EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit

Event-specific Method for the Quantification of Oilseed Rape MON88302 Using Real-time PCR

Validated Method

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Method development:

Monsanto Company

Method validation:

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

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 oilseed rape event MON88302 (unique identifier MON-883Ø2-9) DNA to total oilseed rape 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 oilseed rape event MON88302, a 101-bp fragment of the region spanning the 5' insert-to-plant junction in oilseed rape MON88302 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 (carboxytetramethylrhodamine) as a quencher dye at its 3' end.

For the relative quantification of oilseed rape event MON88302 DNA, an oilseed rape-specific reference system amplifies a 78-bp fragment of oilseed rape endogenous genes *cruciferin (Ccf)*, (Accession number, (EMBL acc: X59294, UniprotKb acc: P33523), using *ccf* gene-specific primers and a *ccf* gene-specific probe labelled with VIC® 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 MON88302 DNA in a test sample, Ct values for the MON88302 and *ccf* systems are determined for the sample. Standard curves are then used to estimate the relative amount of MON88302 DNA to total oilseed rape DNA.

2. Validation and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from mixtures of genetically modified and conventional oilseed rape 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% (related to mass fraction of GM-material) in 200 ng of total oilseed rape DNA. The relative LOD was not assessed in the collaborative study.

2.4 Limit of quantification (LOQ)

According to the method developer, the relative LOQ of the method is at least 0.085% (related to mass fraction of GM-material) in 200 ng of total oilseed rape DNA. The lowest relative GM content of the target sequence included in the collaborative trial was 0.1% (mass fraction of GM-material).

2.5 Molecular specificity

The method exploits a unique DNA sequence in the region spanning the 5' insert-to-plant junction in oilseed rape MON88302; the sequence is specific to event MON88302 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 according to the method described, with genomic DNA extracted from MON88302 as positive control sample and from oilseed rape RT73, RT200; maize GA21, NK603, MON810, MON863, MON88017, LY038, MON89034, MON87460; cotton MON531, MON15985, MON1445, MON88913; soybean 40-3-2, MON89788, MON87769, MON87701; wheat MON71800 and conventional oilseed rape, maize, cotton, soybean, wheat, lentils, sunflower, peanuts, 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 MON88302 event showed no amplification signals following quantitative PCR analysis.

The specificity of the oilseed rape taxon-specific assay *ccf* was assessed by the method developer in real-time PCR according to the method described, with genomic DNA extracted from oilseed rape MON88302, RT73, RT200; maize GA21, NK603, MON810, MON863, MON88017, LY038, MON89034, MON87460; cotton MON531, MON15985, MON1445, MON88913; soybean 40-3-2, MON89788, MON87701; wheat MON71800 and conventional oilseed rape, maize, cotton, soybean, wheat, lentils, sunflower, peanuts, quinoa, millet.

According to the method developer, apart from the positive control reaction with MON88302, RT73, RT200 and conventional oilseed rape, the forward and reverse oligonucleotide primers and the TaqMan® probe of the MON88302 reference system showed no amplification signals following quantitative PCR analysis.

Specificity was further verified and confirmed *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 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 hypochlorite 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 oilseed rape event MON88302

3.2.1 General

The PCR set-up for the taxon-specific target sequence (*ccf*) and for the GMO (event MON88302) 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 50 µL per reaction mixture with the reagents as listed in Table 2 and Table 3.

3.2.2 Ccf reference system

Brassica napus (oilseed rape) is an amphidiploid species (AC genome, n = 19) derived from a hybridization event between *Brassica rapa* (A genome, n = 10) and *Brassica oleracea* (C genome, n = 9) and it probably arose and was selected in human cultivation within the past 10,000 years [1-3].

According to bioinformatics analysis conducted at EU-RL GMFF, the *Ccf* amplification system can recognize an ortholog gene in *B. rapa* and in *B. oleracea*: the two corresponding amplicons are identical, except for one base mismatch, that occurs in the middle of the probe sequence with regard to the *B. rapa* genome. Therefore the *Ccf* reference system should detect two targets in the haploid (AC) genome of *B. napus.*

Tests performed by the EU-RL GMFF with digital PCR are consistent with this hypothesis: a 0.5 ratio between the MON88302 and the *Ccf* targets was found in the positive control sample [4]. This information is coherent with the knowledge of a positive control sample homozygous for MON88302 (applicant's source), and with the above mentioned bioinformatics analysis.

3.2.3 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 contained 10% oilseed rape MON88302 DNA in a total of 200 ng of oilseed rape DNA (corresponding to approximately 173913 oilseed rape genome equivalents, and to 347826 *Ccf* copies, and to 34783 MON88302 copies respectively; with one genome assumed to correspond to 1.15 pg of haploid oilseed rape genomic DNA) [5].

Standards S2-S4 were prepared by serial four-fold dilution in DNA-free buffer of the S1 standard. Standard S5 was prepared as a five-fold dilution of the standard S4.

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	S1	S2	S3	S4	S5
Total amount of DNA in reaction (ng)	200	50	12.5	3.13	0.63
% GM (DNA/DNA)	10	10	10	10	10
Target taxon ccf copies	347826	86957	21739	5435	1087
MON88302 oilseed rape GM copies	34783	8696	2174	543	109

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.

3.2.4 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 MON88302 assay and one for the *ccf* 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 MON88302 assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix 2x	1x	25
88302QF (10 µM)	450 nM	2.25
88302QR (10 µM)	450 nM	2.25
88302QP (10 µM)	200 nM	1
Nuclease free water	#	15.5
DNA	#	4
Total reaction volume:		50 µL

Table 3. Amplification reaction mixture in the final volume/concentration per reaction well for the oilseed rape *ccf* assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix 2X	1x	25
ccf F (10 µM)	300 nM	1.5
ccf R (10 µM)	300 nM	1.5
ccf P (10 µM)	250 nM	1.25
Nuclease free water	#	16.75
DNA	#	4
Total reaction volume:		50 µL

3. Mix well and centrifuge briefly.

4. Prepare two reaction tubes (one for the oilseed rape MON88302 and one for the *ccf* 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 *ccf* reference system and 161 µL for the MON88302 oilseed rape 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) 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 MON88302/*ccf* methods.

Step	Stage		T (°C)	Time (s)	Acquisition	Cycles
1	UNG		50	120	No	1X
2	Initial denaturation		95	600	No	1X
3	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. MON88302) 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. *ccf*).

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 *ccf* and the MON88302 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 MON88302 DNA in the unknown sample, the MON88302 copy number is divided by the copy number of the oilseed rape reference gene (*ccf*) and multiplied by 100 (GM% = MON88302/*ccf* 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, Applied Biosystems Part No 4304437.

4.3 Primers and Probes

Oligonucleotides	Name	DNA Sequence (5′ to 3′)	Length (nt)
MON88302			
Forward primer	88302QF	5′ TCC TTG AAC CTT ATT TTA TAG TGC ACA 3′	27
Reverse primer	88302QR	5′ TCA GAT TGT CGT TTC CCG CCT TCA 3′	24
Probe	88302QP	5′-6FAM- TAG TCA TCA TGT TGT ACC ACT TCA AAC ACT–TAMRA-3′	30
ccf			
Forward primer	ccf R	5′ GCT TCC GTG ATA TGC ACC AGA AAG 3′	24
Reverse primer	ccf F	5′ ATT GGG CTA CAC CGG GAT GTG T 3′	22
Probe	ccf P	5′ VIC-CGA TGG TGT CCC CAG TCC TTA TGT GCT C - TAMRA 3'	28

FAM: 6-carboxyfluorescein; TAMRA: carboxytetramethylrhodamine

5. References

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