Event-specific Method for the Quantification of Oilseed Rape MS1 using Real-time PCR

Protocol

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Content

1. General information and summary of the methodology

This protocol describes an event-specific quantitative real-time PCR procedure for the determination of the relative content of event MS1 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 was developed to determine the amount of DNA of the event MS1 relative to the total oilseed rape (OSR) DNA present in a sample. The real-time PCR method was optimized for use in an ABI Prism® 7700 sequence detection system.

For the detection of event MS1, a 187 bp fragment of the integration region at the 3'-insert-to-plant junction 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-carboxytetramethyilrhodamine) as a quencher dye at its 3' end.

For relative quantification of MS1, an oilseed rape-specific reference system amplifies a 101 bp fragment of the endogenous gene *cruciferin A* gene (*cruA*) using specific primers and a *cruA* 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 quantifying the event MS1 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 MS1 event DNA is estimated.

2. Validation and performance characteristics

2.1 General

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

The precision 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 validated in-house by the EURL-GMFF. The study was undertaken performing eight real-time PCR runs on ABI 7900HT and eight further runs on ABI Prism® 7700.

The samples contained MS1 oilseed rape genomic DNA at five GM contents, ranging from 0.15% to 3.30%.

Each test sample was analyzed twice in triplicate per PCR run. The two replicates of each GM level (six wells) were analyzed on the same PCR plate with both amplification systems. A total of 16 replicates (each replicate being the average of three wells per amplification system) were analysed to compute the validation metrics and evaluate method performance.

A detailed validation report can be found 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 at least 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 at least 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 of the recombination region between the insert and the plant genome. The sequence is specific to MS1 event and thus imparts event-specificity to the method.

The specificity of the event-specific assay was assessed by the applicant in duplicated end-point PCR tests at 35 cycles against 20 ng of genomic DNA extracted from plant materials containing: rice LLRICE62; oilseed rape MS1, MS8, RF1, RF2, RF3, Topas 19/2, T45; soybean A2704-12 and GTS 40-3-2; cotton LLCotton25; maize T25, MON810, Bt11, Bt176, GA21,NK603 and CBH351.

None of the GM lines tested in duplicate experiment, except the positive control MS1, yielded detectable amplicons in ethidium bromide stained agarose gel electrophoresis.

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 and freezer 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 MS1

3.2.1 General

The PCR set-up for the taxon specific target sequence (*cruA*) and for the GMO (MS1 event) target sequence is 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 percentages of MS1 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 standard 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 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 MS1 assay and the other 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* reference assay

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
MDB510– primer forward (10 µM)	200 nM	0.50
MDB511 – primer reverse (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 MS1 assay

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
MLD025 primer forward (10 µM)	400 nM	1.00
MDB175 primer reverse (10 µM)	400 nM	1.00
TM030 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 MS1 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 MS1 and *cruA*

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

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. MS1) 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 system (e.g. *cruA* assay).

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 MS1 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

4.3 Primers and Probes

Name	Oligonucleotide DNA Sequence (5' to 3')
MS1	
MLD025 – primer forward	5'– ACG CTG CGG ACA TCT ACA TT –3'
MDB175 primer reverse	5'– CTA GAT CGG AAG CTG AAG ATG G –3'
TM030 TaqMan® probe	FAM 5'– CTC ATT GCT GAT CCA CCT AGC CGA CTT –3' TAMRA
cruA	
MDB510 – primer forward	5' – GGC CAG GGT TTC CGT GAT–3'
MDB511 – primer reverse	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

5. References

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