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DIRECTORATE GENERAL JRC
JOINT RESEARCH CENTRE
INSTITUTE FOR HEALTH AND CONSUMER PROTECTION
COMMUNITY REFERENCE LABORATORY FOR GM FOOD AND FEED

Event-specific method for the quantitation of

Maize line T25 using real-time PCR

Validated Method

14/06/2005

Corrected version 1- 30/11/2011 (see page 2)

Method development:

Bayer CropScience

Method validation:

Joint Research Centre – European Commission
Biotechnology & GMOs Unit

Correction from the previous versions:

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Page 8 §1:
For relative quantification of event T25 DNA, a maize-specific reference system amplifies a 136-bp fragment
changed by
For relative quantification of event T25 DNA, a maize-specific reference system amplifies a **135**-*bp fragment*

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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 T25 DNA to total maize 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, use of DNA spikes) are recommended.

For specific detection of event T25 genomic DNA, a 102-bp fragment of the recombination region of parts of the construct inserted into the plant genome 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 T25 DNA, a maize-specific reference system amplifies a 135-bp fragment of *Adh*1 (Alcohol dehydrogenase-1) gene, a maize endogenous gene, using a pair of *Adh*1 gene-specific primers and an *Adh*1 gene-specific probe labelled with FAM 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 T25 DNA in a test sample, the normalized ΔCt values of the calibration samples are used to calculate by linear regression a reference curve ΔCt-formula. The normalized ΔCt values of the unknown samples are measured and, by means of the reference ΔCt-formula, the relative amount of T25 event DNA is estimated.

2. Validation status and performance characteristics

2.1 General

The method has been optimised for ground maize seed and grain material, containing mixtures of genetically modified T25 and conventional maize.

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 13 laboratories.

Each participant received twenty unknown samples containing T25 maize genomic DNA at five GM contents, between 0.15 % and 3.3 %.

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 T25 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% GM T25 in 200 ng of total maize DNA. The relative LOD was not assessed in a collaborative trial. The lowest relative GM content of the target sequence included in collaborative trail was 0.15%.

2.4 Limit of quantitation

According to the method developer, the relative LOQ of the method is ≤ 0.09% GM T25 in 200 ng of total maize DNA. The lowest relative GM content of the target sequence included in collaborative trial was 0.15%.

2.5 Molecular specificity

The method utilizes a unique DNA sequence of the recombination region of parts of the construct inserted into the plant genome. The sequence is specific to T25 and thus imparts event-specificity to the detection method.

The specificity was experimentally tested against DNA extracted from plant materials containing the specific targets of LL Rice62, OSR Ms1, Ms8, Rf1, Fr2, Rf3, Topas19/2, T45, Soybean A2704/12 and Cotton LL25, Maize Mon810, Bt11, Bt176, GA21, NK603, CBH351 and Roundup Ready Soybean. None of the materials yielded detectable amplification.

3. Procedures

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 T25 maize

3.2.1 General

The PCR set-up for the taxon specific target sequence (*Adh*1) and for the GMO (T25) 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 T25 DNA in a total amount of 200 ng maize DNA (corresponding to 73,394 maize genome copies with one maize genome assumed to correlate to 2.725 pg of haploid maize genomic DNA) (Arumuganathan & Earle, 1991). 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 T25 system and one for the *Adh*1 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 reference *Adh*1 specific system.

Component	Final concentration	µl/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
KVM182 primer (10 µM)	200 nM	0.5
KVM183 primer (10 µM)	200 nM	0.5
TM014 probe (10 µM)	200 nM	0.5
Nuclease free water	#	6
Template DNA (max 200 ng, see 3.2.1 and 3.2.2)	#	5
Total reaction volume:		25

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for T25 specific system.

Component	Final concentration	µl/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5 µl
MLD143 primer (10 µM)	400 nM	1
MDB551 primer (10 µM)	400 nM	1
TM016 Probe (10 µM)	200 nM	0.5
Nuclease free water	#	5
Template DNA (max 200 ng, see 3.2.1 and 3.2.2)	#	5
Total reaction volume:		25 µl

3. Mix gently and centrifuge briefly.
4. Prepare two reaction tubes (one for the T25 and one for the *Adh*1 master mix) for each DNA sample to be tested (reference 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). Low-speed vortex each tubes at least three times for approx 30 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 T25-*Adh*1 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″	Measure	

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. T25) 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. *Adh*1 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 T25 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 evaluating data after standard curve method (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')
T25 target sequence	
MLD143	5'-ACA AGC GTG TCG TGC TCC AC-3'
MDB551	5'-GAC ATG ATA CTC CTT CCA CCG-3'
TM016 (Probe)	FAM-5'-TCA TTG AGT CGT TCC GCC ATT GTC G-3'-TAMRA
Reference gene Adh1 target sequence	
KVM182	5'-CGT CGT TTC CCA TCT CTT CCT CCT-3'
KVM183	5'-CCA CTC CGA GAC CCT CAG TC-3'
TM014 (Probe)	FAM-5'-AAT CAG GGC TCA TTT TCT CGC TCC TCA-3'-TAMRA

5. References

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