EUROPEAN COMMISSION
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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 MON88302 by Real-time PCR

Validation Report

21 November 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 oilseed rape event MON88302 (unique identifier MON-883Ø2-9). The validation study was conducted according to the EU-RL GMFF validation procedure [http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm] and the internationally accepted guidelines [1, 2].

In accordance with current EU legislation[b], Monsanto has provided the detection method and the positive and negative control samples (genomic DNA extracted from seeds harbouring the MON-883Ø2-9 event as positive control DNA, genomic DNA extracted from seeds of conventional oilseed rape as negative control DNA). The EU-RL GMFF prepared the validation samples (calibration samples and blind samples at different 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 and according to 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 report is published at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm.

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[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, Monsanto 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 MON88302 (unique identifier MON-883Ø2-9) together with genomic DNA as negative and positive control samples (August 2011).

In response to an early submission of the method, the EU-RL GMFF started its step-wise validation procedure (step 1: dossier reception) already in advance to the official dossier (September 2011), before EFSA declared the dossier as complete and valid (March 2012).

The scientific dossier evaluation (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). It was positively concluded in May 2012.

In step 3 of its validation procedure (experimental testing), the EU-RL GMFF verified the purity of the control samples and conducted an in-house testing of the method provided. The positive and negative control DNA samples - submitted in accordance with Art 5(3)(j) and Article 17(3)(j) of Regulation (EC) No 1829/2003 - were found of good quality.

The method characteristics were verified in-house by quantifying five blinded GM levels within the range of 0.05%-9% GM on a copy number basis. The experiments were performed under repeatability conditions and demonstrated that the PCR efficiency, linearity, accuracy 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 positive control sample submitted by investigating the GM- to reference- target ratio, 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% related to mass fraction of GM-material on fifteen replicates. Step 3 was finished in November 2012 with the conclusion to enter into a collaborative trial (step 4).

The collaborative trial (step 4) took place in December 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 MON88302 down to a level of 0.1% (m/m).

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

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[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 reception and acceptance) and step 2 (dossier scientific assessment)

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

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.

In particular, the specificity of the event-specific assay was assessed by the applicant 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.

The specificity of the oilseed rape taxon-specific assay *ccf* was assessed by the applicant 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.

The parameters of the calibration curves (slope, R^2 coefficient) were appropriately determined by the applicant by quantifying three test samples at different GM levels (0.085%, 1% and 10%) expressed in mass fractions of GM material. The assays were performed on ABI 7900 run in '9600 emulation mode' (see Table 1).

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

	MON88302		*Ccf*	
	Slope	R^2	Slope	R^2
Run 1	-3.41	1.00	-3.33	1.00

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 shall be ≥ 0.98.

Table 1 indicates that the slope of the standard curve is − 3.41 and − 3.33 for the MON88302 and for the *Ccf* methods, respectively, and that the R^2 coefficient for the MON88302 and for the oilseed rape specific reference system (*Ccf*) is 1.00 and therefore all values are within the ENGL acceptance criteria.

Table 2 reports precision and trueness for the three GM-levels tested by the applicant. Fifteen values for each GM-level were provided which is fully in line with common practice. Both parameters were established as being within the ENGL acceptance criteria (trueness ± 25%, RSDr ≤ 25% across the entire dynamic range).

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

Expected GMO %*	0.085	1.00	10.00
Measured mean GMO%	0.088	1.1	11
Precision (RSDr %)	14	6.2	6.3
Trueness (bias %)	3.7	7.4	9.2

* GM levels expressed in mass fractions of GM material

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

3.1 DNA extraction

Genomic DNA was isolated by the applicant from ground oilseed rape seeds and grains using a "CTAB-PEG" protocol previously submitted for detection of event RT73. This DNA extraction method was assessed earlier by the EU-RL GMFF in order to confirm its performance characteristics. The protocol for DNA extraction and a report on testing were published in 2007 at http://gmo-crl.jrc.ec.europa.eu/summaries/RT73_DNA-Extr_report.pdf.

In agreement with the ENGL position, which endorses the modularity principle (see also Annex I to Reg. (EC) No 641/2004), and given the similarity in the matrix, the EU-RL GMFF considers the above mentioned DNA extraction protocol applicable for the validation of the method for oilseed rape event MON88302 .

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 1 to this report) and subsequently validated by the EU-RL GMFF (see http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm) is an event-specific, quantitative, real-time TaqMan® PCR procedure for the determination of the relative content of GM event MON88302 DNA to total oilseed rape DNA. The procedure is a simplex system, in which an oilseed rape *Ccf* (*cruciferin*) specific assay, and the target assay (MON88302) are performed in separate wells.

For the detection of GM event MON88302, a 101-bp fragment of the region spanning the 5' plant-to-insert junction in oilseed rape MON88302 is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific

oligonucleotide probe labelled with FAM (6-carboxyfluorescein) as reporter dye at its 5' end and TAMRA (6-carboxytetramethylrhodamine) as quencher dye at its 3' end.

For the relative quantification of GM event MON88302, a oilseed rape specific reference system amplifies a 78-bp fragment of *cruciferin (Ccf)*, an oilseed rape endogenous gene, using *Ccf* gene-specific primers and an *Ccf* gene-specific probe labelled with VIC as reporter dye at its 5' end, and TAMRA as quencher at its 3' end.

Standard curves are to be generated for both the MON88302 and the *Ccf* systems by plotting the Ct values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a regression line into these data. Thereafter, the standard curves are used to estimate the copy numbers in the test sample DNA by interpolation from the standard curves.

For relative quantification of event MON88302 DNA in a test sample, the MON88302 copy number is divided by the copy number of the oilseed rape reference gene (*Ccf*) and multiplied by 100 to obtain the percentage value (GM% = MON88302/*Ccf* x 100).

The absolute copy numbers of the calibration curve samples are calculated by dividing the sample DNA mass (expressed in picograms) by the published average 1C value for the oilseed rape genome (1.15 pg) [3] and considering the presence of two homologs of *Ccf* target amplicon in the amphidiploid oilseed rape *Brassica napus* genome (paragraph 4.1.1 and Validated method paragraph 3.2.2). The copy number values used in the quantification, the GMO contents of the calibration samples and total DNA quantity used in PCR are listed in Table 3.

Table 3. Copy number values of the standard curve samples

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

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." 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.

To this end, the copy number of the MON88302 and of the *Ccf* targets in the positive control sample were determined by digital PCR (dPCR), performed on the BioMark HD System using the 12.765 digital arrays (Fluidigm).

Reaction mixes were prepared in a final volume of 9 µL and contained 1X TaqMan® Universal PCR Master Mix (Applied Biosystems), 1X GE sample loading reagent (Fluidigm), primers and probe at the reaction concentrations indicated in the corresponding Validated Method (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm), 1 µL of DNA at a concentration of 1.5 ng/µL, suitable 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 approximately 4.6 µL were distributed into the 765 chambers (or partitions) constituting one panel. The analysis was repeated three times; five replicates in five panels were run each time for both the GM- and reference-assay, with a total number of fifteen data sets for both targets. No template controls were included. Amplification conditions were as reported in the Validated Method (see Annex). Data analysis and copy number calculation was performed using the BioMark digital PCR Analysis software, the range of Ct retention was from 15 to 40.

Calculations of means and variances were carried out according to the procedure outlined for random variables in 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 by quantifying on a copy number basis five blinded test samples containing a range of 0.05%-9% GM levels. The experiments were performed on an ABI 7900 real-time platform in '9600 emulation mode' and on ABI 7500 '9600 emulation mode' under repeatability conditions and followed the protocol described in the material and method section. Test samples with GM-levels 9.0%, 4.5%, 0.9% and 0.4% 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 0.05% (corresponding to 0.1% in mass fractions of GM-material) was tested in 15 replicates in an additional run. Average values of the slope and of the R^2 coefficient of the standard curves and method trueness and precision over the dynamic range were evaluated against the ENGL method acceptance criteria.

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[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 Reg. (EU) No 619/2011, the EU-RL GMFF estimated, based on 15 replicates, also the method precision (RSDr) at 0.1% GM level in mass fraction (m/m).

3.4 International collaborative study (step 4)

The international collaborative study (EU-RL GMFF step 4) involved twelve laboratories, all being "National reference laboratories, 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:

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

The objective of the international collaborative study was to verify in experienced laboratories the trueness and precision of the PCR analytical method that was provided by the applicant and which is described under 3.2 above and in the attached "Validated Method" (Annex 1).

3.4.1 List of participating laboratories
The 12 participants in the MON88302 validation study (see Table 4) were randomly selected from the 29 national reference laboratories (NRL) that offered to participate.

Clear guidance was given to the selected laboratories to strictly follow the standard operational procedures that were provided for the execution of the protocol (the protocol is detailed in the Validated Method, available in Annex 1 and at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm). The participating laboratories are listed in Table 4.

Table 4. Laboratories participating in the international collaborative validation study of the detection method for oilseed rape MON88302

Laboratory	Country
Agricultural Institute of Slovenia	SI
Crop Research Institute	CZ
DTU Food, National Food Institute	DK
Federal Institute for Risk Assessment	DE
Laboratory for the Detection of GMO in Food – Bad Langensalza	DE
Landeslabor Schleswig-Holstein	DE
National Centre for Food, Spanish Food Safety Agency and Nutrition	ES
National Food Veterinary Risk Assessment Institute	LT
National Institute of Biology	SI
Office for Consumer Protection German Federal State Saarland- Saarbrücken	DE
Service Commun des Laboratoires du MINEFI	FR
Walloon Agricultural Research Centre	BE

3.4.2 Real-time PCR equipment used in the study

Laboratories involved in the collaborative study used a range of real-time PCR equipment: four laboratories used the ABI 7900 HT, four used the ABI 7500, two used the Stratagene Mx 3005P, one used the Roche LC480 and one used the ABI 7300.

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

3.4.3 Material used in the international collaborative study

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

i)   genomic DNA extracted by the applicant from homozygous seeds of oilseed rape (*B. napus*) harbouring the event MON88302, and

ii) genomic DNA extracted by the applicant from conventional seeds of oilseed rape genetically similar to those harbouring the MON88302 event.

The control samples were prepared by the EU-RL GMFF from the genomic DNA provided by the applicant in accordance to Regulation (EC) No 1829/2003, Art 2.11[f].

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[f] Control sample defined as the GMO or its genetic material (positive sample) and the parental organism or its genetic material that has been used for the purpose of the genetic modification (negative sample).

These positive and negative control samples were also used by the EU-RL GMFF to prepare standards (of known GM-content) and test samples (of unknown GM-content), containing mixtures of MON88302 oilseed rape DNA and non-GM oilseed rape DNA, as GM-DNA copy numbers in relation to target taxon-specific DNA copy numbers calculated in terms of haploid genomes.

The calibration sample S1 was prepared by mixing the appropriate amount of MON88302 DNA with control non-GM oilseed rape DNA to obtain a 10% GM solution. Calibration samples S2-S4 were prepared by serial fourfold dilution from the S1 sample and the S5 sample was prepared by fivefold dilution from the S4 sample.

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

- ✓ Five calibration samples with known concentrations of GM-event (140 µL of DNA solution each) labelled from S1 to S5 (Table 3).
- ✓ Twenty blinded test DNA samples (70 µL of DNA solution each at 45 ng/uL) labelled from U1 to U20, representing five GM levels (Table 5).

Table 5. MON88302 GM contents in genome copy number

MON88302 GM%
GM copy number/oilseed rape genome copy number x 100
9.00
4.50
0.90
0.40
0.05

- ✓ Reaction reagents:
    - ▪ TaqMan® Universal PCR Master Mix (2x), three vials: 5 mL
    - ▪ distilled sterile water, one vial: 11 mL

- ✓ Primers and probes (1 tube each) as follows:
    Ccf taxon-specific assay

    - ▪ ccf F   (10 µM):   480 µL
    - ▪ ccfR   (10 µM):   480 µL
    - ▪ ccfP   (10 µM):   400 µL

    MON88302 assay

    - ▪ 88302QF   (10 µM):   720 µL
    - ▪ 88302QR   (10 µM):   720 µL
    - ▪ 88302QP   (10 µM):   320 µL

3.4.4   Design of the collaborative study

Participant 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 MON88302 specific system and for the *Ccf* taxon-specific system. In total, two plates were run per each participating laboratory.

The laboratories prepared the master-mixes for the MON88302 and *Ccf* assays in accordance with the description provided in the validation protocol. Calibration and test samples were loaded on the PCR plates as per determined plate lay-out.

The amplification reactions followed the cycling program specified in the protocol. Participants determined the GM% in the test samples according to the instructions and reported the raw data to the EU-RL on an Excel sheet that was designed, validated and distributed by the EU-RL GMFF. All data are 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

Ten laboratories reported no deviations from the method protocol.
In one laboratory the software of the real-time PCR equipment accepted a reaction volume of 30µL instead of 50µL.

One laboratory informed the EU-RL GMFF that the Stratagene Mx3005P instrument in use did not have filter for the TAMRA dye. The deviation was considered acceptable and the experimental work was carried out.

4.   Results

4.1   EU-RL GMFF experimental testing

4.1.1   Zygosity ratio in the positive control sample

The results of the tests to determine the zygosity ratio in the positive control samples are shown in Table 6.

Table 6. Summary of dPCR analysis conducted on the MON88302 and *Ccf* targets in the positive control sample.

Mean ratio (MON88302/*Ccf*)	0.501
Standard deviation	0.036
RSD_r (%)	7.3
Standard error of the mean	0.009
Upper 95% CI of the mean	0.521
Lower 95% CI of the mean	0.481

In conclusions, the 95% confidence interval (CI) spans around 0.5 and therefore the mean ratio is not significantly different from an expected ratio of 0.5, assuming a homozygous GM target and a two copy reference target, for an alpha = 0.05 (see 3.2).

Hence:

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

The GM level at 0.05%, expressed in terms of GM DNA copy numbers in relation to target taxon specific DNA copy numbers, corresponds to a GM level of 0.1% related to mass fraction of GM material.

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

Test samples with GM-levels 9.0%, 4.5%, 0.9% and 0.4% were tested by the EU-RL GMFF in two real-time PCR runs (run A and B on ABI 7900 and run D and E on ABI 7500) with two replicates for each GM-level on each plate (total of four replicates per GM-level). The sample with a GM-level 0.05% (corresponding to 0.1% in mass fractions of GM-material) was tested in 15 replicates in one run (run C on ABI 7900 and run F on ABI 7500). Tests were carried out in '9600 emulation mode'. The corresponding standard curve parameters are shown in Tables 7a and 7b and in Tables 8a and 8b.

Table 7a. Standard curve parameters of the real-time PCR testing carried out on ABI 790

	MON88302 method			*Ccf* reference method		
	Slope	PCR efficiency*	R^2	Slope	PCR efficiency*	R^2
Run A	-3.30	101	1.00	-3.30	101	1.00
Run B	-3.28	102	1.00	-3.42	96	1.00
Run C	-3.37	98	1.00	-3.42	96	1.00

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

Table 7b. Standard curve parameters of the real-time PCR testing carried out on ABI7500

	MON88302 method			Ccf reference method		
	Slope	PCR efficiency*	R^2	Slope	PCR efficiency*	R^2
Run D	-3.33	100	1.00	-3.30	101	1.00
Run E	-3.39	97	1.00	-3.38	98	1.00
Run F	-3.35	99	1.00	-3.33	100	1.00

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 $\geq$ 0.98. Tables 7a and 7b document that that the slopes of the standard curves, and the R^2 coefficients were within the limits established by the ENGL.

Table 8a. Outcome of the in-house tests, with regards to the quantification of the five test samples. Testing carried out on ABI 7900.

Target GM-level %	Measured GM %	Bias %	Precision (RSDr %)
9.0	9.4	4.3	7.2
4.5	4.7	5.0	13
0.9	0.84	-6.6	8.2
0.4	0.36	-110	9.2
0.05	0.05	-4.4	13

Table 8b. Outcome of the in-house tests, with regards to the quantification of the five test samples. Testing carried out on ABI7500.

Target GM-level %	Measured GM %	Bias %	Precision (RSDr %)
9.0	8.7	-3.7	5.8
4.5	4.0	-10	3.6
0.9	0.79	-13	44.5
0.4	0.33	-18	8.1
0.05	0.04	-21	13

According to the ENGL method acceptance criteria the method's trueness, measured as bias %, should be within ±25% of the target value over the entire dynamic range. The method's precision, estimated as RSDr % (relative standard deviation of repeatability) should be ≤25% over the entire dynamic range. Tables 8a and 8b document that trueness and precision of quantification were within the limits established by the ENGL.

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 indicates that the efficiency of amplification for the MON88302 system ranges from 83 to 103 and the linearity from 0.98 to 1.00; the amplification efficiency for the oilseed rape-specific system ranges from 82% to 98% and the linearity is again about 1.00. The mean PCR efficiency was 92% for both the MON88302 assay and the *Ccf* assay. Both values were within the ENGL acceptance criteria. The average R^2 of the methods was 0.99 and 1.00 for the MON88302 and *Ccf* assays, respectively.

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

Lab	Plate	MON88302			Ccf		
		Slope	PCR Efficiency (%)	R^2	Slope	PCR Efficiency (%)	R^2
1	A	-3.82	83	0.99	-3.70	86	1.00
	B	-3.77	84	0.99	-3.57	90	1.00
2	A	-3.48	94	0.99	-3.43	96	1.00
	B	-3.46	95	0.99	-3.43	96	1.00
3	A	-3.38	97	1.00	-3.37	98	1.00
	B	-3.40	97	1.00	-3.45	95	1.00
4	A	-3.47	94	1.00	-3.52	92	1.00
	B	-3.47	94	0.99	-3.38	97	0.99
5	A	-3.27	102	1.00	-3.48	94	1.00
	B	-3.26	103	1.00	-3.45	95	1.00
6	A	-3.56	91	1.00	-3.59	90	1.00
	B	-3.53	92	1.00	-3.57	90	1.00
7	A	-3.67	87	0.99	-3.60	90	1.00
	B	-3.68	87	0.99	-3.61	89	0.99
8	A	-3.38	98	0.99	-3.40	97	1.00
	B	-3.52	92	0.99	-3.52	92	1.00
9	A	-3.63	88	0.99	-3.55	91	1.00
	B	-3.55	91	0.99	-3.49	93	1.00
10	A	-3.55	91	0.98	-3.84	82	0.97
	B	-3.65	88	0.99	-3.69	87	0.99
11	A	-3.54	92	1.00	-3.59	90	1.00
	B	-3.43	96	1.00	-3.46	94	1.00
12	A	-3.56	91	0.99	-3.42	96	1.00
	B	-3.51	93	0.99	-3.51	93	1.00
	Mean	**-3.52**	**92**	**0.99**	**-3.53**	**92**	**1.00**

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

### 4.2.2 GMO quantification

Table 10 reports the values of the four replicates for each GM level as provided by all laboratories. The % GM content is expressed in terms of GM DNA copy numbers in relation to target taxon-specific DNA copy numbers (copy/copy). The GM level at 0.05% in copy number ratio (copy/copy) corresponds to a GM level of 0.1% expressed in terms of mass fractions of GM material (mass/mass).

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

LAB	0.05				0.4				0.9				4.5				9.0			
	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	0.08	0.07	0.08	0.08	0.51	0.46	0.50	0.49	0.98	0.97	1.07	1.02	5.03	4.71	4.81	4.83	9.04	8.10	9.00	8.76
2	0.04	0.04	0.05	0.04	0.43	0.32	0.36	0.35	0.77	0.77	0.72	0.79	4.45	4.51	4.73	5.26	10.44	7.30	9.38	9.26
3	0.05	0.05	0.06	0.06	0.46	0.41	0.48	0.31	0.95	0.88	0.81	0.87	4.49	5.22	4.53	4.97	9.32	9.26	8.86	8.02
4	0.06	0.05	0.06	0.05	0.41	0.47	0.41	0.39	0.92	0.93	0.80	0.85	4.19	4.93	4.74	3.95	8.50	9.71	8.72	8.23
5	0.05	0.05	0.05	0.06	0.35	0.34	0.35	0.32	0.78	0.75	0.85	0.72	4.21	4.62	4.83	4.43	9.71	9.48	10.41	10.12
6	0.05	0.05	0.06	0.06	0.45	0.40	0.39	0.37	0.92	0.89	0.80	0.90	4.31	5.37	5.05	4.90	9.41	8.93	9.50	9.70
7	0.06	0.05	0.06	0.06	0.47	0.39	0.44	0.45	0.91	0.92	0.95	1.01	4.78	4.61	4.75	4.52	8.09	8.54	7.84	8.38
8	0.05	0.05	0.05	0.05	0.43	0.40	0.33	0.30	0.87	0.93	0.80	0.71	3.91	4.50	4.72	5.04	8.30	9.42	7.54	8.25
9	0.05	0.06	0.06	0.06	0.49	0.36	0.43	0.39	0.90	0.81	0.96	0.86	4.27	4.82	4.12	4.85	8.30	10.30	8.90	8.79
10	0.05	0.09	0.09	0.12	0.78	0.47	0.63	0.25	0.86	0.87	2.24	0.73	4.20	8.06	5.28	6.48	8.84	12.96	8.13	13.78
11	0.04	0.05	0.05	0.06	0.43	0.37	0.38	0.38	0.85	0.84	0.85	0.91	5.10	4.75	4.79	4.76	8.48	8.70	9.45	8.40
12	0.05	0.05	0.05	0.05	0.38	0.40	0.39	0.36	0.95	0.88	0.85	0.83	4.51	4.66	4.48	4.04	9.52	9.74	7.52	9.34

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 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 true value of MON88302 for all laboratories*
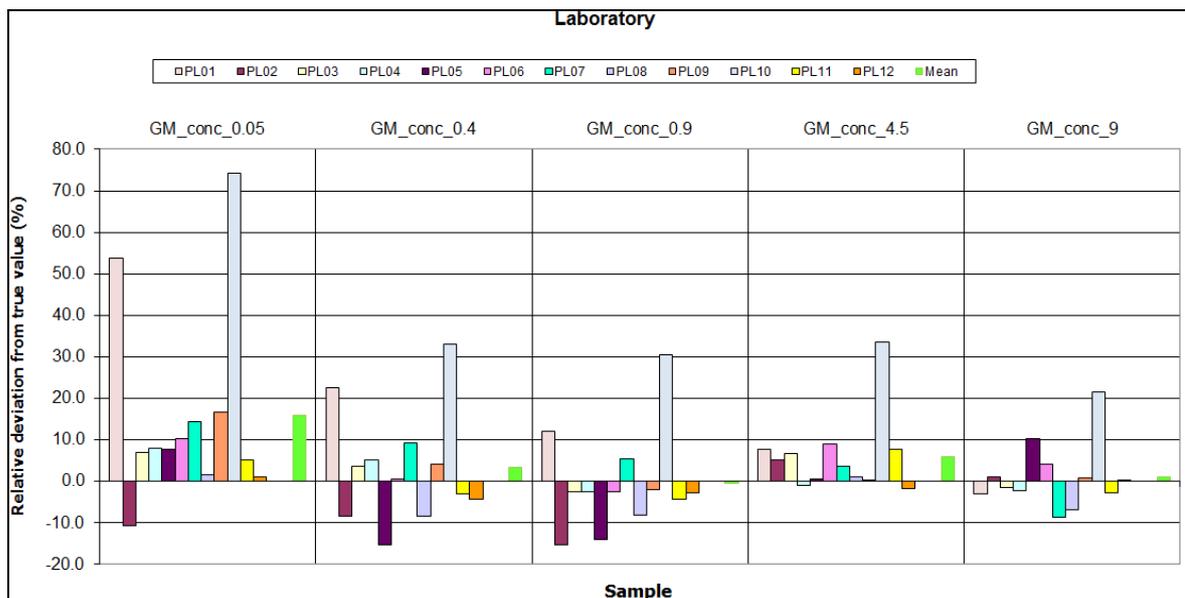
*PL9 at GM level 4.5% and PL 12 at GM level 9.0% had very small relative deviations from the true value and the corresponding histograms do not show up in Figure 1. PL: participating laboratory.

Overall a trend can be observed to overestimate the GM content at the lowest level. PL1 overestimated the GM-content of sample 0.05% by more than 50% and PL10 overestimated the true GM content by more than 30% over most of the entire dynamic range.

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

4.2.3 Method performance requirements

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

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. Indeed, the highest value of RSD_R is 14% at the 0.4% GM level, thus well within the acceptance criterion.

Table 11. Summary of validation results for the MON88302 detection and quantification method, expressed as GM-DNA copy numbers in relation to target taxon specific-DNA copy numbers

	Test Sample Expected GMO %				
	0.05 (*)	0.4	0.9	4.5	9.0
Laboratories having returned valid results	12	12	12	12	12
Samples per laboratory	4	4	4	4	4
Number of outliers	2	1	1	1	1
Reason for exclusion	1C,1G	C	C	C	C
Mean value of measured GM-content	0.05	0.40	0.87	4.7	8.9
Relative repeatability standard deviation, RSD_r (%)	9.1	11	6.4	7.2	8.0
Repeatability standard deviation	0.005	0.043	0.056	0.335	0.718
Relative reproducibility standard deviation, RSD_R (%)	11	14	10	7.3	8.7
Reproducibility standard deviation	0.006	0.056	0.085	0.340	0.777
Bias (absolute value)	0.003	0.002	-0.030	0.160	-0.070
Bias (%)	6.2	0.56	-3.3	3.6	-0.78

C= Cochran's test; G=Grubbs' test; identification and removal of outliers through Cochran and Grubbs tests, according to ISO 5725-2. Bias is estimated according to ISO 5725 data analysis protocol.

* GM level at 0.05% in copy number ratio corresponds to a GM level of 0.1% expressed in terms of mass fractions of GM material.

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 (see 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 repeatability standard deviation is below 25% at all GM levels, with the highest value of 11% at the 0.4% GM level.

The trueness of the method is estimated 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. In this case, the method satisfies this requirement across the dynamic range tested, with the highest value of bias (%) of 6% at the 0.05% GM level.

5. Compliance of the method of detection of event MON88302 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 EU-RL GMFF concluded that it could accept the applicant's data on method performance. Indeed, the RSDr at the level of 0.085% in terms of mass fractions of GM-material resulted to be 14% on 15 replicates (Table 2), hence below 25%;

- at step 3 of the validation process (in-house testing of the method), the EU-RL GMFF determined the RSDr% at the level of 0.05% in terms of GM-DNA copy numbers in relation to target taxon-specific DNA copy numbers. This value corresponds to a GM-level of 0.1% related to mass fraction of GM-material (Paragraph 4.1.1). The experimental testing was carried out under repeatability conditions on fifteen replicates. The RSDr (%) resulted to be 13% when the method was tested in both ABI 7900 and ABI 7500 real-time PCR equipment (Tables 8a and 8b respectively), hence below 25%;

- further to the conclusion of step 4 of the validation process (ring trial), the EU-RL GMFF analysed the data generated by the twelve participating laboratories for determining the method performance parameters. The RSDr (%) of the method at the level of 0.05% in copy number corresponding to a GM-level of 0.1% related to mass fraction of GM-material, was 9.1%, therefore below the limit of 25%.

Table 12. Precision of the event-specific method for quantitative detection of MON88302 at or around 0.1% level related to mass fractions of GM material

Source	RSDr %	GM %
Applicant's method optimisation	14 %	0.085 %
EU-RL GMFF in-house verification	13 %	0.1 %
Collaborative study	9.1 %	0.1 %

Based on the results of the EU-RL GMFF in-house verification and of the collaborative study, it is concluded that the method RSDr is equal or less than 25% at the level of 0.1% related to mass fraction of GM-material, hence the method meets the requirement laid down in Regulation (EU) No 619/2011.

6.   Conclusions

A method for detection, identification and quantification of GM event MON88302 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/statusofdoss.htm and in Annex 1). This method has been fully validated in accordance to the EU-RL GMFF validation scheme, 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 regulatory purposes, including the quantification of low level presence (0.1% m/m) of the GM event. It can be assumed that it is applicable to any appropriately extracted oilseed rape DNA.

7. References

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2. International Standard (ISO) 5725, 1994. Accuracy (trueness and precision) of measurement methods and results. International Organization for Standardization, Genève, Switzerland.
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Annex 1: Event-specific Method for the Quantification of Oilseed Rape MON88302 by Real-time PCR

Validated Method

Method development:

Dow AgroSciences LLC

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) <u>Set the threshold</u>: 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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