Event-specific Method for the Quantification of Oilseed Rape Topas 19/2 Using Real-time PCR

Protocol

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Content

1. GENERAL INFORMATION AND SUMMARY OF THE METHODOLOGY 4

2. VALIDATION AND PERFORMANCE CHARACTERISTICS 5

2.1 GENERAL 5
2.2 IN-HOUSE VALIDATION 5
2.3 LIMIT OF DETECTION (LOD) 5
2.4 LIMIT OF QUANTIFICATION (LOQ) 5
2.5 MOLECULAR SPECIFICITY 5

3. PROCEDURE 6

3.1 GENERAL INSTRUCTIONS AND PRECAUTIONS 6
3.2 REAL-TIME PCR FOR QUANTITATIVE ANALYSIS OF TOPAS 19/2 7
3.2.1 General 7
3.2.2 Calibration 7
3.2.3 Real-time PCR set-up 7
3.3 DATA ANALYSIS 9
3.4 CALCULATION OF RESULTS 9

4. MATERIALS 10

4.1 EQUIPMENT 10
4.2 REAGENTS 10
4.3 PRIMERS AND PROBES 10

5. REFERENCES 11

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 event Topas 19/2 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 use in the PCR assay. Tests for the presence of PCR inhibitors (e.g. monitor run of diluted series, use of DNA spikes) are recommended.

The TaqMan® real-time PCR method has been developed to determine the amount of DNA of the Topas 19/2 event relative to oilseed rape (OSR) DNA present in a sample. The real-time PCR method has been optimized for use in an ABI Prism® 7700 sequence detection system. Other systems may be used, but thermal cycling conditions must be verified.

For specific detection of event Topas 19/2 genomic DNA, a 95-bp fragment of the region of integration between the insert and the plant genome is amplified using specific primers. PCR products are measured at each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with two fluorescent dyes: FAM (6-carboxyfluorescein) as a reporter dye at its 5′ end and TAMRA (6-carboxytetramethylrhodamine) as a quencher dye at its 3′ end.

For relative quantification of event Topas 19/2 DNA, an oilseed rape-specific reference system amplifies a 101 bp fragment of the *cruciferin A* gene (*cruA*), an oilseed rape endogenous gene, using 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 quantification of the amount of event Topas 19/2 DNA in a test sample, the normalised ΔCt values of the calibration samples are used to calculate, by linear regression, a standard curve ΔCt-formula. The normalised ΔCt values of the unknown samples are measured and, by means of the regression formula, the relative amount of Topas 19/2 event DNA is estimated.

2. Validation and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from oilseed rape (OSR) seeds, grains or leaves.

The repeatability and trueness of the method were tested through an in-house validation study using DNA samples at different GM contents.

2.2 In-house validation

The method was in-house validated by the European Union Reference Laboratory for GM Food and Feed (EURL-GMFF). The study was undertaken performing eight real-time PCR runs on ABI Prism® 7700, and seven runs on ABI 7900HT.

The samples contained Topas 19/2 oilseed rape genomic DNA at five GM contents, ranging from 0.15% to 3.30%.

Each test sample was analysed twice by PCR in triplicate. The two replicates of each GM level (six wells) were analysed on the same PCR plate.

A detailed validation report is available at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm

2.3 Limit of detection (LOD)

According to the method developer, the relative LOD of the method is ≤ 0.045% in 200 ng of total oilseed rape DNA. The relative LOD was not assessed in the in-house validation. The lowest relative GM content included in the study was 0.15%.

2.4 Limit of quantification (LOQ)

According to the method developer, the relative LOQ of the method is ≤ 0.09% in 200 ng of total oilseed rape DNA. The lowest relative GM content of the target sequence included in the in-house validation was 0.15%.

2.5 Molecular specificity

According to the method developer, the method exploits a unique DNA sequence in the region between the insert and the plant genome. The sequence is specific to Topas 19/2 event and thus imparts event-specificity to the method.

The specificity of the method was experimentally tested in duplicated end-point PCRs against DNA extracted from plant materials containing: rice LLRICE62; OSR Ms1, Ms8, Rf1, Rf2, Rf3, Topas 19/2, T45; soybean A2704-12, GTS 40-3-2; cotton LL25; maize T25, MON810, BT11, BT176, GA21, NK603, CBH351.

None of the GM lines tested, except the positive control Topas 19/2, yielded detectable amplicons in duplicate experiments.

3. Procedure

3.1 General instructions and precautions

- The procedures require experience working under sterile conditions.
- Laboratory organization, e.g. "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 used should be sterilised prior to use and any residue of DNA should be removed. All material used (e.g. vials, containers, pipette tips, etc.) should be suitable for PCR and molecular biology applications; it should 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 frequently.
- 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 0 - 4 °C.
- In order to avoid repeated freeze/thaw cycles aliquots should be prepared.

3.2 Real-time PCR for quantitative analysis of Topas 19/2

3.2.1 General

The PCR set-up for the taxon-specific target sequence (*cruA*) and for the GMO (Topas 19/2) 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 various amounts of Topas 19/2 DNA in a total amount of 200 ng oilseed rape DNA (5 µL per reaction well at 40 ng/µL). The GM content of the calibration samples ranges from 3.60% to 0.09% (GM% calculated considering the 1C value for oilseed rape genome as 1.15 pg)[1].

A calibration curve is produced by plotting the ΔCt values of the 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 normalized ΔCt 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. To prepare the amplification reaction mixtures add the following components (Tables 1 and 2) in two reaction tubes (one for the Topas 19/2 assay and one for the *cruA* assay) on ice in the order mentioned below (except DNA).

Table 1. Amplification reaction mixture in the final volume/concentration per reaction well for the *cruA* assay.

Component	Final concentration	µL / reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
MDB510– F primer (10 µM)	200 nM	0.50
MDB511 – R primer (10 µM)	200 nM	0.50
TM003 TaqMan® probe (10 µM)	200 nM	0.50
Nuclease free water	#	6.00
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 Topas 19/2 assay.

Component	Final concentration	µL / reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
MDB685 -F primer (10 µM)	400 nM	1.00
KVM180-R primer (10 µM)	400 nM	1.00
TM029 TaqMan® probe (10 µM)	200 nM	0.50
Nuclease free water	#	5
Template DNA (max 200 ng)	#	5
Total reaction volume:		25

3. Mix gently and centrifuge briefly.
4. Prepare two reaction tubes (one for the Topas 19/2 and one for the *cruA* master mixes) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
5. Add to each reaction tube the correct amount of master mix (e.g. 20 µL x 3 = 60 µL Master Mix for three PCR repetitions). Add to each tube the correct amount of DNA (e.g. 5 µL x 3 = 15 µL DNA for three PCR repetitions). Vortex each tubes for approx. 10 sec. This step is mandatory to reduce to a minimum the variability among the repetitions of each sample.
6. Spin down the tubes in a microcentrifuge. 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 Topas 19/2 and *cruA* assays.

Step	Stage		T (°C)	Time (s)	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

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

a) Set the threshold: display the amplification curves of one assay (e.g. Topas 19/2) 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 (or apply)" 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 assay (e.g. *cruA*).

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 value for each reaction.

The standard ΔCt curve is generated by plotting the ΔCt 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 standard ΔCt curve regression formula is used to estimate the relative amount (%) of Topas 19/2 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
- 0.2/1.5/2.0 mL reaction tubes

4. 2 Reagents

- TaqMan® Universal PCR Master Mix (2x), Applied Biosystems Cat. 4304437
- Nuclease free water, Promega Cat. P119C (or equivalent)

4.3 Primers and Probes

Name	Oligonucleotide DNA Sequence (5' to 3')
Topas 19/2	
MDB685 -F primer	5'– GTT GCG GTT CTG TCA GTT CC –3'
KVM180-R primer	5'– CGA CCG GCG CTG ATA TAT GA–3'
TM029 TaqMan® probe	FAM[a] 5'– TCC CGC GTC ATC GGC GG –3' TAMRA[b]
cruA	
MDB510 – F primer	5' – GGC CAG GGT TTC CGT GAT–3'
MDB511 – R primer	5' – CCG TCG TTG TAG AAC CAT TGG –3'
TM003 TaqMan® probe	VIC 5'– AGT CCT TAT GTG CTC CAC TTT CTG GTG CA–3' TAMRA

a) 6-carboxyfluorescein
b) 6-carboxytetramethylrhodamine

5. References

1. Arumuganathan K, Earle ED. 1991. Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter* 9: 208-218.