EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit

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

Validated Method

5 December 2014

Method development:

Syngenta Crop Protection AG

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. 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/