



Event-specific Method for the Quantification of Oilseed Rape Line Rf3 Using Real-time PCR

Protocol
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Corrected version 1 - 25/02/2013 (see page 2)

Method development:
Bayer CropScience

Collaborative trial:
Joint Research Centre – European Commission
Biotechnology & GMOs Unit

Quality assurance

The EU-RL 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 EU-RL GMFF quality system.

The EU-RL GMFF is also ISO 17043:2010 accredited (Proficiency test provider) and apply the corresponding procedures and processes for the management of ring trials during the method validation.

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Correction from the previous version:

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Page 4 §1 :

For specific detection of event Rf3 genomic DNA, a 139-bp fragment of the recombination region between the insert and the plant genome (located at the 5' flanking region) changed by
For specific detection of event Rf3 genomic DNA, a 139-bp fragment of the recombination region between the insert and the plant genome (located at the 3' flanking region)

Page 8 Table 2 :

KVM084 For primer
DPA165 Rev primer
changed by
KVM084 Reverse primer
DPA165 Forward primer

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1. General information and summary of the methodology

This protocol describes an event-specific real-time quantitative TaqMan[®] PCR procedure for the determination of the relative content of event Rf3 DNA to total oilseed rape (OSR) DNA in a sample.

The PCR assay was optimised for the use in real-time PCR instruments for plastic reaction vessels. Glass capillaries are not recommended for the buffer composition described in this method.

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

For specific detection of event Rf3 genomic DNA, a 139-bp fragment of the recombination region between the insert and the plant genome (located at the 3' flanking region) is amplified using two specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with two fluorescent dyes: FAM as a reporter dye at its 5' end and TAMRA as a quencher dye at its 3' end.

For relative quantitation of event Rf3 DNA, an oilseed rape-specific reference system amplifies a 101-bp fragment of the *Cruciferin A* gene (*CruA*), an oilseed rape endogenous gene, using a pair of specific primers and a *CruA* gene-specific probe labelled with VIC and TAMRA as described above.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Ct" value. For quantitation of the amount of event Rf3 DNA in a test sample, the normalised Δ Ct values of the calibration samples are used to calculate by linear regression a reference curve Δ Ct-formula. The normalised Δ Ct values of the unknown samples are measured and, by means of the reference Δ Ct-formula, the relative amount of Rf3 event DNA is estimated.

2. Validation status and performance characteristics

2.1 General

The method has been optimised for suitable DNA extracted from oilseed rape grains or seeds.

The reproducibility and trueness of the method were tested through collaborative trial using DNA samples at different GMO contents.

2.2 Collaborative trial

The method was validated in an international collaborative trial organized by the Joint Research Centre (JRC) of the European Commission, Community Reference Laboratory for GM Food and Feed. The study was undertaken with 13 laboratories in July-August 2006.

Each participant received twenty unknown samples containing Rf3 oilseed rape genomic DNA at five GM contents, between 0.1 % and 3.6 %.

Each test sample was analysed by PCR in three repetitions. The study was designed as a blind quadruplicate collaborative trial; each laboratory received each level of GM Rf3 in four unknown samples. Two replicates of each GM level were analysed on the same PCR plate.

A detailed validation report can be found under <http://gmo-crl.jrc.it/statusofdoss.htm>

2.3 Limit of detection

According to the method developer, the relative LOD of the method is at least 0.045% in 200 ng of total oilseed rape DNA. The relative LOD was not assessed in a collaborative trial. The lowest relative GM content of the target sequence included in collaborative trial was 0.1%.

2.4 Limit of quantitation

According to the method developer, the relative LOQ of the method is at least 0.09% in 200 ng of total oilseed rape DNA. The lowest relative GM content of the target sequence included in collaborative trial was 0.1%.

2.5 Molecular specificity

The method utilises a unique DNA sequence of the recombination region between the insert and the plant genome. The sequence is specific to Rf3 event and thus imparts event-specificity to the method.

The specificity of the forward and reverse oligonucleotide primer was experimentally tested in duplicated end-point PCRs against DNA extracted from plant materials containing the specific targets of maize T25, MON810, Bt11, Bt176, GA21, NK603, CBH351, Roundup

Ready Soybean, soybean A2704-12, oilseed rape Ms1, Ms8, Rf1, Rf2, Rf3, Topas19/2, T45, LLCotton25 and LLRice62.

None of the GM-lines, except the positive control Rf3, yielded detectable amplicons in duplicate experiments.

3. Procedure

3.1 General instructions and precautions

- The procedures require experience of working under sterile conditions.
- Laboratory organisation, e.g. “flow direction” during PCR-setup, should follow the guidelines given by relevant authorities like e.g. ISO, CEN, Codex alimentarius commission.
- PCR-reagents should be stored and handled in a separate room and freezer and in equipment 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 used must be sterilised prior to use and any residue of DNA has to be 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 shall not adsorb protein or DNA.
- In order to avoid contamination, filter pipette tips protected against aerosol should be used.
- Use only powder-free gloves and change them frequently.
- Clean laboratory benches and equipment periodically with 10% sodium hypochloride solution (bleach).
- Pipettes should be checked regularly for precision and calibrated, if necessary.
- All handling steps - unless specified otherwise - shall be carried out at 0 - 4°C.
- In order to avoid repeated freeze/thaw cycles aliquots should be prepared.

3.2 Real-time PCR for quantitative analysis of Rf3 oilseed rape

3.2.1 General

The PCR set-up for the taxon specific target sequence (*CruA*) and for the GMO (Rf3) target sequence should be carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.

The use of maximum 200 ng of template DNA per reaction well is recommended.

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.

3.2.2 Calibration

The calibration curve consists of five samples containing fixed percentages of Rf3 DNA in a total amount of 200 ng oilseed rape DNA. The GM content of the standard samples ranges from 3.6% to 0.09%.

A calibration curve is produced by plotting the ΔC_t -values of calibration samples against the logarithm of the respective GM % contents; the slope (a) and the intercept (b) of the calibration curve ($y = ax + b$) are then used to calculate the mean % GM content of the blind samples based on their normalised ΔC_t values.

3.2.3 Real-time PCR set-up

1. Thaw, mix gently and centrifuge the required amount of components needed for the run. **Keep thawed reagents at 1-4°C on ice.**
2. In two reaction tubes (one for Rf3 system and one for the *CruA* system) on ice, add the following components (Tables 1 and 2) in the order mentioned below (except DNA) to prepare the master mixes.

Table 1. Amplification reaction mixture in the final volume/concentration per reaction well for the OSR *CruA* reference system.

Component	Final concentration	µl/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
MBD510 For primer (10 µM)	200 nM	0.5
MBD511 Rev primer (10 µM)	200 nM	0.5
TM003 probe (10 µM)	200 nM	0.5
Nuclease free water	#	6
Template DNA (max 200 ng)	#	5
Total reaction volume:		25

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the Rf3 specific system.

Component	Final concentration	µl/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
KVM084 Reverse primer (10 µM)	400 nM	1
DPA165 Forward primer (10 µM)	400 nM	1
TM010 Probe (10 µM)	200 nM	0.5
Nuclease free water	#	5
Template DNA (max 200 ng)	#	5
Total reaction volume:		25

- Mix gently and centrifuge briefly.
- Prepare two reaction tubes (one for the Rf3 and one for the *CruA* master mix) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
- Add to each reaction tube the correct amount of master mix (e.g. 20 x 3 = 60 µl master mix for three PCR repetitions). Add to each tube the correct amount of DNA (e.g. 5 x 3 = 15 µl DNA for three PCR repetitions). Vortex each tubes for approx. 10

sec. This step is mandatory to reduce the variability among the repetitions of each sample to a minimum.

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 room temperature) to spin down the reaction mixture.
7. Place the plate into the instrument.
8. Run the PCR with cycling conditions described in Table 3:

Table 3. Cycling program for oilseed rape Rf3/*CruA* systems

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

3.3 Data analysis

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

a) Set the threshold: display the amplification curves of one system (e.g. Rf3) 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 Ct values. 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 geometric 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) Repeat the procedure described in a) and b) on the amplification plots of the other system (e.g. *CruA* system).

e) Save the settings and export all the data into an Excel file 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 Ct-values for each reaction.

The Reference Δ Ct-curve is generated by plotting the Δ Ct-values measured for the calibration points against the logarithm of the GM% content, and by fitting a linear regression line into these data.

Thereafter, the reference Δ Ct-curve formula is used to estimate the relative amount (%) of Rf3 event in the unknown samples of DNA.

4. Materials

4.1 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 run analysis (mostly integrated in the software of the real-time PCR instrument)
- Microcentrifuge
- Micropipettes
- Vortex
- Rack for reaction tubes
- 1.5/2.0 ml tubes

4.2 Reagents

- TaqMan® Universal PCR Master Mix (2X). Applied Biosystems Part No 4304437

4.3 Primers and Probes

Name	Oligonucleotide DNA Sequence (5' to 3')
<i>Rf3 target sequence</i>	
KVM084	5' –AGC ATT TAG CAT GTA CCA TCA GAC A-3'
DPA165	5' – CAT AAA GGA AGA TGG AGA CTT GAG -3'
TM010 (Probe)	FAM 5'- CGC ACG CTT ATC GAC CAT AAG CCC A -3' TAMRA
<i>Reference gene CruA target sequence</i>	
MDB510	5' – GGC CAG GGT TTC CGT GAT -3'
MDB511	5' – CCG TCG TTG TAG AAC CAT TGG -3'
TM003 (Probe)	VIC 5' – AGT CCT TAT GTG CTC CAC TTT CTG GTG CA -3' TAMRA

5. References

Arumuganathan, K., Earle, E.D. (1991). Nuclear content of some important plant species. *Plant Mol Biol Reporter* 9, 208-218.