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INSTITUTE FOR HEALTH AND CONSUMER PROTECTION
COMMUNITY REFERENCE LABORATORY FOR GM FOOD AND FEED

Event-specific Method for the Quantification of Oilseed Rape Line T45 Using Real-time PCR

Protocol

Method development:
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Method validation:
Joint Research Centre – European Commission
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Contents

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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 T45 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 T45 genomic DNA, a 123-bp fragment of the recombination area between the insert and the plant genome (located at the 5′ 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 quantification of event T45 DNA, a 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 T45 DNA in a test sample, the normalized ∆Ct values of the calibration samples are used to calculate by linear regression a reference curve ∆Ct-formula. The normalized ∆Ct values of the unknown samples are measured and, by means of the reference ∆Ct-formula, the relative amount of T45 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 leaves, grains or seeds, containing mixtures of genetically modified and conventional oilseed rape.

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

2.2 Collaborative trial

The method was validated in a collaborative trial by the Joint Research Centre (JRC) of the European Commission. The study was undertaken with 14 laboratories.

Each participant received twenty unknown samples containing T45 oilseed rape genomic DNA at five GM contents, between 0.1 % and 3.6 %.

Each test sample was analyzed by PCR in three repetitions. The study was designed as a blind quadruplicate collaborative trial; each laboratory received each level of GM T45 in four unknown samples. Two replicates of each GM level were analyzed 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 the collaborative trial. The lowest relative GM content of the target sequence included in collaborative trial was 0.10%.

2.4 Limit of quantification

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 0.10%.

2.5 Molecular specificity

The method utilizes a unique DNA sequence of the recombination region between the insert and the plant genome. The sequence is specific to T45 event and thus imparts event-specificity to the method.

The specificity was experimentally tested against DNA extracted from plant materials containing the specific targets of maize T25, MON810, Bt11, Bt176, GA21, NK603, CBH351, Roundup Ready Soybean, OSR Ms1, Ms8, Rf1, Rf2, Rf3, Topas19/2, soybean

A2704-12 and LLCotton25. None of the materials yielded detectable amplification in replicate experiments.

3. Procedure

3.1 General instructions and precautions

- All handling of reagents and controls should occur in an ISO 9001:2000 or ISO 17025 environment or equivalent.
- The procedures require experience of working under sterile conditions.
- Laboratory organization, 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 shall 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 sterilized 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 lab-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 T45 oilseed rape

3.2.1 General

The PCR set-up for the taxon specific target sequence (*CruA*) and for the GMO (T45) 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 T45 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 ∆Ct-values of calibration samples against the logarithm of the respective GM % contents; the slope (a) and the intercept (b) of the standard curve (y = ax + b) are then used to calculate the mean % GM content of the blind samples based on their normalised 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. In two reaction tubes (one for T45 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 T45 specific system.

Component	Final concentration	µl/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
KVM172 For primer (10 µM)	400 nM	1
MDB599 Rev primer (10 µM)	400 nM	1
TM026 Probe (10 µM)	200 nM	0.5
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 T45 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. 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 maize T45/*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. T45) 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. If needed, 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 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 reference ΔCt-curve formula is used to estimate the relative amount (%) of T45 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 (usually 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')
T45 target sequence	
KVM172	5' – CAA TGG ACA CAT GAA TTA TGC -3'
MDB599	5'- GAC TCT GTA TGA ACT GTT CGC -3'
TM026 (Probe)	FAM 5'- TAG AGG ACC TAA CAG AAC TCG CCG T -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

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