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 DP-073496-4 Using Real-time PCR

Validated Method

18 October 2013
Corrected version 1 - 10 November 2014

Method development:

Pioneer Overseas Corporation

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.

Modification form the previous version:

Page 10/11 Table 4. Step 2 (Amplification) number of cycles changed from *45x* to 40x

Address of contact laboratory:

European Commission, Joint Research Centre (JRC)
Institute for Health and Consumer Protection (IHCP)
Molecular Biology and Genomics Unit
European Union Reference Laboratory for GM Food and Feed
Via E. Fermi 2749, 21027 Ispra (VA) – Italy
Functional mailbox: eurl-gmff@jrc.ec.europa.eu

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 (*Brassica napus*) event DP-073496-4 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 rapeseed event DP-073496-4, an 84 bp fragment of the region spanning the 5′ plant-to-insert junction in oilseed rape DP-073496-4 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 with the MGB-NFQ (minor groove binding non-fluorescent quencher) at its 3′ end.

Brassica napus (oilseed rape) is an amphidiploid species (AC genome, n = 19) derived from a recent hybridisation event between *Brassica rapa* (A genome, n = 10) and *Brassica oleracea* (C genome, n = 9) and probably arose and was selected in human cultivation within the past 10,000 years [1, 2, 3].

The *FatA(A)* system is designed to specifically amplify the acyl-ACP-thioesterase gene sequence present in the A genome of *B. napus*, *B. rapa* and *B. juncea*. Specificity tests performed by the method developer confirmed the specificity of the *FatA(A)* system for the acyl-ACP-thioesterasegene sequence present in the A genome of *B. napus*, *B. rapa* and *B. juncea*.

For the relative quantification of oilseed rape event DP-073496-4 DNA, a specific (*Brassica napus*, *Brassica rapa*, *Brassica juncea*) reference system amplifies a fragment of the A-genome copy of acyl-ACP-thioesterase(*FatA(A)*), an oilseed rape endogenous gene, using *FatA(A)* gene-specific primers and a *FatA(A)* gene-specific probe labelled with FAM as reporter dye at its 5′ end, and MGB-NFQ as quencher at its 3′ end.

The amplified FatA(A) fragment is of 126 bp in a majority of *Brassica napus* varieties, in all *Brassica juncea* varieties and in some of the *Brassica rapa* varieties tested; it is of 129 bp in a minority of *Brassica napus* varieties and in some *Brassica rapa* varieties tested. The applicant reported the presence of a single nucleotide polymorphism located at base 4 relative to the end of the amplicon, on the annealing site of the reverse primer, which, according to the method developer, does not have a negative influence on PCR amplification, based on the data on the stability of the Ct value of *FatA(A)* reference system across *Brassica* species. For further details see Annex 1 to the validation report for rapeseed DP-073496-4 (stability of *FatA(A)* Reference PCR System).

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 DP-073496-4

DNA in a test sample, Ct values for the DP-073496-4 and *FatA(A)* systems are determined for the sample. Standard curves are then used to estimate the relative amount of DP-073496-4 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. 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 twelve participating laboratories in September-October 2012.

Each participant received twenty blind samples containing oilseed rape DP-073496-4 genomic DNA at five GM contents, ranging from 0.1% to 5%.
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 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% in 100 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 0.08% in 100 ng of total oilseed rape DNA. The lowest relative GM content of the target sequence included in the collaborative trial was 0.1%.

2.5 Molecular specificity

According to the method developer, the GM-specific method exploits a unique DNA sequence in the region spanning the 5' plant-to-insert junction in oilseed rape DP-073496-4; the sequence is specific to event DP-073496-4 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 (100 ng) extracted from conventional oilseed rape and oilseed rape DP-073496-4 event as positive control sample and rice non-GM and LLRICE62; non-GM wheat; potato non-GM and EH92-527-1; sugarbeet non-GM and H7-1; cotton non-GM, LLCotton25, MON531, MON15985, MON1445, 281-24-236 x 3006-210-23, GHB614, GHB119, T304-40; soybean non-GM, MON89788-1, A2704-12, A5547-127, DP356043, DP305423, 40-3-2; maize non-GM, 3272, TC1507, 59122, T25, MIR604, MON88017, MON89034, NK603, 98140, MIR162, Bt176, Bt11, MON810, GA21, MON863; oilseed rape RF3, MS8, T45 and RT73.

The specificity of the oilseed rape taxon-specific assay was assessed by the method developer by real-time PCR using 100 ng of genomic DNA extracted from conventional oilseed rape (*B. napus*, AC genome), *Brassica oleracea* (C genome), *Brassica carinata* (BC genome), *Brassica nigra* (B genome), soybean and rice, and 200 ng of genomic DNA extracted from conventional maize and sorghum. The oilseed rape-specific reference system did not react with any target DNA except the positive control and 4 out of the 10 *B. nigra* varieties tested. The amplification Ct value obtained for the B-specific genome was however very high. Indeed, the average Ct value for the 17 *B. napus* varieties tested was 23.7, while the average Ct value for the 10 *B. nigra* varieties tested was 38.4.

According to the method developer, apart from the positive control reaction, the forward and reverse oligonucleotide primers and the TaqMan®probe for the DP-073496-4 event showed no amplification signals following quantitative PCR analysis (40 cycles).

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 used 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 oilseed rape event DP-073496-4

3.2.1 General

The PCR set-up for the taxon-specific target sequence (*FatA(A)*) and for the GMO (event DP-073496-4) 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 20 µL per reaction mixture with the reagents as listed in Table 2 and Table 3.

3.2.2 Calibration

The calibration curve has to be established on at least five DNA samples. The first point of the calibration curve (S1) should be established for a sample containing 6% oilseed rape event DP-073496-4 DNA in a total of 100 ng of oilseed rape DNA (GM% calculated considering the 1C value for oilseed rape genome as 1.15 pg) [(4)].

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

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/5µL)	100	100	100	100	100
GM% content	6.0%	3.0%	0.6%	0.3%	0.07%

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 normalized 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 DP-073496-4 assay and one for the *FatA(A)* 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 DP-073496-4 assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x), No UNG	1x	10.0
09-0-2824 (forward primer, 10 µM)	600 nM	1.2
09-0-2825 (reverse primer, 10 µM)	600 nM	1.2
09-QP-83 (probe, 10 µM)	250 nM	0.5
Nuclease free water	#	2.1
Template DNA (100 ng)	#	5.0
Total reaction volume:		20 µL

Table 3. Amplification reaction mixture in the final volume/concentration per reaction well for the oilseed rape *FatA(A)* assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x), No UNG	1x	10.0
09-0-3249 (forward primer, 10 µM)	300 nM	0.6
09-0-3251 (reverse primer, 10 µM)	900 nM	1.8
09-QP-87 (probe, 10 µM)	150 nM	0.3
Nuclease free water	#	2.3
Template DNA (100 ng)	#	5.0
Total reaction volume:		20 µL

3. Mix well and centrifuge briefly.

4. Prepare two reaction tubes (one for the oilseed rape DP-073496-4 and one for the *FatA(A)* 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. (52.5 µL for the *FatA(A)* reference system and 52.5 µL for the DP-073496-4 oilseed rape 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 minimise the variability among the repetitions of each sample.
6. Spin down the tubes in a micro-centrifuge. Aliquot 20 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 DP-073496-4/*FatA(A)* assays.

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. DP-073496-4) 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. *FatA(A)*).

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 DP-073496-4 event in the unknown samples of DNA.

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

- TaqMan® Universal PCR Master Mix, No AmpErase® UNG (2x). Applied Biosystems (cat. 4324018).

4.3 Primers and Probes

Oligonucleotides	Name	DNA Sequence (5′ to 3′)	Length (nt)
DP-073496-4			
Forward primer	09-0-2824	5′ GTT CTT CTC TTC ATA GCT CAT TAC AGT TTT 3′	30
Reverse primer	09-0-2825	5′ CAA ACC TCC ATA GAG TTC AAC ATC TTA A 3′	28
Probe	09-QP-83	6-FAM-5' TTA GTT AGA TCA GGA TAT TCT TG-MGBNFQ-3'	23
FatA(A)			
Forward primer	09-0-3249	5′ ACA GAT GAA GTT CGG GAC GAG TAC 3′	24
Reverse primer	09-0-3251	5′ CAG GTT GAG ATC CAC ATG CTT AAA TAT 3′	27
Probe	09-QP-87	6-FAM-5' AAG AAG AAT CAT CAT GCT TC-MGBNFQ-3'	20

FAM: 6-carboxyfluorescein; MGBNFQ: Minor Groove Binder/Non-Fluorescent Quencher

5. References

1. The *Brassica rapa* Genome Sequencing Project Consortium. The genome of the mesopolyploid crop species *Brassica rapa. Nature Genetics 2011*, 42: 1035-1039

2. Nagaharu U. Genome-analysis in *Brassica* with special reference to the experimental formation of *B. napus* and peculiar mode of fertilization. *Jap J Bot 1935*, 7: 389-452

3. Wang J, Lydiate DJ, Parkin IA, Falentin C, Delourme R, Carion PW, King GJ. Integration of linkage maps for the Amphidiploid Brassica napus and comparative mapping with Arabidopsis and Brassica rapa. *BMC Genomics 2011*, 12: 101

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