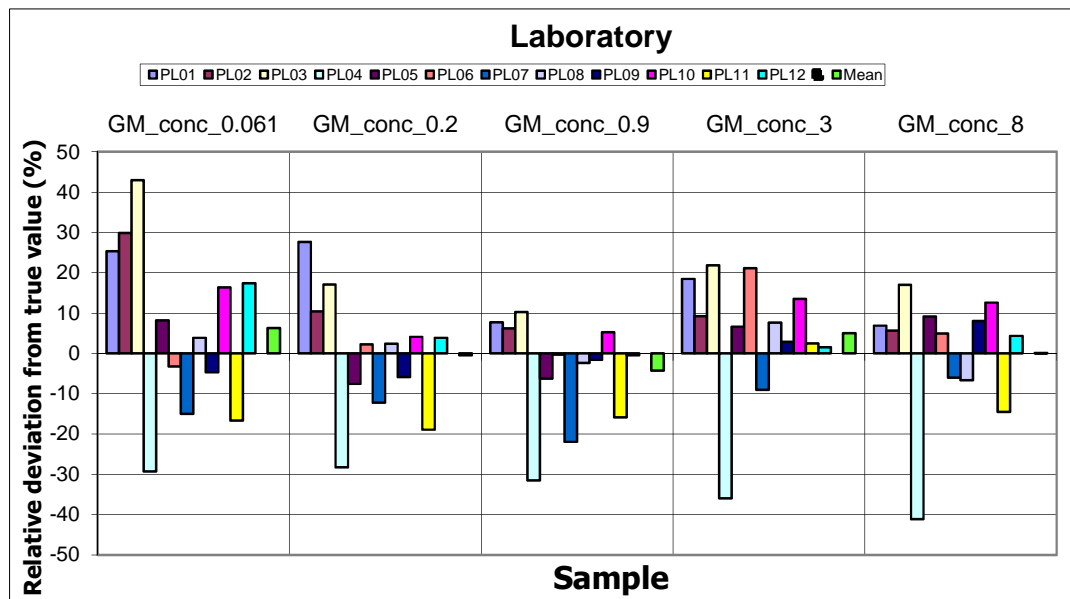
Table 11. GM% values determined by laboratories for test samples

	GMO content (%) *																			
LAB	0.061				0.2				0.9				3.0				8.0			
	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4
1	0.08	0.08	0.08	0.07	0.24	0.25	0.26	0.27	0.98	0.98	1.02	0.91	3.55	3.26	3.70	3.70	7.81	8.09	9.44	8.86
2	0.07	0.08	0.09	0.08	0.24	0.21	0.22	0.21	0.92	1.01	0.96	0.93	3.28	3.16	3.40	3.27	8.07	7.58	9.63	8.54
3	0.08	0.08	0.11	0.09	0.26	0.24	0.23	0.21	0.96	1.01	0.98	1.01	3.71	4.07	3.34	3.51	9.29	9.83	9.41	8.91
4	0.04	0.07	0.04	0.03	0.08	0.14	0.19	0.16	0.67	0.50	0.75	0.54	2.28	1.63	1.87	1.90	6.42	4.67	3.49	4.25
5	0.07	0.06	0.07	0.07	0.17	0.18	0.20	0.19	0.82	0.82	0.86	0.87	3.15	3.19	3.27	3.18	9.01	8.69	8.75	8.46
6	0.04	0.06	0.07	0.06	0.24	0.22	0.17	0.19	0.82	1.00	0.83	0.94	4.16	4.43	3.06	2.88	7.72	7.88	8.71	9.28
7	0.04	0.04	0.07	0.06	0.17	0.16	0.18	0.19	0.66	0.71	0.73	0.71	2.96	2.59	2.77	2.60	7.55	5.48	9.05	7.99
8	0.05	0.06	0.08	0.06	0.22	0.24	0.19	0.17	0.99	0.86	0.85	0.81	3.58	3.25	3.10	2.99	6.48	7.72	7.82	7.85
9	0.05	0.06	0.06	0.06	0.21	0.21	0.17	0.17	0.86	0.86	0.89	0.94	3.19	3.35	2.94	2.86	8.75	8.83	8.33	8.66
10	0.07	0.05	0.08	0.08	0.22	0.23	0.18	0.20	0.92	0.97	0.90	1.01	3.35	3.81	3.20	3.27	9.28	10.20	8.00	8.57
11	0.06	0.05	0.03	0.06	0.08	0.22	0.15	0.20	0.61	0.71	0.76	0.95	4.11	2.81	2.62	2.76	4.10	7.67	7.10	8.47
12	0.06	0.06	0.08	0.08	0.22	0.21	0.19	0.21	0.83	0.95	0.89	0.91	3.42	3.00	2.83	2.94	8.28	7.34	9.61	8.14

* GMO% = (GMO copy number/maize genome copy number) x 100

A graphical representation of the data reported in Table 11 is provided in Figure 1 that shows the relative deviation from the true value for each GM level tested for each laboratory. The coloured bars represent the deviation of the GM level measured in % of the true GM level; the green bar on the right represents the mean relative deviation over all twelve participating laboratories for each true GM level.

Figure 1. Relative deviation (%) from the true value of GM level for all laboratories*



*PL6 at GM level 0.9% and the mean at GM level 0.2% and 8.0% had very small relative deviations from the true value and the corresponding histograms do not show up in Figure 1. PL = participating laboratory.

Overall no trend for over or under-estimation of the amount of the GM-target over the dynamic range can be observed. The mean bias generated by all laboratories ranges between -0.44% and +6.3%. The method is well within the accepted limits established by the ENGL ($\pm 25\%$ over the entire dynamic range).

All data were retained for the statistical analysis and for tests of outliers (Cochran and Grubbs) whose results are reported in Table 12.

4.2.3 Method performance requirements

Among the performance requirements established by ENGL and adopted by the EURL GMFF (<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>), repeatability and reproducibility are to be assessed through an international collaborative trial. Table 12 illustrates the estimation of repeatability and reproducibility at the various GM levels tested during the study (see Table 4 for a list of the participant laboratories). No outliers were identified further to the application of the Cochran's and Grubbs' tests for identification and removal of outliers.

According to the ENGL method performance requirements, the relative reproducibility standard deviation (RSD_R), that describes the inter-laboratory variation, should be below 35% at the target concentration and over the majority of the dynamic range, while it should be below 50% at the lower end of the dynamic range.

As it can be observed in Table 12, the method satisfies this requirement at all GM levels tested. Indeed, the highest value of RSD_R is 25% at the 0.061% GM level, thus within the acceptance criterion.

Table 12. Summary of validation results for the MON 87427 method, expressed as GM copy numbers in relation to target taxon copy numbers

	Test Sample Expected GMO %				
	0.061	0.2	0.9	3.0	8.0
Laboratories having returned valid results	12	12	12	12	12
Samples per laboratory	4	4	4	4	4
Number of outliers	0	0	0	0	0
Reason for exclusion	-	-	-	-	-
Mean value	0.06	0.20	0.86	3.2	8.00
Relative repeatability standard deviation, RSD_r (%)	17	15	8.2	12	12
Repeatability standard deviation	0.011	0.029	0.071	0.366	0.990
Relative reproducibility standard deviation, RSD_R (%)	25	20	15	18	19
Reproducibility standard deviation	0.016	0.040	0.130	0.569	1.519
Bias* (absolute value)	0.004	-0.001	-0.038	0.151	0.001
Bias (%)	6.3	-0.44	-4.3	5.0	0.01

*Bias is estimated according to ISO 5725 data analysis protocol.

Table 12 also documents the relative repeatability standard deviation (RSD_r) estimated for each GM level. In order to accept methods for collaborative study, the EURL GMFF and ENGL require that the RSD_r value indicated by the applicant and confirmed by the EURL GMFF through in-house experiments, is below 25% (see ENGL document "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" <http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>). As it can be observed from the values reported, the repeatability standard deviation is below 25% at all GM levels, with the highest value of 17% at the 0.061% GM level.

The trueness of the method is estimated using the measures of the method bias for each GM level. According to ENGL method performance requirements, trueness should be $\pm 25\%$ across the entire dynamic range. The method satisfies this requirement across the dynamic range tested, with the highest value of bias (%) of 6.3% at the 0.061% GM level.

5. Compliance of the method for detection and quantification of event MON 87427 with the requirements of Regulation (EU) No 619/2011

To verify the compliance of the method under validation with the requirements of Regulation (EU) No 619/2011, the following steps were carried out and their outcome is summarised in Table 13:

- at step 2 of the validation process (scientific assessment of the dossier), the EURL GMFF acknowledged that the RSDr value at the 0.085% level shown by the applicant's dossier (expressed as mass fraction of GM-material) was 20%, based on 15 replicates (Table 2), hence below the maximum value of 25% required by the ENGL. The EURL GMFF therefore concluded that it could accept the applicant's data on method performance;
- at step 3 of the validation process (experimental testing of samples and methods), the EURL GMFF determined the RSDr% value at the level of 0.1% in mass fraction of GM-material (corresponding to 0.061% expressed in terms of copy number ratio). The experiments were carried out under repeatability conditions on fifteen replicates. The RSDr resulted to range between 16% and 24% (Table 8 and 9) depending on the qPCR platform applied, hence also below 25%;
- the collaborative study (step 4 of the validation process) established that over the twelve participating laboratories at the level of 0.1% related to mass fraction of GM-material the RSDr of the method was 17%, therefore also below 25% and well in line with the previous data.

The outcome of the different steps is summarised Table 13.

Table 13. Precision of the event-specific method for quantitative detection of MON 87427 at or around 0.1% level related to mass fractions of GM material.

Source	RSDr %	GM %
Applicant' method optimisation*	20%	0.085%
EURL GMFF tests	16-24%	0.1%
Collaborative study	17%	0.1%

Based on the results of the EURL GMFF in-house verification and of the international collaborative study, it is concluded that the method RSDr % is lower than 25% at the level of 0.1% related to mass fraction of GM material, hence the method meets the requirement laid down in Regulation (EU) No 619/2011.

6. Conclusion

The method provided by the applicant and described in detail under 3.2 (and available as "Validated Method" at <http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx> and in Annex 1) has been validated in accordance to the EURL GMFF validation scheme, respecting all requirements of the relevant EU legislation and international standards for method validation.

This validation study confirmed that the method is applicable to the control samples provided by the applicant (see paragraph 3.4.3), in accordance with the requirements of Annex I-3.C.2 to Commission Regulation (EU) No 503/2013 and (EU) No 619/2011 and meets all method performance requirements established by the ENGL. The method is therefore valid to be used for regulatory purposes, including the quantification of low level presence of 0.1% (m/m) of the GM event. It can be assumed that it is applicable to any appropriately extracted maize DNA.

7. References

1. Horwitz W. Protocol for the design, conduct and interpretation of method- performance studies, *Pure and Appl. Chem.* 1995; 67: 331-343.
2. International Standard (ISO) 5725-1, 1994. Accuracy (trueness and precision) of measurement methods and results. Part 1: General principles and definitions. International Organization for Standardization, Genève, Switzerland.
3. ISO 5725-1:1994/Cor 1:1998.
4. International Standard (ISO) 5725-2, 1994. Accuracy (trueness and precision) of measurement methods and results. Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method. International Organization for Standardization, Genève, Switzerland.
5. ISO 5725-2:1994/Cor 1:2002.
6. Plant DNA C-values Database, <http://data.kew.org/cvalues/>

Annex 1: Event-specific Method for the Quantification of Maize MON 87427 Using Real-time PCR

Validated Method

Method development:

Monsanto Company

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	19279	5073	1335	351
MON 87427 copies	7326	1928	507	134	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

- Vortex for approx. 5 seconds and spin down.
- 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).
- 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.
- 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.
- 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	Denaturation	95	15	No	45
	Amplification 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:

- 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.
- 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).
- Save the settings.
- Repeat the procedure described in a), b) and c) on the amplification plots of the other system (e.g. *hmg*).
- 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 ($GM\% = \text{MON 87427} / \text{hmg} \times 100$).

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)
MON 87427			
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/>

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