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Event-specific Method for the Quantification of Oilseed rape MON 94100 Using Real-time PCR

Validation Report

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EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Directorate F - Health, Consumers and Reference Materials (Geel)

Food and Feed Compliance

EURL European Union Reference Laboratory for GM Food & Feed

Event-specific Method for the Quantification of Oilseed rape MON 94100 Using Real-time PCR

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24/08/2022

European Union Reference Laboratory for GM Food and Feed

Executive Summary

In line with its mandate [1] the European Union Reference Laboratory for GM Food and Feed (EURL GMFF), in collaboration with the European Network of GMO Laboratories (ENGL), validated an event-specific real-time polymerase chain reaction (qPCR) method for detecting and quantifying oilseed rape MON 94100 (unique identifier MON-941ØØ-2). The validation study was conducted according to the EURL GMFF validation procedure [http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm] and the relevant internationally accepted guidelines [2-6].

In accordance with current EU legislation [7], Bayer Agriculture BV provided the detection method and the positive and negative control samples (genomic DNA from seeds of oilseed rape MON 94100 as positive control DNA, and genomic DNA from seeds of conventional oilseed rape as negative control DNA). The EURL GMFF verified the method performance data provided by the applicant, where necessary experimentally, prepared the validation samples (calibration samples and blind samples at different GM percentage (copies GM/total oilseed rape haploid genome copies), organised an international collaborative study and analysed the results.

The EURL GMFF in-house verification and the collaborative study confirmed that the method meets the method performance requirements as established by the EURL GMFF and the ENGL, in line with the provisions of Annex III-3.C.2 to Regulation (EU) No 503/2013 [7], and it fulfils the analytical requirements of Regulation (EU) No 619/2011 [8]. This validation report is published at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx.

This corrected version edited the FatA(A) probe sequence, adding a missing triplet (page 33)

Content

Quality assurance

The EURL GMFF is ISO/IEC 17025:2017 accredited [certificate number: BELAC 268 TEST (Flexible Scope for determination of Genetically Modified content in % (m/m) and % (cp/cp) in food and feed by DNA extraction, DNA identification and Real-time PCR and for determination of Genetically Modified content in % (cp/cp) in food and feed by DNA extraction and digital PCR)].

The original version of the document containing evidence of internal checks and authorisation for publication is archived within the EURL GMFF quality system.

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1. Introduction

In line with Regulation (EC) No 1829/2003 [1], Bayer Agriculture BV provided the EURL GMFF with an event-specific method for detection and quantification of oilseed rape event MON 94100 (unique identifier MON-941ØØ-2) together with genomic DNA as positive and negative control samples.

The dossier was found complete (step 1 of the EURL GMFF validation procedure) and the scientific dossier assessment (step 2) concluded that the reported method performance characteristics, assessed against the ENGL method acceptance criteria [9], allowed moving the method forward to step 3 of the procedure (experimental testing), where the EURL 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 Regulation (EC) No 1829/2003, were found of good quality.

Step 3 was completed with the conclusion that the method could be submitted to the collaborative study (step 4). This study confirmed that the method is well suited for quantifying genomic DNA of GM oilseed rape MON 94100, appropriately extracted from food or feed, down to a GM content level of 0.1 % m/m.

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

2. Dossier reception and acceptance (step 1)

Bayer Agriculture BV submitted the identification and quantification method, data demonstrating its adequate performance when applied to genomic DNA extracted from oilseed rape MON 94100 GM event and from non GM oilseed rape, and the corresponding positive and negative control DNA samples.

The dossier was found to be complete and was thus moved to step 2.

3. Scientific assessment and bioinformatics analysis (step 2)

Documentation and data supplied by the applicant were evaluated by the EURL GMFF for compliance with the ENGL method acceptance criteria.

The specificity of the event-specific assay was verified by the applicant and confirmed by the EURL GMFF by means of bioinformatics analysis, on the basis of the sequence data provided by the applicant.

3.1. Specificity assessment conducted by the applicant

The specificity of the event-specific assay was assessed by the applicant in duplicate real-time Polymerase Chain Reactions (PCR) reactions, according to the method described in Annex 1 (Tables 1, 2, and 4), using 2500 copies of genomic DNA of the target DNA (MON 94100) and of the following non-target DNA samples:
oilseed rape MS1, MS8, RF1, RF2, RF3, Topas19/2, T45, RT73, MON 88302, event 73496; **maize** T25, Bt176, Bt11, MON 810, GA21, NK603, MON 863, TC1507, 3272, MIR604, MIR162, DAS-59122, 98140, MON 88017, MON 89034, MON 87403, MON 87411, MON 87419, MON 87427, MON 87429, MON 87460, DAS-40278-9, VCO-01981-5, DP-004114-3; **cotton** LLCotton25, T304-40, GHB614, GHB119, MON 1445, MON 531, MON 15985, MON 88701, MON 88702, MON 88913, 281-24-236 x 3006-210-23, COT102; **soybean** A2704-12, A5547-127, GTS 40-3-2, MON 89788, MON87701, MON 87708, CV127, MON 87769, MON 87705, MON 87751, DAS-68416-4, DAS-81419-2, DAS-44406-6, FG72, 356043, 305423, BPS-CV127-9; **potato** EH92-527-1, AM04-1020, AV43-6-G7; **alfalfa** J101, J163, KK179; **rice** LLRICE62; **sugar beet** H7-1 and conventional oilseed rape, maize, cotton, soybean, potato, alfalfa, rice, sugar beet and wheat. According to the method developer the MON 94100 assay did not react with any sample except the positive control. All non-target DNA samples were amplifiable when tested with the respective taxon-specific reference methods.

In addition, the applicant performed an *in-silico* specificity analysis, checking the 89 base pair (bp) amplicon sequence of MON 94100 against public sequences of the National Center for Biotechnology Information (NCBI) and the Patent Genbank databases. No sequence showed full alignment to both forward and reverse primers or to the full-length probe.

A previously validated oilseed rape-specific PCR method (https://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-02-12VR-EFSA-Corr1.pdf), which amplifies a 126 bp fragment of the acyl-ACP-thioesterase (*FatA(A)*) gene of *Brassica napus* (and can reach 129 bp in a minority of *Brassica napus* varieties and in some *Brassica rapa* ones), was used as a reference method.

3.2. Specificity assessment conducted by the EURL GMFF

The detection method spans the 5' plant-to-insert junction in oilseed rape MON 94100. The forward primer "MON 94100 primer 1" binding site was found in the oilseed rape (*Brassica napus*) genomic border adjacent to the insertion. The reverse primer "MON 94100 primer 2" aligns with the insert. The probe "MON 94100 probe" aligns with the junction between the 5' genomic region of *Brassica napus* and the insert.

The amplicon size is expected to be 89 bp, consistent to what reported by the applicant. The sequence of the amplicon was analysed by BLAST (NCBI) against local copies of the "nt" and "patents" databases, and no significant similarity was found with any other published sequence. In addition, the primers were tested against the sequences of the other GMO events present in the

Central Core Sequence Information System (CCSIS) of the JRC, as well as the whole genomes of more than 80 plants (including *Brassica rapa*, *Glycine max*, *Oryza sativa*, *Solanum lycopersicum* and *Zea mays*) using the e-PCR prediction tool (NCBI). No potential amplicon was identified, except for one of 78 bp generated by the reverse primer only on the 3rd insertion site of Florigene Moonvista Carnation FLO-40685-2 GM Event (but deemed unlikely to produce unspecific signal).

3.3. Verification of the ENGL acceptance parameters

The applicant prepared the calibration curve from a DNA solution (S1) of 10 % oilseed rape event MON 94100 genomic DNA (expressed as copy number ratio) which was serially diluted (1:5) in TE (0.1x) to obtain solutions S2, S3, S4 and S5. The parameters (slope, R^2 coefficient) of five runs of the calibration curve are reported as provided by the applicant (Table 1).

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

	MON 94100		***FatA(A)***	
Run	**Slope**	**R^2**	**Slope**	**R^2**
1	-3.27	0.99	-3.37	0.99
2	-3.33	0.99	-3.40	0.99
3	-3.40	0.99	-3.37	0.99
4	-3.39	0.99	-3.32	0.99
5	-3.43	0.99	-3.34	0.99
Mean	**-3.36**	**0.99**	**-3.36**	**0.99**

According to the ENGL method acceptance criteria, the average value of the slope of the standard curve shall range from -3.1 to -3.6 and the R^2 coefficient shall be ≥ 0.98.

Table 1 indicates that the slope and R^2 coefficient of the standard curves for the GM-system (MON 94100) and the oilseed rape-specific *FatA(A)* system, as established by the applicant, were within the ENGL acceptance criteria.

Precision and trueness of the method were established by the applicant and 15 values for each of four GM levels (expressed as copy number ratio) were provided. Table 2A reports trueness and precision values for the four GM-levels, each analysed in 3 replicates in 5 runs. Both parameters were within the ENGL acceptance criteria (trueness ± 25 %, RSD_r ≤ 25 % across the entire dynamic range).

Table 2A. Mean %, trueness and precision values provided by the applicant

	Test results			
Expected GM %**	**10**	**1**	**0.085**	**0.05**
Measured mean GM %	9.83	1.08	0.093	0.059
Precision (RSD_r %)	4.82	6.52	16.44	14.69
Trueness (bias %)	-1.68	7.76	8.90	18.02

* Numbers are not rounded but are presented as reported by the applicant

The method met the ENGL acceptance criteria for trueness and precision at the lowest GM level i.e. 0.05 % (in copy number ratio), which contains 52 copies of MON 94100 in 120 ng of total DNA per reaction. The GM content of this sample is in line with the requirements for testing the Limit of Quantification (LOQ, below or equal to 0.09 % or 50 copies).

The absolute limit of detection (LOD_{abs}) of the MON 94100 event specific real-time PCR method was assessed by the applicant in 60 PCR replicates at 10, 5 and 1 haploid genome copies per reaction of MON 94100 event DNA. The LOD_{abs} was found to be below 5 haploid genome copies for the MON 94100 event-specific method. The LOD_{abs} is in line with the ENGL acceptance criteria (below 0.045 % or 25 copies with a level of confidence of 95 %).

The robustness of the method was assessed in 8 combinations of the following variations to the method: unchanged/+/-5 % enzyme mix concentration, unchanged/-/+10 % primer concentration, unchanged/+/-10 % probe concentration, unchanged/+/-1 µL reaction volume, +/-1 °C in annealing temperature. The RSD_r and the trueness calculated for each combination of variations on a sample at the LOQ level (0.085 %) did not exceed 30 %, thus meeting the ENGL acceptance criteria.

Precision and trueness of the method were tested in a transferability study involving one laboratory (Eurofins BioDiagnostics, Inc.) using an ABI QS6-Pro instrument. For each of the 3 GM levels (expressed as copies GM/total haploid genome copies), 15 values (5 replicates in 3 runs) were provided. Table 2B reports precision and trueness values for the three GM-levels as provided by a laboratory different from the method developer. Both parameters were within the ENGL acceptance criteria (trueness ≤ 25 %, RSDr ≤ 25 % across the entire dynamic range).

Table 2B Mean %, precision and trueness values obtained by the applicant in the transferability study

	Test results		
Expected GM %	**10**	**1.0**	**0.085**
Measured mean GM %	9.4	1.1	0.09
Precision (RSD_r %)	7.6	9.0	17
Trueness (bias %)	-6.3	9.7	9.6

3.4. DNA extraction

Genomic DNA was isolated from ground *Brassica napus* seeds, using a CTAB-based protocol (relying on the elimination of lipids and proteins via chloroform extraction followed by an isopropanol precipitation of DNA). This protocol has already been validated in-house by the EURL GMFF and has been published, along with a report on testing, at https://gmo-crl.jrc.ec.europa.eu/summaries/CRL-VL-26-04-XP-Corrected-version-1.pdf. According to the experimental data submitted by the applicant, the protocol for DNA extraction produced DNA of suitable quantity and quality for PCR based applications when applied to ground seeds from the oilseed rape event MON 94100.

In agreement with the ENGL position, endorsing the modularity principle (see also Annex III to Regulation (EU) No 503/2013), and given the similarity in the matrix, the EURL GMFF considers the above mentioned DNA extraction protocol applicable in the context of the validation of the method for oilseed rape event MON 94100.

Annex III to Regulation (EU) No 503/2013 [7] requires the applicant to discuss the validity and limitations of the detection methods in the various types of foods and feeds (matrices) expected to be placed on the market. To this regard, the applicant stated that "*the processing of oilseed rape grain involves varying degrees of mechanical, enzymatic, solvent, heat, acid treatment or combinations of these steps* [10-12]*. These steps can influence the quality and intactness of DNA contained in the final processed oilseed rape products* [13-14]*. After extraction from these processed matrices, the DNA may need additional rounds of purification to eliminate PCR inhibitors, and achieve a quality suitable for PCR* [15-16]*. Regardless of the DNA extraction method employed, studies have shown that the processing steps for oilseed rape result in significant degradation of high molecular weight DNA and in failure of PCR amplification for products greater than 100 base pairs* [13-14]*. Random DNA fragmentation may lead to variability in quantitating DNA targets by qPCR* [17]*. This is affecting the ability to accurately quantify the presence of a GM event in processed fractions. Finally, as developers of GM seeds and grains, Bayer is responsible for developing methods to test the produced materials as such, and not food and feed products produced by third parties. It will be a daunting task to validate DNA extraction methods for each different food or feed matrices, and equally challenging for the end user to know what DNA extraction method to apply".*

Whenever DNA is extracted from more complex and difficult matrices, a thorough control of the quality of the DNA is recommended in order to ensure that it has the required quality for subsequent PCR analysis.

4. Materials and method

4.1. Samples

The following positive and negative control samples were provided by the applicant to the EURL GMFF in accordance to Regulation (EC) No 1829/2003 Art 2.11[a]:

- genomic DNA extracted by the applicant from hemizygous oilseed rape seeds harbouring the MON 94100 event, and
- genomic DNA extracted by the applicant from conventional oilseed rape seeds genetically similar to those harbouring the MON 94100 event.

4.2. Method for the PCR analysis

The PCR method provided by the applicant is an event-specific, quantitative, real-time TaqMan® PCR procedure for the determination of the relative content of GM event MON 94100 DNA to total oilseed rape DNA. The procedure is a simplex system, in which an oilseed rape specific assay targeting the endogenous gene acyl-ACP-thioesterase (*FatA(A)*), and the GM target assay for MON 94100 are performed in separate wells. The validated method protocol is published by the EURL GMFF at https://gmo-crl.jrc.ec.europa.eu/method-validations and can be found in Annex 1 to this report.

For the detection of GM event MON 94100, an 89 bp fragment of the region spanning the 5' plant-to-insert junction in oilseed rape MON 94100 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 MGBNFQ (Minor Groove Binder Non-Fluorescent Quencher) as non-fluorescent quencher dye at its 3' end.

For the relative quantification of GM event MON 94100, an oilseed rape taxon-specific system amplifies a 126 bp fragment of an oilseed rape acyl-ACP-thioesterase (*FatA(A)*) endogenous gene, using *FatA(A)* gene-specific primers and a *FatA(A)* gene-specific probe labelled with FAM (6-carboxyfluorescein) as reporter dye at its 5' end and MGBNFQ as non-fluorescent quencher dye at its 3' end.

Standard curves are generated for both the MON 94100 and the *FatA(A)* systems by plotting the Cq (quantification cycle) 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

[a] 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).

used to estimate the copy numbers in the test sample DNA by interpolation from the standard curves.

For the relative quantification of event MON 94100 DNA in a test sample, the MON 94100 copy number is divided by the copy number of the oilseed rape haploid genome and multiplied by 100 to obtain the percentage value (GM % = MON 94100/ oilseed rape haploid genome x 100).

The absolute copy numbers of the calibration curve samples are calculated by dividing the sample DNA mass (nanograms) by the published average 1C value for the oilseed rape genome (1.15 pg) [18]. The copy number values used in the quantification, the GMO contents of the calibration samples, and the total DNA quantity used in the PCR reactions are listed in Table 3.

Note: Numerical values presented in the following tables were rounded keeping two digits for values ≤ 1, one digit for values between 1 and 10 and no digit for values ≥ 10, unless otherwise stated. The calculations in the MS Excel files however were done over not rounded data. This approach might create small inconsistencies in the numerical values reported in the tables but it allows a higher precision in the final results.

Table 3. Copy number values of the standard curve samples

Sample code	S1	S2	S3	S4	S5
Total amount of oilseed rape DNA in the reaction (ng)	200	40	8	1.6	0.32
Target taxon (haploid genome copies)	173913	34783	6957	1391	278
Target MON 94100 copies	17391	3478	696	139	28

4.3. EURL GMFF experimental testing (step 3)

4.3.1. Determination of the zygosity ratio in the positive control sample

The EURL GMFF experimentally verified the zygosity ratio (GM-target to reference target ratio) in the positive control sample to assess the method performance at 0.1% GM level -expressed as mass fraction of GM material- in relation to the provisions of Regulation (EU) No 619/2011 [8].

The copy number of the MON 94100 and of the FatA(A) targets in the positive control sample were determined by digital PCR (dPCR) performed on the BioMark HD System using the 37K digital arrays (Fluidigm).

Reaction mixes were prepared in order to test the zygosity for a total number of eighteen data sets for the GM target and eighteen for the reference target in final volume of 6 µL and contained 1X TaqMan® Universal PCR Master Mix (Applied Biosystems, Cat. Number 4318157), 1X GE sample loading reagent (Fluidigm PN 85000746), primers and probes at concentrations indicated in the

corresponding validated method (400 nM MON 94100 primer 1 and 2, 200nM MON 94100 probe for MON 94100 assay and 300nM FatA(A) primer 1, FatA(A) 900nM primer 2, 150 nM FatA(A) probe for *FatA(A)* assay) and 1.8 µL of genomic DNA from the positive control sample at a concentration of 1.5 ng/µL.

Loading of the digital chip was performed according to the manufacturer's instructions by using the IFC controller (Fluidigm). Approximately 4 µL of the reaction mixes were loaded into each well and distributed into the 770 partitions constituting one panel. 'No template controls' were included. Amplification conditions were as reported in Annex 1 or in the Validated Method document at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx. Data analysis and copy number calculations were performed using the BioMark digital PCR Analysis software. The range of Cq retention was from 15 to 40.

Calculations of means and variances were 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 - Version 2'[19].

4.3.2. 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 blind test samples distributed over a range of GM levels (5 % - 0.05 %, see Table 4). The blind test samples were prepared by the EURL GMFF from the genomic DNA provided by the applicant (see 4.1 for details) by mixing oilseed rape MON 94100 DNA and non-GM oilseed rape DNA.

Table 4. MON 94100 blinded samples GM % contents

MON 94100 GM % (GM copy number/oilseed rape haploid genome copy number x 100)
5.00
2.00
0.90
0.45
0.05

The calibration sample S1 was prepared from the genomic DNA provided by the applicant by mixing the appropriate amount of MON 94100 DNA with control non-GM oilseed rape DNA to obtain a 10 % (in copy number ratio related to haploid genome copies) GM sample. Calibration samples S2-S5 were prepared by 5-fold serial dilutions from the S1 sample (see Table 3).

The experiments were performed on an ABI 7500, a Q7 (QuantStudio 7 Flex System) and a Roche LC480 real-time platform under repeatability conditions and followed the protocol provided by the applicant.

Test samples with GM levels 5 %, 2 %, 0.9 % and 0.45 % 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 % was tested in 15 replicates in an additional run for each platform. 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.

4.4. International collaborative study (step 4)

The international collaborative trial involved twelve randomly selected laboratories, all being "national reference laboratories, assisting the EURL GMFF for testing and validation of methods for detection", as listed in annex to Regulation (EC) No 120/2014 [20] who had expressed their interest in participation. 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) [2]
> ISO 5725 "Accuracy (trueness and precision) of measurement methods and results", Part 1 and Part 2 (ISO, 1994); ISO 5725-1:1994/Cor 1 (ISO 1998) and ISO 5725-2:1994/Cor 1 (ISO, 2002) [3-6]

The objective of the international collaborative study was to verify in experienced laboratories the trueness and precision of the PCR analytical method provided by the applicant and verified in-house by the EURL GMFF.

4.4.1. List of participating laboratories

The twelve laboratories participating in MON 94100 international collaborative study were randomly selected from 30 national reference laboratories (NRL) that offered to participate.

Clear guidance was given to the selected laboratories for strictly following the validation protocol that was provided to them. The participating laboratories are listed in Table 5.

Table 5. Laboratories participating in the validation of the detection method for oilseed rape event MON 94100

Laboratory	Country
Plant Breeding and Acclimatization Institute	PL
Voivodeship Sanitary and Epidemiological Station in Rzeszów	PL
State Veterinary and Food Institute Dolny Kubin	SK
Italian National Institute of Health (ISS)	IT
CREA-DC Sede di Tavazzano	IT
BioGEVES - Groupe d'Etude et de contrôle des Variétés et des Semences	FR
National Health Laboratory	LU
Wageningen Food Safety Research (WFSR)	NL
Walloon Agricultural Research Centre	BE
Center for Agricultural Technology Augustenberg	DE
Laboratory for the Detection of GMO in Food - Bad Langensalza	DE
Helenic Agricultural Organisation	GR

4.4.2. Real-time PCR equipment used in the study

Laboratories involved in the collaborative study used the following real-time PCR equipment: two ABI QuantStudio 5, one QuantStudio 7 Flex, three Bio-Rad CFX-96, three ABI 7500, one ABI 7900, one LC480II and one PCRmax ECO48.

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

4.4.3. Materials used in the international collaborative study

For the validation of the quantitative event-specific method, calibration samples (of known GMO content) and blind test samples (of undisclosed GM content = blind samples) were provided by the EURL GMFF to the participating laboratories (for test samples preparation see 4.3.2).

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

- ✓ Five calibration samples with known concentrations of GM-event (150 µL of DNA solution each) labelled from S1VL0420 to S5VL0420 (Table 3).

- ✓ Twenty blinded test DNA samples (75 µL of DNA solution, each at 30 ng/µL) labelled from U1VL0420 to U20VL0420, representing five GM levels, each in four replicates (Table 4)

- ✓ Reaction reagents:
  - TaqMan® Universal PCR Master Mix (2x), one vial: 8 mL
  - distilled sterile water, one vial: 4 mL

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

FatA(A) taxon-specific assay
- Fat(A) primer 1 (10 µM): 240 µL
- Fat(A) primer 2 (10 µM): 720 µL
- Fat(A) probe (10 µM): 120 µL

MON 94100 assay
- MON 94100 primer 1 (10 µM): 320 µL
- MON 94100 primer 2 (10 µM): 320 µL
- MON 94100 probe (10 µM): 160 µL

4.4.4. Design of the collaborative study

Participating laboratories received a detailed validation protocol that included the exact design of the PCR plates, ensuring that on each PCR plate the samples were analysed for the MON 94100 event-specific system and for the *FatA(A)* taxon-specific system. In total, two plates were run by each participating laboratory.

The laboratories prepared the PCR master-mixes for the oilseed rape event MON 94100 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 layout.

The amplification reaction followed the cycling program specified in the protocol. Participants determined the GM % in the test samples according to the instructions and also reported the raw data to the EURL GMFF on an Excel sheet that was designed, validated and distributed by the EURL GMFF. All data are stored by the EURL GMFF on a dedicated and protected server.

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

4.4.5. Deviations reported from the protocol

None of the participating laboratories reported deviations from the validation protocol.

5. Results

5.1. EURL GMFF experimental testing

5.1.1. Zygosity ratio in the positive control sample

The results of the digital PCR analysis conducted by the EURL GMFF on the MON 94100 and *FatA(A)* targets to determine the zygosity ratio in the positive control samples are shown in Table 6.

Table 6. Zygosity ratio of the MON 94100 and *FatA(A)* targets in the positive control sample.

Mean ratio (MON 94100 / *FatA(A)*)	0.51
Standard deviation	0.06
RSD_r (%)	11
Standard error of the mean	0.01
Upper 95 % CI of the mean	0.53
Lower 95 % CI of the mean	0.48

The mean ratio (MON 94100/*FatA(A)*) was 0.51. The 95 % confidence interval (CI) spans around 0.50, the expected ratio for a oilseed rape control sample, hemizygous for the GM-locus, and assuming a single - copy endogenous gene target. Therefore, the measured mean ratio is not significantly different from the expected ratio, for an alpha = 0.05. Hence, 0.05 % GM in haploid genome copy numbers = 0.1 % in mass fraction of GM DNA

Note: the zygosity ratio herein reported is valid for the positive control sample DNA in the context of the present validation study. It is used to assess the method performance at 0.1% GM level - expressed as mass fraction of GM material- in relation to the provisions of Regulation (EU) No 619/2011.

When analytical results of official laboratories are primarily expressed as ratio of GM- DNA copy numbers, they shall be translated into mass fraction results by means of the specific conversion factor published in the document "Conversion factors (CF) for certified references materials (CRM)" (https://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm).

5.1.2. In-house verification of method performance against ENGL method acceptance criteria

Test samples with GM levels from 5 % to 0.45 % (GM copies/copies haploid genomes) 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 sample at 0.05 % GM-level (copies/copies haploid genomes, equal to 0.1% in mass fraction) was tested for its precision in quantification in 15 replicates in separate runs.

Tests were conducted on ABI 7500, a Q7 (QuantStudio 7 Flex System) and a Roche LC480 for robustness.

The standard curve parameters and the results of efficiency, linearity, trueness and precision obtained in the three real-time PCR runs with the test samples are shown in Tables 7A, 7B, 8, 9 and 10.

According to the ENGL method acceptance criteria, the average value of the slope of the standard curve shall range from -3.1 to -3.6 and the R^2 coefficient shall be $\geq$ 0.98. Table 7A and 7B document that the slopes of the standard curves and the R^2 coefficients were within the limits established by the ENGL. The EURL GMFF in-house results confirm the data provided by the applicant.

Table 7A. Standard curve parameters of the real-time PCR tests, carried out on ABI 7500, Q7 and Roche LC480 to quantify GM-levels in the range 5 % to 0.45 % in four replicates each. Slope and R^2 coefficient values were rounded to two digits.

	MON 94100			***FatA(A)***		
	Slope	PCR efficiency*	R^2	Slope	PCR efficiency*	R^2
Run A	-3.4	96	1.0	-3.5	94	1.0
Run B	-3.4	97	1.0	-3.4	95	1.0
Run C	-3.4	96	1.0	-3.4	98	1.0
Run D	-3.4	96	1.0	-3.4	96	1.0
Run E	-3.3	99	1.0	-3.4	97	1.0
Run F	-3.3	101	1.0	-3.4	98	1.0

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

Runs A-B were carried out on ABI 7500; runs C-D were carried out on Q7; runs E and F were carried out on Roche LC480.

Table 7B. Standard curve parameters of the real-time PCR tests, carried out on ABI 7500, Q7 and Roche LC480 to quantify the GM-level 0.05 % (copy number, equal to 0.1% in mass fraction) in 15 replicates. Slope and R^2 coefficient values were rounded to two digits.

	MON 94100			***FatA(A)***		
	Slope	PCR efficiency*	R^2	Slope	PCR efficiency*	R^2
Run G	-3.4	98	1.0	-3.4	95	1.0
Run H	-3.2	104	1.0	-3.4	96	1.0
Run I	-3.4	98	1.0	-3.4	96	1.0

* PCR efficiency (%) is calculated using the formula Efficiency = $(10^{(-1/slope)} - 1) \times 100$
Run G was carried out on ABI 7500; run H was carried out on Q7; run I was carried out on Roche LC480.

According to the ENGL method acceptance criteria the method trueness (measured as bias in % of the target GM level) should be within ± 25 % of the accepted reference value over the entire dynamic range and the precision, expressed as RSD_r % (relative standard deviation of repeatability), should be ≤ 25 %, also over the entire dynamic range. Tables 8, 9 and 10 show that trueness and precision of quantification were within the limits established by the ENGL for the PCR machines used.

Table 8. Values of trueness and precision as established by the EURL GMFF in its in-house verification using an ABI 7500. GM % in GM copies/copies haploid genomes.

Target GM-levels %	**Measured GM-level %**	**Bias % of the target GM-level**	**Precision (RSD_r %)**
5.0	4.8	-3.3	1.9
2.0	1.9	-3.8	5.1
0.90	0.81	-9.5	3.6
0.45	0.40	-11	4.7
0.05	0.04	-15	17

Table 9. Values of trueness and precision as established by the EURL GMFF in its in-house verification using a Q7. GM % in GM copies/copies haploid genomes.

Target GM-levels %	**Measured GM-level %**	**Bias % of the target GM-level**	**Precision (RSD_r %)**
5.0	5.1	2.5	1.6
2.0	2.0	1.1	0.29
0.90	0.90	-0.42	3.2
0.45	0.44	-1.5	2.2
0.05	0.04	-21	13

Table 10. Values of trueness and precision as established by the EURL GMFF in its in-house verification using a Roche LC480. GM copies/copies haploid genomes.

Target GM-levels %	Measured GM-level %	Bias % of the target GM-level	Precision (RSD_r %)
5.00	5.0	0.50	1.1
2.00	1.9	-2.7	4.6
0.90	0.84	-6.8	3.6
0.45	0.40	-11	6.8
0.05	0.04	-13	12

5.2. Results of the international collaborative study

5.2.1. 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 11. The PCR efficiency (%) was calculated from the standard curve slopes using the formula:

$$\text{Efficiency (\%)} = \left(10^{\frac{-1}{slope}} - 1\right) \times 100$$

Table 11 indicates that the efficiency of amplification for the MON 94100 system ranges from 95 % to 105 % and the linearity is 1.0; the amplification efficiency for the oilseed rape-specific system ranges from 94 % to 109 % and the linearity is equal to 1.0. The mean PCR efficiency was 99 % for the MON 94100 assay and 98 % for the *FatA(A)* one. The mean R^2 of the methods was 1.0 for both the MON 94100 and *FatA(A)* assays. Both PCR efficiency and linearity values were within the ENGL acceptance criteria.

Table 11. Values of slope, PCR efficiency and R^2 obtained during the international collaborative trial. Slope and R^2 coefficient values were rounded to two digits.

		MON 94100			*FatA(A)*		
Lab	**Plate**	**Slope**	**PCR Efficiency (%)**	**R^2**	**Slope**	**PCR Efficiency (%)**	**R^2**
1	A	-3.4	98	1.0	-3.4	96	1.0
	B	-3.3	99	1.0	-3.4	95	1.0
2	A	-3.4	98	1.0	-3.4	98	1.0
	B	-3.4	96	1.0	-3.4	98	1.0
3	A	-3.4	97	1.0	-3.2	106	1.0
	B	-3.2	105	1.0	-3.1	109	1.0
4	A	-3.4	97	1.0	-3.4	96	1.0
	B	-3.4	98	1.0	-3.4	96	1.0
5	A	-3.4	98	1.0	-3.3	102	1.0
	B	-3.4	99	1.0	-3.3	100	1.0
6	A	-3.3	100	1.0	-3.4	98	1.0
	B	-3.4	95	1.0	-3.4	96	1.0
7	A	-3.4	98	1.0	-3.3	99	1.0
	B	-3.2	104	1.0	-3.3	99	1.0
8	A	-3.3	99	1.0	-3.4	97	1.0
	B	-3.4	98	1.0	-3.3	99	1.0
9	A	-3.3	102	1.0	-3.3	98	1.0
	B	-3.2	103	1.0	-3.3	100	1.0
10	A	-3.5	95	1.0	-3.5	94	1.0
	B	-3.4	96	1.0	-3.3	100	1.0
11	A	-3.4	96	1.0	-3.4	97	1.0
	B	-3.4	98	1.0	-3.5	94	1.0
12	A	-3.3	101	1.0	-3.4	95	1.0
	B	-3.3	101	1.0	-3.4	96	1.0
	Mean	-3.3	99	1.0	-3.4	98	1.0

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

5.2.2. GMO quantification

Table 12 reports the values of quantification for the four replicates of each GM level as reported by each of the twelve participating laboratories.

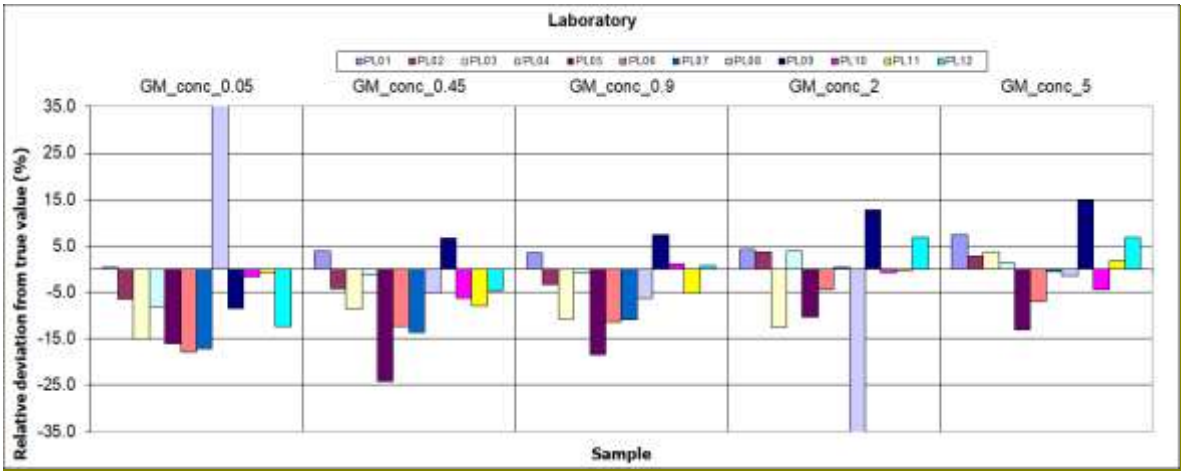
Table 12. GM % values determined by laboratories for test samples

	GMO content (%) *																			
LAB	0.05				0.45				0.90				2				5			
	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	0.05	0.05	0.05	0.05	0.47	0.48	0.47	0.45	0.94	0.94	0.93	0.91	2.1	2.1	2.0	2.1	5.6	5.2	5.2	5.4
2	0.06	0.05	0.04	0.04	0.45	0.41	0.47	0.39	0.86	0.90	0.85	0.87	2.0	2.1	1.9	2.2	4.9	5.3	5.5	4.8
3	0.03	0.02	0.07	0.05	0.38	0.38	0.44	0.44	0.73	0.72	0.91	0.85	2.0	1.5	1.6	1.9	4.3	4.1	6.7	5.5
4	0.04	0.04	0.06	0.05	0.42	0.47	0.47	0.42	0.87	0.93	0.82	0.94	2.2	2.0	2.1	2.0	5.0	5.3	5.1	4.8
5	0.04	0.04	0.04	0.03	0.32	0.31	0.36	0.37	0.63	0.77	0.78	0.76	1.50	1.7	1.9	2.1	4.0	4.1	4.6	4.7
6	0.04	0.04	0.04	0.04	0.40	0.42	0.41	0.35	0.79	0.83	0.83	0.73	1.74	2.0	1.9	2.0	4.5	4.2	5.3	4.7
7	0.04	0.04	0.05	0.04	0.36	0.40	0.41	0.38	0.82	0.79	0.83	0.77	1.88	2.0	2.0	2.1	5.2	4.8	4.8	5.1
8	0.04	0.04	2.0	0.04	0.38	0.39	0.50	0.44	0.77	0.79	0.89	0.92	2.2	2.0	0.00	0.06	3.3	5.0	5.3	6.0
9	0.04	0.04	0.04	0.06	0.49	0.51	0.46	0.47	0.87	0.91	0.98	1.1	2.5	2.1	2.1	2.3	5.7	5.9	5.5	5.8
10	0.04	0.05	0.06	0.05	0.39	0.39	0.45	0.45	0.95	0.81	0.96	0.91	2.0	1.9	1.8	2.2	4.8	4.3	4.9	5.1
11	0.05	0.04	0.05	0.05	0.42	0.41	0.42	0.40	0.86	0.86	0.82	0.87	2.0	2.0	2.1	1.9	5.1	5.3	5.0	4.9
12	0.04	0.04	0.04	0.05	0.42	0.42	0.45	0.44	0.94	0.89	0.91	0.89	2.1	2.1	2.2	2.1	5.3	5.1	5.5	5.5

* GMO % = (GMO copy number/oilseed rape haploid genome copy number) x 100

A graphical representation of the data reported in Table 12 is provided in Figure 1 that shows the relative deviation from the true value for each GM level tested for the participating laboratory. The coloured bars represent the deviation of the GM level measured in % of the true GM level.

Figure 1. Relative deviation (%) from the true value of GM level



PL = participating laboratory.

Overall, almost all laboratories' mean relative deviations from the true values were within a maximum of ± 25 %. A few outliers were noticeable; one laboratory (PL08) overestimated the GM-level 0.05 % by more than 900 %; the same laboratory also underestimated the GM-level 2 % by 46 %. These results suggest this laboratory might have mixed-up the two samples.

5.2.3. Method performance requirements

Among the performance requirements established by ENGL and adopted by the EURL GMFF [9] repeatability and reproducibility are to be assessed through an international collaborative trial. Table 13 illustrates the estimation of repeatability and reproducibility at the various GM levels tested during the study (see Table 5 for a list of the participant laboratories).

According to the ENGL method performance requirements the relative reproducibility standard deviation (RSD_R), that describes the inter-laboratory variation, should be below 35 % at the target concentration and 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 13, the method satisfies this requirement at all GM levels tested. Indeed, the highest value of RSD_R % is 14.6 % at the 0.05 % GM level, thus within the acceptance criterion.

Table 13. Summary of validation results for the MON 94100 method, expressed as GM copy numbers in relation to target taxon haploid genome copy numbers.

	Test Sample Expected GMO %				
	0.05	**0.45**	**0.90**	**2.0**	**5.0**
Laboratories having returned valid results	12	12	12	12	12
Samples per laboratory	4	4	4	4	4
Number of outliers	2	0	0	1	2
Reason for exclusion	C*	-	-	C*	C*
Mean value	**0.05**	**0.42**	**0.85**	**2.0**	**5.0**
Relative repeatability standard deviation, RSD_r (%)	14	7.0	7.0	7.4	5.6
Repeatability standard deviation	0.01	0.03	0.06	0.15	0.28
Relative reproducibility standard deviation, RSD_R (%)	14	11	9.9	9.7	9.2
Reproducibility standard deviation	0.01	0.04	0.08	0.19	0.46
Bias** (absolute value)	-0.00	-0.03	-0.04	0.01	0.05
Bias (%)	-8.8	-6.5	-4.5	0.3	1.0

* C= Cochran's 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.

Table 13 also documents the relative repeatability standard deviation (RSD_r) estimated for each GM level. In order to accept methods for collaborative study, the EURL GMFF and ENGL require that the RSD_r value indicated by the applicant and confirmed by the EURL GMFF through in-house experiments is below 25 % (see ENGL document "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" [9]). 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 14 % at the 0.05 % 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. The method satisfies this requirement across the dynamic range tested, with the highest value of bias (%) of -8.8 % at the 0.05 % GM level.

6. Compliance of the method for detection and quantification of event MON 94100 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 steps were carried out and their outcome is summarised in Table 14:

- at step 2 of the validation process (scientific assessment of the dossier), the EURL GMFF acknowledged that the RSD_r value at the 0.1 % level (in mass fraction of GM-material) shown by the applicant's dossier was 15 %, based on 15 replicates (Table 2A). In the transferability study, the RSD_r at the 0.17 % level (in mass fraction of GM-material; or 0.085 % in terms of copy number ratio to haploid genome copy numbers) reached 17 %, based on 15 replicates (Table 2B). Both values were hence below the maximum value of 25 % required by the ENGL. The EURL GMFF therefore concluded that it could accept the applicant's data on method performance;

- at step 3 of the validation process (experimental testing of samples and methods), the EURL GMFF determined the RSD_r % value at the level of 0.1 % in mass fraction of GM-material (corresponding to 0.05 % expressed in terms of GM copy number ratio to haploid genome copy numbers). The experiments were carried out under repeatability conditions on fifteen replicates. The RSD_r resulted to range between 12 % and 17 % (Table 8, 9 and 10) depending on the qPCR platform applied, hence also below 25 %;

- the collaborative study (step 4 of the validation process) established that over the twelve participating laboratories at the level of 0.1 % related to mass fraction of GM-material, the RSD_r of the method was 14 %, therefore also below 25 % and well in line with the previous data.

Table 14. Precision of the event-specific method for quantitative detection of MON 94100 at or around 0.1 % level related to mass fractions of GM material.

Source	**GM %**	**RSD_r %**
Applicant's method optimisation	0.1 %	15 %
Applicant's transferability study	0.17 %	17 %
EURL GMFF tests	0.1 %	12 - 17 %
Collaborative study	0.1 %	14 %

Based on the results of the EURL GMFF in-house verification and of the international collaborative study, it is concluded that the method RSD_r % is lower 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.

7. Conclusion

The method provided by the applicant has been validated in accordance to the EURL GMFF validation process, 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 4.1), in accordance with the requirements of Annex I-3.C.2 to Commission Regulation (EU) No 503/2013 and (EU) No 619/2011 and meets all method performance requirements established by the ENGL and the EURL GMFF. The method is therefore valid to be used for regulatory purposes, including the quantification of low-level presence of 0.1 % (m/m) of the GM event. It can be assumed that it is applicable to any appropriately extracted oilseed rape genomic DNA.

In any case, the user of the method is advised to verify the quality of the extracted genomic DNA in order to ensure that it is suitable for the subsequent PCR analysis. This is particularly relevant for more complex matrices of samples from food and feed products.

The validated method is described in detail as "Validated Method" at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx and in Annex 1.

8. References

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Annex 1: Event-specific Method for the Quantification of Oilseed rape MON 94100 by Real-time PCR

Validated Method

Method development:

Bayer Agriculture BV

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 MON 94100 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 assays. Tests for the presence of PCR inhibitors (e.g. monitor run of diluted series, use of DNA spikes) are also recommended to ensure the suitability of the extracted DNA.

For the detection of GM event MON 94100, an 89 bp fragment of the region spanning the 5' plant-to-insert junction in oilseed rape MON 94100 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 MGBNFQ (Minor Groove Binder Non-Fluorescent Quencher) as non-fluorescent quencher dye at its 3' end.

For the relative quantification of GM event MON 94100, a *Brassica napus* taxon-specific system amplifies a 126 bp fragment (can reach 129 bp in a minority of *Brassica napus* varieties and in some *Brassica rapa* ones) of an acyl-ACP-thioesterase (*FatA(A)*) endogenous gene (Accession number, GeneBank: X87842.1), using *FatA(A)* gene-specific primers and a *FatA(A)* gene-specific probe labelled with FAM (6-carboxyfluorescein) as reporter dye at its 5' end and MGBNFQ as non-fluorescent quencher dye at its 3' end.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Cq" value. For quantification of the amount of MON 94100 DNA in a test sample, Cq values for the MON 94100 and the *FatA(A)* systems are determined for the sample. Standard curves are then used to estimate the relative amount of MON 94100 DNA to total oilseed rape DNA.

2. Validation and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from genetically modified and conventional oilseed rape seeds and grains. 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 (EURL GMFF). The study was undertaken with twelve participating laboratories in November - December 2021.

A detailed validation report can be found at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx.

2.3 Limit of detection (LOD)

According to the method developer, the absolute LOD of the method is at least 5 haploid genome copies for MON 94100 event-specific method in 200 ng of total suitable 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 % (expressed as copies GM/total haploid genome copies) in 200 ng of total suitable 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 MON 94100 and is therefore event-specific for the event MON 94100. This was confirmed in the validation study.

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 should have been 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 regularly
- 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 room temperature.
- In order to avoid repeated freeze/thaw cycles, aliquots should be prepared.

3.2 Real-time PCR for quantitative analysis of oilseed rape event MON 94100

3.2.1 General

The real-time PCR set-up for the taxon (*FatA(A)*) and the GMO (event MON 94100) target sequences are carried out in separate vials. Multiplex qPCR (using differential fluorescent labels for the probes) has not been tested or validated by the EURL GMFF.

The method is developed for a total volume of 25 µL per reaction mixture for the GM (event MON 94100) and the taxon (*FatA(A)*) assay with the reagents as listed in Table 2 and Table 3.

3.2.2 Calibration

The calibration curves have to be established on at least five samples. The first point of the calibration curve (S1) should be established for a sample containing 10 % oilseed rape MON 94100 DNA in a total of 200 ng of oilseed rape DNA (corresponding to 173913 oilseed rape haploid genome copies with one haploid genome assumed to correspond to 1.15 pg of oilseed rape genomic DNA) [(1)]. Standards S2 to S5 are to be prepared by serial dilutions (dilution factor 5) according to Table 1 below.

Table 1. Copy number values of the standard curve samples

Sample code	S1	S2	S3	S4	S5
Total amount of oilseed rape DNA in reaction (ng)*	200	40	8	1.6	0.32
oilseed rape haploid genome copies	173913	34783	6957	1391	278
MON 94100 copies	17391	3478	696	139	28

* Total nanograms are rounded to the integral value

A calibration curve is produced by plotting the Cq values against the logarithm of the target copy number for the calibration points. This can be done by means of spreadsheet software, e.g. Microsoft Excel, or directly by options available with the software.

The copy number measured for each unknown sample DNA is obtained by interpolation from the standard curves.

3.2.3 Real-time PCR set-up

1. Thaw, mix and centrifuge the components needed for the run. Keep thawed reagents on ice.
2. In two tubes on ice, add the components in the order mentioned below (except DNA) to prepare the reaction mixes for the MON 94100 oilseed rape specific system (Table 2) and the *FatA(A)* reference gene system (Table 3). Please note that additional volume is included in the total to cover pipetting variability due to the viscosity of the solution.

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the MON 94100 assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
MON 94100 primer 1 (10 µM)	400 nM	1
MON 94100 primer 2 (10 µM)	400 nM	1
MON 94100 probe* (10 µM)	200 nM	0.5
Nuclease free water	-	6
DNA	≤200 ng	4
Total reaction volume:		25 µL

*probe labelled with 6-FAM at its 5'-end and MGBNFQ at its 3'-end

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)	1x	12.5
FatA(A) primer 1 (10 µM)	300 nM	0.75
FatA(A) primer 2 (10 µM)	900 nM	2.25
FatA(A) probe* (10 µM)	150 nM	0.38
Nuclease free water	-	5.12
DNA	≤200 ng	4
Total reaction volume:		25 µL

*probe labelled with 6-FAM at its 5'-end and MGBNFQ at its 3'-end

3. Mix well and centrifuge briefly.
4. Prepare two 0.5 mL reaction tubes (one for the oilseed rape MON 94100 and one for the *FatA(A)* system) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
5. Add into each reaction tube the amount of reaction mix for 3.5 PCR repetitions (73.5 µL for both the MON 94100 oilseed rape and the *FatA(A)* systems). Add to each tube the correct

amount of DNA for 3.5 PCR repetitions (14 µL DNA). The volume for the additional 0.5 repetition will ensure adequate volume when loading the samples. Vortex each tube for approx. 10 seconds. This step is mandatory to reduce to a minimum the variability among the repetitions of each sample.

6. Spin down the tubes. Aliquot 25 µL for the MON 94100 system and for the *FatA(A)* reference system in each well.
7. Place an optical cover on the reaction plate and briefly centrifuge the plate.
8. Place the reaction plate in the real-time PCR apparatus (possibly apply a compression pad, depending on the model), according to the manufacturer's instructions and your Standard Operating Procedures and start the run.
9. For both the MON 94100 and the *FatA(A)* systems, select FAM as reporter dye and MGBNFQ (or non-fluorescent) as quencher dye. Select ROX (6-carboxy-X-rhodamine) as the passive reference dye. Enter the correct reaction volume (25 µL).
10. Run the PCR with the cycling program described in Table 4.

Table 4. Cycling program for MON 94100 / *FatA(A)* assays.

Step	Stage		T (°C)	Time (s)	Acquisition	Cycles
1	UNG*		50	120	No	1
2	Initial denaturation		95	600	No	1
3	Amplification	Denaturation	95	15	No	45
		Annealing & Extension	60	60	Yes	

*UNG: Uracil-N-glycosylase

3.3 Data analysis

After the real-time PCR, analyse the run following the procedure below:

a) Set the threshold following the automatic or the manual mode. In the manual mode, display the amplification curves of the event specific assay in logarithmic mode. Locate the threshold line in the area where the amplification profiles are parallel (exponential phase of PCR) and where there is no "fork effect" between repetitions of the same sample. Press the "update" button to ensure changes affect Cq values (only needed for some analysis software). Switch to the linear view mode by clicking on the Y axis of the amplification plot and check that the threshold previously set falls within the exponential phase of the curves.

b) Set the baseline following the automatic or the manual mode. In the manual mode: determine the cycle number at which the threshold line crosses the first amplification curve and set the baseline three cycles before that value (e.g. earliest Cq = 25, set the baseline crossing at Cq = 25 – 3 = 22).

c) Save the settings.

d) Repeat the procedure described in a), b) and c) on the amplification plots of the taxon specific system.

e) Save the settings and export all the data for further calculations.

3.4 Calculation of results

After having defined a threshold value within the logarithmic phase of amplification as described above, the instrument's software calculates the Cq values for each reaction.

The standard curves are generated both for the *FatA(A)* and the MON 94100 specific assays by plotting the Cq 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 samples.

To obtain the percentage value of event MON 94100 DNA in the unknown sample, the MON 94100 copy number is divided by the copy number of the oilseed rape endogenous gene *FatA(A)* and multiplied by 100 (GM% = MON 94100 / *FatA(A)* x 100).

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) and appropriate analysis software
- 96-well reaction plates
- Optical caps/adhesion covers

- Microcentrifuge
- Micropipettes
- Standard bench top centrifuge with rotor or standard microfuge fit for 0.5 mL reaction tubes, centrifuge for 96-Well reaction plates
- Vortex
- Racks for reaction tubes, also cooled
- 0.5, 1.5 mL and 5 or 15 mL DNAse free reaction tubes

4.2 Reagents

- Nuclease-free water Sigma Cat. No. W-4502
- TaqMan® universal PCR master mix (2X), ABI Part No. 4304437

4.3 Primers and Probes

Table 5. Primers and probes for the MON 94100 and *FatA(A)* methods

	Name	DNA Sequence (5' to 3')	Length (nt)
MON 94100			
Forward primer	MON 94100 primer 1	CAC CAT CTA ATG AAT AGT CAC CAA AAT AAC G	31
Reverse primer	MON 94100 primer 2	CTA TTC GGG CCT AAC TTT TGG TGT G	25
Probe	MON 94100 probe	6-FAM-TGA TGC TGA CTG GTG TCA A-MGBNFQ	19
FatA(A)			
Forward primer	FatA(A) primer 1	ACA GAT GAA GTT CGG GAC GAG TAC	24
Reverse primer	FatA(A) primer 2	CAG GTT GAG ATC CAC ATG CTT AAA TAT	27
Probe	FatA(A) probe	6-FAM-AAG AAG AAT CAT CAT GCT TC-MGBNFQ	20

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

5. References

1. CEN/TS 17329-1:2021 (Foodstuffs - General guidelines for the validation of qualitative real-time PCR methods - Part 1: Single-laboratory validation)

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