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

Event-specific method for the quantitation of maize line TC1507 using real-time PCR

Protocol

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Method validation:
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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 TC1507 DNA to total maize DNA in a sample.

The PCR assay has been 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 ad 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 TC1507 genomic DNA, a 58-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 TC1507 DNA, a maize-specific reference system amplifies a 79-bp fragment of HMG (High Mobility Group) gene, a maize endogenous gene, using a pair of HMG gene-specific primers and an HMG 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 TC1507 DNA in a test sample, event TC1507 and HMG Ct values are determined for the sample. Standard curves are then used to calculate the relative content of event TC1507 DNA to total maize DNA.

2. Validation status and performance characteristics

2.1 General

The method has been optimised for ground maize seed, containing mixtures of genetically modified TC1507 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 14 laboratories.

Each participant received twelve unknown samples. The samples consisted of DNA mixtures of 0% and 100% TC1507 maize genomic DNA at six GMO levels, between 0.0 % and 5.0 %.

Each test sample was analyzed by PCR in three repetitions. The study was designed as a blind duplicate collaborative trial; each laboratory received each level of GM TC1507 in two unknown samples, and the two replicates for 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 the method developer, the absolute LOD of the method is 1.25 copies (8 positives out of 10 replicates). The relative LOD was not assessed in a collaborative trail. The lowest relative concentration of the target sequence included in collaborative trail was 0.1%.

2.4 Limit of quantitation

According the method developer, the relative LOQ of the method is ≤ 0.08%. The absolute LOQ for the individual systems is ≤ 10 copies (TC1507) and ≤ 40 copies (HMG). The lowest relative concentration of the target sequence included in collaborative trail was 0.1%.

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 TC1507 and thus imparts event-specificity to the detection method.

The specificity was assessed by Blastsearch on 16/11/2002. No 100% match with other maize GMO sequences was found.

The specificity was experimentally tested against DNA extracted from plant materials containing the specific targets of TC1360, Bt176, GA21, NK603, MON810, Bt11, Starlink,

T25, MON 863 maize, Roundup Ready® soybean, conventional rapeseed, rice and wheat. None of the materials yielded detectable amplification.

The target sequence is a single copy sequence in the haploid TC1507 genome.

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 TC1507 maize

3.4.1 General

The PCR set-up for the taxon specific target sequence (HMG) and for the GMO (TC1507) 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.

3.4.2 Calibration

Separate calibration curves with each primer/probe system are generated in the same analytical amplification run.

The calibration curves consist of four dilutions of a DNA sample containing 10% TC1507. A series of one to five dilution intervals at a starting concentration of 73,394 maize genome copies may be used (corresponding to 200 ng of DNA with one maize genome assumed to correlate to 2.725 pg of haploid maize genomic DNA) (Arumuganathan & Earle, 1991).

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

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

3.4.3 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 TC1507 system and one for the HMG 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 HMG specific system.

Component	Final concentration	µl/reaction
Buffer 10x (including Rox)	1x	2.5 µl
Primer MaiJ-F1	300 nM	-
Primer mhmg-rev	300 nM	-
Probe mhmg	180 nM	-
$MgCl_2$ 25 mM	4.5 mM	4.5 µl
dNTPs[a] 10/20 mM	200/400 µM	0.5 µl
AmpliTaq Gold Polymerase	1U/reaction	-
Nuclease free water		up to 25 µl
[Template DNA (maximum 200 ng, see 3.4.1 and 3.4.2)]		(5 µl)
Total reaction volume:		25 µl

[a] dATP (10 mM), dCTP (10 mM), dGTP (10 mM), dUTP (20 mM)

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for TC1507 specific system.

Component	Final concentration	µl/reaction
Buffer 10x (including Rox)	1x	2.5 µl
Primer MaiY-F1	300 nM	-
Primer MaiY-R3	300 nM	-
Probe MaiY-S1	150 nM	-
$MgCl_2$ 25 mM	5.5 mM	5.5 µl
dNTPs[a] 10/20 mM	200/400 µM	0.5 µl
AmpliTaq Gold Polymerase	1U/reaction	
Nuclease free water		up to 25 µl
[Template DNA (maximum 200 ng, see 3.4.1 and 3.4.2)]		(5 µl)
Total reaction volume:		25 µl

[a] dATP (10 mM), dCTP (10 mM), dGTP (10 mM), dUTP (20 mM)

3. Mix gently and centrifuge briefly.
4. Prepare two reaction tubes (one for the TC1507 and one for the HMG 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). 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. Reaction conditions.

Step	Stage		T°C	Time (sec)	Acquisition	Cycles
1	Initial denaturation		95 °C	600″	No	1x
2a	Amplification	Denaturation	95 °C	15″	No	45x
2b		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. TC1507) 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. HMG 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 instruments software calculated the Ct-values for each reaction.

The standard curves are generated both for the HMG and TC1507 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 by interpolation from the standard curves.

For the determination of the amount of TC1507 DNA in the unknown sample, the TC1507 copy number is divided by the copy number of the maize reference gene (HMG) and multiplied by 100 to obtain the percentage value (GM% = TC1507/HMG * 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 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

(equivalents may be substituted)

- TRIS pH=8.0: Tris[hydroxymethyl] aminomethane hydrochloride (Molecular Biology grade)
- KOAc (SIGMA Part No P1190)
- Gelatine (VWR Part No 1.04078.1000)
- Tween 20 (SIGMA Part No P9416-50ML)
- Glycerol (SIGMA Part No P5516-100ML)
- Rox (Applied Biosystems Part No 434925)
- dATP (GeneCraft Part No GC-013-007)
- dCTP (GeneCraft Part No GC-013-009)
- dGTP (GeneCraft Part No GC-013-006)
- dUTP (GeneCraft Part No GC-013-010)
- $MgCl_2$ (SIGMA Part No M1028-1ML)
- Ampli Taq Gold (Applied Biosystems Part No N8080242)

4.3 Primers and Probes

Name	Oligonucleotide DNA Sequence (5' to 3')
TC1507 target sequence	
MaiY-F1	TAG TCT TCG GCC AGA ATG G
MaiY-R3	CTT TGC CAA GAT CAA GCG
MaiY-S1	6-FAM-TAA CTC AAG GCC CTC ACT CCG-TAMRA
Reference gene HMG target sequence	
MaiJ-F2	TTG GAC TAG AAA TCT CGT GCT GA
mhmg-rev	GCT ACA TAG GGA GCC TTG TCC T
Mhmg-probe	6-FAM-CAA TCC ACA CAA ACG CAC GCG TA-TAMRA

5. Buffers and Solutions

The following describes the preparation of the buffers used in this procedure. Volume may be scaled as needed. Equivalent reagent may be substituted.

- **Preparation of the 10x Buffer**

  a) Mix the following chemicals at the final concentration indicated and adjust the buffer to pH = 8.0

Component	Final concentration
Tris pH = 8.0	0.5 M
KOAc	0.5 M
Gelatine	0.5%
Tween 20	0.1%
Glycerol	0.8%
Rox	0.2 µl/reaction
Water	-

6. References

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