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Event-Specific Method for the Detection of Oilseed Rape Oxy-235 Using Real-Time PCR

European Union Reference Laboratory for Genetically Modified Food and Feed

2016

Joint Research Centre

EURL European Union Reference Laboratory for GM Food & Feed

European Commission
Joint Research Centre
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This publication is a Validated Methods, Reference Methods and Measurements Report
by the Joint Research Centre of the European Commission.

JRC100496

Printed in Italy

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE
Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit

Event-Specific Method for the Detection of Oilseed Rape Oxy-235 Using Real-Time PCR

Validation Report

5 May 2016

European Union Reference Laboratory for GM Food and Feed

Executive Summary

Further to the detection by UK authorities of the unauthorised oilseed rape Oxy-235 (Unique Identifier ACS-BNØ11-5) in conventional oilseed rape seed lots imported from France, a notification was sent to the European Commission and other Competent Authorities in October 2015.

The European Union Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF) received from Bayer CropScience AG an event-specific method for the detection of oilseed rape Oxy-235. The method developer declared this method specific for event Oxy-235 as it targets the junction between the transgenic insert and the 3' genomic region.

On 10th November 2015, the EURL GMFF received from Bayer CropScience AG Oxy-235 positive and negative control samples in the form of genomic DNA. The EURL GMFF carried out experiments, using the control samples received, in order to verify the specificity and the limit of detection (LOD) of the event-specific method.

The Oxy-235 event-specific assay generates a PCR amplification product of 119 bp. The testing of the specificity indicated that the Oxy-235 event-specific assay does not detect other genetically modified events under the conditions reported. The limit of detection (LOD) is established between 1 and 5 haploid genome copies of Oxy-235, corresponding to 0.003% mass/mass (m/m) with 200 ng of oilseed rape DNA per PCR reaction, or to 0.006% m/m with 100 ng oilseed rape DNA per reaction.

This report is available at http://gmo-crl.jrc.ec.europa.eu/emerg-unauth.html.

Quality assurance

The EURL GMFF is ISO 17025:2005 accredited certificate number: ACCREDIA 1172 (Flexible Scope for DNA extraction and qualitative/quantitative PCR). Accredited tests are available at http://www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7.

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

The EURL GMFF conducts its activities under the certification ISO 9001:2008 of the Institute for Health and Consumer Protection IHCP provided by SGS.

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Content

1. Introduction

UK authorities reported the detection of the unauthorised oilseed rape Oxy-235 (Unique Identifier ACS-BNØ11-5) in conventional oilseed rape seed lots imported from France. A notification was sent to the European Commission and to other Competent Authorities in October 2015.

The European Union Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF) received from Bayer CropScience AG a real-time PCR method for event-specific quantification of oilseed rape event Oxy-235.

The real-time PCR method targets the junction between the transgenic insert and the 3' genomic region in oilseed rape Oxy-235.

On 10th November 2015, further to a request from the European Commission, the EURL GMFF received from Bayer CropScience AG Oxy-235 positive and negative genomic DNA as control samples.

The EURL GMFF carried out experiments on the control samples received in order to verify the specificity and sensitivity (limit of detection) of the event-specific method received.

This report describes the outcome of the tests performed.

2. Experimental design, materials and methods

2.1 DNA concentration, purity and quality

The EURL GMFF received two DNA control samples from Bayer CropScience AG: a positive control sample consisting of 100% Oxy-235 homozygous genomic DNA, and a negative control sample, consisting of non-GM genomic DNA extracted from the parental line of Oxy-235. The concentration of the two DNA samples was determined with the PicoGreen dsDNA Quantitation Kit (Molecular Probes) on a Versafluor fluorometer (BioRad), on the basis of a four-point standard curve ranging from 1 to 500 ng/mL. DNA integrity was verified by 1% agarose-gel electrophoresis. The absence of DNA inhibitors in the DNA control samples was verified with an inhibition run on a dilution series[1]. The purity of the DNA control samples was verified using pre-spotted plates[2] and real-time PCR, to verify the absence of maize events 3272, 98140, Bt11, Bt176, DAS-40278-9, DAS-59122-7, GA21, MIR162, MIR604, MON810, MON863, MON87460, MON88017, MON89034, NK603, T25, TC1507; soybean events A2704-12, A5547-127, CV127, DP-305423-1, DP-356043-5, FG72, GTS 40-3-2, MON87701, MON89788; cotton events 281-24-236, 3006-210-23, GHB119, GHB614, LLCOTTON25, MON1445, MON15985, MON531, MON88913, T304-40 (positive control DNA) and for oilseed rape events GT73, Ms1, Ms8, Rf1, Rf2, Rf3, T45, Topas19/2; conventional maize, soybean, oilseed rape and cotton (positive and negative control DNA).

Absolute haploid genome copy number contents for subsequent analyses were calculated by dividing the sample DNA mass (nanograms) by the average 1C value for the oilseed rape genome (1.15 pg)[3].

2.2 Method protocol for the PCR analysis

The PCR method provided by Bayer CropScience AG is an event-specific real-time TaqMan® PCR method. Although the method can be used for the determination of the relative content of GM event Oxy-235 DNA to total oilseed rape DNA, the EURL GMFF describes in this report the verification of the method for its qualitative use.

For the detection of GM event Oxy-235, a 119-bp fragment of the region spanning the 3' insert-to-plant junction in oilseed rape Oxy-235 is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with FAM (6-carboxyfluorescein) as reporter dye at its 5' end and TAMRA (6-carboxytetramethylrhodamine) as quencher dye at its 3' end.

Further information on the Oxy-235 event-specific assay is available in Annex 1 ("Oxy-235 Detection Method").

2.3 Specificity

According to the method developer the method exploits a unique DNA sequence in the region spanning the junction region between the transgenic insert and the 3' genomic region in oilseed rape Oxy-235 and is therefore event-specific for the event Oxy-235.

2.3.1 Bioinformatics analyses

The specificity of the event-specific assay was verified by the EURL GMFF with bioinformatics analyses, on the basis of the sequence data provided by the method developer. Bioinformatics analyses were conducted by BLAST[4], with the sequences of the primers/probe and amplicon of the Oxy-235 event-specific assay developed by Bayer CropScience AG against local copies of the "nt" and "patents" databases, the sequences of the GMO events present in the Central Core Sequence Information System of the JRC, as well as the whole genomes of more than 100 plants (including *Brassica rapa*, *Glycine max*, *Oryza sativa*, *Solanum lycopersicum* and *Zea mays*) using the e-PCR prediction tool[5,6].

2.3.2 Experimental testing conducted by the method developer

The specificity of the event-specific assay was assessed by the method developer in duplicate real-time PCR reactions on approximately 200 ng genomic DNA from oilseed rape Ms1, Ms8, Rf1, Rf2, Rf3, Topas19/2, T45, RT73; rice LLRICE62; maize Bt176, Bt11, MON810, GA21, NK603, MON863, TC1507, event 3272, MIR604, DAS-59122-7, event 98140, MON88017, MON89034 T25; soybean A2704-12, A5547-127; FG72, 40-3-2, MON89788, DP-356043-5, DP-305423-1, MON87701; cotton LLCOTTON25, T304-40, GHB614, GHB119, MON1445, MON531, MON 15985, MON88913, 281-24-236x3006-210-23 and conventional oilseed rape, soybean, rice, cotton and maize.

2.3.3 Experimental testing conducted by the EURL GMFF

The EURL GMFF assessed the specificity of the Oxy-235 event-specific assay in triplicate real-time PCR reactions on DNA extracted from 100% GM oilseed rape MS8xRf3xGT73, Ms1, Rf1, Rf2, Topas19/2, T45, MON88302; carnation IFD-25958-3, FLO-40685-2, from 50% GM DP-073496-4 oilseed rape and from conventional oilseed rape, soybean, rice, cotton, maize, potato, sugar beet and carnation. The specificity was assessed with approximately 200 ng genomic DNA per reaction except for T45 oilseed rape and conventional potato (approx. 150 ng); Topas 19/2 oilseed rape (approx. 130 ng) and conventional carnation (approx. 100 ng). The DNA amounts tested are all well above 10 000 haploid genome copies, thus fully satisfying the requirement to test specificity of a PCR assay on a minimum of 2500 copies of non-target DNA outlined in the document on Minimum Performance Requirements for analytical methods of GMO testing[7]. The same DNA samples were also tested with their respective target-taxon reference systems, i.e. *hmg* for maize, Le1 for soybean, *cruA* for oilseed rape, adhC for cotton, GS for sugar beet, PLD for rice and UGPase for potato, according to the methods submitted to the EURL GMFF under Regulation (EC) No 1829/2003.

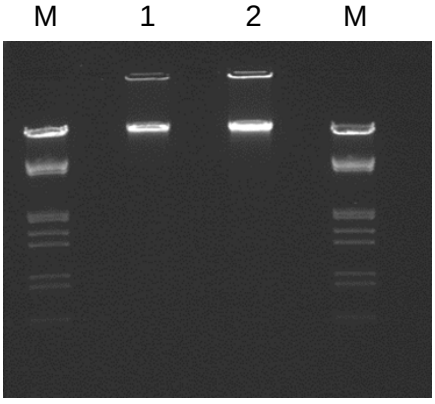
2.4 Limit of Detection

To determine the limit of detection (LOD), defined as the amount of analyte that is detected at least 95% of the times, thus ensuring ≤5% false negative results, 60 replicates per level were tested by the EURL GMFF. The LOD was set at the lowest concentration yielding at least 59 positive results[8,9]. The oilseed rape Oxy-235 DNA sample received by the EURL GMFF was tested at 1, 5, 10 and 20 copies per reaction, where the 1 copy per reaction should yield approximately 36% negative results, if the DNA dilution is performed correctly[10].

3. Results

3.1 DNA concentration, purity and quality

The concentration of the positive and negative control DNA samples were quantified as 89.3 ng/µL and 179.5 ng/µL, respectively. The agarose gel electrophoresis showed a high molecular weight band, with no signs of DNA degradation (Figure 1).



M = *Lambda/EcoRI+HindIII* DNA marker
Lane 1= Oxy-235 positive control DNA
Lane 2= Conventional oilseed rape negative control DNA

Figure 1. Agarose gel electrophoresis of Oxy-235 positive (1) and negative (2) control DNAs.

Inhibition run results demonstrated that the control samples were not inhibited at 300 ng per reaction.
Purity verification confirmed the absence of detectable contamination from the events and species listed under paragraph 2.1.

The control samples were therefore considered suitable for the purpose of determining the LOD and specificity of the Oxy-235 event-specific method.

3.2 Molecular specificity

3.2.1 Bioinformatics analyses

The "MDB110" (forward) primer binds in the insert, in a region that contains the T-DNA border region. The probe "TM033" binds near the junction region, spanning from the T-DNA border region in the transgenic insert to the 3' genomic region. The "SHA016" (reverse) primer-binding site was found in the genomic border adjacent to the insertion.
According to the bioinformatics analysis, the amplicon size is expected to be 119 bp, consistent to what is reported by the method developer.

The sequence of the amplicon was analysed by BLAST[4] against local copies of the "nt" and "patents" databases, and no significant similarity was found with any other published sequence. Using the e-PCR prediction tool[5,6] one potential amplicon was identified: when using only 2x the forward primer "MDB110" - located in the very common T-DNA region - e-PCR predicts a potential amplicon for GM event FLO-40685-2 carnation 3rd insertion site, based on the dossier provided by Florigene Limited, due to the palindromic structure of this insert of the carnation event. The probe "TM033" also matches 100% for this 168 bp amplicon. It is not known whether the potential target in the 3rd insertion site of FLO-40685-2 is stable, or might be lost. The generation of a potential amplicon in Florigene FLO-40685-2 carnation was therefore experimentally verified (paragraph 3.2.3).

3.2.2 Experimental testing conducted by the applicant

According to the method developer the Oxy-235 event-specific assay did not react with any sample except the positive control.

3.2.3 Experimental testing conducted by the EURL GMFF

The experimental testing performed by the EURL GMFF confirmed that the Oxy-235 event-specific assay did not react with any of the DNA samples tested, except the positive control. Results of the specificity test are shown in Table 1.

Table 1. Results of experimental testing of Oxy-235 event-specific assay specificity

Event Name	Species	Oxy-235 assay Cq value (SD)	Taxon-specific assay Cq value (SD)/ Ref system
Oxy-235	oilseed rape	20.66 (0.10)	22.05 (0.11) / *cruA*
Ms8xRf3xGT73	oilseed rape	-	23.29 (0.14) / *cruA*
Ms1	oilseed rape	-	22.19 (0.12) / *cruA*
Rf1	oilseed rape	-	22.92 (0.15) / *cruA*
Rf2	oilseed rape	-	23.35 (0.13) / *cruA*
Topas 19/2	oilseed rape	-	22.42 (0.02) / *cruA*
T45	oilseed rape	-	22.91 (0.15) / *cruA*
MON88302	oilseed rape	-	22.21 (0.13) / *cruA*
DP-073496-4	oilseed rape	-	22.18 (0.21) / *cruA*
IFD-25958-3	carnation	-	+ (conventional PCR) / ANS
FLO-40685-2	carnation	-	+ (conventional PCR) / ANS
-	oilseed rape	-	22.64 (0.19) / *cruA*
-	soybean	-	20.90 (0.15) / Le1
-	rice	-	18.58 (0.18) / PLD
-	Cotton	-	21.54 (0.13) / adhC
-	Maize	-	21.76 (0.10) / *hmg*
-	Potato	-	13.06 (0.52) / UGPase
-	sugar beet	-	24.55 (2.40) / GS
-	Carnation	-	+ (conventional PCR) / ANS

To investigate the possibility that an amplicon is produced when the method is used on a sample containing FLO-40685-2 carnation GM event, Oxy-235 primers were assessed in real-time PCR with TaqMan® Universal PCR Master Mix (Applied Biosystems) with an annealing/ extension temperature gradient ranging from 54°C to 60°C. No amplification was identified, except for positive controls that amplified as expected; no template controls were negative.

The Oxy-235 event-specific assay can thus be considered specific, under the conditions tested.

3.3 Limit of detection

The sensitivity of the Oxy-235 event-specific assay was evaluated through the determination of the LOD, assessed on the Oxy-235 positive control DNA sample received by the EURL GMFF from Bayer CropScience AG. Table 2 reports the results of the LOD determination.

Table 2. Result of the LOD for the Oxy-235 event-specific assay

Oxy-235 copies / reaction	Positives / total reactions (% negatives)	Average Cq value (SD)
20	60/60 (0%)	35.16 (0.42)
10	59/59 (0%)*	36.36 (0.62)
5	60/60 (0%)	37.72 (0.84)
1	38/60 (36.7%)	38.34 (1.00)

* one replicate was discarded

Considering the results presented in Table 2, the LOD of the Oxy-235 event-specific assay is between 1 and 5 haploid genome copies of Oxy-235, based on the 1C value for the oilseed rape genome (1.15 pg)[3]. The reactions containing 1 copy of Oxy-235 per reaction yielded 36.7% negative results, in line with an expected theoretical value of 36%[10], therefore confirming the correctness of the dilutions used for the determination of the LOD of the assay.

4. Conclusions

Based on the bioinformatics analyses and experimental tests described above, the EURL GMFF concludes that the specificity and sensitivity (LOD) of Oxy-235 event-specific method developed by Bayer CropScience AG is meeting the method performance criteria determined by the ENGL and the EURL GMFF.

The potential amplification of carnation FLO-40685-2 event indicated by bioinformatics analyses does not occur experimentally.

The LOD of the method is established between 1 and 5 copies of Oxy-235, which corresponds to 0.003% in mass/mass (m/m) when 200 ng oilseed rape DNA are used per reaction, or to 0.006% m/m with 100 ng oilseed rape DNA per reaction.

In view of its specificity and sensitivity the method provided by Bayer CropScience AG for the detection of oilseed rape OXY-235 is fit for purpose, i.e. it can detect very low concentrations of GMO contamination.

5. References

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Annex 1: Event-specific Method for the Detection of Oilseed Rape Oxy-235 using Real-time PCR

Method development:

Bayer CropScience AG

Method verification:

European Reference Laboratory for GM Food and Feed
Joint Research Centre of the European Commission

General information and summary of the methodology

This protocol describes an event-specific real-time TaqMan® PCR (polymerase chain reaction) method for the detection of oilseed rape event Oxy-235.

The method was optimised for suitable DNA extracted from mixtures of genetically modified and conventional oilseed rape seeds. Specificity and limit of detection of the Oxy-235 event-specific assay were assessed by the applicant and the EURL GMFF.

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 specific detection of oilseed rape event Oxy-235, a 119-bp fragment of the region spanning the 3′ insert-to-plant junction in oilseed rape Oxy-235 event is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with the fluorescent dye FAM (6-carboxyfluorescein), as a reporter at its 5′ end, and the dye TAMRA (6-carboxytetramethylrhodamine) as a quencher at its 3′ end.

Protocol

General instructions and precautions

- The procedures require working under sterile conditions.
- Laboratory organization, 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 separate rooms 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
- Laboratory benches and equipment should be cleaned with 10% sodium hypochloride solution (bleach).
- Pipettes should be checked for precision and calibrated, if necessary.

- All handling steps, unless specified otherwise, should be carried out at room temperature (approximately 18-20 °C).

Real-time PCR for detection of oilseed rape event Oxy-235

The method is developed for a total volume of 25 µL per reaction mixture with the reagents as listed in Table 1 and Table 2.

Real-time PCR set-up

1. Thaw, mix and spin down all the components needed for the run. Keep thawed reagents on ice.
2. To prepare the amplification reaction mixture add the components listed in Table 1 in a reaction tube on ice in the order mentioned below (except DNA).

Table 1. Amplification reaction mixture in the final volume/concentration per reaction well for the Oxy-235 method.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
SHA016 primer (10 µM)	400 nM	1.0
MDB110 primer (10 µM)	400 nM	1.0
TM033 probe (10 µM)	200 nM	0.50
Nuclease free water	-	5.0
DNA (max 300 ng)	-	5
Total reaction volume:		25 µL

3. Vortex for approx. 5 seconds and spin down.
4. Prepare one reaction tube for each DNA sample to be tested.
5. Add to each reaction tube the correct amount of reaction mix for 3.5 PCR repetitions (70 µL). 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. 5 seconds. This step is mandatory to reduce to a minimum the variability among the repetitions of each sample.
6. Spin down the tubes in a micro-centrifuge. Aliquot 25 µL in each well. Seal the reaction plate with optical cover or optical caps. Centrifuge the plate at low speed (e.g. approximately 250 x *g* for 1 minute at 4 °C) to spin down the reaction mixture.
7. Place the plate into the instrument.
8. Run the PCR with the cycling program described in Table 3.

Table 3. Cycling program for Oxy-235 method.

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

Data analysis

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

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

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

c) Save the settings.

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

Equipment and Materials

Equipment

- Real-time PCR instrument for plastic reaction vessels (glass capillaries are not recommended for the described buffer composition)
- Plastic reaction vessels suitable for real-time PCR instrument (enabling undisturbed fluorescence detection)
- Software for analysis of the runs (mostly integrated in the software of the real-time PCR instrument)
- Microcentrifuge
- Micropipettes
- Centrifuge for PCR plates
- Vortex
- Rack for reaction tubes
- 0.2/1.5/2.0 mL reaction tubes

Reagents

- TaqMan® Universal PCR Master Mix. Applied Biosystems Part No 4304437.

Primers and Probes

Table 5. Primers and probes for the Oxy-235 method

Name		DNA Sequence (5' to 3')	Length (nt)
Oxy-235			
Forward primer	SHA016 primer	5' – CgC CgT gCT TCA TAA CCg – 3'	18
Reverse primer	MDB110 primer	5'– CgA ATT Tgg CCT gTA gAC CTC AAT TgC gAg C – 3'	31
Probe	TM033 probe	FAM 5' – CAg CAT CAT CAC ACC AAA AgT TAg gCC – 3' TAMRA	27

FAM: 6-carboxyfluorescein; TAMRA: carboxytetramethylrhodamine

References

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

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European Commission
Joint Research Centre – Institute for Health and Consumer Protection

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Author(s): European Union Reference Laboratory for GM Food and Feed

2016 – 20 pp. – 21.0 x 29.7 cm

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JRC100496

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