EUROPEAN COMMISSION
JOINT RESEARCH CENTRE
Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit

Event-specific Method for the Quantification of Cotton MON 88701 Using Real-time PCR

Validated Method

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Method development:

Monsanto Comapany

Method validation:

European Union Reference Laboratory for GM Food and Feed (EURL GMFF)

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 cotton event MON 88701 (unique identifier MON-887Ø1-3) DNA to total cotton 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 cotton event MON 88701, an 84-bp fragment of the region spanning the 3' insert-to-plant junction in cotton MON 88701 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 TAMRA (6-carboxytetramethylrhodamine) as quencher at its 3' end.

For the relative quantification of cotton event MON 88701 DNA, a cotton-specific reference system amplifies a 76-bp fragment of *acyl carrier protein 1* (*acp1*), a cotton endogenous gene (GeneBank accession number: U48777.1), using *acp1* gene-specific primers and a *acp1* 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 "Cq" value. For quantification of the amount of MON 88701 DNA in a test sample, Cq values for the MON 88701 and *acp1* systems are determined for the sample. Standard curves are then used to estimate the relative amount of MON 88701 DNA to total cotton DNA.

2. Validation and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from mixtures of genetically modified and conventional cotton seeds. Precision and trueness of the method were tested through an international collaborative trial using DNA samples at different GM levels.

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).

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 cotton DNA. The relative LOD was not assessed by the EURL 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 0.09% (related to mass fraction of GM material) in 200 ng of total cotton 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 3' insert-to-plant junction in cotton MON 88701; the sequence is specific to event MON 88701 and thus imparts event-specificity to the method.

The specificity of the event-specific assay was assessed by the applicant in real-time PCR using genomic DNA samples (200 ng), extracted from MON 88701 (positive control sample) and from maize NK603, MON 863, MON 810, MON 88017, MON 89034, MON 87460, MON 87427, conventional maize; cotton MON 531, MON 1445, MON 15985, MON 88913, conventional cotton; soybean MON 89788, GTS 40-3-2, MON 87708, MON 87705, MON 87769, MON 87701, MON 87712, and conventional soybean; canola RT73, MON 88302 and conventional canola; wheat MON 71800 and conventional wheat, lentil, sunflower, peanut, quinoa, millet.

According to the method developer, apart from the positive control reaction, the forward and reverse oligonucleotide primers and the TaqMan® probe of the MON 88701 event showed no amplification signals following quantitative PCR analysis (45 cycles).

The specificity of the cotton taxon-specific assay, previously validated by the EURL GMFF was demonstrated by the method developer contextually to the event-specific assay, on maize NK603, MON 863, MON 810, MON 88017, MON 89034, MON 87460, MON 87427, conventional maize; cotton MON 531, MON 1445, MON 15985, MON 88913, conventional cotton; soybean MON 89788, GTS 40-3-2, MON 87708, MON 87705, MON 87769, MON 87701, MON 87712, and conventional soybean; canola RT73, MON 88302 and conventional canola; wheat MON 71800 and conventional wheat, lentil, sunflower, peanut, quinoa, millet.

Specificity was further verified and confirmed *in silico* by the EURL 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 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 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 cotton event MON 88701

3.2.1 General

The PCR set-up for the taxon-specific target sequence (*acp1*) and for the GMO (event MON 88701) target sequence has 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 50 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 trial.

For the collaborative trial, the calibration curve was established on the basis of five samples. The first point of the calibration curve contained 10% (mass/mass) cotton MON 88701 DNA in a total of 200 ng of cotton DNA.

Standards S2 and S3 were prepared by serial three-fold dilution of the S1 sample. Standards S4 and S5 were prepared by serial four-fold dilution of the standard S3.

The GM contents of the calibration samples and the total DNA quantity used in PCR are provided in Table 1.

Table 1. GM% values of the standard curve samples.

Sample code	S1	S2	S3	S4	S5
Amount of total DNA in reaction (ng)	200	67	22	5.6	1.4
% GM-DNA	10	10	10	10	10
Amount of GM DNA (ng)	20	6.7	2.2	0.56	0.14

A calibration curve is produced by plotting the Cq values for the calibration points against the logarithm of the amount of MON 88701 DNA. This can be done by means of spreadsheet software, e.g. Microsoft Excel, or directly by options available with the software of the real-time PCR equipment.

3.2.3 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 MON 88701 assay and one for the *acp1* assay) on ice and in the order mentioned below (except DNA).

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the **MON 88701** assay.

Component	**Final concentration**	**µL/reaction**
TaqMan® Universal PCR Master Mix UNG (2x)	1x	25
MON 88701 primer 1 (10 µM)	300 nM	1.50
MON 88701 primer 2 (10 µM)	300 nM	1.50
MON 88701 probe (10 µM)	250 nM	1.25
Nuclease free water	/	16.75
DNA	/	(4)
Total reaction volume:		50 µL

Table 3. Amplification reaction mixture in the final volume/concentration per reaction well for the cotton ***acp1*** assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix UNG (2x)	1x	25
acp1 primer 1 (10 µM)	150 nM	0.75
acp1 primer 2 (10 µM)	150 nM	0.75
acp1 probe (10 µM)	50 nM	0.25
Nuclease free water	/	19.25
DNA	/	(4)
Total reaction volume:		50 µL

3. Mix well and centrifuge briefly.
4. Prepare two reaction tubes (one for the cotton MON 88701 and one for the *acp1* 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. (161 µL for the *acp1* reference system and 161 µL for the MON 88701 cotton system). Add to each tube the correct amount of DNA for 3.5 PCR repetitions (e.g. 14 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 50 µL in each well. Seal the reaction plate with optical cover or optical caps. Centrifuge the plate at low speed (e.g. approximately 250 x *g* for 1 minute at 4 °C) to spin down the reaction mixture.
7. Place the plate into the instrument.
8. Run the PCR with the cycling program described in Table 4.

Table 4. Cycling program for MON 88701/*acp1* methods.

Step	Stage		T °C	Time (sec)	Acquisition	Cycles
1	UNG		50 °C	120	No	1x
2	Initial denaturation		95 °C	600	No	1x
3	Amplification	Denaturation	95 °C	15	No	45x
		Annealing & Extension	60 °C	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. MON 88701) 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 Cq 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 Cq = 25, set the baseline crossing at Cq = 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. *acp1*).

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 Cq values for each reaction.

The standard curves are generated both for the *acp1* and the MON 88701 specific assays by plotting the Cq values measured for the calibration points against the logarithm of the amount of MON 88701 DNA (ng) and by fitting a linear regression line into these data.

Thereafter, the standard curves are used to estimate the amount of MON 88701 DNA in the unknown samples.

The amount of event MON 88701 is divided by the amount of the cotton reference gene (*acp1*) and multiplied by 100 to obtain the percentage value (GM% = MON 88701/ *acp1* 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, UNG. Applied Biosystems Cat. 4304437.

4.3 Primers and Probes

Oligonucleotides	Name	DNA Sequence (5′ to 3′)	Length (nt)
MON 88701			
Forward primer	MON 88701 primer 1	5′ CAT ACT CAT TgC TgA TCC Atg TAg A 3′	25
Reverse primer	MON 88701 primer 2	5′ AgT gTT AAA CAA gTT Atg TTC TAg AgC 3′	27
Probe	MON 88701 probe	5′-6FAM- TTC CCg gAC Atg Aag CCT TAA TTC AAT – TAMRA-3′	27
acp1			
Forward primer	*acp1* primer 1	5′ ATT gTg ATg ggA CTT gAg gAA gA 3′	23
Reverse primer	*acp1* primer 2	5′ CTT gAA CAg TTg TgA Tgg ATT gTg 3′	24
Probe	*acp1* probe	5′-6FAM- ATT gTC CTC TTC CAC CgT gAT TCC gAA –TAMRA-3'	27

6FAM: 6-carboxyfluorescein; TAMRA: 6-carboxytetramethylrhodamine

Annex 2: Troubleshooting

After analysis of the results of the collaborative study additional in-house experiments were performed with the aim to further investigate the slope values and the bias at the 0.1% level obtained during the collaborative study. During in-house testing of the GM and reference systems conducted before the collaborative study, the EURL GMFF did not identify specific problems, both in terms of slope values and bias % (4.1.1). In fact, all parameters were within ENGL acceptance criteria.

One experiment, consisting in a repetition of the GM quantification on the samples used for the collaborative trial, showed acceptable values of trueness and precision with a slight worsening in the slopes for both the GM and the reference system (slope -3.6). For this reason, the loss of efficiency reported for the reference amplification system was further investigated.

Two following experiments performed in November 2013, aiming at evaluating the effect of the storage temperature and the influence of the PCR instrument, gave indication of a possible effect of the storage temperature on the efficiency of PCR amplification. Mean values were anyway within the ENGL acceptance criteria.

In January-February 2014, the EURL GMFF conducted additional tests to determine the impact of various factors (temperature, plastic ware and addition of background DNA) on DNA short term stability (3 weeks) but these tests (data not shown) did not produce clear indication on the causes of the method underperformance at the 0.1% level.

Based on these elements the EURL GMFF decided to recall the left-over samples from the laboratories that participated to the collaborative study, to test the samples in-house and to further investigate this issue. Results obtained on samples recalled from the collaborative study are shown in Table 13.

Table 13. Comparison between slope values obtained during the collaborative study and slope values obtained at the EURL GMFF after recall of samples.

Slope values of the *acp1* reference system	**Collaborative study**	**Tests using recalled validation samples**	**Tests using validation counter-samples* and recalled primers/probes**
PL1	-3.86	-3.45	-
PL4	-3.77	-3.60	-3.27
PL5	-3.88	-4.00	-3.40
PL6	-3.69	-3.89	-
PL8	-3.64	-3.36	-
PL11	-3.72	-3.48	-
PL12	-3.83	-3.89	-3.30

* stored at the EURL GMFF

After evaluation of these results on samples recalled from the collaborative study, it appeared that in about 50% of cases the returned samples produced results in line with the performance demonstrated at the EURL GMFF during step 3; in the other cases an efficiency similar to the one obtained during the validation study was obtained by the EURL GMFF after re-testing the samples recalled. However, the amount of samples recalled was not sufficient to repeat the experiment and further investigate the issue. Although the collected information is therefore not conclusive, we hypothetise that genomic DNA may have been damaged during the delivery of the ring-trial kits to the participating laboratories.