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

EURL
European Union Reference Laboratory
for GM Food & Feed

Event-specific Method for the Quantification of Oilseed Rape DP-073496-4 Using Real-time PCR

Validation Report

1 October 2013

European Union Reference Laboratory for GM Food and Feed

Executive Summary

In line with its mandate[a], the European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF), in collaboration with the European Network of GMO Laboratories (ENGL), has validated an event-specific polymerase chain reaction (PCR) method for detecting and quantifying the oilseed rape event DP-073496-4 (unique identifier DP-Ø73496-4). The validation study was conducted according to the EU-RL GMFF validation procedure [http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm] and internationally accepted guidelines [1, 2].

In accordance with current EU legislation[b], Pioneer Overseas Corporation provided the detection method and the samples (genomic DNA extracted from oilseed rape seeds harbouring the DP-073496-4 event as positive control DNA, genomic DNA extracted from conventional oilseed rape seeds as negative control DNA). The EU-RL GMFF prepared the validation samples (calibration samples and blind samples at test GM percentage [DNA/DNA]), organised an international collaborative study and analysed the results.

The study confirms that the method meets the method performance requirements as established by the EU-RL GMFF and the ENGL, in line with the provisions of Annex I-2.C.2 to Regulation (EC) No 641/2004 and it fulfils the analytical requirements of Regulation (EU) No 619/2011[c].

This validation report is published at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.htm.

[a] Regulation (EC) No 1829/2003 of 22 September 2003 "on genetically modified food and feed".

[b] Regulation (EC) No 641/2004 of 6 April 2004 "on detailed rules for the implementation of Regulation (EC) No 1829/2003".

[c] Regulation (EU) No 619/2011 of 24 June 2011 laying down the methods of sampling and analysis for the official control of feed as regards presence of genetically modified material for which an authorisation procedure is pending or the authorisation of which has expired.

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. Timeline

In line with Regulation (EC) No 1829/2003, Pioneer Overseas Corporation provided the EU-RL GMFF with a copy of the official application for authorisation of an event-specific method for the detection and quantification of oilseed rape (*Brassica napus*) event DP-073496-4 (unique identifier DP-Ø73496-4) together with negative and positive control samples (February 2012).

In response to an earlier submission of the method, the EU-RL GMFF started its step-wise validation procedure (step 1: dossier reception) before the formal approval by EFSA of the official dossier (December 2012).

The scientific dossier assessment (step 2) focused on the reported method performance characteristics assessed against the ENGL method acceptance criteria[d] (see http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf for a summary of method acceptance criteria and method performance requirements) and it was positively concluded in July 2012.

In step 3 of its validation procedure (experimental testing), the EU-RL GMFF verified the purity of the control samples provided and conducted an in-house testing of samples and method. The positive and negative control DNA, submitted in accordance with Art 5(3)(j) and Article 17(3)(j) of Reg. (EC) No 1829/2003, were found of good quality. The method characteristics were verified by quantifying five blind GM levels within the range 0.1%-5.0% on a genome copy number basis. The experiments were performed under repeatability conditions and demonstrated that the PCR efficiency, linearity, trueness and precision were within the limits established by the ENGL.

In addition, and in line with the requirements of Reg. (EU) No 619/2011, the EU-RL GMFF also verified *i)* the zygosity ratio of the submitted positive control sample in order to determine the conversion factor between copy numbers and mass fractions, and *ii)* the method's precision (relative repeatability standard deviation, RSDr) at the 0.1% level related to mass fraction of GM material. Step 3 was completed in August 2012 and concluded that the method could be finally tested in a collaborative study.

The collaborative study (step 4) took place in September-October 2012. It demonstrated that the method is well suited for analysing DNA, appropriately extracted from food or feed, and for identifying and quantifying the presence of GM event DP-073496-4 down to a level of 0.1% m/m. The method is therefore applicable for this purpose.

The preparation of the report (step 5) was aligned with the timelines communicated by EFSA for its risk assessment.

[d] EURL/ENGL guidance doc "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm)

2. Step 1 (dossier acceptance) and step 2 (scientific dossier assessment)

Documentation and data supplied by the applicant were evaluated by the EU-RL GMFF for completeness (step 1) and compliance with the ENGL method acceptance criteria (step 2).

The specificity was verified by the applicant and confirmed by the EU-RL GMFF by means of bioinformatics analysis, on the basis of the sequence data provided by the applicant.

The parameters of the calibration curve (slope, R^2 coefficient) were determined by the applicant by quantifying in three runs three test samples at different GM levels (see Table 1).

Table 1. Values of slope and R^2 obtained by the applicant

	Slope	R^2
Run 1	-3.2	1.00
Run 2	-3.3	1.00
Run 3	-3.1	0.99

According to the ENGL method acceptance criteria, the average value of the slope of the standard curve shall be within the range of -3.1 to -3.6, and the R^2 coefficient shall be ≥ 0.98. Table 1 indicates that the average slope of the standard curves is 3.2 and the R^2 coefficient is 1.00 and therefore both values are within the ENGL acceptance criteria.

Table 2 reports precision (measured as relative repeatability standard deviation RSDr) and trueness (bias) for the five GM levels tested by the applicant. Fifteen values for each GM level were provided. The mean values of trueness and precision were within the ENGL acceptance criteria (trueness ± 25%, RSDr ≤ 25% across the entire dynamic range).

Table 2. Mean % (copy/copy), precision and trueness (measured at five GM levels by the applicant)

	Test results				
Expected GMO%	**0.08**	**0.50**	**0.90**	**2.0**	**5.0**
Measured mean GMO%	0.07	0.43	0.82	1.8	4.5
Precision (RSDr %)	14	4.7	4.9	3.8	6.0
Trueness (bias %)	-13	-14	-8. 9	-8.0	-10

3. Step 3 (experimental testing of the samples and method)

3.1 DNA extraction

A "CTAB-Anion-Exchange" method was used by the method developer for extracting genomic DNA from DP-073496-4 and non-GM oilseed rape seeds. This DNA extraction method was previously assessed by the EU-RL GMFF. The assessment report is published at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx.

The positive and negative DNA control samples submitted to the EU-RL GMFF by the applicant were isolated from ground oilseed rape using a large-scale version of the above mentioned DNA extraction procedure.

3.2 Method protocol for the PCR analysis

The PCR method provided by the applicant (see the corresponding Validated Method at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx and in Annex 2 to this report) and subsequently validated in a collaborative trial by the EU-RL GMFF is an event-specific, quantitative, real-time TaqMan® PCR procedure for the determination of the relative content of GM event DP-073496-4 DNA to total oilseed rape DNA. The procedure is a simplex system, in which an oilseed rape acyl-ACP-thioesterase (*FatA(A)*) assay specific for the *Brassica* A genome (*Brassica napus*, *Brassica rapa*, *Brassica juncea)* and the GM target assay (DP-073496-4) are performed in separate wells.

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

For the relative quantification of oilseed rape event DP-073496-4 DNA, a *Brassica* reference system specific to the A-genome (*Brassica napus*, *Brassica rapa*, *Brassica juncea)* amplifies a fragment of the acyl-ACP-thioesterase (*FatA(A)*) 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 126 bp long in a majority of *Brassica napus* varieties, in all *Brassica juncea* varieties and in some of the *Brassica rapa* varieties tested; it is 129 bp long 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 (stability of *FatA(A)* Reference PCR System).

For relative quantification of event DP-073496-4 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 DP-073496-4 event DNA). The normalised ΔCt values of the unknown samples are measured and, by means of the regression formula, the relative amount of DP-073496-4 event DNA is estimated.

The GM% content of the calibration samples is calculated considering the 1C value for oilseed rape genomes as equivalent to 1.15 pg (Arumuganathan & Earle, 1991[3]). The total DNA amount used in the PCR reactions and the corresponding GM content of the calibration samples are listed in Table 3.

Table 3. DNA amount and GM% content of the standard curve samples, as determined by the EU-RL GMFF

Sample code	S1	S2	S3	S4	S5
Total amount of DNA in reaction (ng/5 µL)	100	100	100	100	100
Total amount of DNA copies in reaction	86957	86957	86957	86957	86957
GM% content (copy/copy)	6.0%	3.0%	0.6%	0.3%	0.07%

3.3 EU-RL GMFF experimental testing (step 3)

3.3.1 Determination of the zygosity ratio in the positive control sample

Annex II of Reg. (EU) No 619/2011 requires that "when results are primarily expressed as GM-DNA copy numbers in relation to target taxon-specific DNA copy numbers calculated in terms of haploid genomes, they shall be translated into mass fraction in accordance with the information provided in each validation report of the EU-RL GMFF." In order to satisfy this requirement, the EU-RL GMFF conducted an assessment of the zygosity (GM-target to reference target ratio) in the positive control sample submitted by the applicant.

The copy number of the DP-073496-4 and *FatA(A)* targets was determined by digital PCR (dPCR) on the BioMark HD System using the 12.765 digital arrays (Fluidigm).

One and a half micrograms of control genomic DNA were digested for one hour at 37 ºC with 20 units of restriction enzyme EcoRI that does not cleave within the annealing sites of the primers for the DP-073496-4 or *FatA(A)* amplification systems. Bioinformatics analysis confirmed that EcoRI restriction sites are located outside the respective targeted sequences.

Further to digestion, the DNA was precipitated with ammonium acetate (2.5 M final) and two volumes of absolute ethanol. The outcome of the enzymatic digestion was controlled by running approximately 200 ng of digested and undigested DNA alongside DNA molecular markers in 1% agarose-gel electrophoresis.

The digested DNA was then used as a template for the digital PCR experiments. Reaction mixes were prepared in a final volume of 9 µL and contained 1X TaqMan® Universal PCR Master Mix No AmpErase® UNG (Applied Biosystems), 1X GE sample loading reagent (Fluidigm), primers and probe at the reaction concentrations indicated in the corresponding Validated Method document (http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx) and 1 µL of DNA at a concentration of 1.1 ng/µL, to avoid panel saturation after analysis (optimal between 200<positive partitions<700).

Loading of the digital chip was performed according to the manufacturer's instructions by using the IFC controller (Fluidigm). A volume of 9 µL of reaction mix was loaded into each well of which only 4.6 µL were distributed into the 765 partitions (or chambers) constituting one panel. Five replicates of the same dilution were loaded in five panels for both the GM- and the reference assay. The experiments were repeated three times for a total number of fifteen data sets for both targets. No template controls were included. Amplification conditions were as reported in the Validated Method document. Data analysis and copy number calculation were performed using the BioMark digital PCR Analysis software using a range of Ct retention from 15 to 45.

Calculations of mean and variances where carried out according to the procedure outlined for random variables in the Annex 4 of the ENGL guidance document 'Verification of analytical methods for GMO testing when implementing inter-laboratory validated methods[e].

3.3.2 In-house verification of the method performance against ENGL method acceptance criteria

The method performance characteristics were verified (EU-RL GMFF step 3) by quantifying on a copy number basis five blinded test samples with known GM levels, within the range 0.1%-5.0% (copy/copy, equivalent to mass/mass). The experiments were performed on an ABI 7900 real-time platform under repeatability conditions. Test samples with GM levels 0.1%, 0.4%, 0.9%, 2.5% and 5.0% were tested in two real-time PCR runs with two replicates for each GM-level on each plate (total of four replicates per GM level). The test sample with GM level 0.1% was tested in 15 replicates in one real-time PCR run. Average values of the slope and of the R^2 coefficient of the standard curves and method trueness and precision of quantification over the dynamic range were evaluated for compliance against the ENGL method acceptance criteria.

[e] Verification of analytical methods for GMO testing when implementing interlaboratory validated methods. European Network of GMO laboratories (ENGL), 2011.
http://gmo-crl.jrc.ec.europa.eu/doc/ENGL%20MV%20WG%20Report%20July%202011.pdf

In order to assess the method compliance with Regulation (EU) No 619/2011, the EU-RL GMFF estimated the method precision (RSDr) at 0.1% GM level on the basis of 15 replicates.

3.4 International collaborative study (step 4)

The international collaborative study (EU-RL GMFF step 4) involved twelve laboratories, all being "National Reference Laboratories (NRL), assisting the CRL for testing and validation of methods for detection", as listed in annex to Regulation (EC) No 1981/2006. The study was carried out in accordance with the following internationally accepted guidelines:

- The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" (Horwitz, 1995) (1)
- ISO 5725 (1994) (2)

The objective of the international collaborative study was to verify in experienced laboratories the trueness and reproducibility of the PCR analytical method that was provided by the applicant and which is described under 3.2 above and in the "Validated method" (Annex 2).

3.4.1 List of participating laboratories

The 12 participants in the DP-073496-4 validation study (see table 4) were randomly selected from the 35 NRLs that offered to participate.

Table 4. Laboratories participating in the international validation study of the detection method for oilseed rape DP-073496-4

Laboratory	Country
Central Control and Testing Institute of Agriculture	SK
DTU-Food, National Food Institute	DK
Finnish Customs Laboratory	FI
Institute for Hygiene and Environment- Hamburg	DE
Italian National Institute for Health - Department of Veterinary Public Health and Food Safety - Unit GMOs and Mycotoxins	IT
Laboratory Agroalimentary of the Spanish Ministry of Agriculture, Food and Environment	ES
National Food Chain Safety Office, Food and Feed Safety Directorate, GMO Laboratory	HU
National Research Institute of Animal Production, National Feed Laboratory in Lublin	PL
Office for Consumer Protection of the German Federal State Saarland - Saarbrücken	DE
Service Commun des Laboratoires du MINEFI - Laboratoire de Strasbourg	FR
State Institute of Chemical and Veterinarian Analysis - Freiburg	DE
Walloon Agricultural Research Centre - Department Valorisation of Agricultural Products (D4) - Unit 16 - Authentication and traceability	BE

Clear guidance was given to the selected laboratories for strictly following the standard operational procedures provided for the execution of the protocol (the "Validated Method" is available in Annex 2 and at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx).

3.4.2 Real-time PCR equipment used in the study

Laboratories involved in the collaborative study used the following real-time PCR equipment: three laboratories used ABI 7900, eight laboratories used ABI 7500 and one laboratory used ABI 7700.

This variability of equipment, with its known potential influence on the PCR results, reflects the real-life situation in the control laboratories and provides additional assurance that the method is robust and useable under real conditions.

3.4.3 Materials used in the international collaborative study

For the validation of the quantitative event-specific method, control samples were provided by the EU-RL GMFF to the participating laboratories. They were derived from:

i) genomic DNA extracted by the applicant from oilseed rape seeds harbouring the event DP-073496-4 homozygously, and
ii) genomic DNA extracted by the applicant from conventional oilseed rape seeds genetically similar to those harbouring the DP-073496-4 event.

The control samples were used by the EU-RL GMFF for preparing standard and test samples (of unknown GM-content) containing mixtures of DP-073496-4 oilseed rape and non-GM oilseed rape. The content was expressed in terms of haploid genomes ratios between the calculated GM-DNA and target taxon-specific DNA copy numbers.

The calibration samples S1-S5 were prepared by mixing the appropriate amount of DP-073496-4 DNA with control non-GM oilseed rape DNA to obtain 6.0% to 0.07% (copy/copy) solutions of GM DP-073496-4.

The total amount of DNA/reaction and the GM% content of standards S1 to S5 are reported in Table 3.

The twelve NRLs participating in the validation study received the following materials:

- ✓ Five calibration samples with known concentrations of GM-event (190 µL of DNA solution each) labelled from S1 to S5 (Table 3),
- ✓ Twenty blinded test DNA samples (90 µL of DNA solution each at 20 ng/µL) labelled from U1 to U20, representing five GM levels (Table 5).

Table 5. DP-073496-4 GM contents in blinded DNA samples

DP-073496-4 GM% GM copy number/oilseed rape genome copy number x 100
0.1
0.4
0.9
2.5
5.0

- ✓ Reaction reagents:
  - TaqMan® Universal PCR Master Mix, No AmpErase® UNG (2x), one vial: 6.5 mL
  - distilled sterile water, one vial: 1.5 mL

- ✓ Primers and probes (1 tube each) as follows:

FatA(A) taxon-specific assay
- 09-0-3249 (10 µM): 200 µL
- 09-0-3251 (10 µM): 580 µL
- 09-QP-87 (10 µM): 100 µL

DP-073496-4 assay provided by the applicant
- 09-0-2824 (10 µM): 400 µL
- 09-0-2825 (10 µM): 400 µL
- 09-QP-83 (10 µM): 170 µL

3.4.4 Design of the collaborative study

Participating laboratories received a detailed validation protocol that included, inter alia, the exact design of the PCR plates, ensuring that on each PCR plate the samples were analysed for the DP-073496-4 specific system and for the *FatA(A)* taxon-specific system. In total, two plates were run per participating laboratory.

The laboratories prepared the master-mixes for the DP-073496-4 and the *FatA(A)* assay in accordance with the description provided in the validation protocol. Calibration and test samples were loaded on the PCR plates as per pre-determined plate lay-out and amplified following the cycling program specified in the protocol. The raw data were reported to the EU-RL GMFF on an excel sheet that was designed, validated and distributed by the EU-RL GMFF. Participants determined the GM% in the test samples according to the instructions and using the excel sheet provided. All data were stored by the EU-RL GMFF on a dedicated and protected server.

The EU-RL GMFF analysed the data against the parameters and the limits set by the ENGL, i.e. trueness, precision, amplification efficiency and linearity.

3.4.5 Deviations reported from the protocol

One laboratory reported that one PCR replicate (D12) of the no template control of plate B was eliminated due to a potential pipetting error.

4. Results

4.1 EU-RL GMFF experimental testing

4.1.1 Zygosity ratio in the positive control sample

A summary of the dPCR analysis conducted on the positive control sample for both the DP-073496-4 and the *FatA(A)* targets is shown in Table 6. The results were determined on a total of fifteen data sets.

Table 6. Results of the tests to determine the zygosity ratio in the positive control sample.

Mean ratio (DP-073496-4/*FatA(A)*)	0.97*
Standard deviation	0.073
RSD_r %	7.5
Standard error of the mean	0.019
Upper 95% CI of the mean	1.01
Lower 95% CI of the mean	0.93

* Mean of fifteen datasets

The 95% confidence interval (CI) spans around 1 and therefore the mean ratio is not significantly different from an expected ratio of 1, assuming a GM homozygous and a single-copy reference target, for an alpha = 0.05.

Hence:

GM % in DNA copy number ratio = GM % in mass fraction

4.1.2 Result of the in-house verification of method performance against ENGL method acceptance criteria

Test samples with GM levels 0.4%, 0.9%, 2.5%, 5.0% (copy/copy), were tested in two real-time PCR runs (run A and B) with two replicates for each GM level on each plate (total of four replicates per GM-level), using an ABI 7900 platform.
The test sample with GM level 0.1% (mass/mass, equivalent to 0.1% copy/copy) was tested in 15 replicates in one run (run C).

The corresponding standard curve parameters and the results of efficiency, linearity, trueness and precision are shown in Table 7 and 8.

Table 7. Standard curve parameters

	073496		
	Slope	PCR efficiency*	R^2
Run A	-3.3	99	1.00
Run B	-3.5	95	1.00
Run C	-3.4	96	1.00
mean	- 3.4	96	1.00

* PCR efficiency (%) is calculated using the formula Efficiency = $(10^{(-1/slope)}) - 1) \times 100$

According to the ENGL method acceptance criteria, the average value of the slope of the standard curve shall fall into the range of -3.1 to -3.6 and the R^2 coefficient shall be ≥ 0.98.
Table 7 documents that the slope of the standard curve and the R^2 coefficient were within the limits established by the ENGL. The EU-RL GMFF *in-house* results confirmed the data provided by the applicant.

Table 8. Outcome of the *in-house* verification of trueness (bias %) and precision (RSDr). GM % expressed as copy/copy, equivalent to mass/mass.

Target GM-levels %	**Measured GM level %**	**Bias % of the target GM-level**	**Precision (RSDr %)**
0.1	0.10	1.02	15
0.4	0.40	0.33	4.9
0.9	0.88	-2.6	4.8
2.5	2.47	-1.4	6.5
5.0	4.67	-6.5	5.0

According to the ENGL method acceptance criteria, the method trueness (measured as bias %) should be within ±25% of the target value over the entire dynamic range. The method's precision estimated through relative standard deviation of repeatability (RSDr) should be ≤25% over the entire dynamic range. Table 8 documents that trueness and precision of quantification were within the limits established by the ENGL. The EU-RL *in-house* results confirmed the data provided by the applicant.

4.2 Results of the international collaborative study

4.2.1 PCR efficiency and linearity

The PCR efficiency (%) and R^2 values (expressing the linearity of the regression) for the standard curve, reported by participating laboratories are displayed in Table 9. The PCR efficiency (%) was calculated from the standard curve slopes using the formula:

$$\text{Efficiency} = (10 * (-1/\text{slope})) - 1) \times 100.$$

Table 9. Values of slope, PCR efficiency and R^2 obtained during the validation study

Lab	Plate	Slope	PCR Efficiency (%)	R^2
1	A	-3.5	93	1.00
	B	-3.5	95	1.00
2	A	-3.5	92	1.00
	B	-3.6	91	1.00
3	A	-3.4	95	1.00
	B	-3.5	95	1.00
4	A	-3.3	101	1.00
	B	-3.4	99	1.00
5	A	-3.5	94	1.00
	B	-3.5	94	1.00
6	A	-3.5	93	1.00
	B	-3.5	94	1.00
7	A	-3.4	95	1.00
	B	-3.5	94	1.00
8	A	-3.3	102	1.00
	B	-3.5	93	1.00
9	A	-3.6	90	1.00
	B	-3.4	96	1.00
10	A	-3.4	96	1.00
	B	-3.4	96	1.00
11	A	-3.3	100	1.00
	B	-3.5	95	1.00
12	A	-3.5	92	1.00
	B	-3.5	92	1.00
	Mean	-3.5	95	1.00

Table 9 indicates that the efficiency of amplification for the standard curve ranges from 90 to 102% and that the linearity is 1.00. The mean PCR efficiency was 95%, a value within the ENGL acceptance criteria. The average R^2 of the method was equal to 1.00.

These results confirm the appropriate performance of the method tested in terms of efficiency and linearity.

4.2.2 GMO quantification

Table 10 reports the values of quantification for the four replicates of each GM level as generated by each of the twelve participating laboratories, before application of the Cochran and Grubbs tests, which according to ISO 5725 are to be performed for identifying outlying values.

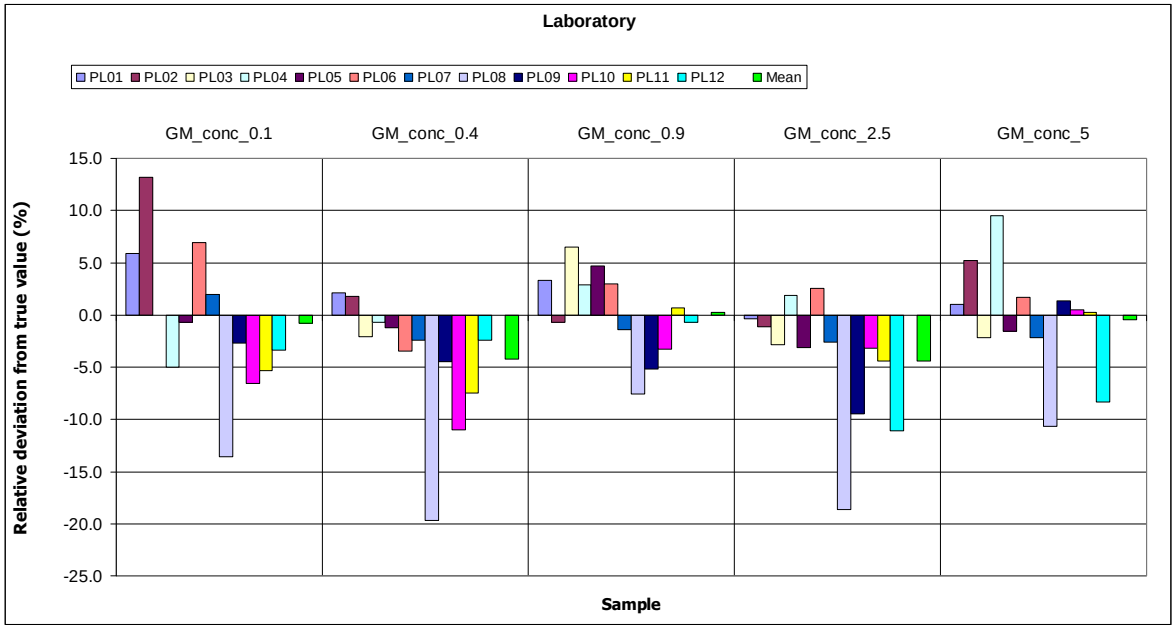
Table 10. GM% values determined by laboratories for test samples, including outliers

	GMO content (%) *																			
LAB	0.1				0.4				0.9				2.5				5.0			
	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4
1	0.12	0.09	0.10	0.11	0.40	0.42	0.40	0.40	0.98	0.89	0.97	0.88	2.47	2.50	2.42	2.57	4.84	5.32	5.09	4.96
2	0.12	0.10	0.12	0.11	0.41	0.40	0.41	0.40	0.94	1.00	0.81	0.82	2.26	2.46	2.81	2.35	5.02	5.56	5.20	5.25
3	0.10	0.09	0.11	0.10	0.40	0.38	0.40	0.39	0.90	0.91	0.96	1.06	2.38	2.45	2.52	2.37	4.74	4.78	4.93	5.11
4	0.10	0.10	0.09	0.09	0.39	0.39	0.43	0.38	0.89	0.87	0.92	1.02	2.43	2.65	2.51	2.59	5.56	5.07	5.70	5.59
5	0.10	0.10	0.10	0.10	0.41	0.39	0.40	0.38	0.91	1.00	0.91	0.95	2.52	2.41	2.28	2.49	4.95	5.15	4.66	4.93
6	0.11	0.11	0.10	0.11	0.38	0.37	0.41	0.38	0.97	0.90	0.98	0.86	2.40	2.58	2.62	2.65	4.93	5.25	5.05	5.11
7	0.11	0.09	0.10	0.11	0.38	0.41	0.39	0.39	0.83	0.96	0.92	0.85	2.35	2.59	2.23	2.57	5.13	4.84	4.94	4.66
8	0.06	0.10	0.10	0.09	0.36	0.31	0.31	0.31	0.99	0.79	0.89	0.66	2.18	2.29	1.97	1.69	4.67	4.52	4.63	4.06
9	0.10	0.08	0.10	0.11	0.38	0.38	0.41	0.36	0.83	0.81	0.89	0.88	2.32	2.22	2.43	2.08	6.00	4.97	4.96	4.35
10	0.09	0.09	0.10	0.09	0.36	0.36	0.38	0.33	0.91	0.83	0.89	0.85	2.46	2.60	2.33	2.28	5.34	5.19	4.93	4.64
11	0.09	0.10	0.10	0.09	0.38	0.39	0.35	0.36	0.95	0.86	0.94	0.88	2.45	2.28	2.47	2.37	5.01	5.01	5.01	5.02
12	0.10	0.10	0.09	0.10	0.38	0.37	0.41	0.40	0.92	0.86	0.94	0.86	2.23	2.13	2.23	2.30	4.63	4.50	4.73	4.48

* GMO% = (GMO copy number/oilseed rape genome copy number) x 100, equivalent to GMO % expressed as mass/mass

A graphical representation of the data reported in Table 10 is provided in Figure 1 where the relative deviation from the target value for each GM level tested is shown for each laboratory. The coloured bars represent the deviation of the GM level measured by the respective laboratory in % of the true GM level; the light green bar on the right represents the mean relative deviation over all twelve participating laboratories for each true GM level.

Figure 1. Relative deviation (%) from the target value of concentration (% copy/copy, equivalent to mass/mass) of DP-073496-4 for all laboratories*



*PL03 at GM level 0.1% had very small relative deviations from the target value and the corresponding histogram does not show up in Figure 1.

Overall, all relative deviations from the target values were within a maximum of ±20%, with a slight trend towards underestimation for all laboratories at all GM levels. Relative deviations from the target values of the general mean were within a maximum of 5%.

All data were retained for the statistical analysis and for tests of outliers (Cochran and Grubbs) whose results are reported in Table 11.

4.2.3 Method performance requirements

According to the method performance requirements established by the ENGL and adopted by the EU-RL GMFF (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm), reproducibility is assessed through an international collaborative trial. Table 11 illustrates the estimation of reproducibility at the various GM levels tested during the collaborative trial.

According to the ENGL method performance requirements, the relative reproducibility standard deviation (RSD_R), that describes the inter-laboratory variation, should be below 35% over the majority of the dynamic range, while it should be below 50% at the lower end of the dynamic range.

As it can be observed in Table 11, the method satisfies this requirement at all GM levels tested. In fact, the highest value of RSD_R is 11% at the 0.1% GM level, thus well within the acceptance criterion.

Table 11. Summary of validation results for the DP-073496-4 method

	Test Sample GMO % (*)				
	0.1	**0.4**	**0.9**	**2.5**	**5.0**
Laboratories having returned valid results	12	12	12	12	12
Samples per laboratory	4	4	4	4	4
Number of outliers	0	0	0	0	0
Reason for exclusion	-	-	-	-	-
Mean value of measured GM content (%)	**0.1**	**0.38**	**0.9**	**2.39**	**5.0**
Relative repeatability standard deviation, RSD_r (%)	9.0	4.4	7.5	6.1	5.7
Repeatability standard deviation	0.01	0.02	0.07	0.15	0.28
Relative reproducibility standard deviation, RSD_R (%)	11	7.4	7.7	8.2	7.3
Reproducibility standard deviation	0.01	0.03	0.07	0.20	0.37
Bias (absolute value)	0.00	-0.02	0.00	-0.11	-0.02
Bias (%)	-0.8	-4.3	0.2	-4.4	-0.4

* GMO % expressed as copy/copy, equivalent to mass/mass.
Bias is estimated according to ISO 5725 data analysis protocol [2].

Table 11 also documents the relative repeatability standard deviation (RSD_r) estimated for each GM level. In order to accept methods for a collaborative study, the EU-RL GMFF requires the RSD_r value to be below 25%, as indicated by the ENGL (Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm). As it can be observed from the values reported, the method showed a relative repeatability standard deviation below 25% at all GM levels, with the highest value of RSD_r being 9% at the 0.1% GM level.

The trueness of the method is estimated in the collaborative trial using the measures of the method bias for each GM level. According to ENGL method performance requirements, trueness should be ± 25% across the entire dynamic range. The method satisfies this requirement across the dynamic range tested, with the highest value of bias (%) being -4.4% at the 2.5% GM level.

5. Compliance of the method for detection of event DP-073496-4 with the requirements of Regulation (EU) No 619/2011

To verify the compliance of the method under validation with the requirements of Regulation (EU) No 619/2011, the following was carried out:

- at step 2 of the validation process (scientific assessment of the dossier), the applicant's data (Table 2) indicated that the RSDr at the level of 0.08%, expressed as ratio of GM-DNA copy numbers to target taxon-specific DNA copy numbers, was 14%, hence below 25%. The results had been determined on 15 replicates. The EU-RL GMFF therefore accepted the applicant's data

- at step 3 of the validation process (*in-house* testing of the method), the EU-RL GMFF determined the RSDr at the level of 0.1% m/m (expressed as mass fraction of GM-material). The measurements were carried out under repeatability conditions on fifteen replicates. The RSDr resulted to be about 15% (Table 8), hence similar to the value obtained by the applicant, and below 25%,

- the collaborative study (step 4 of the validation process) established that (over the twelve participating laboratories) the mean RSDr of the method at the level of 0.1% (m/m) was 9%, i.e. lower than the RSDr established by the applicant or by the EURL GMFF and in any case well below 25%.

The outcome of these tests is summarised in Table 12.

Table 12. Precision of the method for quantitative detection of event DP-073496-4 around 0.1%

Source	**RSDr %**	**GM %**
Applicant's method optimisation	14%	0.08%*
EU-RL GMFF in-house verification	15%	0.1%**
Collaborative study	9%	0.1%**

* copy/copy: GM- DNA copy numbers in relation to target taxon specific DNA copy numbers
** m/m: mass fraction of GM material in relation to non-GM material

Based on the results of the *in-house* verification and of the collaborative study, it is concluded that the method RSDr% is lower than 25% at the level of 0.1% related to mass fraction of GM-material, hence the method for quantitative detection of oilseed rape event DP-073496-4 meets the requirement laid down in Regulation (EU) No 619/2011.

6. Conclusion

A method for detection, identification and quantification of GM event DP 073496-4 was provided by the applicant. It is described in detail under 3.2 (and available as "Validated Method" at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx and in Annex 1). This method has been fully validated in accordance to the EU-RL GMFF validation scheme (step 1, 2, 3 and 4), respecting all requirements of the relevant EU legislation and international standards for method validation.

This validation study confirmed that the method is applicable to the control samples provided by the applicant (see paragraph 3.4.3), in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004 and (EU) No 619/2011. The method is therefore valid to be used for control purposes, including the quantification of low level presence of 0.1% (m/m). It can be assumed that it is applicable to any appropriately extracted oilseed rape DNA.

7. References

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Annex 1: Stability of *FatA(A)* Reference PCR System

4.2 Stability of FatA(A) Reference PCR System

To test for the absence of allelic variation/copy number variation of the *Brassica* A-genome-specific reference PCR system, the Ct values were compared across several varieties of non-GM *B. juncea, B. napus and B. rapa.* All genomic DNAs were tested in triplicate with 100 ng genomic DNA per reaction. The mean Ct value of each variety was compared to the mean of all varieties tested within the species. In all cases the Ct value deviated from the mean Ct value calculated for the species by <1 Ct value (Table 7) indicating that this FatA(A) reference PCR system is quantitatively stable in the different gene

Table 7. Stability of Ct Value Across Species

Species	Source	Country	Ct Mean	Mean Ct of Varieties Tested Within the Species	Deviation from Mean
B. napus	Pioneer	Canada	23.91	23.66	0.26
B. napus	Pioneer	Canada	23.52		-0.14
B. napus	Pioneer	Canada	23.29		-0.36
B. napus	Pioneer	Canada	23.77		0.12
B. napus	Pioneer	Canada	24.22		0.56
B napus	Pioneer	France	23.10		-0.55
B. napus	Pioneer	France	23.81		0.15
B. napus	Pioneer	France	23.30		-0.36
B. napus	USDA	China	24.38		0.73
B. napus	USDA	France	23.54		-0.12
B. napus	USDA	Germany	24.01		0.35
B. napus	USDA	Hungary	23.59		-0.07
B. napus	USDA	Japan	23.09		-0.56
B. napus	USDA	Korea, South	23.31		-0.35
B. napus	USDA	New Zealand	24.09		0.44
B. napus	USDA	Poland	23.77		0.11
B. napus	USDA	Sweden	23.44		-0.21
B. rapa	Pioneer	Canada	23.51	23.08	0.43
B. rapa	Pioneer	Canada	22.55		-0.53
B. rapa	Pioneer	Canada	22.88		-0.20
B. rapa	Pioneer	Canada	23.28		0.20
B. rapa	USDA	China	22.75		-0.33
B. rapa	USDA	France	23.87		0.79
B. rapa	USDA	Germany	22.93		-0.15
B. rapa	USDA	India	22.57		-0.51
B. rapa ssp dichotoma	USDA	India	23.83		0.75
B. rapa ssp oleifera	USDA	Belgium	23.19		0.11
B. rapa ssp trilocularis	USDA	India	22.81		-0.27
B. rapa var. parachinensis	USDA	Hong Kong	22.77		-0.31
B juncea	Pioneer	Canada	24.39	23.87	0.52
B juncea	Pioneer	Canada	23.84		-0.02
B juncea	Pioneer	Canada	23.67		-0.20
B juncea	Pioneer	Canada	23.21		-0.65
B juncea	Pioneer	Canada	23.72		-0.14
B juncea	Pioneer	Canada	23.91		0.04
B. juncea	USDA	China	23.92		0.05
B. juncea	USDA	Germany	24.58		0.71
B. juncea	USDA	India	23.55		-0.31

Annex 2: Event-specific Method for the Quantification of Oilseed Rape DP-073496-4 Using Real-time PCR

Validated Method

Method development:

Pioneer Overseas Corporation

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	45x
		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

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