Event-specific Method for the Quantification of Maize Line Bt11 Using Real-time PCR

Protocol

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Content

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 Bt11 DNA to total maize DNA in a sample.

The PCR assay was optimised for use in real-time PCR instruments for plastic reaction vessels.

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.

For the specific detection of event Bt11 DNA, a 68-bp fragment of the integration region of the construct inserted into the plant genome (located at the 5′ flanking DNA region) is amplified using two 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 as a reporter dye at its 5′ end and TAMRA as a quencher dye at its 3′ end.

For the relative quantification of event Bt11 DNA, a maize-specific reference system amplifies a 135-bp fragment of the maize endogenous *alcohol dehydrogenase 1* gene (*adh1*), using two specific primers and an *adh1* 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 Bt11 DNA in a test sample, the normalised ∆Ct values of the calibration samples are used to calculate by linear regression a reference curve ∆Ct-formula. The normalised ∆Ct values of the unknown samples are measured and, by means of the regression formula, the relative amount of Bt11 event DNA is estimated.

2. Validation status and performance characteristics

2.1 General

The method has been optimised for suitable DNA extracted from maize seeds containing mixtures of genetically modified and conventional maize.

The reproducibility and trueness of the method were tested through an international collaborative study using DNA samples at different GM% contents.

2.2 Collaborative trial

The method was validated in an international collaborative study by the Joint Research Centre (JRC) of the European Commission. The study was undertaken with twelve laboratories in November 2007.

Each participant received twenty unknown samples containing Bt11 maize genomic DNA at five GM% contents, ranging from 0.09% to 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 Bt11 in four unknown samples. Two replicates of each GM level were analysed on the same PCR plate.

A detailed validation report can be found at 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 250 ng of total maize DNA. The relative LOD was not assessed in collaborative study.

2.4 Limit of quantification (LOQ)

According to the data provided by the applicant, the relative LOQ of the method is at least 0.08% in 250 ng of total maize DNA. The lowest relative GM content of the target sequence included in the international collaborative study was 0.09%.

2.5 Molecular specificity

The method exploits a unique DNA sequence in the region of recombination between the insert and the plant genome. The sequence is specific to Bt11 event and thus imparts event-specificity to the method.

The specificity of the Bt11 assay (forward/reverse primers and probe) was experimentally tested in real-time PCR by the applicant against DNA extracted from samples containing GM maize Bt11, Bt10, NK603, MON810, MON863, MON810 x MON863, TC1507, MIR604, Bt176, GA21, MON88017, T25 and Herculex RW (59122).

According to the applicant, none of the above mentioned GM lines tested, except the positive control Bt11, produced amplification signals in replicated samples when 100 ng total DNA per reaction were used.

3. Procedure

3.1 General instructions and precautions

- The procedures require sterile conditions working experience.
- Laboratory organisation, 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 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.
- 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 Bt11 maize

3.2.1 General

The PCR set-up for the taxon specific target sequence (*adh1*) and for the GMO (Bt11) 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 250 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 Bt11 DNA in a total amount of 250 ng maize DNA. The GM content of the standard samples ranges from 10% to 0.08%.

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 calibration 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 on ice**.
2. In two reaction tubes (one for Bt11 system and one for the *adh1* 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 maize *adh1* reference system.

Component	Final concentration	µL / reaction
Sigma Jumpstart ReadyMix (2x)	1x	12.5
Zm *adh1* – F primer (10 µM)	300 nM	0.75
Zm *adh1* – R primer (10 µM)	300 nM	0.75
Zm *adh1* – P probe (10 µM)	200 nM	0.50
Nuclease free water	#	5.50
Template DNA (max 250 ng)	#	5
Total reaction volume:		25

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the Bt11 specific system.

Component	Final concentration	µL / reaction
Sigma Jumpstart ReadyMix (2x)	1x	12.5
Bt11-ev-f1 primer (10 µM)	200 nM	0.50
Bt11-ev-r5 primer (10 µM)	200 nM	0.50
Bt11-ev-p1 probe (10 µM)	150 nM	0.38
Nuclease free water	#	6.12
Template DNA (max 250 ng)	#	5
Total reaction volume:		25

3. Mix gently and centrifuge briefly.
4. Prepare two reaction tubes (one for the Bt11 and one for the *adh1* 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 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 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 maize Bt11/*adh1* 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	40
		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 system (e.g. Bt11) 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. *adh1* 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 ∆Ct-curve is generated by plotting the ∆Ct-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 regression formula is used to estimate the relative amount (%) of Bt11 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
- 1.5/2.0 mL reaction tubes

4. 2 Reagents and solutions

- Sigma JumpStart Taq ReadyMix (2x), Sigma Aldrich Ltd Cat No P-2893
- Sulforhodamine 101, Sigma Cat No S-7635
- 1 M $MgCl_2$, Sigma Aldrich Ltd Cat No M-1028

10000x Sulforhodamine 101 stock:

Resuspend 227.5 mg of Sulforhodamine 101 in 250 mL nuclease free water to make a 1.5 mM stock solution.
Vortex well and store at -20 ºC.

Supplemented 2x Sigma JumpStart ReadyMix:

For 50 mL: to Sigma Jumpstart Taq ReadyMix (2X), add:

- 550 μL of 1 M $MgCl_2$
- 20 μL 10000x Sulforhodamine 101.

Vortex well and store at 4 ºC for up to 1 year.

4.3 Primers and Probes

Name	Oligonucleotide DNA Sequence (5' to 3')
Bt11 target sequence	
Bt11-ev-f1 primer	5' – TGT GTG GCC ATT TAT CAT CGA -3'
Bt11-ev-r5 primer	5' – CGC TCA GTG GAA CGA AAA CTC -3'
Bt11-ev-p1 probe	FAM 5'- TTC CAT GAC CAA AAT CCC TTA ACG TGA GT -3' TAMRA
Reference gene *adh1* target sequence	
Zm *adh1* – F primer	5' – CGT CGT TTC CCA TCT CTT CCT CC-3'
Zm *adh1* – R primer	5' – CCA CTC CGA GAC CCT CAG TC -3'
Zm *adh1* - P probe	VIC 5' – AAT CAG GGC TCA TTT TCT CGC TCC TCA-3' TAMRA