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Event-specific Method for the Quantification of Maize 5307 by Real-time PCR

Validation Report

European Union Reference Laboratory for Genetically Modified Food and Feed

2014

Joint Research Centre

EURL European Union Reference Laboratory for GM Food & Feed

European Commission
Joint Research Centre
Institute for Health and Consumer Protection

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5 December 2014

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Executive Summary

In line with its mandate[a], the European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF), in collaboration with the European Network of GMO Laboratories (ENGL), has validated an event-specific polymerase chain reaction (PCR) method for detecting and quantifying the maize event 5307 (unique identifier SYN Ø53Ø7-1). The validation study was conducted according to the EU-RL GMFF validation procedure [http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm] and internationally accepted guidelines [1 - 5].

In accordance with current EU legislation[b], Syngenta Crop Protection AG provided the detection method and the positive and negative control samples (genomic DNA extracted from maize seeds harbouring the 5307 event as positive control DNA, genomic DNA extracted from conventional maize seeds as negative control DNA). The EU-RL GMFF verified the 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 EU-RL GMFF in-house verification and the collaborative study confirmed that the method meets the method performance requirements as established by the EU-RL GMFF and the ENGL, in line with the provisions of Annex I-2.C.2 to Regulation (EC) No 641/2004 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 641/2004 of 6 April 2004 "on detailed rules for the implementation of Regulation (EC) No 1829/2003".

[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 EU-RL 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 EU-RL GMFF quality system.

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

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Content

1. Timeline

In line with Regulation (EC) No 1829/2003, Syngenta Crop Protection AG provided the EU-RL GMFF with a copy of the official application for authorisation of an event-specific method for the detection and quantification of maize (*Zea mays* L.) event 5307 (unique identifier SYN-Ø53Ø7-1) together with negative and positive control samples (April 2011).

In response to an earlier submission of the method, the EU-RL GMFF started its step-wise validation procedure (step 1: dossier reception) before the formal approval by EFSA of the official dossier (June 2011).

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 a summary of method acceptance criteria and method performance requirements) and it was positively concluded in September 2012.

In step 3 of the procedure (experimental testing), the EU-RL 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 Reg. (EC) No 1829/2003, were found to be of good quality.

The method characteristics were verified in-house by quantifying five blind samples with GM levels within the range 0.04%-5.0% on a copy number basis. The experiments were performed under repeatability conditions and found suboptimal behaviour of the amplification curves on one model of real-time PCR platform. In response to a request for further optimisation, a modified method was submitted by Syngenta to the EU-RL GMFF in August 2013. The experiments conducted by the EU-RL GMFF demonstrated that the PCR efficiency, linearity, trueness and precision of the optimised method were within the limits established by the ENGL.

In addition, and in line with the requirements of Reg. (EU) No 619/2011, the EU-RL 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 October 2013 with the conclusion that the method could be submitted to collaborative study (step 4).

The collaborative study (step 4) took place in November-December 2013. It demonstrated that the method is well suited for analysing DNA of GM maize 5307, appropriately extracted from food or feed, down to a GM level of 0.1% m/m.

[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)

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

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 EU-RL 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 EU-RL 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 3' genomic region. The "5307 3-prime-reverse" primer binding site was found in the genomic border adjacent to the insertion. The "5307 3-prime-forward" primer binds in the insert, in a region that corresponds to T-DNA border region. The "5307 3-prime-s2" probe binds in the junction between the T-DNA border region and the 3' genomic region. The amplicon size is expected to be 107 bp, consistent to that reported by the applicant. The sequence of the amplicon was analysed by BLAST against local copies of the "nt" and "patents" databases, and no significant similarity was found with any other published sequence. 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, Triticum aestivum and Zea mays) using the e-PCR prediction tool and no potential amplicon was identified.

In the initial submission of the event-specific method for the detection and quantification of maize (*Zea mays* L.) event 5307 (April 2011), the applicant determined the parameters slope and R^2 coefficient on eight calibration curves and the bias and precision of quantification over five levels, respectively 5.0%, 2.0%, 0.9%, 0.5% and 0.08% expressed in copy numbers. All parameters were within ENGL acceptance criteria (data not shown). The EU-RL GMFF tested *in-house* the method for detection and quantification of event 5307 maize on different real-time PCR platforms and found a suboptimal behaviour of the amplification curves on ABI 7900 platform, while regular plots were produced on ABI 7500. The EU-RL GMFF requested an optimisation of the method to the applicant in order to make it applicable on the different thermal cycler models in common use, in accordance with the requirement of 'robustness' [e]. In response to this request, a revised method for quantitative detection of event 5307 was sent to the EU-RL GMFF in August 2013; it only concerned an increase of the passive reference dye (sulforhodamine 101) concentration in reaction, thus intensifying the background fluorescence.

The parameters of the calibration curve (slope, R^2 coefficient) were appropriately determined by the applicant by quantifying in two runs two test samples at the upper and lower end of the

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

previously established dynamic range (5% - 0.08%, see Table 1). The assays were performed on ABI 7900HT.

Table 1. Values of slope and R^2 obtained by the applicant

	Slope	**R^2**
Run 1	-3.34	1.00
Run 2	-3.44	1.00
Mean	-3.39	1.00

According to the ENGL method acceptance criteria, the average value of the slope of the standard curve shall be within the range of -3.1 to -3.6, and the R^2 coefficient shall be ≥ 0.98. Table 1 indicates that the mean slope of the standard curve is -3.39 and the R^2 coefficient is 1.00 and therefore both values are within the ENGL acceptance criteria.

Table 2 reports precision (measured as relative repeatability standard deviation RSDr) and trueness (bias) for the two GM levels tested by the applicant. Twelve values for each GM level were provided. The mean values of trueness and precision were within the ENGL acceptance criteria (trueness ± 25%, RSDr ≤ 25% across the entire dynamic range).

Table 2. Mean %, precision and trueness (measured by the applicant)

	Test results	
Expected GMO%	**0.08**	**5**
Measured mean GMO%	0.086	4.8
Precision (RSDr %)	14	5.6
Trueness (bias %)	7.5	-4

Acceptable performance was also observed when the revised method was tested on ABI® 7500Fast and Agilent Mx3005P (data not shown).

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

3.1 DNA extraction

A DNA extraction method from maize seeds was used by the method developer for extracting genomic DNA from event 5307 and non-GM maize seeds. This DNA extraction method was previously assessed by the EU-RL GMFF and the report is published at http://gmo-crl.jrc.ec.europa.eu/summaries/MIR604_DNAExtr.pdf. In agreement with the ENGL position, which endorses the modularity principle (see also Annex I to Reg. (EC) No 641/2004), and given the similarity in the matrix, the EU-RL GMFF considers the above mentioned DNA extraction protocol applicable for the validation of the method for maize event 5307.

The positive and negative DNA control samples provided to the EU-RL GMFF were prepared by the applicant using a large-scale DNA extraction method that combines cetyltrimethyl ammonium bromide (CTAB)-based lysis, phenol/chloroform/isoamylalcohol and chloroform/isoamylalcohol extraction of the lysate, and isopropanol precipitation of the DNA. After re-suspension, the precipitated DNA was further purified by anion exchange chromatography.

3.2 Method protocol for the PCR analysis

The optimised PCR method provided by the applicant (see the corresponding Validated Method at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx and Annex 1 of this report) and subsequently validated by the EU-RL GMFF is an event-specific, quantitative, real-time TaqMan® PCR procedure for the determination of the relative content of GM event 5307 DNA to total maize DNA. The procedure is a simplex system, in which a maize alcohol dehydrogenase 1 (*adh1*) specific assay and the GM target assay (5307) are performed in separate wells.

For the specific detection of maize event 5307, a 107 bp fragment of the region spanning the 3' insert – to - plant junction in maize event 5307 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 TAMRA (carboxytetramethylrhodamine) as a quencher at its 3' end.

As a *Zea mays L* specific system a fragment of the alcohol dehydrogenase 1 (*adh1*) endogenous gene (Entrez® Database Accession No. AY691949 [NCBI 2011]), is amplified using gene-specific primers and a probe, labelled with VIC as reporter dye at its 5' end, and TAMRA as quencher at its 3' end. The amplified *adh1* fragment is 135 bp long. This maize-specific PCR method had been previously validated (http://gmo-crl.jrc.ec.europa.eu/summaries/MIR604_validated_Method_correctedversion1.pdf and http://gmo-crl.jrc.ec.europa.eu/summaries/MIR604_val_report_correctedVersion1.pdf).

For relative quantification of event 5307 in a test sample, the normalised ΔCt values of calibration samples are used to calculate, by linear regression, a standard curve (plotting ΔCt values against the logarithm of the relative amount of 5307 event DNA). The normalised ΔCt values of the unknown samples are measured and, by means of the regression formula, the relative amount of 5307 event DNA is estimated.

The GM% content of the calibration samples is calculated considering the 1C value for maize genomes as equivalent to 2.73 pg (Plant DNA C-values Database [6]). The total DNA amount used in the PCR reactions and the corresponding GM content of the calibration samples 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 DNA in reaction (ng)	250	250	250	250	250
Target taxon *adh1* copies	91575	91575	91575	91575	91575
5307 maize GM copies	9158	4579	916	82	27
5307 maize GM %	10.0	5.0	1.0	0.09	0.03

3.3 EU-RL GMFF experimental testing (step 3)

3.3.1 Determination of the zygosity ratio in the positive control sample

Annex II of Reg. (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 EU-RL GMFF." In order to satisfy this requirement, the EU-RL GMFF conducted an assessment of the zygosity (GM-target to reference-target ratio) in the positive control sample submitted by the applicant.

To this end, the copy number of the 5307 and *adh1* 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), 1X GE sample loading reagent (Fluidigm), primers and probe at the reaction concentrations indicated in the corresponding Validated Method document (http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx) and 1 µL of DNA at a concentration of 2.5 ng/µL, to avoid panel saturation after analysis (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). A volume of 9 µL of reaction mix was loaded into each well of which only 4.6 µL were distributed into the 765 partitions (or chambers) constituting one panel. Five replicates of the same dilution were loaded in five panels for both the GM- and the taxon-specific assay. The experiments were 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 the Validated Method document (see Annex 1). Data analysis and copy number calculation were performed using the BioMark digital PCR Analysis software using a range of Ct retention from 15 to 45.

Calculations of mean and variances where 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'[f].

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 with known GM levels, within the range 0.04%- 5.0% copy/copy (equivalent to 0.1%-12.5% mass/mass). The experiments were performed on an ABI 7900 and on ABI 7500 real-time platforms under repeatability conditions. Test samples with GM levels 5.0%, 2.5%, 0.9% and 0.1% 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 0.04% copy/copy (corresponding to 0.1% in mass fractions of GM material) was tested in 15 replicates in an additional real-time PCR run. Average values of the slope and of the R^2 coefficient of the standard curves and method trueness and precision of quantification over the dynamic range were evaluated for compliance against the ENGL method acceptance criteria.

In order to assess the method compliance with Reg. (EU) No 619/2011, the EU-RL GMFF estimated, based on 15 replicates, also the method precision (RSDr) at 0.1% GM level in mass fraction (m/m).

3.4 International collaborative study (step 4)

The international collaborative study (EU-RL GMFF step 4) involved 12 laboratories, all being "National Reference Laboratories (NRL), assisting the EU-RL GMFF for testing and validation of methods for detection", as listed in annex to Regulation (EC) No 1981/2006 (now amended by Regulation (EU) No 120/2014). The study was carried out in accordance with the following internationally accepted guidelines:

- 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, 3, 4, 5)

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

[f] Verification of analytical methods for GMO testing when implementing interlaboratory validated methods. European Network of GMO Laboratories (ENGL), 2011.
http://gmo-crl.jrc.ec.europa.eu/doc/ENGL%20MV%20WG%20Report%20July%202011.pdf

3.4.1 List of participating laboratories

The 12 participants in the 5307 validation study were randomly selected from the 30 National Reference Laboratories (NRLs) 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 international validation study of the detection method for maize event 5307

Laboratory	Country
Austrian Agency for Health and Food Safety	AT
Crop Research Institute - Reference Laboratory for GMO Detection and DNA fingerprinting	CZ
Danish Veterinary and Food Administration, Laboratory for Diagnostics in Plants, Seed, and Feed	DK
Institute of Chemical Technology Prague	CZ
Italian National Institute for Health - Department of Veterinary Public Health and Food Safety - Unit GMOs and Mycotoxins	IT
Laboratory of DNA analysis - Department of Gene Technology - Tallinn University of Technology	EE
Laboratorio Arbitral Agroalimentario	ES
Ministry of Finance, General Secretariat for Tax and Customs Issues, General Chemical State Laboratory (GCSL), Food Division- Athens	GR
National Centre for Food, Spanish Food Safety Agency and Nutrition	ES
National Institute of Biology	SI
State Institute of Chemical and Veterinarian Analysis - Freiburg	DE
Veterinary Public Health Institute for Lazio and Toscana Regions; National Reference Centre for GMO Analysis	IT

3.4.2 Real-time PCR equipment used in the study

The laboratories involved in the collaborative study used a range of real-time PCR equipment: four laboratories used the ABI 7500, five used the ABI 7900, one used the ABI 7700, one the Roche LC480 and one used the Mx 3000 Agilent.

This variability of equipment, with its known potential influence on the PCR results, reflects the real-life situation in the control laboratories and the fact that in this case it did not significantly influence the performance of the method provides additional assurance that the method is useable under real conditions.

3.4.3 Materials used in the international collaborative study

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

i) genomic DNA extracted by the applicant from maize seeds harbouring the event 5307 in hemizygous status with GM male contribution in the parental crossing

ii) genomic DNA extracted by the applicant from conventional maize seeds genetically similar to those harbouring the 5307 event.

The control samples were prepared by the EU-RL GMFF from the genomic DNA provided by the applicant in accordance to Regulation (EC) No 1829/2003, Art 2.11[9].

These positive and negative control samples were also used by the EU-RL GMFF to prepare standards (at known GM content) and test samples (at unknown GM content), containing mixtures of 5307 maize DNA and non-GM maize DNA, as GM-DNA copy numbers in relation to target taxon-specific DNA copy numbers.

The calibration samples S1-S5 were prepared by mixing the appropriate amount of 5307 DNA with control non-GM maize DNA to obtain 10%, 5%, 1%, 0.09% and 0.03% (copy/copy) solutions of GM 5307.

The total amount of DNA/reaction of standards S1 to S5 are reported in Table 3.

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

- ✓ Five calibration samples with known concentrations of GM-event (175 µL of DNA solution each) labelled from S1 to S5 (Table 3),
- ✓ Twenty blinded test DNA samples (87.5 µL of DNA solution each at 50 ng/µL) labelled from U1 to U20, representing five GM levels (Table 5).

Table 5. 5307 GM contents in blinded DNA samples

5307 GM% GM copy number/maize genome copy number x 100
5.00
2.50
0.90
0.10
0.04

- ✓ Reaction reagents:
  - 2x Sigma JumpStart™ Taq ReadyMix™ (supplemented with 1500 nM sulforhodamine 101 and 11 mM $MgCl_2$), one vial: 8 mL

[9] 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).

- distilled sterile water, one vial: 3.5 mL

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

adh1 taxon-specific method

- Zm *adh1* primer F (10 µM): 240 µL
- Zm *adh1* primer R (10 µM): 240 µL
- Zm *adh1* probe (10 µM): 160 µL

5307 assay

- 5307i3' forward primer (10 µM): 280 µL
- 5307i3' reverse primer (10 µM): 280 µL
- 5307i3'-s2 probe (10 µM): 100 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 5307 event-specific system and for the *adh1* taxon-specific system. In total, two plates were run by each participating laboratory.

Each participant received twenty blind samples containing mixtures of maize 5307 genomic DNA and non-GM maize genomic DNA at five GM contents, ranging from 0.04% to 5% (copy/copy). Each laboratory received each GM level in four blind replicates. Each test sample was analysed by PCR in three repetitions. Two replicates of each GM level were analysed on the same PCR plate.

The laboratories prepared the PCR master-mixes for the maize event 5307 and the *adh1* 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 lay-out.

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 EU-RL GMFF on an Excel sheet that was designed, validated and distributed by the EU-RL GMFF. All data are stored by the EU-RL GMFF on a dedicated and protected server.

The EU-RL 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

One laboratory was not able to analyse the data because of a software failure in showing the threshold line; in this case the data were analysed by the EU-RL GMFF.

One laboratory making use of an Mx3000 real-time PCR platform informed that the data were not normalised for sulforhodamine 101 because it was not required by the instrument.

One laboratory reported that, at the low threshold setting, one data point of GM S5 was excluded. Data sets from three laboratories were analysed using ROX as the passive reference dye instead of sulforhodamine 101; fluorochrome ROX shows partially overlapping excitation spectra and somehow similar emission spectra compared to sulforhodamine. In all three cases the parameters of the standard curves demonstrated to be in line with the ENGL acceptance criteria and were able to quantify the unknown samples with acceptable trueness and precision.

4. Results

4.1 EU-RL GMFF experimental testing

4.1.1 Zygosity ratio in the positive control sample

The results of the tests to determine the zygosity ratio in the positive control sample for both the event 5307 and the *adh1* targets is shown in Table 6.

Table 6. Summary of dPCR analysis conducted on the 5307 and *adh1* targets in the positive control sample.

Mean ratio (5307/*adh1)*	0.413*
Standard deviation	0.038
RSD_r %	9.1
Standard error of the mean	0.010
Upper 95% CI of the mean	0.434
Lower 95% CI of the mean	0.392

* Mean of fifteen datasets

The 95% confidence interval (CI) spans around 0.4 (from 0.392 to 0.434). This is in agreement with knowledge on the male contribution for the transgenic event, [7 – 9] in seeds, such as the case of the material used for the extraction of the positive control DNA 5307 (3.4.3), assuming a single copy reference target per haploid genome, for an alpha = 0.05 (see 3.3.1).

Hence:

2.5 x GM % in DNA copy number ratio = GM % in mass fraction

The GM concentration of 0.04%, expressed in terms of GM DNA copy numbers in relation to target taxon specific DNA copy numbers, corresponds to a GM level of 0.1% related to mass fraction of GM material.

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

Test samples with GM levels 0.1%, 0.9%, 2.5%, 5.0% (copy/copy) were tested in two real-time PCR runs (run A and B on ABI 7900 and run D and E on ABI 7500) with two replicates for each GM level on each plate (total of four replicates per GM-level).

The sample with GM level 0.1% mass/mass (equivalent to 0.04% copy/copy) was tested in 15 replicates in one run (run C on ABI 7900 and run F on ABI 7500).

The corresponding standard curve parameters and the results of efficiency, linearity, trueness and precision are shown in Table 7a and 7b and in Tables 8a and 8b.

Table 7a. Standard curve parameters of the real-time PCR testing carried out on ABI 7900

	Slope	PCR efficiency*	R^2
Run A	-3.40	97	1.00
Run B	-3.48	94	1.00
Run C	-3.42	96	0.99

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

Table 7b. Standard curve parameters of the real-time PCR testing carried out on ABI 7500

	Slope	PCR efficiency*	R2
Run D	-3.41	97	1.00
Run E	-3.48	94	1.00
Run F	-3.38	98	0.99

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

According to the ENGL method acceptance criteria, the average value of the slope of the standard curve shall fall into the range -3.1 to -3.6 and the R^2 coefficient shall be ≥ 0.98.
Tables 7a and 7b document that the slope of the standard curve and the R^2 coefficient were within the limits established by the ENGL.

Table 8a. Outcome of the *in-house* tests, with regards to the quantification of the five test samples. Testing carried out on ABI 7900.

Target GM-levels % (copy/copy)	Measured GM level %	Bias %	Precision (RSDr %)
5.0	5.1	1.5	1.7
2.5	2.5	-0.06	7.2
0.9	0.86	-4.4	3.0
0.1	0.11	7.2	9.2
0.04	0.040	-0.52	10

Table 8b. Outcome of the *in-house* tests, with regards to the quantification of the five test samples. Testing carried out on ABI 7500.

Target GM-levels % (copy/copy)	Measured GM level %	Bias %	Precision (RSDr %)
5.0	5.2	3.2	2.7
2.5	2.7	6.9	1.5
0.9	0.92	2.4	4.0
0.1	0.11	4.8	3.5
0.04	0.040	-1.1	23

According to the ENGL method acceptance criteria, the method trueness, measured as bias %, should be within ±25% of the target value over the entire dynamic range. The method's precision estimated through relative standard deviation of repeatability (RSDr) should be ≤25% over the entire dynamic range. Tables 8a and 8b document that trueness and precision of quantification were within the limits established by the ENGL for both PCR machines used.

4.2 Results of the international collaborative study

4.2.1 PCR efficiency and linearity

The PCR efficiency (%) and R^2 values (expressing the linearity of the regression) for the standard curve, reported by participating laboratories are displayed in Table 9. The PCR efficiency (%) was calculated from the standard curve slopes using the formula:

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

Table 9. Values of slope, PCR efficiency and R^2 obtained during the validation study

Lab	Plate	Slope	PCR Efficiency (%)	R^2
1	A	-3.38	98	1.00
	B	-3.40	97	1.00
2	A	-3.38	98	1.00
	B	-3.43	96	1.00
3	A	-3.21	105	1.00
	B	-3.18	106	1.00
4	A	-3.34	99	1.00
	B	-3.45	95	1.00
5	A	-3.58	90	1.00
	B	-3.48	94	0.99
6	A	-3.43	96	1.00
	B	-3.37	98	1.00
7	A	-3.37	98	1.00
	B	-3.33	100	1.00
8	A	-3.37	98	1.00
	B	-3.32	100	1.00
9	A	-3.51	93	1.00
	B	-3.34	99	1.00
10	A	-3.37	98	1.00
	B	-3.29	101	1.00
11	A	-3.47	94	1.00
	B	-3.38	98	1.00
12	A	-3.26	103	1.00
	B	-3.33	100	1.00
	Mean	**-3.37**	**98**	**1.00**

Table 9 indicates that the efficiency of amplification for the standard curve ranges from 90 to 106% and the linearity from 0.09 to 1.00. The mean PCR efficiency was 98%, a value within the ENGL acceptance criteria. The average R^2 of the method was equal to 1.00.

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

4.2.2 GMO quantification

Table 10 reports the values of quantification for the four replicates of each GM level as provided by all laboratories.

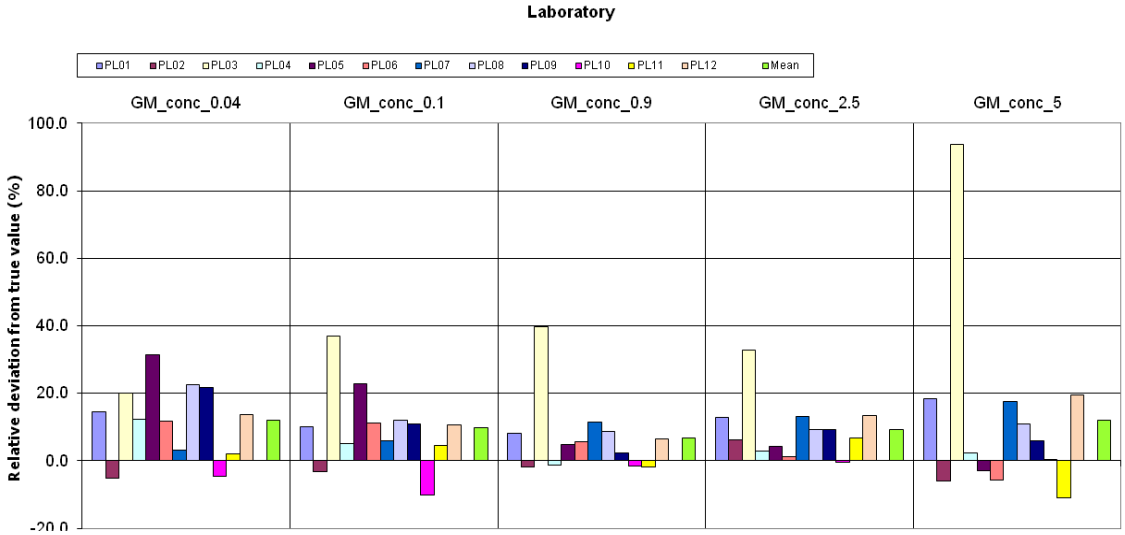
Table 10. GM% values determined by laboratories for test samples, including outliers

	GMO content (%) *																			
LAB	**0.04**				**0.1**				**0.9**				**2.5**				**5.0**			
	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	0.05	0.04	0.05	0.05	0.11	0.11	0.11	0.11	1.02	1.12	0.83	0.92	3.00	2.83	2.58	2.88	6.11	6.59	5.59	5.36
2	0.03	0.04	0.04	0.03	0.10	0.10	0.10	0.09	0.90	0.90	0.90	0.83	2.71	2.55	2.83	2.53	4.37	4.72	4.78	4.94
3	0.07	0.03	0.04	0.05	0.11	0.13	0.13	0.18	1.34	1.49	1.05	1.16	3.23	3.50	2.89	3.66	8.16	13.26	7.10	10.26
4	0.04	0.05	0.05	0.05	0.11	0.10	0.10	0.10	0.97	0.99	0.77	0.81	2.68	2.76	2.36	2.50	5.59	5.53	4.50	4.82
5	0.06	0.05	0.05	0.05	0.11	0.13	0.12	0.13	1.01	0.85	0.98	0.95	2.54	2.57	2.77	2.53	5.95	4.03	4.93	4.51
6	0.05	0.05	0.04	0.05	0.09	0.11	0.11	0.14	0.91	0.97	1.08	0.84	2.55	2.55	2.58	2.44	4.61	4.82	4.71	4.74
7	0.04	0.04	0.04	0.04	0.11	0.12	0.09	0.11	1.11	0.97	0.98	0.95	2.87	2.76	2.75	2.93	6.43	6.10	5.66	5.33
8	0.05	0.05	0.05	0.05	0.11	0.10	0.12	0.12	0.97	1.02	0.92	1.00	2.79	2.66	2.70	2.77	5.65	5.79	5.21	5.55
9	0.04	0.05	0.05	0.06	0.10	0.11	0.13	0.10	0.85	0.92	1.00	0.91	2.66	2.72	2.77	2.78	5.46	5.26	5.03	5.43
10	0.04	0.04	0.04	0.03	0.10	0.08	0.08	0.09	0.92	0.97	0.86	0.80	2.60	2.69	2.31	2.37	5.63	5.55	4.51	4.41
11	0.05	0.04	0.04	0.04	0.11	0.11	0.10	0.10	0.90	0.84	0.87	0.94	2.75	2.55	2.68	2.70	5.01	4.89	3.85	4.05
12	0.05	0.05	0.04	0.04	0.11	0.11	0.11	0.11	0.94	1.09	0.81	1.00	3.40	3.03	2.33	2.58	6.48	6.51	5.52	5.42

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

A graphical representation of the data reported in Table 10 is provided in Figure 1, where the relative deviation from the true value for each GM level tested is shown for each laboratory. The coloured bars represent the deviation of the GM level measured by the respective laboratory in % (copy/copy) of the true GM level; the light green bar on the right represents the mean relative deviation for each true GM level over all twelve participating laboratories.

Figure 1. Relative deviation (%) from the target value of event 5307 concentration (% copy/copy,) for all laboratories*



*PL10 at GM level 2.5% and 5.0% had very small relative deviations from the target value and the corresponding histograms do not show up in Figure 1.

One laboratory clearly overestimated the GM content of more than 25% in the range from 5.0% to 0.1%; one laboratory overestimated of more than 25% the GM content at the 0.04% level. Overall the relative deviations from the target values were within a maximum of ±25%, with a trend towards overestimation for all laboratories at all GM levels. Relative deviations of the general mean from the target values were within a maximum of 12%.

All data were retained for the statistical analysis and for tests of outliers (Cochran and Grubbs) whose results are reported in Table 11.

One laboratory was excluded from the analysis, in accordance with ISO 5725, as measurements obtained from 4 out of 5 levels were regarded as outliers according to Grubbs and/or Cochran's test, and the only level that was not flagged as outlier (0.1%) was characterised by a poor reproducibility (37.5%) and relevant overestimation of GM target (0.14%). Further evaluations using approaches for multivariate outlier detection [10 - 11] based on evaluations of inter-laboratory distances provided evidence that this laboratory can be considered an outlier.

4.2.3 Method performance requirements

Among the method performance requirements established by the ENGL and adopted by the EU-RL GMFF (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm), repeatability and reproducibility are assessed through an international collaborative trial. Table 11 illustrates the estimation of repeatability and reproducibility at the various GM levels tested during the study.

Table 11. Summary of validation results for the 5307 method

	Test Sample expected GMO % (*)				
	0.04	**0.1**	**0.9**	**2.5**	**5.0**
Laboratories having returned valid results	12	12	12	12	12
Samples per laboratory	4	4	4	4	4
Number of outliers	1	1	1	2	1
Reason for exclusion	C	^	G	1C,1G	C
Mean value of measured GM content (%)	0.04	0.11	0.93	2.7	5.2
Relative repeatability standard deviation, RSD_r (%)	13	9.7	8.8	4.7	9.6
Repeatability standard deviation	0.006	0.010	0.083	0.124	0.503
Relative reproducibility standard deviation, RSD_R (%)	15	12	8.9	5.9	13
Reproducibility standard deviation	0.007	0.012	0.084	0.158	0.693
Bias (absolute value)	0.005	0.007	0.034	0.164	0.226
Bias (%)	11	7.2	3.8	6.6	4.5

* GMO % expressed as copy/copy.
C= Cochran's test; G= Grubbs' test; DG= Double Grubbs test; identification and removal of outliers through Cochran and Grubbs tests, according to ISO 5725-2 and bias is estimated according to ISO 5725 data analysis protocol [2].
^ One level for one laboratory removed further to the considerations explained in 4.2.2

According to the ENGL method performance requirements, the relative reproducibility standard deviation (RSD_R), that describes the inter-laboratory variation, should be below 35% 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 11, the method satisfies this requirement at all GM levels tested. In fact, the highest value of RSD_R is 15% at the 0.04% GM level, thus within the acceptance criterion.

Table 11 also documents the relative repeatability standard deviation (RSD_r), estimated for each GM level. In order to accept methods for a collaborative study, the EU-RL GMFF requires the RSD_r value to be below 25%, as indicated by the ENGL (see "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 relative repeatability standard deviation is below 25% at all GM levels, with the highest value of 13% at the 0.04% 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 ± 25% across the entire dynamic range. The method satisfies this requirement across the dynamic range tested, with the highest value of bias (%) being 11% at the 0.04% GM level.

5. Compliance of the method for detection of event 5307 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 assessments were carried out:

- at step 2 of the validation process (scientific assessment of the dossier), the EU-RL GMFF concluded that it could accept the applicant's data on method performance. Indeed, the RSDr at the level of 0.08% in terms of GM DNA copy numbers in relation to the target taxon-specific DNA copy numbers was 14%, hence below the acceptance criterion of 25%;

- at step 3 of the validation process (*in-house* testing of the method), the EU-RL GMFF determined the RSDr % value at the level of 0.1% in mass fraction of GM material (corresponding to 0.04% expressed in terms of copy number ratio). The experiments were carried out under repeatability conditions on fifteen replicates. The RSDr resulted to be about 10% on real-time PCR platform ABI 7900 and 23% on real-time PCR platform ABI 7500, respectively (Table 8a and 8b), hence below 25%;

- further to the conclusion of step 4 of the validation process (collaborative study), the EU-RL GMFF analysed the data generated by the participating laboratories for determining the method performance parameters. The RSDr of the method at the level of 0.1% of mass fraction of GM material was 13%, therefore below the limit of 25%.

The outcome of the different steps is summarised in Table 12.

Table 12. Precision of the event-specific method for quantitative detection of 5307 at and around 0.1% level related to mass fractions of GM material

Source	Real-time PCR	**RSDr %**	**GM %**
Applicant's method optimisation	ABI 7900	14%	0.08%*
EU-RL GMFF in-house verification	ABI 7900	10	0.1%**
	ABI 7500	23	
Collaborative study	§ 4.2.3	13%	0.1%**

* copy/copy: GM- DNA copy numbers in relation to target taxon specific DNA copy numbers
** m/m: mass fraction of GM material

Based on the results of the EU-RL 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

A method for detection, identification and quantification of GM event 5307 was provided by the applicant. It is 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). This method has been validated in accordance to the EU-RL 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-2.C.2 to Commission Regulation (EC) No 641/2004 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

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Annex 1: Event-specific Method for the Quantification of Maize 5307 by Real-time PCR

Validated Method

Method development:

Syngenta Crop Protection AG

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 (*Zea mays* L.) event 5307 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 a PCR assay. Tests for the presence of PCR inhibitors (e.g. monitor run of diluted series, use of DNA spikes) are recommended.

For the specific detection of maize event 5307, a 107 bp fragment of the region spanning the 3' insert -to- plant junction in maize event 5307 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 TAMRA (carboxytetramethylrhodamine) as a quencher at its 3' end.

For the relative quantification of maize event 5307 DNA, a *Zea mays L.* specific system amplifies a fragment of the alcohol dehydrogenase 1 *(adh1)* endogenous gene, using *(adh1)* gene-specific primers and a *(adh1)* gene-specific probe, labelled with VIC as reporter dye at its 5' end, and TAMRA as quencher at its 3' end. The amplified *adh1* fragment is 135 bp long.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Ct" value. For relative quantification of event 5307 in a test sample, the normalised ΔCt values of calibration samples are used to calculate, by linear regression, a standard curve (plotting ΔCt values against the logarithm of the relative amount of 5307 event DNA). The normalised ΔCt values of the unknown samples are measured and, by means of the regression formula, the relative amount of 5307 event DNA is estimated.

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. 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 an international collaborative study by the European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF). The study was undertaken with 12 participating laboratories in November-December 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% (GM DNA copy numbers to target taxon-specific DNA copy numbers) in 250 ng of total maize DNA. The relative LOD was not assessed by the EU-RL GMFF in the collaborative study.

2.4 Limit of quantification (LOQ)

According to the method developer, the relative LOQ of the method is at least 0.08% (GM-DNA copy numbers to target taxon-specific DNA copy numbers) in 250 ng of total maize DNA. The lowest relative GM content of the target sequence included in the collaborative trial was 0.1% (mass/mass) equivalent to level 0.04% expressed in terms of copy number ratio.

2.5 Molecular specificity

The method exploits a unique DNA sequence in the region spanning the 3' insert -to- plant junction in maize event 5307; the sequence is specific to event 5307 and thus imparts event-specificity to the method.

The specificity of the event-specific assay was assessed by the method developer in real-time PCR using genomic DNA (100 ng) extracted from maize event 5307, Bt11, NK603, MON88017, MON863, MON810, MON89034, event 3272, TC1507, MIR604, Bt176, GA21, T25, DAS59122 and conventional maize, soybean, oilseed rape, rice, wheat, potato, sugar beet, cotton.

According to the method developer, the forward and reverse oligonucleotide primers and the TaqMan® probe of the 5307 method show no amplification signals following quantitative PCR analysis, apart from the maize event 5307.

The specificity was further verified *in silico* by the EU-RL GMFF by means of bioinformatics analysis, on the basis of the sequence data provided by the applicant.

3. Procedure

3.1 General instructions and precautions

- The procedure requires 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 employed previously. All handling of PCR reagents and controls requires dedicated equipment, especially pipettes.

- All 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 hypochloride solution (bleach).
- Pipettes should be checked regularly for precision and calibrated, if necessary.
- All handling steps, unless specified otherwise, should be carried out at 0 – 4°C.
- In order to avoid repeated freeze/thaw cycles, aliquots should be prepared.

3.2 Real-time PCR for quantitative analysis of maize event 5307

3.2.1 General

The PCR set-up for the taxon-specific target sequence (*adh1)* and for the GMO (event 5307) target sequence is carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated and is therefore not recommended.

The method is developed and validated 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

To establish the calibration curve five samples should be prepared and analysed. The range of GM contents in the calibration curve should be equal or included in the range validated during the international collaborative trail.

For the collaborative trial, the calibration curve was established on the basis of five samples.
The first point of the calibration curve (S1) contained 10% maize event 5307 DNA (GM-DNA copy numbers to target taxon-specific DNA copy numbers) in a total of 250 ng of maize DNA (GM% calculated considering the 1C value for maize genome as 2.73 pg) [(1)].

The total amount of DNA/reaction and the GM% content of standards S1 to S5 are reported in Table 1.

Table 1. Total amount of DNA in PCR reaction and GM% content of the standard curve samples.

Sample code	**S1**	**S2**	**S3**	**S4**	**S5**
Total amount of DNA in reaction (ng)	250	250	250	250	250
GM% content	10%	5.0%	1%	0.09%	0.03%

A calibration curve is produced by plotting the ΔCt values of calibration samples against the logarithm of the respective GM % contents; the slope (a) and the intercept (b) of the calibration curve (y = ax + b) are then used to calculate the mean GM % content of the blind samples based on their normalised ΔCt values.

3.3 Real-time PCR set-up

1. Thaw, mix and centrifuge the components needed for the run. Keep thawed reagents on ice.

2. To prepare the amplification reaction mixtures add the following components (Table 2 and 3) in two reaction tubes (one for the 5307 assay and one for the *adh1* 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 5307 assay.

Component	**Final concentration**	**µL/reaction**
Supplemented 2x JumpStart™ Taq ReadyMix™ (supplemented with 1500 nM sulforhodamine 101 and 11 mM $MgCl_2$)	1x	12.5
5307i3' forward primer (10 µM)	350 nM	0.875
5307i3' reverse primer (10 µM)	350 nM	0.875
5307i3'-s2 probe (10 µM)	125 nM	0.313
Nuclease free water	/	5.437
Template DNA (50 ng/µL)	/	5.0
Total reaction volume:		25 µL

Table 3. Amplification reaction mixture in the final volume/concentration per reaction well for the maize *adh1* assay.

Component	Final concentration	µL/reaction
Supplemented 2x JumpStart™ Taq ReadyMix™ (supplemented with 1500 nM sulforhodamine 101 and 11 mM $MgCl_2$)	1x	12.5
Zm *adh1* primer F (10 µM)	300 nM	0.75
Zm *adh1* primer R (10 µM)	300 nM	0.75
Zm *adh1* probe (10 µM)	200 nM	0.5
Nuclease free water	/	5.5
Template DNA (50 ng/µL)	/	5.0
Total reaction volume:		25 µL

3. Mix well and centrifuge briefly.
4. Prepare two reaction tubes (one for the maize 5307 and one for the *adh1* 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 (e.g. (70 µL for the *adh1* reference system and 70 µL for the 5307 maize system). Add to each tube the correct amount of DNA for 3.5 PCR repetitions (e.g. 17.5 µL DNA). The additional 0.5 repetition included will ensure adequate volume when loading the samples. Vortex each tube for approx. 10 sec. This step is mandatory for minimising 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 5307/*adh1*.

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

3.4 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. 5307) 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. *adh1*).

e) Save the settings and export all the data to a text file for further calculations.

3.5 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 ΔCt curve is generated by plotting the ΔCt values measured for the calibration points against the logarithm of the GM % content, and by fitting a linear regression line into these data.

Thereafter, the standard ΔCt curve regression formula is used to estimate the relative amount (%) of event 5307 in the unknown DNA samples.

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 instruments (enabling undisturbed fluorescence detection)

- Software for run analysis (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

- JumpStart™ Taq ReadyMix™ (2x), Sigma Aldrich Ltd Cat No P2893
- Sulforhodamine 101, Sigma Cat No S-763

10000x Sulforhodamine 101 stock:

Resuspend 227.5 mg of Sulforhodamine 101 in 250 mL nuclease free water to make a 1.5 mM stock solution.
Vortex well and store at -20 ºC.

Supplemented 2x JumpStart™ Taq ReadyMix™

For 50 ml: To JumpStart™ Taq ReadyMix™ (2x), add
550 µL of 1 M MgCl2
50 µL 10,000 X sulforhodamine 101 stock
Solution should be vortexed and stored at 4°C for up to one year

4.3 Primers and Probes

Oligonucleotides	**Name**	**DNA Sequence (5' to 3')**	**Length (nt)**
Event 5307			
Forward primer	5307i3' forward primer	5'- CAT GGC CGT ATC CGC AAT GTG –3'	21
Reverse primer	5307i3' reverse primer	5'- TGC ACC CTT TGC CAG TGG –3'	18
Probe	5307i3'-s2 probe	5'-6FAM- ACC ACA ATA TAC CCT CTT CCC TGG GCC AG-TAMRA-3'	29
adh1			
Forward primer	Zm *adh1* primer F	5'- CGT CGT TTC CCA TCT CTT CCT CC –3'	23
Reverse primer	Zm *adh1* primer R	5'- CCA CTC CGA GAC CCT CAG TC –3'	20
Probe	Zm *adh1* probe	5'-VIC-AAT CAG GGC TCA TTT TCT CGC TCC TCA-TAMRA-3'	27

FAM: 6-carboxyfluorescein; TAMRA: carboxytetramethylrhodamine

5. References

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

European Commission
Joint Research Centre – Institute for Health and Consumer Protection

Title: **Event-specific Method for the Quantification of Maize 5307 by Real-time PCR**

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Abstract

In line with its mandate , the European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF), in collaboration with the European Network of GMO Laboratories (ENGL), has validated an event-specific polymerase chain reaction (PCR) method for detecting and quantifying the maize event 5307 (unique identifier SYN Ø53Ø7-1). The validation study was conducted according to the EU-RL GMFF validation procedure [http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm] and internationally accepted guidelines (1 - 5).

In accordance with current EU legislation , Syngenta Crop Protection AG provided the detection method and the positive and negative control samples (genomic DNA extracted from maize seeds harbouring the 5307 event as positive control DNA, genomic DNA extracted from conventional maize seeds as negative control DNA). The EU-RL GMFF verified the 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 EU-RL GMFF in-house verification and the collaborative study confirmed that the method meets the method performance requirements as established by the EU-RL GMFF and the ENGL, in line with the provisions of Annex I-2.C.2 to Regulation (EC) No 641/2004 and it fulfils the analytical requirements of Regulation (EU) No 619/2011.

JRC Mission

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