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Event-specific Methods for the Quantification of Oilseed Rape LBFLFK Locus1 and Locus2 Using Real-time PCR

Validation Report

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Event-specific Methods for the Quantification of Oilseed Rape LBFLFK Locus1 and Locus2 Using Real-time PCR

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24 August 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 two event-specific real-time polymerase chain reaction (qPCR) methods for detecting and quantifying oilseed rape event LBFLFK (unique identifier BPS-BFLFK-2) Locus1 and Locus2. The two loci are not in genetic linkage. 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 [1], BASF Plant Science Company GmbH provided the detection methods and the positive and negative control samples (genomic DNA from seed of LBFLFK oilseed rape as positive control DNA, and genomic DNA from seed 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), organised an international collaborative study and analysed the results.

The EURL GMFF in-house verification and the collaborative study confirmed that the methods meet 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 [2], and fulfil the analytical requirements of Regulation (EU) No 619/2011 [3]. 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 two missing triplets (page 46).

Content

Quality assurance

The EURL GMFF is ISO 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], BASF Plant Science Company GmbH provided the EURL GMFF with two event-specific methods for detection and quantification of the two inserts, respectively Locus1 and Locus2 in oilseed rape event LBFLFK (unique identifier BPS-BFLFK-2), together with genomic DNA as positive and negative control samples. The two loci are not in genetic linkage and follow independent Mendelian inheritance.

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 [4], 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 methods could be submitted to the collaborative study (step 4). This study confirmed that the methods are suited for the quantification of genomic DNA of oilseed rape LBFLFK, respectively at Locus1 and Locus2, 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)

BASF Plant Science Company GmbH submitted the identification and quantification methods, data demonstrating their adequate performance when applied to genomic DNA extracted from oilseed rape LBFLFK 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 two event-specific methods was documented 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 LBFLFK Locus1-specific and the LBFLFK Locus2-specific methods was assessed by the applicant in triplicate real-time PCR reactions, according to the methods described in Annex 1 (Tables 1 to 7), using approximately 2500 copies per reaction of non-target DNA and at least 250 copies per reaction of target DNA, in accordance with the ENGL requirements.
The LBFLFK Locus1 and Locus2 methods were tested against DNA extracted from: oilseed rape LBFLFK, LBFDHG (sister event), 73496, Ms1, Rf1, Rf2, Topas 19/2, T45, Ms8, Rf3, GT73, MON88302; rice LLRice62; soybean 40-3-2, 356043, 305423, DAS-68416-4, DAS-44406-6, DAS-81419-2, FG72, A2704-12, A5547-127, MON 87701, MON 87769, MON 89788, CV-127-9, MON 87708, MON 87705; cotton 281-24-236 x 3006-210-23, GHB119, T304-40, GHB614, LLCotton25, MON1445, MON 531, MON 15985-7, MON 88913, MON 88701, DAS-81910-7; maize 3272, MIR604, 59122, 98140, DAS-40278-9, T25, MON 88017, MON 87460, MON 89034, MIR162, 5307, Bt176, Bt11, MON 810, GA21, NK603, MON 863, TC1507, MON 87427, VCO-1981-5; sugar beet H7-1; potato EH-92-527-1, AM04-1020, AV43-6-G7, PH05-026-0048 and conventional cotton, maize, oilseed rape, *Brassica napus* var. Kumily (parental line to LBFLFK), rice, potato, sugar beet, soybean. According to the method developer, the LBFLFK Locus1 and Locus2 methods did not react with any sample except the positive control.

In addition, the applicant performed an *in silico* specificity analysis by using the amplicon sequence as a query for BLAST® algorithm search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) against the nucleotide collection and GenBank® patent sequences with the National Center for Biotechnology Information (NCBI). The method developer indicated that no 100% match with LBFLFK Locus1 and Locus2 amplicons was found other than patent sequences including the LBFLFK inserts.

A validated PCR method, which amplifies a fragment of the A-genome copy of acyl-ACP-thioesterase (*FatA(A)*) gene of *Brassica*, was used as a reference method.

3.2. Specificity *in silico* assessment conducted by the EURL GMFF

The LBFLFK Locus1 method spans the 3' insert1-to-plant junction in oilseed rape LBFLFK. The forward primer "leftFST_ev1_loc1-f4" aligns to the insert 1. The reverse primer "leftFST_ev1_loc1-r5" annealing site is placed in the oilseed rape (*Brassica napus*) genomic border adjacent to insertion 1. The probe annealing site is located in the insert.

The amplicon size is expected to be 128 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 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), and no potential amplicon was identified.

The LBFLFK Locus2 method spans the 5' insert-to-plant junction in oilseed rape LBFLFK. The forward primer "rightFST_ev1_loc2-f4" aligns to the oilseed rape (*Brassica napus*) genomic border adjacent to the insertion 2. The reverse primer "rightFST_ev1_loc2-r1" annealing site was found in the insert 2. The probe annealing site is located in the insert.

The amplicon size is expected to be 123 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 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), and no potential amplicon was identified.

The ePCR predicted the capability of the primer "rightFST_ev1_loc2-r1" to prime amplification on insert 3 of Florigene carnation Moonvista, potentially generating an amplicon of 170 bp. The LBFLFK Locus2 probe was predicted to match with the putative amplicon. Experimental testing conducted at EURL GMFF showed absence of amplification of the LBFLFK Locus2 method on Moonvista DNA, under the experimental conditions described in the LBFLFK protocol.

3.3. Assessment of the ENGL acceptance parameters

The applicant prepared the calibration curves for the LBFLFK Locus1 and Locus2 methods from a DNA solution (S1) of 10 % event LBFLFK in non-GM oilseed rape Kumily genome (expressed as mass fractions of GM DNA equivalent to copy number ratio). Sample S1 was serially diluted (1:5) to obtain samples S2 and S3. Sample S4 was obtained by 1:10 dilution of sample S3. The parameters (slope, R2 coefficient) of eight runs of the calibration curve are reported as provided by the applicant (Table 1A and 1B).

The applicant made use of a Bio-Rad CFX96 Touch™ Real-Time PCR Detection System to carry out the optimisation and in-house validation of the methods for identification and quantification of the LBFLFK Locus1 and Locus2.

Table 1A. Summary of the slope and R^2 values obtained by the applicant with the LBFLFK Locus1 method

	LBFLFK Locus1		***FatA(A)***	
Run	**Slope**	**R^2**	**Slope**	**R^2**
1	-3.23	1.00	-3.22	1.00
2	-3.22	1.00	-3.28	1.00
3	-3.25	1.00	-3.29	1.00
4	-3.25	1.00	-3.29	1.00
5	-3.30	1.00	-3.29	1.00
6	-3.32	1.00	-3.25	1.00
7	-3.22	1.00	-3.26	1.00
8	-3.27	1.00	-3.28	1.00
Mean	**-3.26**	**1.00**	**-3.27**	**1.00**

Table 1B. Summary of the slope and R^2 values obtained by the applicant with the LBFLFK Locus2 method

	LBFLFK Locus2		***FatA(A)***	
Run	**Slope**	**R^2**	**Slope**	**R^2**
1	-3.31	1.00	-3.27	1.00
2	-3.20	1.00	-3.24	1.00
3	-3.29	1.00	-3.23	1.00
4	-3.30	1.00	-3.29	1.00
5	-3.24	1.00	-3.30	1.00
6	-3.29	1.00	-3.25	1.00
7	-3.33	1.00	-3.24	1.00
8	-3.40	1.00	-3.24	1.00
Mean	**-3.30**	**1.00**	**-3.27**	**1.00**

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.

Tables 1A and 1B indicate that the slope and R^2 coefficient of the standard curves for the GM-systems of LBFLFK Locus1 and Locus2 and the taxon-specific (*FatA(A)* system, as established by the applicant, were within the ENGL acceptance criteria.

Precision and trueness of the LBFLFK Locus1 and Locus2 methods were established by the applicant and 16 values for each of five GM levels (expressed as copy number ratio) were provided for each method. Tables 2A and 2B report precision and trueness values respectively of the LBFLFK Locus1 and Locus2 methods as provided by the applicant for the five GM-levels, each analysed in 2 replicates in 8 runs. Both parameters were within the ENGL acceptance criteria (trueness ± 25 %, $RSD_r \leq 25$ % across the entire dynamic range).

Table 2A. Mean %, precision and trueness values provided by the applicant estimated for single measurements with the LBFLFK Locus1 method

	Test results				
Expected GM %	**0.058 %**	**0.10 %**	**0.90 %**	**2.00 %**	**5.00 %**
Measured mean GM %	0.052	0.097	0.881	1.99	5.16
Precision (RSD_r %)	8.7	7.3	9.2	5.5	5.2
Trueness (bias %)	-10.3	-3.0	-2.1	-0.5	3.2

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

Table 2B. Mean %, precision and trueness values provided by the applicant estimated for single measurements with the LBFLFK Locus2 method

	Test results				
Expected GM %	**0.058 %**	**0.10 %**	**0.90 %**	**2.00 %**	**5.00 %**
Measured mean GM %	0.056	0.099	0.888	2.06	5.11
Precision (RSD_r %)	14.5	12.8	8.1	9.8	7.7
Trueness (bias %)	-3.4	-1.0	-1.3	3.0	2.2

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

The methods met the ENGL acceptance criteria for trueness and precision at the lowest GM level i.e. 0.058 %, which contains 50 copies of LBFLFK Locus1 and Locus2 in 100 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 relative limit of detection (LOD_{rel}) of the LBFLFK Locus1 and Locus2 methods were assessed by the applicant in 60 PCR replicates at 0.025 % and 0.010 %, containing respectively 22 and 9 haploid genome copies per reaction of LBFLFK event. The relative LOD (LOD_{rel}) of the LBFLFK Locus1 and Locus2 methods were at least 0.010 % (related to copies GM/total haploid genome copies) in 100 ng of total oilseed rape DNA per reaction. The LOD_{abs} is reported to be at least 9 haploid genome copies for both the LBFLFK Locus 1 and Locus 2 methods.

The LBFLFK Locus1 and Locus2 methods were also in-house validated by the applicant for absolute quantification of the respective targets. Test samples containing 4348, 1739, 783, 87, and 50 copies of LBFLFK DNA were analysed in eight replicates with two tests each per run, resulting in a total number of sixteen test results for each method. Data reported by the applicant indicated that both trueness and precision (RSDr) were within the ENGL acceptance criteria.

The Fat(A)A method was also in-house validated by the applicant for absolute quantification of the oilseed rape DNA with a calibration curve ranging from 108696 to 44 haploid genome copies. Test samples containing 56000, 14000, 7000, 700, and 50 copies of haploid oilseed rape genome were analysed four times with four tests per analysis for a total of sixteen test results.

Data reported by the applicant indicated that both trueness and precision (RSDr) were within the ENGL acceptance criteria.

The LOD_{abs} of the FatA(A) method was found to be at least 20 haploid genome copies, thus in line with the ENGL acceptance criteria (below 0.045 % or 25 copies with a level of confidence of 95 %).

The robustness of the methods was assessed in sixteen combinations of the following variations to the methods (LBFLFK Locus1 and Locus2): unchanged/+10 %/-10 % enzyme mix concentration; unchanged/+30 %/-30 % primer concentration; unchanged/+30 %/-30 % probe concentration; unchanged/+1 µL/-1 µL master mix volume; +1 °C/-1 °C in annealing temperature.

The precision RSD_r and the trueness (bias%) calculated for each combination of variations on a sample at the LOQ level (0.058 %) did not exceed 30 %, thus meeting the ENGL acceptance criteria.

Precision and trueness of the methods were tested in a transferability study involving four laboratories (including the method developer) making use respectively of Bio-Rad CFX96 Touch™ Real-Time PCR system (two laboratories), Applied Biosystems® ViiA 7 and Applied Biosystems® 7500 Real-Time PCR.

Two values (each as the mean of a set of triplicates) for each of the five GM levels (expressed as mass fractions of GM DNA or copies GM/total haploid genome copies) were provided by each laboratory.

Table 2C reports precision and trueness values for the five GM-levels as provided by the applicant for the four laboratories testing the LBFLFK Locus1 method.

Table 2D reports precision and trueness values for the five GM-levels as provided by the applicant for the four laboratories testing the LBFLFK Locus2 method. The data presented indicated that both parameters were within the ENGL acceptance criteria (trueness ≤ 25 %, RSDr ≤ 25 % across the entire dynamic range).

Table 2C. Mean %, precision and trueness values obtained by the applicant in the transferability study of LBFLFK Locus1 method

	Test results					Real-time PCR equipment
Expected GM %	**0.058**	**0.100**	**0.900**	**2.00**	**5.00**	
Measured mean GM %	0.055	0.091	0.861	1.87	5.03	Lab 1 - Bio-Rad CFX96 Touch™
Precision (RSD_r %)	6.4	5.4	12.3	1.9	6.2	
Trueness (bias %)	-5.2	-9.0	-4.3	-6.5	0.6	
Measured mean GM %	0.049	0.095	0.830	2.16	5.20	Lab 2 - Applied Biosystems® ViiA 7
Precision (RSD_r %)	4.3	3.7	3.7	15.7	8.0	
Trueness (bias %)	-15.5	-5.0	-7.8	8.0	4.0	
Measured mean GM %	0.047	0.098	0.835	1.89	4.70	Lab 3 - Bio-Rad CFX96 Touch™
Precision (RSD_r %)	10.4	6.5	1.9	3.8	3.6	
Trueness (bias %)	-19.0	-2.0	-7.2	-5.5	-6.0	
Measured mean GM %	0.048	0.077	0.860	2.02	5.49	Lab 4 - Applied Biosystems® 7500
Precision (RSD_r %)	16.3	2.7	3.8	9.8	14.7	
Trueness (bias %)	-17.2	-23.0	-4.4	1.0	9.8	

Table 2D. Mean %, precision and trueness values obtained by the applicant in the transferability study of LBFLFK Locus2 method

	Test results					Real-time PCR equipment
Expected GM %	**0.058**	**0.100**	**0.900**	**2.00**	**5.00**	
Measured mean GM %	0.053	0.084	0.840	2.10	5.06	Lab 1 - Bio-Rad CFX96 Touch™
Precision (RSD_r %)	0.0	6.8	9.4	8.1	16.1	
Trueness (bias %)	-8.6	-16.0	-6.7	5.0	1.2	
Measured mean GM %	0.057	0.097	0.890	2.08	5.28	Lab 2 - Applied Biosystems® ViiA 7
Precision (RSD_r %)	11.2	8.8	4.8	0.7	6.6	
Trueness (bias %)	-1.7	-3.0	-1.1	4.0	5.6	
Measured mean GM %	0.052	0.090	0.854	1.90	4.70	Lab 3 - Bio-Rad CFX96 Touch™
Precision (RSD_r %)	1.3	7.9	0.6	4.5	2.1	
Trueness (bias %)	-10.3	-10.0	-5.1	-5.0	-6.0	

Measured mean GM %	0.053	0.098	0.924	2.22	5.21	Lab 4 - Applied Biosystems® 7500
Precision (RSD_r %)	4.0	2.9	12.8	10.2	6.0	
Trueness (bias %)	-8.6	-2.0	2.7	11.0	4.2	

3.4. DNA extraction

Genomic DNA was isolated from ground oilseed rape seeds, using a CTAB-based protocol with removal of lipids and proteins via chloroform and an isopropanol precipitation of DNA, followed by purification of DNA with an ion exchange-based column and a gel filtration-based column to eliminate PCR inhibitors. 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 LBFLFK. This method was validated by the EURL GMFF for the extraction of DNA from ground canola seeds. The protocol for DNA extraction and a report on testing are published at http://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-02-19-XP.pdf).

Annex III to Regulation (EU) No 503/2013 [(2)] requires the applicant to discuss the validity and limitations of the detection methods in the various types of foods and feeds (matrices) that are expected to be placed on the market. To this regard, the applicant stated that the submitted method for DNA extraction is suitable for the isolation of genomic DNA from a wide variety of matrices (e.g. canola seed, grain and other canola tissues). The suitability of isolated DNA as an analyte for PCR-based detection of GMOs depends on the quality, purity, and quantity of the DNA. Although the DNA extraction method can be applied to different food and feed matrices, the application of the method to certain complex and difficult processed matrices may require adaptation. In fact, food processes can influence the quality and intactness of the DNA contained in the final processed products [(5-8)]. Another challenge of working with processed food and feed matrices is the presence of PCR inhibitors, which can reduce the efficiency and/or reproducibility of PCR and thus may contribute to inaccurate PCR results [(9-10)]. Therefore, DNA extraction from certain of these processed matrices may require additional rounds of purification in order to achieve the quality standards needed for quantitative real-time PCR [(9-10)].

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 methods

4.1. Samples used at EURL GMFF

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 oilseed rape seeds homozygous for both the LBFLFK loci;
- genomic DNA extracted by the applicant from conventional oilseed rape seed var. Kumily genetically similar to the LBFLFK event;
- Heat-devitalised intact seeds from non-GM var. Kumily rapeseed and from 100 % LBFLFK seeds for the validation of the DNA extraction method (§ 3.4).

4.2. Methods for the PCR analysis

The PCR methods provided by the applicant are two event-specific, quantitative, real-time TaqMan® PCR procedures for the determination of the relative content of respectively GM event LBFLFK Locus1 and Locus 2 DNA to total oilseed rape DNA. The procedures consist in simplex systems, in which the GM target methods for LBFLFK Locus1 and Locus2 and the oilseed rape specific method targeting the endogenous gene (*FatA(A)*), are performed in separate wells. The validated method protocol is published by the EURL GMFF at http://gmo-crl.jrc.ec.europa.eu/method-validations and can be found in Annex 1 to this report.

For the detection of GM event LBFLFK Locus1, a 128 bp fragment of the region spanning the 3' insert-to-plant junction in oilseed rape LBFLFK is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonudeotide probe labelled with FAM (6-carboxyfluorescein) as reporter dye at its 5' end and Black Hole Quencher (BHQ1), as non-fluorescent quencher at its 3' end.

For the detection of GM event LBFLFK Locus2, a 123 bp fragment of the region spanning the 5' insert-to-plant junction in oilseed rape LBFLFK is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonudeotide probe labelled with FAM (6-carboxyfluorescein) as reporter dye at its 5' end and Black Hole Quencher (BHQ1), as non-fluorescent quencher at its 3' end.

For the relative quantification of GM event LBFLFK Locus1 and Locus2, an oilseed rape taxon-specific system amplifies a 126 bp fragment of the A-genome copy of acyl-ACP-thioesterase (*FatA(A)*), (GenBank Accession No.: X87842.1), using *FatA(A)* gene-specific primers and a *FatA(A)*

[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).

gene-specific probe labelled with FAM as reporter dye at its 5' end and MGB (minor groove binder) as non-fluorescent quencher dye at its 3' end.

Standard curves are generated for both the LBFLFK Locus1 and Locus 2 and the *FatA(A)* systems by plotting the Cq 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 the relative quantification of event LBFLFK Locus1 or Locus2 DNA in a test sample, the LBFLFK Locus1 or Locus2 copy number is divided by the copy number of the oilseed rape haploid genome and multiplied by 100 to obtain the percentage value (GM % = LBFLFK Locus1 (or Locus2)/ 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) [(11)]. The total DNA quantity used in the PCR reactions, the copy number values used in the quantification and the GMO contents of the calibration samples 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 shown. 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
Total amount of oilseed rape DNA in the reaction (ng)	125	25	5	0.5
Target taxon [haploid genome copies]	108696	21739	4348	435
Target LBFLFK copies*	10870	2174	435	43

* Applicable to LBFLFK Locus1 and Locus2

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 [(3)].

The copy number of the LBFLFK Locus1 in relation to the *FatA(A)* target and the copy number of the LBFLFK Locus2 in relation to the *FatA(A)* target per haploid genome in the positive control sample were determined by digital PCR (dPCR) performed on the Bio Rad QX200 Droplet Reader.

The zygosity was tested in simplex resulting in eighteen data sets for both the LBFLFK Locus1 and Locus2 GM targets and eighteen data sets for the reference target.
Reaction mixes were prepared in order to obtain a final volume of 22 µL and contained 1X ddPCR Super Mix (Bio Rad, Cat. number 1863024), primers and probes at concentrations indicated in the corresponding methods and 2 µL of genomic DNA from the positive control sample at a concentration of 25 ng/µL. HindIII was added to the reaction mix (3U/reaction) to digest genomic DNA.

Reaction mixes were loaded into a semi skirt 96-well plate. 'No template controls' were included. After sealing with a sealing aluminium foil using the PX1™ PCR Plate Sealer, the plate was briefly centrifuged (1 min at 1000 rpm) and placed on a Bio Rad Automated Droplet Generator (AutoDG). The instrument added the Automated Droplet Generation oil for Probes (Bio Rad, Cat. number 1864110), generated the droplets in a final volume of 40 µL of the emulsion containing droplets. The AutoDG then transferred all the emulsions into a new semi skirt 96-well plate. The new plate was sealed with a sealing foil with the PX1™ PCR Plate Sealer and run in a Bio Rad C1000 TouchThermal Cycler. The thermal cycling conditions in a final volume of 40µL were as indicated below.

Step	Temperature (°C)	Time (mm:ss)	Ramp rate	Number of cycles
Polymerase activation	95	10:00	2°C/sec	1
DNA denaturation	94	00:30		40
Annealing/extension	60	01:00		
Enzyme deactivation	98	10:00		1
Hold	4	infinite		1

The sealed 96-well plate was then placed in the QX200 Droplet Reader to determine, through cytofluorimetry, the fraction of fluorescent PCR-positive droplets with respect of the total number of droplets in the original sample by selecting the proper fluorescent dye used. Data analysis and copy number calculations were performed using the Bio-Rad QX200 Droplet Reader Analysis software (QuantaSoft version 1.7.4).

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 interlaboratory validated methods - Version 2'[12].

4.3.2. In-house verification of LBFLFK locus1 and locus2 methods performance against ENGL method acceptance criteria

The methods performance characteristics were verified by quantifying on a copy number basis five blind samples distributed over a range of GM levels (5.0 % - 0.10 %, see Table 4). The samples were prepared by the EURL GMFF from the genomic DNA provided by the applicant (see 4.1 for details) by mixing LBFLFK oilseed rape DNA and non-GM oilseed rape DNA.

Table 4. LBFLFK samples GM % contents for both loci

LBFLFK GM % GM copy number/oilseed rape (haploid genome copy number*) x 100
5.0
2.0
0.90
0.50
0.10

* Corresponding to mass fractions of GM DNA (§ 5.1.2)

The calibration sample S1 was prepared from the genomic DNA provided by the applicant by mixing the appropriate amount of LBFLFK DNA with control non-GM oilseed rape DNA to obtain a 10 % (in mass fractions of GM DNA or in copy number ratio related to haploid genome copies) GM sample. Sample S1 was serially diluted (1:5) to obtain samples S2 and S3. Sample S4 was obtained by 1:10 dilution of sample S3 (see Table 3).

The experiments were performed on an ABI 7500, an ABI 7900HT and a Roche LC480 real-time platform under repeatability conditions and followed the protocols provided by the applicant. Test samples with GM levels 5.0 %, 2.0 %, 0.90 % and 0.50 % 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) for each LBFLFK locus1 and locus2 method. The test sample with GM level 0.10 % was tested in 15 replicates in an additional run for each platform and for each LBFLFK method. 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. On Roche LC480 platform the methods were run at 45 cycles as described in the validated methods published in Annex 1 to this report, and analysed with the second derivative maximum method.

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 (EU) No 120/2014 [13] 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) [14]
> ISO 5725 "Accuracy (trueness and precision) of measurement methods and results", ISO 5725-1:1994/Cor 1 (ISO 1998) and ISO 5725-2:2021 [15-17]

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

4.4.1. List of participating laboratories

The twelve laboratories participating in the LBFLFK international collaborative study were randomly selected from 25 national reference laboratories (NRLs) 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 methods for oilseed rape event LBFLFK

Laboratory	Country
AGES -Austrian Agency for Health and Food Safety	AT
Center for Agricultural Technology Augustenberg	DE
Environment Agency Austria	AT
Hellenic Agricultural Organisation	GR
Institute for Hygiene and Environment- Hamburg	DE
Institute of Food Safety, Animal Health and Environment "BIOR"	LV
Lower Saxony State Office for Consumer Protection and Food Safety	DE
National Food and Veterinary Risk Assessment Institute	LT
National Food Chain Safety Office	HU
National Institute of Biology	SI
Service commun des laboratoires des ministères économiques et financiers	FR
Wageningen Food Safety Research (WFSR)	NL

4.4.2. Real-time PCR equipment used in the study

Laboratories involved in the collaborative study used the following real-time PCR equipment: four laboratories used ABI 7500 (one of these was an ABI 7500 FAST), two laboratories used a Roche LC480, two laboratories used Bio-Rad CFX-96, one used ABI QuantStudio™ 7, one used ABI QuantStudio™ 5, one used ABI ViiA 7 and one used 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 methods are robust and usable under real conditions.

4.4.3. Materials used in the international collaborative study

For the validation of the quantitative event-specific methods, 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:

- ✓ Four calibration samples with known concentrations of GM-event (175 µL of DNA solution each) labelled from S1VL0219loc1 to S4VL0219loc1 (Table 3), marked with a yellow label.
- ✓ Twenty blinded test DNA samples (87.5 µL of DNA solution, each at 20 ng/µL) labelled from U1VL0219loc1 to U20VL0219loc1, representing five GM levels, each in four replicates (Table 4), marked with a yellow label.
- ✓ Four calibration samples with known concentrations of GM-event (175 µL of DNA solution each) labelled from S1VL0219loc2 to S4VL0219loc2 (Table 3), marked with a green label.
- ✓ Twenty blinded test DNA samples (87.5 µL of DNA solution, each at 20 ng/µL) labelled from U1VL0219loc2 to U20VL0219loc2, representing five GM levels, each in four replicates (Table 4), marked with a green label.

✓ Reaction reagents:

- Jumpstart™ Taq ReadyMix™ (2x), one vial: 13 mL
- distilled sterile water, one vial: 2 mL
- Reference Dye (100X): one vial: 260 µL
- $MgCl_2$ (50 mM), one vial: 1600 µL

✓ Primers and probes (2 tubes each) as follows:

FatA(A) taxon-specific method

- 09-0-3249 primer, two vials (10 µM): 200 µL
- 09-0-3251 primer, two vials (10 µM): 600 µL
- 09-QP-87 probe, two vials (10 µM): 100 µL

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

LBFLFK Locus1 method

- leftFST_ev1_loc1-f4 primer, one vial (10 µM): 200 µL
- leftFST_ev1_loc1-r5 primer, one vial (10 µM): 200 µL
- leftFST_ev1_loc1-s2 probe, one vial (10 µM): 100 µL

LBFLFK Locus2 method

- rightFST_ev1_loc2-f4 primer, one vial (10 µM): 200 µL
- rightFST_ev1_loc2-r1 primer, one vial (10 µM): 200 µL
- rightFST_ev1_loc2-s1 probe, one vial (10 µM): 100 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 LBFLFK Locus1 or the Locus2 event-specific system and for the *FatA(A)* taxon-specific system. In total, four plates were run by each participating laboratory.

The laboratories prepared the PCR master-mixes for the oilseed rape LBFLFK Locus1 and Locus2 and the *FatA(A)* methods 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

Nine laboratories reported no deviations from the validation protocol.
In one laboratory, one well (out of three) corresponding to one of the two replicates/plate for the 0.9 % sample of LBFLFK Locus2 was mixed with the NTC well. Consequently, the laboratory did not report the results of the two wells.
For Locus 1 two participants did not load, in one of the two plates, one set of no template controls (NTCs) for both the GM- and references-specific methods. However, since the study design provided for two sets of NTCs for each method in each plate, one set was still available and evaluated.

5. Results

5.1. EURL GMFF experimental testing

5.1.1. DNA extraction and purification

The method for DNA extraction provided by the applicant was not previously validated on the specific matrix. Hence, the EURL GMFF carried out an in-house validation of the DNA extraction method from ground canola seeds. The protocol for DNA extraction and a report on testing are published at http://gmo-crl.jrc.ec.europa.eu/summaries/EURL-<VL-02-19-XP.pdf.

5.1.2. Zygosity ratio in the positive control sample

The results of the digital PCR analysis conducted by the EURL GMFF to determine the zygosity ratio in the positive control samples for the LBFLFK locus1 and locus2 in relation to *FatA(A)* are shown, respectively, in Tables 6 and 7.

Table 6. Zygosity ratio of the LBFLFK Locus1 and *FatA(A)* targets in the positive control sample.

Mean ratio (LBFLFK locus1/*FatA(A)*)	1.00
Standard deviation	0.02
RSD_r (%)	2.5
Standard error of the mean	0.01
Upper 95 % CI of the mean	1.01
Lower 95 % CI of the mean	0.99

Table 7. Zygosity ratio of the LBFLFK Locus2 and *FatA(A)* targets in the positive control sample.

Mean ratio (LBFLFK locus2/*FatA(A)*)	0.97
Standard deviation	0.03
RSDr (%)	2.9
Standard error of the mean	0.01
Upper 95 % CI of the mean	0.98
Lower 95 % CI of the mean	0.96

The mean ratio of LBFLFK Locus1/*FatA(A)* is 1.00. The 95 % confidence interval (CI) spans around 1.00, the expected ratio for an oilseed rape control sample, homozygous for the GM-locus, assuming single-copy endogenous gene target. The measured mean ratio is not significantly different from the expected ratio, for an alpha = 0.05.

The mean ratio of LBFLFK Locus2/FatA(A) is 0.97. The upper bound of the 95 % confidence interval (CI) is close to 1.00. This is well in agreement with the value estimated for the ratio LBFLFK Locus1/FatA(A) and with the applicant's statement about the homozygosity of the positive control sample.

Hence, these data confirm that the positive control sample is homozygous for the two LBFLFK loci. The relative (%) GM content of the samples expressed in haploid genome copy numbers corresponds to the GM content expressed in mass fraction. For example, the 0.1 GM % in DNA copy number ratio corresponds to a 0.1 GM % in mass fraction.

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/guidance-documents).

5.1.3. In-house verification of the performance of the LBFLFK Locus1 method against ENGL method acceptance criteria

Test samples with GM levels from 5.00 % to 0.50 % (GM % mass fractions of GM DNA, equivalent to 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 0.10 % GM-level was tested for precision with 15 replicates in separate runs.

Tests were conducted on ABI 7500, ABI 7900HT and Roche LC480 for robustness.

The standard curve parameters and the results of efficiency, linearity, trueness and precision obtained in the three real-time PCR equipment with the test samples are reported in Tables 8, 9, 10, 11 and 12.

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 8 and 9 document that the slopes of the standard curves and the R^2 coefficients for the LBFLFK locus1 method were within the limits established by the ENGL. The EURL GMFF in-house results confirm the data provided by the applicant for the locus1 method.

Table 8. Standard curve parameters of the real-time PCR tests for the LBFLFK Locus1 method, carried out on ABI 7500, ABI 7900HT and Roche LC480 to quantify GM-levels in the range 5.0 % to 0.5 %, four replicates each. Slope and R^2 coefficient values were rounded to two digits.

	LBFLFK Locus1			***FatA(A)***		
	Slope	PCR efficiency*	R^2	Slope	PCR efficiency*	R^2
Run A	-3.28	102	1.00	-3.32	100	1.00
Run B	-3.33	100	1.00	-3.32	100	1.00
Run C	-3.21	105	1.00	-3.33	99	1.00
Run D	-3.38	98	1.00	-3.33	100	1.00
Run E	-3.29	101	1.00	-3.18	106	1.00
Run F	-3.45	95	1.00	-3.43	96	1.00

* 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 ABI 7900HT; runs E and F were carried out on Roche LC480.

Table 9. Standard curve parameters of the real-time PCR tests for the LBFLFK Locus1 method, carried out on ABI 7500, ABI 7900HT, and Roche LC480 to quantify the GM-level 0.10 % in 15 replicates. Slope and R^2 coefficient values were rounded to two digits.

	LBFLFK Locus1 system			***FatA(A)* system**		
	Slope	PCR efficiency*	R^2	Slope	PCR efficiency*	R^2
Run G	-3.35	99	1.00	-3.35	99	1.00
Run H	-3.27	102	0.99	-3.25	103	1.00
Run I	-3.34	99	1.00	-3.21	105	1.00

* 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 ABI 7900HT; 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 10, 11 and 12 show that trueness and precision of quantification for the LBFLFK locus1 method were within the limits established by the ENGL for the PCR machines used.

Table 10. Values of trueness and precision as established by the EURL GMFF by in-house verification of the LBFLFK Locus1 method using an ABI 7500. GM % mass fractions of GM DNA, equivalent to GM copies /copies haploid genomes.

Target GM-levels %	Measured GM-level %	Bias % of the target GM-level	Precision (RSD_r %)
5.0	5.0	-0.14	2.7
2.0	1.9	-3.5	4.3
0.90	0.88	-2.3	4.1
0.50	0.46	-7.0	6.7
0.10	0.10	0.96	17

Table 11. Values of trueness and precision as established by the EURL GMFF by in-house verification of the LBFLFK Locus1 method using an ABI 7900HT. GM % mass fractions of GM DNA, equivalent to GM copies /copies haploid genomes.

Target GM-levels %	Measured GM-level %	Bias % of the target GM-level	Precision (RSD_r %)
5.0	5.1	2.1	4.9
2.0	2.3	13	9.1
0.90	0.99	10	2.9
0.50	0.58	16	14
0.10	0.09	-11	17

Table 12. Values of trueness and precision as established by the EURL GMFF by in-house verification of the LBFLFK Locus1 method using a Roche LC480. GM % mass fractions of GM DNA, equivalent to 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	7.4
2.0	2.0	2.1	14
0.90	1.00	11	8.0
0.50	0.53	6.6	17
0.10	0.10	-4.9	16

5.1.4. In-house verification of the performance of the LBFLFK Locus2 method against ENGL method acceptance criteria

Test samples with GM levels from 5.00 % to 0.50 % 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

0.10 % GM-level was tested for precision with 15 replicates in separate runs. Tests were conducted on ABI 7500, ABI 7900HT and Roche LC480 for robustness.

The standard curve parameters and the results of efficiency, linearity, trueness and precision obtained in the three real-time PCR equipment with the test samples are shown in Tables 13, 14, 15, 16 and 17.

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. Tables 13 and 14 document that the slopes of the standard curves and the R^2 coefficients for the LBFLFK locus2 method were within the limits established by the ENGL. The EURL GMFF in-house results confirm the data provided by the applicant.

Table 13. Standard curve parameters of the real-time PCR tests for the LBFLFK Locus2 method, carried out on ABI 7500, ABI 7900HT and Roche LC480 to quantify GM-levels in the range 5.0 % to 0.5 % in four replicates each. Slope and R^2 coefficient values were rounded to two digits.

	LBFLFK Locus2 system			***FatA(A)* system**		
	Slope	PCR efficiency*	R^2	Slope	PCR efficiency*	R^2
Run J	-3.32	100	1.00	-3.29	101	1.00
Run K	-3.35	99	1.00	-3.30	101	1.00
Run L	-3.40	97	1.00	-3.30	101	1.00
Run M	-3.26	102	0.99	-3.28	102	1.00
Run N	-3.38	98	1.00	-3.43	96	1.00
Run O	-3.28	102	1.00	-3.40	97	1.00

* PCR efficiency (%) is calculated using the formula Efficiency = $(10^{(-1/slope)}) - 1) \times 100$
Runs J-K were carried out on ABI 7500; runs L-M were carried out on ABI 7900HT; runs N and O were carried out on Roche LC480.

Table 14. Standard curve parameters of the real-time PCR tests for the LBFLFK Locus2 method, carried out on ABI 7500, ABI 7900HT, and Roche LC480 to quantify the GM-level 0.10 % in 15 replicates. Slope and R^2 coefficient values were rounded to two digits.

	LBFLFK Locus2 system			***FatA(A)* system**		
	Slope	PCR efficiency*	R^2	Slope	PCR efficiency*	R^2
Run P	-3.48	94	1.00	-3.27	102	1.00
Run Q	-3.47	94	1.00	-3.31	101	1.00
Run R	-3.33	100	1.00	-3.18	106	1.00

* PCR efficiency (%) is calculated using the formula Efficiency = $(10^{(-1/slope)}) - 1) \times 100$
Run P was carried out on ABI 7500; run Q was carried out on ABI 7900HT; run R 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 15, 16 and 17 show that trueness and precision of quantification for the LBFLFK locus2 method were within the limits established by the ENGL for the PCR machines used.

Table 15. Values of trueness and precision as established by the EURL GMFF in its in-house verification of the LBFLFK Locus2 method using an ABI 7500. GM % in mass fractions of GM DNA equivalent to GM copies /copies haploid genomes.

Target GM-levels %	Measured GM-level %	Bias % of the target GM-level	Precision (RSD_r %)
5.0	4.9	-2.3	6.9
2.0	2.0	0.37	3.7
0.90	0.87	-3.6	5.6
0.50	0.45	-11	11
0.10	0.12	16	14

Table 16. Values of trueness and precision as established by the EURL GMFF in its in-house verification of the LBFLFK Locus2 method using an ABI 7900HT. GM % mass fractions of GM DNA equivalent to GM copies /copies haploid genomes.

Target GM-levels %	Measured GM-level %	Bias % of the target GM-level	Precision (RSD_r %)
5.0	5.4	7.1	4.7
2.0	2.1	6.3	8.1
0.90	1.1	17	8.8
0.50	0.54	7.5	5.5
0.10	0.08	-23	19

Table 17. Values of trueness and precision as established by the EURL GMFF in its in-house verification of the LBFLFK Locus2 method using a Roche LC480. GM % mass fractions of GM DNA equivalent to GM copies /copies haploid genomes.

Target GM-levels %	Measured GM-level %	Bias % of the target GM-level	Precision (RSD_r %)
5.0	5.4	8.0	7.9
2.0	2.3	13	8.5
0.90	1.1	17	6.9
0.50	0.59	19	11
0.10	0.10	0.45	17

5.2. Results of the international collaborative study

5.2.1. PCR efficiency and linearity of the LBFLFK Locus1 method

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 11A. 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 18 indicates that the efficiency of amplification for the LBFLFK Locus1 system ranges from 93 % to 105 % and the linearity from 0.99 to 1.00; the amplification efficiency for the oilseed rape-specific system ranges from 86 % to 105 % and the linearity from 0.99 to 1.00.

Table 18. Values of slope, PCR efficiency and R^2 obtained during the international collaborative trial for the LBFLFK Locus1 method. Slope and R^2 coefficient values were rounded to two digits.

		LBFLFK Locus1			***FatA(A)***		
Lab	**Plate**	**Slope**	**PCR Efficiency (%)**	**R^2**	**Slope**	**PCR Efficiency (%)**	**R^2**
1	A	-3.32	100	1.00	-3.37	98	1.00
	B	-3.38	98	1.00	-3.38	98	1.00
2	A	-3.33	100	1.00	-3.22	105	1.00
	B	-3.32	100	1.00	-3.22	104	1.00
3	A	-3.42	96	1.00	-3.39	97	1.00
	B	-3.20	105	1.00	-3.36	98	1.00
4	A	-3.38	98	0.99	-3.28	102	1.00
	B	-3.42	96	1.00	-3.33	100	1.00
5	A	-3.32	100	1.00	-3.28	102	1.00
	B	-3.27	102	1.00	-3.71	86	0.99
6	A	-3.40	97	1.00	-3.35	99	1.00
	B	-3.30	101	1.00	-3.29	101	1.00
7	A	-3.35	99	0.99	-3.31	101	1.00
	B	-3.34	99	1.00	-3.37	98	1.00
8	A	-3.32	100	1.00	-3.31	100	1.00
	B	-3.38	98	1.00	-3.36	99	1.00
9	A	-3.41	97	1.00	-3.43	96	1.00
	B	-3.41	96	1.00	-3.45	95	1.00
10	A	-3.50	93	1.00	-3.49	94	1.00
	B	-3.47	94	1.00	-3.42	96	1.00
11	A	-3.35	99	1.00	-3.32	100	1.00
	B	-3.39	97	1.00	-3.32	100	1.00
12	A	-3.39	97	1.00	-3.45	95	1.00
	B	-3.42	96	1.00	-3.42	96	1.00
	Mean	**-3.37**	**98**	**1.00**	**-3.37**	**98**	**1.00**

The mean PCR efficiency was 98 % for both the LBFLFK Locus1 and the *FatA(A)* systems. The average R^2 was 1.00 for both the LBFLFK Locus1 and *FatA(A) systems*. Both PCR efficiency and linearity values were within the ENGL acceptance criteria.
These results confirm the appropriate performance characteristics of the methods tested in terms of efficiency and linearity.

5.2.2. GMO quantification of the LBFLFK Locus1 method

Table 19 reports the values of quantification for the four replicates of each GM level as reported by each of the twelve participating laboratories.

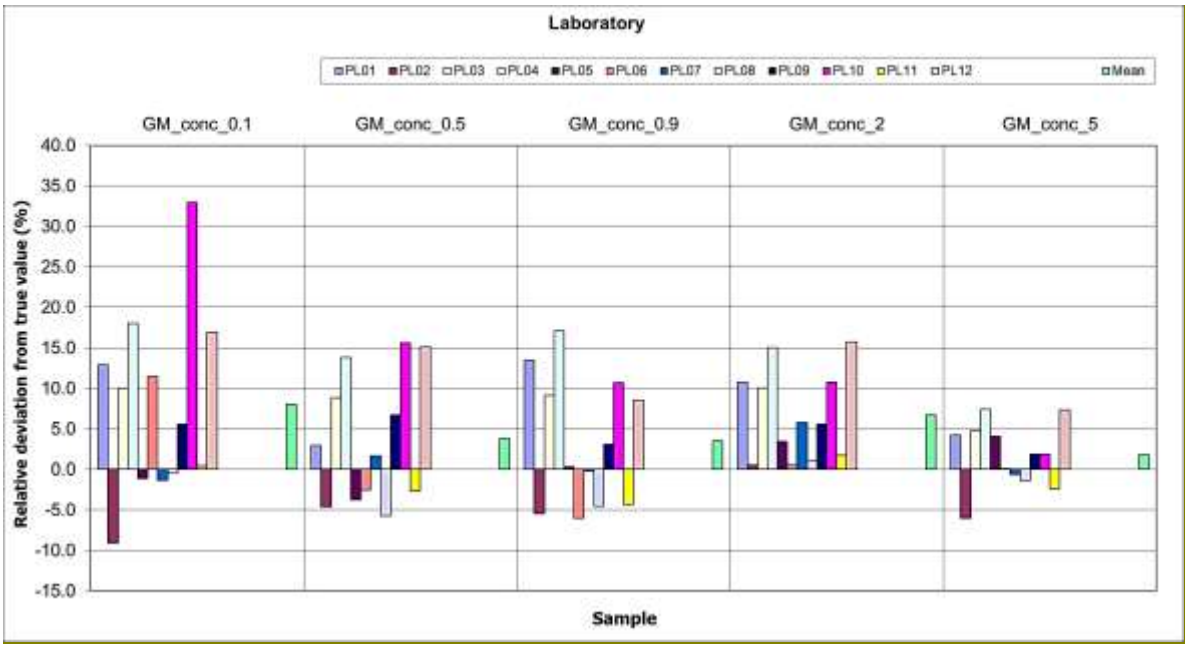
Table 19. GM % values determined by laboratories for test samples

	GMO content (%) *																			
LAB	0.10				0.50				0.90				2.0				5.0			
	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4
1	0.12	0.12	0.09	0.11	0.53	0.55	0.51	0.47	1.1	1.0	0.98	0.99	2.3	2.2	2.2	2.1	5.2	5.2	5.0	5.4
2	0.09	0.09	0.10	0.08	0.48	0.54	0.44	0.44	0.83	0.89	0.84	0.85	2.0	2.1	1.9	2.0	5.1	4.5	5.0	4.3
3	0.10	0.09	0.12	0.13	0.51	0.50	0.56	0.60	0.96	0.90	0.98	1.1	2.4	2.0	2.2	2.2	5.0	5.1	5.3	5.6
4	0.12	0.11	0.14	0.11	0.56	0.62	0.56	0.54	1.1	1.08	0.94	1.1	2.2	2.5	2.3	2.2	5.6	5.4	5.3	5.3
5	0.11	0.09	0.09	0.11	0.52	0.52	0.43	0.45	0.97	0.94	0.90	0.81	1.8	2.2	2.2	2.0	5.2	6.1	4.9	4.7
6	0.11	0.09	0.13	0.11	0.48	0.44	0.54	0.50	0.85	0.78	0.90	0.87	1.9	2.1	1.8	2.3	4.8	4.8	5.5	4.8
7	0.11	0.10	0.08	0.10	0.53	0.52	0.49	0.50	0.89	0.90	0.90	0.89	2.1	2.2	2.2	2.0	5.1	5.1	4.7	5.0
8	0.10	0.10	0.10	0.10	0.45	0.51	0.47	0.45	0.90	0.86	0.82	0.86	2.0	2.1	1.9	2.0	4.8	5.0	5.0	4.8
9	0.10	0.12	0.11	0.10	0.50	0.56	0.53	0.55	0.93	0.94	0.89	0.96	2.1	2.1	2.1	2.1	5.1	5.2	5.0	5.1
10	0.12	0.12	0.14	0.15	0.53	0.58	0.61	0.59	1.0	0.95	1.1	0.95	2.3	2.0	2.1	2.4	5.1	4.9	5.2	5.1
11	0.08	0.10	0.11	0.11	0.49	0.49	0.47	0.50	0.93	0.87	0.73	0.92	2.0	2.2	1.9	2.1	4.9	4.6	5.2	4.9
12	0.11	0.11	0.12	0.13	0.58	0.52	0.56	0.64	0.95	0.80	1.2	0.96	2.6	2.1	2.1	2.5	5.0	5.2	5.5	5.8

* GM % in mass fractions of GM DNA or in copy/copy haploid genomes.

A graphical representation of the data reported in Table 19 is provided in Figure 1A 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; the green bar on the right represents the mean relative deviation over all data before eliminating outliers for each GM level.

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



* For PL07 at level 0.90 % and PL06 at 5.0 % a very small relative deviation from the true value was observed and therefore the corresponding histogram does not show up in Figure 1A. PL = participating laboratory.

Overall, almost all laboratories' mean relative deviations from the true values were within a maximum of ± 25 %. One laboratory overestimated GM-level 0.10 % by more than 25 %.

5.2.3. Method performance requirements for the LBFLFK Locus1 method.

Among the performance requirements established by ENGL and adopted by the EURL GMFF (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm), repeatability and reproducibility are to be assessed through an international collaborative trial. Table 13A illustrates the estimation of repeatability and reproducibility at the various GM levels tested with the LBFLFK Locus1 method 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 20, the LBFLFK Locus1 method satisfies this requirement at all GM levels tested. Indeed, the highest value of RSD_R is 14 % at the 0.10 % GM level, thus within the acceptance criterion.

Table 20. Summary of validation results for the LBFLFK Locus1 method.

	Test Sample Expected GMO %*				
	0.10	**0.50**	**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	-	-	1	-	-
Reason for exclusion	-	-	C**	-	-
Mean value	0.11	0.52	0.93	2.1	5.1
Relative repeatability standard deviation, RSD_r (%)	11	7.2	6.2	7.1	5.8
Repeatability standard deviation	0.01	0.04	0.06	0.15	0.29
Relative reproducibility standard deviation, RSD_R (%)	14	9.9	9.7	8.1	6.4
Reproducibility standard deviation	0.02	0.05	0.09	0.17	0.33
Bias*** (absolute value)	0.01	0.02	0.03	0.13	0.09
Bias (%)	8.0	3.8	3.0	6.7	1.8

*expressed as mass fractions of GM DNA equivalent to GM copy numbers in relation to target taxon haploid genome copy numbers

** 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 20 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" [4]. 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 0.10 % 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 8.0 % at the 0.10 % GM level.

5.2.4. PCR efficiency and linearity of the LBFLFK Locus2 method

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 21.

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 21 indicates that the efficiency of amplification for the LBFLFK Locus2 system ranges from 94 % to 105 % and the linearity from 0.99 to 1.00; the amplification efficiency for the oilseed rape-specific system ranges from 96 % to 105 % and the linearity is always 1.00. The mean PCR efficiency was 98 % and 99 % respectively for the LBFLFK Locus2 and the *FatA(A)* systems. The average R^2 was 1.00 for both the LBFLFK Locus2 and *FatA(A) systems*, respectively. Both PCR efficiency and linearity values were within the ENGL acceptance criteria.

Table 21. Values of slope, PCR efficiency and R^2 obtained during the international collaborative trial for the LBFLFK Locus2 method. Slope and R^2 coefficient values were rounded to two digits.

		LBFLFK Locus2			***FatA(A)***		
Lab	**Plate**	**Slope**	**PCR Efficiency (%)**	**R^2**	**Slope**	**PCR Efficiency (%)**	**R^2**
1	A	-3.41	96	1.00	-3.37	98	1.00
	B	-3.27	102	1.00	-3.33	100	1.00
2	A	-3.37	98	1.00	-3.24	104	1.00
	B	-3.21	105	1.00	-3.20	105	1.00
3	A	-3.42	96	1.00	-3.40	97	1.00
	B	-3.28	102	1.00	-3.37	98	1.00
4	A	-3.34	99	1.00	-3.25	103	1.00
	B	-3.31	100	1.00	-3.28	102	1.00
5	A	-3.48	94	1.00	-3.41	97	1.00
	B	-3.47	94	1.00	-3.43	96	1.00
6	A	-3.32	100	1.00	-3.29	101	1.00
	B	-3.34	99	1.00	-3.39	97	1.00
7	A	-3.32	100	1.00	-3.38	97	1.00
	B	-3.34	99	1.00	-3.37	98	1.00
8	A	-3.45	95	1.00	-3.40	97	1.00
	B	-3.41	97	1.00	-3.34	99	1.00
9	A	-3.37	98	1.00	-3.42	96	1.00
	B	-3.39	97	1.00	-3.39	97	1.00
10	A	-3.45	95	1.00	-3.39	97	1.00
	B	-3.49	94	1.00	-3.39	97	1.00
11	A	-3.34	99	1.00	-3.25	103	1.00
	B	-3.33	100	0.99	-3.33	100	1.00
12	A	-3.38	97	1.00	-3.38	98	1.00
	B	-3.45	95	1.00	-3.37	98	1.00
	Mean	**-3.37**	**98**	**1.00**	**-3.35**	**99**	**1.00**

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

5.2.5. GMO quantification of the LBFLFK Locus2 method

Table 22 reports the values of quantification for the four replicates of each GM level as reported by each of the twelve participating laboratories.

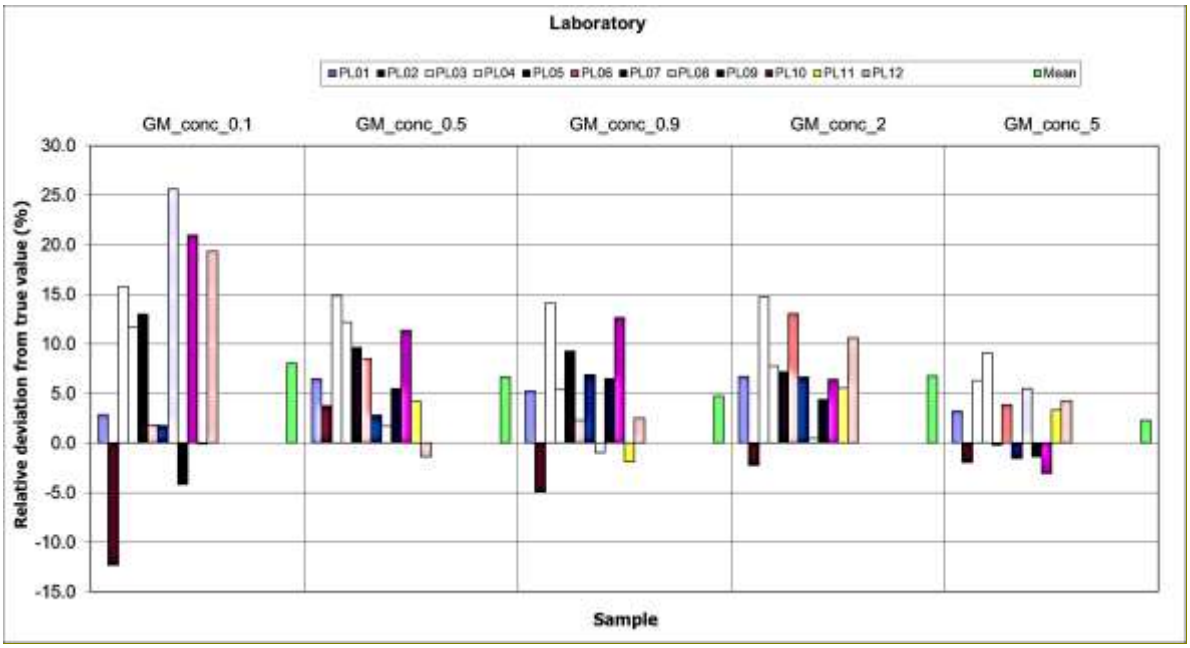
Table 22. GM % values determined by laboratories for test samples

	GMO content (%) *																			
LAB	0.10				0.50				0.90				2.0				5.0			
	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4
1	0.09	0.09	0.10	0.12	0.46	0.53	0.54	0.60	0.93	0.93	0.94	0.99	2.0	2.2	2.3	2.0	5.4	5.2	5.0	5.0
2	0.07	0.08	0.10	0.10	0.44	0.56	0.48	0.59	0.79	0.82	0.94	0.87	2.0	1.8	2.2	1.9	5.1	5.0	4.5	5.1
3	0.11	0.11	0.13	0.12	0.61	0.54	0.57	0.58	1.07	0.99	0.95	1.10	2.1	2.4	2.4	2.3	5.5	5.2	5.2	5.4
4	0.10	0.11	0.13	0.11	0.52	0.62	0.54	0.56	0.86	1.03	0.96	0.94	2.4	2.0	2.3	2.0	5.2	5.6	5.4	5.6
5	0.11	0.12	0.12	0.11	0.54	0.54	0.56	0.56	1.0	1.1	0.93	0.89	2.1	2.1	2.3	2.1	4.6	5.2	5.1	5.0
6	0.10	0.11	0.11	0.09	0.43	0.63	0.46	0.65	0.79	0.80	1.30	0.79	2.1	2.1	2.2	2.7	6.2	5.0	4.6	4.9
7	0.10	0.10	0.11	0.10	0.51	0.49	0.51	0.55	0.96	0.94	0.99	0.96	2.1	2.2	2.1	2.1	5.0	4.9	4.7	5.1
8	0.10	0.11	0.18	0.11	0.43	0.56	0.53	0.52	0.85	0.95	0.84	0.93	2.1	1.9	2.2	1.8	5.1	6.2	4.9	4.9
9	0.11	0.11	0.09	0.08	0.55	0.52	0.54	0.50	0.93	0.97	0.95	0.98	2.1	2.0	2.1	2.1	4.9	4.8	5.0	5.0
10	0.12	0.14	0.10	0.12	0.57	0.54	0.57	0.56	0.98	1.0	0.99	1.1	2.1	2.2	2.1	2.1	4.8	4.8	4.9	4.9
11	0.09	0.10	0.11	0.10	0.47	0.53	0.55	0.54	0.90	0.90	0.90	0.83	2.2	1.9	2.2	2.1	5.4	5.0	5.2	5.0
12	0.14	0.12	0.10	0.12	0.37	0.55	0.55	0.50	0.92	0.93	0.96	0.88	2.2	2.1	2.3	2.3	5.2	5.1	5.2	5.3

* GM % in mass fractions of GM DNA or in copy/copy haploid genomes.

A graphical representation of the data reported in Table 22 is provided in Figure 1B that shows the relative deviation from the true value for each GM level tested with the LBFLFK Locus2 method for each participating laboratory. The coloured bars represent the deviation of the GM level measured in % of the true GM level; the green bar on the right represents the mean relative deviation over all data before eliminating outliers for each GM level.

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



* For PL11 at level 0.10 % and PL05 at 5.0 % a very small relative deviation from the true value was observed and therefore the corresponding histogram does not show up in Figure 1B. PL = participating laboratory.

Overall, almost all laboratories' mean relative deviations from the true values were within a maximum of ± 25 %. One laboratory overestimated GM-level 0.10 % by more than 25 %.

5.2.6. Method performance requirements for the LBFLFK Locus2 method.

Among the performance requirements established by ENGL and adopted by the EURL GMFF [4], repeatability and reproducibility are to be assessed through an international collaborative trial. Table 23 illustrates the estimation of repeatability and reproducibility at the various GM levels tested with the LBFLFK Locus2 method 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 23, the LBFLFK Locus2 method satisfies this requirement at all GM levels tested. Indeed, the highest value of RSD_R is 14 % at the 0.10 % GM level, thus within the acceptance criterion.

Table 23. Summary of validation results for the LBFLFK Locus2 method

	Test Sample Expected GMO %*				
	0.10	**0.50**	**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	1	-	1	-	-
Reason for exclusion	C**	-	C	-	-
Mean value	0.11	0.53	0.94	2.1	5.1
Relative repeatability standard deviation, RSD_r (%)	11	10	5.6	6.9	6.2
Repeatability standard deviation	0.01	0.06	0.05	0.15	0.31
Relative reproducibility standard deviation, RSD_R (%)	14	10	7.5	7.4	6.5
Reproducibility standard deviation	0.01	0.06	0.07	0.16	0.33
Bias*** (absolute value)	0.01	0.03	0.04	0.14	0.11
Bias (%)	6.4	6.6	5.0	6.8	2.3

* mass fractions of GM DNA equivalent to GM copy numbers in relation to target taxon haploid genome copy numbers.

** 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 23 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" [4]. 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 0.10 % 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 6.8 % at the 2.0 % GM level.

6. Compliance of the method for detection and quantification of event LBFLFK 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 24:

- at step 2 of the validation process (scientific assessment of the dossier), the EURL GMFF acknowledged that for the LBFLFK locus1 method, the RSD_r value at the 0.10 % level shown by the applicant's dossier (expressed as haploid genome copies number ratio, corresponding to mass fraction of GM material) was 7.3 %, based on 16 replicates (Table 2A) and ≤6.5 % based on 2 replicates by four laboratories in the transferability study (Table 2C).
For the LBFLFK locus2 method the RSD_r value at the 0.10 % level shown by the applicant's dossier was 12.8 %, based on 16 replicates (Table 2B) and ≤8.8 % based on 2 replicates by four laboratories in the transferability study (Table 2D). Hence, the RSD_r was 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 0.1 % expressed in terms of copy number ratio to haploid genome copy numbers).
The experiments were carried out under repeatability conditions on fifteen replicates for the LBFLFK Locus1 method. The RSD_r resulted to range between 16 % and 17 % (Table 10, 11 and 12) depending on the qPCR platform applied, hence below 25 %; similarly, for the LBFLFK locus2 method, the RSD_r resulted to range between 14 % and 19 % (Table 15, 16 and 17) depending on the qPCR platform applied, hence also below 25 %

- the collaborative study (step 4 of the validation process) established that at the level of 0.1 % related to mass fraction of GM-material (corresponding to 0.1 % expressed in terms of copy number ratio to haploid genome copy numbers), the RSD_r of the LBFLFK locus1 method was 11 % and the RSD_r of the LBFLFK Locus2 method was 11 %, therefore also below 25 % and well in line with the previous data.

The outcome of the different steps is summarised in Table 24.

Table 24. Precision of the event-specific methods for quantitative detection of LBFLFK locus1 and locus2 at or around 0.1 % level related to mass fractions of GM material.

Source	LBFLFK Locus1		LBFLFK Locus2	
	RSD_r %	GM %	RSD_r %	GM %
Applicant's method optimisation	7.3	0.10	12.8	0.10
Applicant's transferability study	≤6.5	0.10	≤8.8	0.10
EURL GMFF tests	16 - 17	0.10	14 - 19	0.10
Collaborative study	11	0.10	11	0.10

Based on the results of the EURL GMFF in-house verification and of the international collaborative study, it is concluded that the methods RSD_r are lower than 25 % at the level of 0.1 % related to mass fraction of GM material, hence the methods meet the requirement laid down in Regulation (EU) No 619/2011[3].

7. Conclusion

The methods provided by the applicant were validated in accordance to the EURL GMFF validation process, in line with the requirements of the relevant EU legislation and international standards for method validation.

This validation study confirmed that the methods are 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 meet all method performance requirements established by the ENGL and the EURL GMFF. The methods are 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 they are applicable to any appropriately extracted oilseed rape genomic DNA.

In any case the user of the methods 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 methods are described in detail as "Validated Methods" at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx and in Annex 1.

8. References

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Annex 1: event-specific Methods for the Quantification of Oilseed Rape LBFLFK Locus1 and Locus2 by Real-time PCR

1. General information and summary of the methodology

This protocol describes two event-specific real-time quantitative TaqMan® PCR (polymerase chain reaction) procedures for the determination of the relative content of oilseed rape event LBFLFK Locus1 and Locus2 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 suitability of the extracted DNA.

For the detection of GM event LBFLFK Locus1, a 128 bp fragment of the region spanning the 3' insert-to-plant junction in oilseed rape LBFLFK is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonudeotide probe labelled with FAM (6-carboxyfluorescein) as reporter dye at its 5' end and Black Hole Quencher (BHQ1), as non-fluorescent quencher at its 3' end.

For the detection of GM event LBFLFK Locus2, a 123 bp fragment of the region spanning the 5' insert-to-plant junction in oilseed rape LBFLFK is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonudeotide probe labelled with FAM (6-carboxyfluorescein) as reporter dye at its 5' end and Black Hole Quencher (BHQ1), as non-fluorescent quencher at its 3' end.

For the relative quantification of GM event LBFLFK Locus1 and Locus2, an oilseed rape taxon-specific system amplifies a 126 bp fragment of the A-genome copy of acyl-ACP-thioesterase (FatA) gene (GenBank Accession No.: X87842.1), using *FatA(A)* gene-specific primers and a *FatA(A)* gene-specific probe labelled with FAM as reporter dye at its 5' end and minor groove binder (MGB) 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 LBFLFK Locus1 and Locus2 DNA in a test sample, Cq values for the LBFLFK Locus1 and Locus2 and the *FatA(A)* systems are determined for the sample. Standard curves are then used to estimate the relative amount of LBFLFK Locus1 and Locus2 DNA to total oilseed rape DNA.

2. Validation and performance characteristics

2.1 General

The methods were optimised for suitable DNA extracted from genetically modified and conventional oilseed rape seeds and grain. Precision and trueness of the methods were tested through an international collaborative ring trial using DNA samples at different GM contents.

2.2 Collaborative trial

The methods were 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 September – October 2020.

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 relative LOD of the LBFLFK Locus1 and Lcous2 methods is at least 0.010 % (related to haploid genome copies, corresponding to 0.010 % in mass fraction of GM material) in 100 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 LBFLFK Locus1 and Locus2 methods is at least 0.058 % (related to haploid genome copies, corresponding to 0.058 % in mass fraction of GM material) in 100 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 LBFLFK Locus1 and Locus2 methods exploit a unique DNA sequence in the region spanning respectively the 3' insert-to-plant junction and the 5' insert-to-plant junction in oilseed rape LBFLFK and are therefore event-specific for the event LBFLFK Locus1 and Locus2. This was confirmed by the applicant's specificity studies and by *in silico* analysis performed by the applicants and the EURL GMFF.

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 LBFLFK Locus1 and Locus2

3.2.1 General

The real-time PCR set-up for the taxon (*FatA(A)*) and the GMO (event LBFLFK Locus1 and Locus2) 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 methods are developed for a total volume of 20 µL per reaction mixture for the GM (event LBFLFK Locus1 and Locus2) and the taxon (*FatA(A)*) assay with the reagents as listed respectively in Table 2, 3 and 4.

3.2.2 Calibration

The calibration curves are established based on at least four samples. The first point of the calibration curve (S1) should be established for a sample containing 10 % oilseed rape LBFLFK Locus1 and Locus2 DNA in a total of 125 ng of oilseed rape DNA (corresponding to 108696 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 S4 are to be prepared by serial dilutions (dilution factor 5 for samples S2-S3 and dilution factor 10 for standard S4) according to Table 1 below.

Table 1. Copy number values of the standard curve samples

Sample code	S1	S2	S3	S4
Total amount of oilseed rape DNA in reaction (ng)	125	25	5.0	0.5
Oilseed rape haploid genome copies	108696	21739	4348	435
LBFLFK Locus1 copies	10870	2174	435	43
LBFLFK Locus2 copies	10870	2174	435	43

A calibration curve is to be 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 three tubes on ice, add the components in the order mentioned below (except DNA) to prepare the reaction mixes for the oilseed rape LBFLFK Locus1 and Locus2 specific systems as shown respectively in Tables 2 and 3 and the *FatA(A)* reference gene system (Table 4). 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 LBFLFK Locus1 method.

Component	Final concentration	µL/reaction
Sigma JumpStart™ Taq ReadyMix™ (2x)	1x	10
Reference dye (100x)	0.1x (1x**)	0.20
$MgCl_2$ (50nM)	3.0 mM	1.20
leftFST_ev1_loc1-f4 (10 µM)	300 nM	0.60
leftFST_ev1_loc1-r5 (10 µM)	300 nM	0.60
leftFST_ev1_loc1-s2* (10 µM)	150 nM	0.30
Nuclease free water	-	2.10
DNA	-	5.0
Total reaction volume:		20 µL

*TaqMan® probe labelled with 6-FAM at its 5'-end and BHQ1 at its 3'-end

** § Table 5

Table 3. Amplification reaction mixture in the final volume/concentration per reaction well for the LBFLFK Locus2 method.

Component	Final concentration	µL/reaction
Sigma JumpStart™ Taq ReadyMix™ (2x)	1x	10
Reference dye (100x)	0.1x (1x**)	0.20
$MgCl_2$ (50 mM)	3.0 mM	1.20
rightFST_ev1_loc2-f4 (10 µM)	300 nM	0.60
rightFST_ev1_loc2-r1 (10 µM)	300 nM	0.60
rightFST_ev1_loc2-s1* (10 µM)	150 nM	0.30
Nuclease free water	-	2.10
DNA	-	5.0
Total reaction volume:		20 µL

*TaqMan® probe labelled with 6-FAM at its 5'-end and BHQ1 at its 3'-end

** § Table 5

Table 4. Amplification reaction mixture in the final volume/concentration per reaction well for the oilseed rape *FatA(A)* assay.

Component	Final concentration	µL/reaction
Sigma JumpStart™ Taq ReadyMix™	1x	10
Reference dye (100x)	0.1x (1x**)	0.20
$MgCl_2$ (50 mM)	3.0 mM	1.20
09-0-3249 (10 µM)	300 nM	0.60
09-0-3251 (10 µM)	900 nM	1.80
09-QP-87* (10 µM)	150 nM	0.30
Nuclease free water	-	0.90
DNA	-	5.0
Total reaction volume:		20 µL

*TaqMan® probe is labelled with FAM at its 5'-end and MGB at its 3'-end

** § Table 5

Table 5. Reference dye working concentrations per real-time PCR equipment recommended by the applicant

Real-time PCR equipment	Reference dye concentration
ABI 7500	0.1x
ABI 7900	1x
Roche LC480	0.1x
QuantStudio	0.1x
BioRad CFX96	0.1x
ViiA7	0.1x

3. Mix well and centrifuge briefly.
4. Prepare three 0.5 mL reaction tubes (two for the oilseed rape LBFLFK Locus1 and Locus2 systems respectively and one for the *FatA(A)* system) for each DNA sample to be tested (standard curve samples, blind samples and control samples).
5. Add into each reaction tube the amount of reaction mix for 3.5 PCR repetitions (52.5 µL for the oilseed rape LBFLFK Locus1 or Locus2 system and for the *FatA(A)* system). Add to each tube the correct amount of DNA for 3.5 PCR repetitions (17.5 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 20 µL for the LBFLFK Locus1 or Locus2 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. Select FAM as reporter dye for the LBFLFK Locus1 and Locus2 and for the *FatA(A)* reference system. Define BHQ1 or non-fluorescent as quencher dye for LBFLFK Locus1 and Locus2 system and MGB or non-fluorescent for *FatA(A)* reference system. Select ROX as the passive reference dye. Enter the correct reaction volume (20 µL).
10. Run the PCR with the cycling program described in Table 6. Users who plan to use Roche LC480 instrument with the second derivative maximum analysis method are advised to program 45 cycles instead of 40, in order to be able to quantify down to Cq 40.

Table 6. Cycling program for LBFLFK Locus1 and Locus2/*FatA(A)* assays.

Step	Stage		T (°C)	Time (s)	Acquisition	Cycles
1	Initial denaturation		95	600	No	1
2	Amplification	Denaturation	95	15	No	40*
		Annealing & Extension	60	60	Yes	

* see comment above for users of second derivative maximum analysis method

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 assays 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 LBFLFK Locus1 and Locus2 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 LBFLFK Locus1 and Locus2 DNA in the unknown sample, the LBFLFK Locus1 or Locus2 copy numbers are respectively divided by the copy number of the oilseed rape endogenous gene *FatA(A)* and multiplied by 100 (GM% = LBFLFK Locus1 or Locus2/*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

Jumpstart™ Taq ReadyMix™ (2x). Sigma Cat. No. D7440
ROX Reference Dye (100x) from Jumpstart™. Sigma Cat. No. D7440

4.3 Primers and Probes

Table 7. Primers and probes for the LBFLFK Locus1, Locus2 and *FatA(A)* methods

		DNA Sequence (5' to 3')	Length (nt)
LBFLFK Locus1			
Forward primer	leftFST_ev1_loc1-f4	ACC ATC ATA CTC ATT gCT gAT CC	23
Reverse primer	leftFST_ev1_loc1-r5	TTC CTg TAT ACg CAC ACA TAg TAT A	25
Probe	leftFST_ev1_loc1-s2	6FAM- ATg TAg ATT TCC Cgg ACA TgA AgC CA-BHQ1	26
LBFLFK Locus2			
Forward primer	rightFST_ev1_loc2-f4	gAT Tgg TAA TAT gTA AAT AAC ggg ATC C	28
Reverse primer	rightFST_ev1_loc2-r1	gCg AAT TTg gCC TgT AgA CC	20
Probe	rightFST_ev1_loc2-s1	6FAM- CAT CAC ACC AAA AgT TAg gCC CgA A-BHQ1	25
FatA(A)			
Forward primer	09-0-3249	ACA gAT gAA gTT Cgg gAC gAg TAC	24
Reverse primer	09-0-3251	CAg gTT gAg ATC CAC ATg CTT AAA TAT	27
Probe	09-QP-87	6-FAM-AAG AAG AAT CAT CAT GCT TC-MGBNFQ	20

FAM: 6-carboxyfluorescein; BHQ1: black hole quencher; MGB NFQ: minor groove binder non-fluorescent quencher.

5. References

1. Plant DNA C-values Database. Royal Botanic Gardens, Kew, http://data.kew.org/cvalues/

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