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JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit

Event-specific Method for the Quantification of Maize DAS-40278-9 using Real-time PCR

Validated Method

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Method development:

Dow AgroSciences LLC

Method validation:

European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF)

Quality assurance

The EU-RL GMFF is ISO 9001:2008 certified (certificate number: CH-32232) and 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.

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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 event DAS-40278-9 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 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 DAS-40278-9, a 98-bp fragment of the region spanning the 5′ insert-to-plant junction in maize DAS-40278-9 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 dye at its 3′ end.

For the relative quantification of maize event DAS-40278-9 DNA, a maize-specific reference system amplifies a 79-bp fragment of *high mobility group (hmg)*, a 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 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 DAS-40278-9 DNA in a test sample, Ct values for the DAS-40278-9 and *hmg* systems are determined for the sample. Standard curves are then used to estimate the relative amount of DAS-40278-9 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 (EU-RL GMFF).

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

2.3 Limit of detection (LOD)

According to the method developer, the relative LOD of the method is at least 0.04% in 200 ng of total 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 0.08% in 200 ng of total maize DNA. The lowest relative GM content of the target sequence included in the collaborative trial was 0.1%.

2.5 Molecular specificity

The method exploits a unique DNA sequence in the region spanning the 5' insert-to-plant junction in maize DAS-40278-9; the sequence is specific to event DAS-40278-9 and thus imparts event-specificity to the method.

The specificity of the maize taxon-specific assay was assessed by the method developer in real-time PCR using 100 ng of conventional genomic DNA extracted from maize, soybean, rapeseed, cotton, wheat, rice, potato, tomato, sugarbeet, oat, barley, spelt, rye and sorghum. According to the method developer the maize-specific reference system did not react with any target DNA except the positive control.

The specificity of the event-specific assay was assessed by the applicant in real-time PCR using genomic DNA (100 ng) extracted from DAS-40278-9 (1%) as positive control sample and from maize DAS-40474-7, GA21, Bt176, Bt11, NK603, MON863, TC1507, MIR604, 59122, T25, MON810, MON88017, 3272, MON89034; sugarbeet H7-1; rapeseed Ms8, Rf3, T45, RT73; cotton MON531, MON15985, MON1445, GHB614, LLcotton 25; potato EH-92-527-1; soybean DAS-64209-9, DAS-68416-4, A2704-12, DP-305423-1, DP-356043-5, GTS 40-3-2, MON89788, A5547-127; rice LL62; conventional maize, soybean, rapeseed, rice, cotton, wheat, potato and sugarbeet.

According to the method developer, apart from the positive control reaction, the forward and reverse oligonucleotide primers and the TaqMan® probe of the DAS-40278-9 event showed no amplification signals following quantitative PCR analysis (45 cycles).

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 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 DAS-40278-9

3.2.1 General

The PCR set-up for the taxon-specific target sequence (*hmg*) and for the GMO (event DAS-40278-9) target sequence is to be 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 1 and Table 2.

3.2.2 Calibration

The calibration curve has to be established on at least four samples. The first point of the calibration curve should be established for a sample containing 10% maize DAS-40278-9 DNA in a total of 200 ng of maize DNA (corresponding to approximately 73394 maize genome copies with one genome assumed to correspond to 2.725 pg of haploid maize genomic DNA)[(1)]. Standards S2 to S3 are to be prepared by serial 5-fold dilution of the 10% standard. Standard S4 is to be prepared as a 6-fold dilution of the standard S3.

The copy number values of the calibration samples and total DNA quantity used in PCR are reported in Table 1.

Table 1. Copy number values of the standard curve samples.

Sample code	S1	S2	S3	S4
Total amount of DNA in reaction (ng)	200	40	8	1.33
Target taxon *hmg* copies	73394	14679	2936	489
DAS-40278-9 Maize GM copies	7339	1468	294	49

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 e.g. by means of spreadsheet software, e.g. Microsoft Excel, or directly by options available within the sequence detection system software of the method user.

3.2.2 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 DAS-40278-9 assay and one for the *hmg* assay) on ice and in the order mentioned below (<u>except DNA</u>).

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the DAS-40278-9 assay.

Component	Final concentration	µL / reaction
TaqMan® Universal PCR Master Mix no UNG 2x	1x	12.5
DAS-40278-9_5'-f1 (10 µM)	350 nM	0.875
DAS-40278-9_5'-r3 (10 µM)	350 nM	0.875
DAS-40278-9_5'-S2 (10 µM)	150 nM	0.375
Nuclease free water	#	5.375
DNA	#	5
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 no UNG 2X	1x	12.5
MaiJ-F (10 µM)	300 nM	0.75
mhmg-R (10 µM)	300 nM	0.75
mhmg-P (10 µM)	180 nM	0.45
Nuclease free water	#	5.55
DNA	#	5
Total reaction volume:		25 µL

3. Mix well and centrifuge briefly.

4. Prepare two reaction tubes (one for the maize DAS-40278-9 and one for the *hmg* system) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).

5. Add to each reaction tube the correct amount of reaction mix for 3.5 PCR repetitions (e.g. (70 µL for the *hmg* reference system and 70 µL for the DAS-40278-9 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 to reduce to a minimum the variability among the repetitions of each sample.

6. Spin down the tubes in a micro-centrifuge. Aliquot 25 µL in each well. Seal the reaction plate with optical cover or optical caps. Centrifuge the plate at low speed (e.g. approximately 250 x *g* for 1 minute at 4°C) to spin down the reaction mixture.

7. Place the plate into the instrument.

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

Table 4. Cycling program for DAS-40278-9/*hmg* methods.

Step	Stage		T (°C)	Time (s)	Acquisition	Cycles
1	Initial denaturation		95	600	No	1X
2	Amplification	Denaturation	95	15	No	45X
		Annealing & Extension	60	60	Yes	

3.3 Data analysis

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

a) Set the threshold: display the amplification curves of one assay (e.g. DAS-40278-9) in logarithmic mode. Locate the threshold line in the area where the amplification profiles are parallel (exponential phase of PCR) and where there is no "fork effect" between repetitions of the same sample. Press the "update" button to ensure changes affect Ct values (only needed for some analysis software). Switch to the linear view mode by clicking on the Y axis of the amplification plot and check that the threshold previously set falls within the exponential phase of the curves.

b) Set the baseline: determine the cycle number at which the threshold line crosses the first amplification curve and set the baseline three cycles before that value (e.g. earliest Ct = 25, set the baseline crossing at Ct = 25 – 3 = 22).

c) Save the settings.

d) Repeat the procedure described in a), b) and c) on the amplification plots of the other system (e.g. *hmg*).

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

3.4 Calculation of results

After having defined a threshold value within the logarithmic phase of amplification as described above, the instrument's software calculates the Ct-values for each reaction.

The standard curves are generated both for the *hmg* and the DAS-40278-9 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 sample.

To obtain the percentage value of event DAS-40278-9 DNA in the unknown sample, the DAS-40278-9 copy number is divided by the copy number of the maize reference gene (*hmg*) and multiplied by 100 (GM% = DAS-40278-9/*hmg* x 100).

4. 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 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

- TaqMan® Universal PCR Master Mix, No AmpErase® UNG. Applied Biosystems Part No 4326614.

4.3 Primers and Probes

Oligonucleotides	Name	DNA Sequence (5' to 3')	Length (nt)
DAS-40278-9			
Forward primer	DAS-40278-9_5'-f1	5' CAC gAA CCA TTg AgT TAC AAT C 3'	22
Reverse primer	DAS-40278-9_5'-r3	5' Tgg TTC ATT gTA TTC Tgg CTT Tg 3'	23
Probe	DAS-40278-9_5'-S2	5' 6FAM- CgT AgC TAA CCT TCA TTg TAT TCC g –TAMRA 3'	25
hmg			
Forward primer	MaiJ-F	5' TTg gAC TAg AAA TCT CgT gCT gA 3'	23
Reverse primer	mhmg-R	5' gCT ACA TAg ggA gCC TTg TCC T 3'	22
Probe	mhmg-P	5' 6FAM- CAA TCC ACA CAA ACg CAC gCg TA -TAMRA 3'	23

FAM: 6-carboxyfluorescein; TAMRA: carboxytetramethylrhodamine;

5. References

1. Arumuganathan K., Earle E.D., 1991. Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter,* 9: 208-218.