EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

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

Food and Feed Compliance

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

Validated Method

This corrected version edited the FatA(A) probe sequence, adding a missing triplet (page 8).

Method development:

Bayer Agriculture BV

1. General information and summary of the methodology

This protocol describes an event-specific real-time quantitative TaqMan® PCR (polymerase chain reaction) procedure for the determination of the relative content of oilseed rape event MON 94100 DNA to total oilseed rape DNA in a sample.

Template DNA extracted by means of suitable methods should be tested for quality and quantity prior to use in PCR assays. Tests for the presence of PCR inhibitors (e.g. monitor run of diluted series, use of DNA spikes) are also recommended to ensure the suitability of the extracted DNA.

For the detection of GM event MON 94100, an 89 bp fragment of the region spanning the 5' plant-to-insert junction in oilseed rape MON 94100 is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with FAM (6-carboxyfluorescein) as reporter dye at its 5' end, and MGBNFQ (Minor Groove Binder Non-Fluorescent Quencher) as non-fluorescent quencher dye at its 3' end.

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

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

2. Validation and performance characteristics

2.1 General

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

2.2 Collaborative trial

The method was validated in an international collaborative study by the European Union Reference Laboratory for GM Food and Feed (EURL GMFF). The study was undertaken with twelve participating laboratories in November - December 2021.

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

2.3 Limit of detection (LOD)

According to the method developer, the absolute LOD of the method is at least 5 haploid genome copies for MON 94100 event-specific method in 200 ng of total suitable oilseed rape DNA. The relative LOD was not assessed in the collaborative study.

2.4 Limit of quantification (LOQ)

According to the method developer, the relative LOQ of the method is at least 0.085 % (expressed as copies GM/total haploid genome copies) in 200 ng of total suitable oilseed rape DNA. The lowest relative GM content of the target sequence included in the collaborative trial was 0.1 % (mass fraction of GM-material).

2.5 Molecular specificity

The method exploits a unique DNA sequence in the region spanning the 5' insert-to-plant junction in oilseed rape MON 94100 and is therefore event-specific for the event MON 94100. This was confirmed in the validation study.

3. Procedure

3.1 General instructions and precautions

- The procedures require experience of working under sterile conditions.
- Laboratory organisation, e.g. "forward flow direction" during PCR-setup, should follow international guidelines, e.g. ISO 24276:2006.
- PCR reagents should be stored and handled in a separate room where no nucleic acids (with exception of PCR primers or probes) or DNA degrading or modifying enzymes have been handled previously. All handling of PCR reagents and controls requires dedicated equipment, especially pipettes.
- All the equipment should be sterilised prior to use and any residue of DNA should have been removed. All material used (e.g. vials, containers, pipette tips, etc.) must be suitable for PCR and molecular biology applications. They must be DNase-free, DNA-free, sterile and unable to adsorb protein or DNA.
- Filter pipette tips protected against aerosol should be used.
- Powder-free gloves should be used and changed regularly
- Laboratory benches and equipment should be cleaned periodically, with 10% sodium hypochlorite solution (bleach).
- Pipettes should be checked regularly for precision and calibrated, if necessary.

- All handling steps, unless specified otherwise, should be carried out at room temperature.
- In order to avoid repeated freeze/thaw cycles, aliquots should be prepared.

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

3.2.1 General

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

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

3.2.2 Calibration

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

Table 1. Copy number values of the standard curve samples

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

* Total nanograms are rounded to the integral value

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

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

3.2.3 Real-time PCR set-up

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

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

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

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

Table 3. Amplification reaction mixture in the final volume/concentration per reaction well for the oilseed rape *FatA(A)* assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
FatA(A) primer 1 (10 µM)	300 nM	0.75
FatA(A) primer 2 (10 µM)	900 nM	2.25
FatA(A) probe* (10 µM)	150 nM	0.38
Nuclease free water	-	5.12
DNA	≤200 ng	4
Total reaction volume:		25 µL

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

3. Mix well and centrifuge briefly.
4. Prepare two 0.5 mL reaction tubes (one for the oilseed rape MON 94100 and one for the *FatA(A)* system) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).

5. Add into each reaction tube the amount of reaction mix for 3.5 PCR repetitions (73.5 µL for both the MON 94100 oilseed rape and the *FatA(A)* systems). Add to each tube the correct amount of DNA for 3.5 PCR repetitions (14 µL DNA). The volume for the additional 0.5 repetition will ensure adequate volume when loading the samples. Vortex each tube for approx. 10 seconds. This step is mandatory to reduce to a minimum the variability among the repetitions of each sample.
6. Spin down the tubes. Aliquot 25 µL for the MON 94100 system and for the *FatA(A)* reference system in each well.
7. Place an optical cover on the reaction plate and briefly centrifuge the plate.
8. Place the reaction plate in the real-time PCR apparatus (possibly apply a compression pad, depending on the model), according to the manufacturer's instructions and your Standard Operating Procedures and start the run.
9. For both the MON 94100 and the *FatA(A)* systems, select FAM as reporter dye and MGBNFQ (or non-fluorescent) as quencher dye. Select ROX (6-carboxy-X-rhodamine) as the passive reference dye. Enter the correct reaction volume (25 µL).
10. Run the PCR with the cycling program described in Table 4.

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

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

*UNG: Uracil-N-glycosylase

3.3 Data analysis

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

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

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

c) Save the settings.

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

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

3.4 Calculation of results

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

The standard curves are generated both for the *FatA(A)* and the MON 94100 specific assays by plotting the Cq values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a linear regression line into these data.
Thereafter, the standard curves are used to estimate the DNA copy number in the unknown samples.

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

4. Equipment and Materials

4.1 Equipment

- Real-time PCR instrument for plastic reaction vessels (glass capillaries are not recommended for the described buffer composition) and appropriate analysis software
- 96-well reaction plates
- Optical caps/adhesion covers

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

4.2 Reagents

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

4.3 Primers and Probes

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

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

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

5. References

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