EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

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

Food and Feed Compliance

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

Validated Methods

This corrected version edited the FatA(A) probe sequence, adding two missing triplets (page 9).

Method development:

BASF Plant Science Company GmbH

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/