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COMMUNITY REFERENCE LABORATORY FOR GM FOOD AND FEED

Event-specific Method for the Quantification of Oilseed Rape Line RT73 Using Real-time PCR

Protocol

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Collaborative trial:

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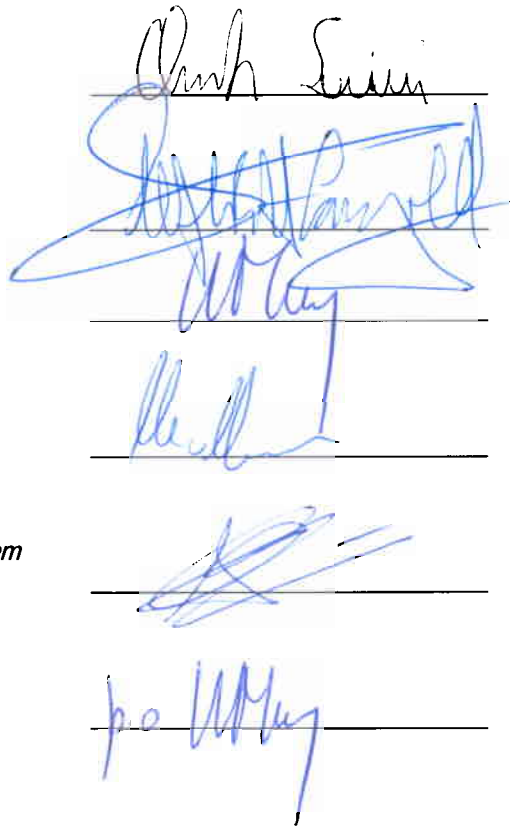

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Contents

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 RT73 DNA to total oilseed rape (OSR) DNA in a sample.

The PCR assay was optimised for 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 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 RT73 genomic DNA, a 108-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 RT73 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 quantification of the amount of event RT73 DNA in a test sample, event RT73 and *CruA* Ct values are determined for the sample. Standard curves are then used to calculate the relative content of event RT73 DNA to total oilseed rape DNA.

2. Validation status and performance characteristics

2.1 General

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

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

2.2 Collaborative trial

The method was validated in an international collaborative trial by the Joint Research Centre (JRC) of the European Commission. The study was undertaken with 14 laboratories in October-November 2006.

Each participant received twenty unknown samples containing RT73 oilseed rape genomic DNA at five GM contents, between 0.1 % and 8.0 %.

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 RT73 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 (LOD)

According to the data provided by the applicant, the relative LOD of the method is at least 0.04% in 200 ng of total oilseed rape DNA.

The relative LOD was not assessed in the collaborative trial.

2.4 Limit of quantification (LOQ)

According to the data provided by the applicant, the relative LOQ of the method is 0.085% in 200 ng of total oilseed rape DNA.

The lowest relative GM content of the target sequence included in the international 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 RT73 event and thus imparts event-specificity to the method.

The specificity of the event-specific system was analysed by the RT73-specific real-time PCR against DNA extracted from plant materials containing the specific GM targets of oilseed rape RT73, RT200, maize GA21, NK603, MON810, MON863, wheat MON71800, soybean 40-3-2, cotton 15985 and non-transgenic oilseed rape, maize, wheat, soybean, cotton, amaranth, rice, barley, lentil, millet, oat, peanut, pine nuts, rye berries and sunflower.
None of the non-transgenic plant lines and none of the GM lines, except the positive control RT73, provided positive signals.

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 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 unable to 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 RT73 oilseed rape

3.2.1 General

The PCR set-up for the taxon specific target sequence (*CruA*) and for the GMO (RT73) 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 for the taxon specific reaction mixture and for a total volume of 50 µl for the RT73 specific reaction mixture, with the reagents as listed in Table 1 and Table 2.

3.2.2 Calibration

The calibration curves consist of five samples. The first point of the calibration curves is a 10% RT73 in non-GM oilseed rape DNA for a total of 200 ng of DNA (corresponding to 173910 oilseed rape genome copies with one genome assumed to correspond to 1.15 pg of haploid oilseed rape genomic DNA) [(1)].

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

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

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 RT73 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
MDB510 For primer (10 µM)	200 nM	0.5
MDB511 Rev primer (10 µM)	200 nM	0.5
TM003 probe (10 µM)	200 nM	0.5
Nuclease free water	#	7
Template DNA (max 200 ng)	#	4
Total reaction volume:		25

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the RT73 specific system.

Component	Final concentration	µl / reaction
TaqMan® Universal PCR Master Mix (2x)	1x	25
RT73 primer 1 (10 µM)	150 nM	0.75
RT73 primer 2 (10 µM)	150 nM	0.75
RT73 Probe (5 µM)	50 nM	0.50
Nuclease free water	#	19
Template DNA (max 200 ng)	#	4
Total reaction volume:		50

3. Mix gently and centrifuge briefly.
4. Prepare two reaction tubes (one for the RT73 and one for the *CruA* master mix) 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. 21 x 3 = 63 µl master mix for three PCR repetitions for each sample of the *CruA* reference system and 46 x 3 = 138 µl master mix for three PCR repetitions for each sample of the RT73 system). Add to each tube the correct amount of DNA (e.g. 4 x 3 = 12 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 of the *CruA* system and 50 µl in each well of the RT73 system. 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 RT73/*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. RT73) 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 standard curves are generated both for the *CruA* and the RT73 specific systems by plotting the Ct 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 copy numbers in the unknown sample DNA.

For the determination of the amount of RT73 DNA in the unknown sample, the RT73 copy number is divided by the copy number of the oilseed rape reference gene (*CruA*) and multiplied by 100 to obtain the percentage value (GM% = oilseed rape/*CruA* * 100).

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
- 1.5/15 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')
RT73 target sequence	
RT73 primer 1	5' – CCA TAT TGA CCA TCA TAC TCA TTG CT-3'
RT73 primer 2	5' –GCT TAT ACG AAG GCA AGA AAA GGA-3'
RT73 probe	FAM 5'-TTC CCG GAC ATG AAG ATC ATC CTC CTT-3' TAMRA
Reference gene CruA target sequence	
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

1. Royal Botanic Garden, Kew. Plant DNA C-values Database (release 4.0, October 2005). MD Bennett and IJ Leitch (http://www.rbgkew.org.uk/cval/homepage.htm