GMO specific real-time PCR system

Protocol for event-specific quantitation of Bt11 in maize

Method development:
National Veterinary Institute (Norway) and INRA (France)

Method Validation:
European Commission – DG JRC
Community Reference Laboratory

Contents

1. General information

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. The procedure is a simplex system, in which a maize *adh*1 endogenous assay (reference gene) and the target assay (Bt11) are amplified in separate wells. The PCR assay has been optimized for use in an ABI Prism® 7700 sequence detection system. Other systems may be used, but thermal cycling conditions must be verified.

The method has been developed and pre-validated within the EU shared cost action project QPCRGMOFOOD (contract no. QLK1–1999–01301). The Bt11 specific part has been developed by the National Veterinary Institute of Norway and the *adh*1 part by the Institut National de la de la Recherche Agronomique (INRA PMDV/MDO), France. The Bt11 specific system has been firstly developed and published for the LightCycler equipment (Rønning *et al*. 2003). The scientific paper describing the *adh*1 system is J. Agric. Food Chem. 2004. 52: 4632-4637 (Hernández *et al*.).

The method has been validated by the DG Joint Research Centre of the European Commission in 2003. The results from the collaborative study have been reported in a separate document.

2. Protocol

2.1 Short description

Two types of quantitation are performed in this method: one for the maize *adh*1 reference gene and one for the Bt11 maize specific junction region. The following sets of primers and probes are used:

***Adh*1 method**	
*A*DH-F3 forward primer	5'-CgT CgT TTC CCA TCT CTT CCT CC-3'
*A*DH-R4 reverse primer	5'- CCA CTC CgA gAC CCT CAg TC-3'
*A*DH1-MDO probe	5'-*FAM*-AAT CAg ggC TCA TTT TCT CgC TCC TCA-*TAMRA*-3'
Bt11 maize method	
Bt113JFor primer	5'-gCg gAA CCC CTA TTT gTT TA-3'
Bt113JRev primer	5'-TCC AAg AAT CCC TCC ATg Ag-3'
Bt113JFT FAM probe	5'-*FAM*-AAA TAC ATT CAA ATA TgT ATC CgC TCA-*TAMRA*-3'

2.2 General indications and rules

- The procedure requires experience of working under sterile conditions.

- Maintain separate working areas for DNA preparation, reaction set-up and amplification.

- Use filter-plugged pipette tips in order to avoid possible cross-contamination.

- Use only powder-free gloves and change them frequently.

- Clean lab-benches and equipment periodically with 10% sodium hypochlorite solution (bleach).

- To obtain reliable quantitation results all pipettes have to be checked regularly for precision and, if necessary, have to be calibrated.

2.3 Bt11 and *adh*1 detection system – operative protocol

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 on ice, add the components in the order mentioned below (except DNA) to prepare the master mixes for the *adh*1 gene specific method (Table 1) and the Bt11 maize junction specific system (Table 2).

Table 1. Preparation of the master-mix for the *adh*1 system.

Component	Final concentration in PCR	µl/reaction
TaqMan Universal Master Mix 2X	1x	12.5
*A*DH-F3 primer (20 µM)	300 nM	0.375
*A*DH-R4 primer (20 µM)	300 nM	0.375
ADH1-MDO Probe (10 µM)	200 nM	0.5
Nuclease free water	#	5.25
Template DNA (maximum 250 ng)	#	6
Total volume:		25 µl

Table 2. Preparation of the master-mix for the Bt11 maize junction system.

Component	Final concentration in PCR	µl/reaction
TaqMan Buffer A 10x	1x	2.5
$MgCl_2$ (25 mM)	4 mM	4
dNTP[a] 10 mM each	0.2 mM each	0.5 each
dUTP 20 mM	0.4 mM	0.5
Bt113JFor primer (20 µM)	750 nM	0.9375
Bt113JRev primer (20 µM)	750 nM	0.9375
Bt113JFT probe (10 µM)	250 nM	0.625
AmpErase UNG (1 U/µl)	0.3 U	0.25
Ampli Taq Gold (5 U/µl)	1.5 U	0.25
Nuclease free water	#	7.5
Template DNA (≤250 ng DNA for standards, maximum 200 ng for unknowns)	#	6
Total volume:		25 µl

[a] dNTP = dATP, dCTP and dGTP.

3. Mix well and centrifuge briefly.

4. Prepare and label two reaction tubes (one for the Bt11 and one for the *adh*1 quantitation) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).

5. Add into each reaction tube the amount of master mix needed for the number of repetitions chosen (e.g. 57 µl master-mix for triplicate reaction). Add into each tube the proper amount of DNA for the correspondent number of repetitions (e. g. 18 µl DNA for triplicate reaction). It is advisable to include additional volumes of master-mix and DNA solution to ensure adequate excess volume when loading the samples. Vortex at least three times for approx 10 sec. each tube. This step is of mandatory importance to reduce to a minimum the variability between the replicates of each sample.

6. Spin down in a micro-centrifuge. Aliquot 25 µl in each well according to the scheme provided on page 10.

7. Place the plate into the ABI Prism 7700/7900 instrument.

8. Label the samples as appropriate.

9. Please programme the real-time equipment as follows:

- Select FAM as the reporter dye. Define TAMRA as the quencher dye and ROX as the passive reference dye.
- Select a 'Real Time' detection programme for the run, select 'Single Reporter' and 'Real Time', for fluorescence measurement use the default settings of the instrument.
- Cycle the samples as described in Table 3.

Table 3. Cycling program for the maize Bt11 – *adh*1 system.

Set: Real-time PCR modus
25 µl reaction volume
Probe labelling: FAM

Step	Stage		T°C	Time (sec)	Acquisition	Cycles
1	UNG		50 °C	120"	no	1x
2	Initial denaturation		95°C	600"	no	1x
3	Amplification	denaturation	95°C	15"	no	50x
4		Annealing & Extension	60°C	60"	Measure	

3. Data analysis

The standard curves are generated both for the *adh*1 and Bt11 specific system by plotting the Ct-values measured for the standard curve samples against the logarithm of the DNA copy numbers, and by fitting a linear regression line into these data. Thereafter, the standard curve can be used to estimate the copy numbers in the unknown sample DNA. For the determination of the amount of Bt11 DNA in the test sample, the Bt11 copy number is divided by the copy number of the maize reference gene (*adh*1) and multiplied by 200 to obtain the percentage value.

Note: GM maize is normally heterozygous, in which case the ratio of GM to reference per diploid genome is 1:2. Therefore the conversion factor should logically be 200.

4. References

Rønning, S.B., Vaïtilingom, M, Berdal, K.G. & Holst-Jensen, A. 2003. Event specific real-time quantitative PCR for genetically modified Bt11 maize (*Zea mays*). Eur Food Res Technol 216: 347 – 354.

Hernández M., Duplan M-N, Berthier G., Vaitilingom M., Hauser W., Freyer R., Pla M. and Bertheau Y. Development and comparison of four RTi-PCR systems for specific detection and quantification of *Zea mays* L. J. Agric. Food Chem. 2004. 52: 4632-4637