



Event-specific Method for the Quantification of Maize Line MIR604 Using Real-time PCR

Protocol

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**Joint Research Centre
Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit**

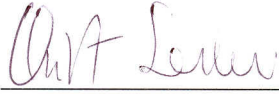
Method development:

Syngenta Seeds S.A.S.

Collaborative trial:

Community Reference Laboratory for GM Food and Feed (CRL-GMFF)
Molecular Biology and Genomics Unit

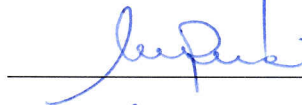
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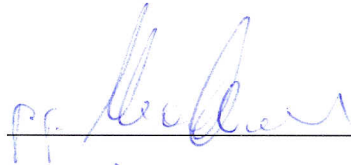
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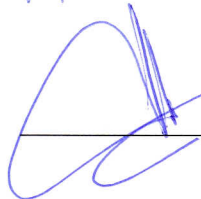
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1. General information and summary of the methodology

This protocol describes an event-specific real-time quantitative TaqMan® PCR procedure for the determination of the relative content of event MIR604 DNA to total maize DNA in a sample.

The PCR assay was 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 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 MIR604 DNA, a 76-bp fragment of the recombination region between the insert and 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 MIR604 DNA, a maize-specific reference system amplifies a ~~136-bp~~ 135-bp fragment of the maize endogenous *Alcohol dehydrogenase* gene (*Adh1*), using a pair of 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 MIR604 DNA in a test sample, the normalised ΔCt values of the calibration samples are used to calculate by linear regression a reference curve ΔCt -formula. The normalised ΔCt values of the unknown samples are measured and, by means of the regression formula, the relative amount of MIR604 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.

The reproducibility and trueness of the method were tested through an international collaborative study using DNA samples at different GMO 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 14 laboratories in December 2006.

Each participant received twenty unknown samples containing MIR604 maize genomic DNA at five GM contents, ranging from 0.1 % to 6.0 %.

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 MIR604 in four unknown samples. Two replicates of each GM level were analysed 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 (LOD)

According to the data provided by the applicant, the relative LOD of the method is < 0.045% 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 < 0.09% in 250 ng of total maize DNA. The lowest relative GM content of the target sequence included in the international collaborative study was 0.1 %.

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 MIR604 event and thus imparts event-specificity to the method.

The specificity of the MIR604 assay (forward/reverse oligonucleotide primers and probe) was tested in real-time PCR against DNA extracted from samples containing the specific targets of maize MON810, Bt11, Bt176, GA21, NK603 and MIR604.

None of the above mentioned GM lines tested, except the positive control MIR604, produced amplification signals in replicated samples when 250 ng total DNA per reaction were applied. A relatively weak amplification, with Ct values of 39.76 and 39.04 (average of three samples), was reported with 100% GA21 DNA at 250 ng and 500 ng per reaction (Ct values for reference system of 23.26 and 22.35, respectively). However, no signal was reported when both 50 ng and 1000 ng of GA21 DNA per reaction were used (Ct=40 for MIR604 assay and 22.36 and 21.46 for the reference assay, respectively). In an additional set of experiments, no amplification signal was

obtained with MIR604 assay on GA21 samples (three replicates per concentration level) at 200 and 400 ng per reaction, while the reference assay showed amplifications in the expected range.

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 the guidelines given by relevant authorities as e.g. ISO, CEN, Codex Alimentarius Commission.
- 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.
- All the equipment used should be sterilised 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 unable to adsorb protein or DNA.
- In order to avoid contamination, 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 hypochloride 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 MIR604 maize

3.2.1 General

The PCR set-up for the taxon specific target sequence (*Adh1*) and for the GMO (MIR604) 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 MIR604 DNA in a total amount of 250 ng maize DNA. The GM content of the standard samples ranges from 10.0% to 0.1%.

A calibration curve is produced by plotting the ΔC_t -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 ΔC_t 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 MIR604 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
50x Zm <i>Adh1</i> Endogenous Assay stock	1x	0.5
Nuclease free water	#	7
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 MIR604 specific system.

Component	Final concentration	µl/reaction
Sigma Jumpstart ReadyMix (2x)	1x	12.5
50x Event MIR604 Assay Stock	1x	0.5
Nuclease free water	#	7
Template DNA (max 200 ng)	#	5
Total reaction volume:		25

3. Mix gently and centrifuge briefly.
4. Prepare two reaction tubes (one for the MIR604 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. $20 \times 3 = 60$ µl master mix for three PCR repetitions). Add to each tube the correct amount of DNA (e.g. $5 \times 3 = 15$ 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 $250 \times g$ 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 MIR604/*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

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. MIR604) 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. *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 Δ Ct-curve is generated by plotting the Δ 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 MIR604 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 RedTaq PCR master mix (2X). Sigma Aldrich Ltd P-2893
- Sulforhodamine 101, Sigma Cat No S-7635
- 50x Zm Adh1 Endogenous Assay Stock:
 - For 1ml of 50x Zm Adh1 Endogenous Assay Stock:
 - 15 μ l of Zm Adh1 primer F (1000 pmol/ μ l)
 - 15 μ l of Zm Adh1 primer R (1000 pmol/ μ l)
 - 100 μ l of Zm Adh1 probe (100 pmol/ μ l)
 - 870 μ l of nuclease-free water

The 1x Zm Adh1 Endogenous Assay Stock contains: 300nM Zm Adh1 primer F, 300nM Zm Adh1 primer R and 200nM Zm Adh1 probe.

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

- 50x Event *MIR604* Assay Stock:
 - For 1ml of 50x Event *MIR604* Assay Stock:
 - 30 μ l of *MIR604* primer F (1000 pmol/ μ l)
 - 15 μ l of *MIR604* primer R (1000 pmol/ μ l)
 - 100 μ l of *MIR604* probe (100 pmol/ μ l)
 - 855 μ l of nuclease-free water

The 1x Event MIR604 Assay Stock contains: 600 nM MIR 604 primer F, 300 nM MIR604 primer R and 200 nM MIR604 probe.

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

- 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°C.

- Sigma Jumpstart ReadyMix 2x:

- For 50 ml: to Sigma Jumpstart RedTaq PCR master mix (2X), add:
 - 550 µl of 1M MgCl₂
 - 10 µl 10000x Sulforhodamine 101

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

4.3 Primers and Probes

Name	Oligonucleotide DNA Sequence (5' to 3')
<i>MIR604 target sequence</i>	
MIR604 primer F	5' –GCG CAC GCA ATT CAA CAG-3'
MIR604 primer R	5' –GGT CAT AAC GTG ACT CCC TTA ATT CT-3'
MIR604 probe	FAM 5'- AGG CGG GAA ACG ACA ATC TGA TCA TG-3' TAMRA
<i>Reference gene Adh1 target sequence</i>	
Zm <i>Adh1</i> primer F	5' –CGT CGT TTC CCA TCT CTT CCT CC-3'
Zm <i>Adh1</i> primer R	5' –CCA CTC CGA GAC CCT CAG TC -3'
Zm <i>Adh1</i> (Probe)	VIC 5' –AAT CAG GGC TCA TTT TCT CGC TCC TCA-3' TAMRA