EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit

Event-specific Method for the Quantification of Maize VCO-01981-5 Using Real-time PCR

Validated Method

Method development:

Genective SA

1. General information and summary of the methodology

This protocol describes an event-specific real-time quantitative TaqMan® PCR (polymerase chain reaction) procedure for the determination of the relative content of maize (*Zea mays* L.) event VCO-01981-5 DNA to total maize DNA in a sample.

Template DNA extracted by means of suitable methods should be tested for quality and quantity prior to use in 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 maize event VCO-01981-5, an 85 bp fragment of the region spanning the 5' insert-to-plant junction in maize event VCO-01981-5 is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with the fluorescent dye FAM (6-carboxyfluorescein), as a reporter at its 5' end, and MGB (Minor Groove Binding) as a quencher at its 3' end.

For the relative quantification of maize event VCO-01981-5 DNA, a *Zea mays* L. specific system amplifies a fragment of the aldolase endogenous gene, using gene-specific primers and a gene-specific probe, labelled with VIC® as reporter dye at its 5' end, and TAMRA (5-Carboxytetramethylrhodamine) as quencher at its 3' end. The amplified aldolase fragment is 69 bp long.

The measured fluorescence signal passes a threshold value after a certain number of cycles. The cycle number when the fluorescence crosses the threshold is called the "Cq" value; Cq values for the VCO-01981-5 and aldolase systems are determined for each sample.

For relative quantification of event VCO-01981-5 in a test sample, the ΔCq values of calibration samples (Cq_{GM}-Cq_{Ref}) are used to calculate, by linear regression, a standard curve (plotting ΔCq values against the logarithm of the relative amount of VCO-01981-5 event DNA). The ΔCq values of the unknown samples are measured and, by means of the regression formula, the relative amount of VCO-01981-5 event DNA is estimated.

2. Validation and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from mixtures of genetically modified and conventional maize seeds, grains and flour. The reproducibility and trueness of the method were tested through an international collaborative ring trial using DNA samples at different GM contents.

2.2 Collaborative trial

The method was validated in an international collaborative study by the European Union Reference Laboratory for GM Food and Feed (EURL GMFF). The study was undertaken with twelve participating laboratories in July-August 2013.

Each participant received twenty blind samples containing maize VCO-01981-5 genomic DNA at five GM contents, ranging from 0.02% to 4.5% (copy/copy). Each laboratory received each GM level in four blind replicates. Each test sample was analysed by PCR in three repetitions. Two replicates of each GM level were analysed on the same PCR plate.

The validation report can be found at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx.

2.3 Limit of detection (LOD)

According to the method developer, the relative LOD of the method is at least 0.01% in 200 ng of total maize DNA, tested in 60 replicates. The relative LOD was not assessed in the collaborative study.

2.4 Limit of quantification (LOQ)

According to the method developer, the relative LOQ of the method is at least 0.09% in 200 ng of total maize DNA. The lowest relative GM content of the target sequence included in the collaborative trial was 0.02% expressed as ratio of GM-DNA copy numbers to target taxon-specific DNA copy numbers and corresponding to 0.1% (mass/mass).

2.5 Molecular specificity

The method exploits a unique DNA sequence in the region spanning the 5' insert-to-plant junction in maize VCO-01981-5; the sequence is specific to event VCO-01981-5 and thus imparts event-specificity to the method. This was confirmed in the validation study.

3. Procedure

3.1 General instructions and precautions

- The procedure requires experience of working under sterile conditions.
- Laboratory organisation, e.g. "forward 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 employed previously. All handling of PCR reagents and controls requires dedicated equipment, especially pipettes.
- All 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.
- 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 maize event VCO-01981-5

3.2.1 General

The PCR set-up for the taxon-specific target sequence (aldolase) and for the GMO (event VCO-01981-5) target sequence is carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated and is therefore not recommended.

The method is developed and validated for a total volume of 25 µL per reaction mixture with the reagents as listed in Table 2 and Table 3.

3.2.2 Calibration

To establish the calibration curve five samples should be prepared and analysed. The range of GM contents in the calibration curve should be equal or included in the range validated during the international collaborative trial. For the collaborative trial, the calibration curve was established on the basis of five samples.

The first point of the calibration curve (S1) contained 5.6% maize event VCO-01981-5 DNA in a total of 200 ng of maize DNA (GM% calculated considering the 1C value for maize genome as 2.73 pg) [1].

The total amount of DNA/reaction, and the GM% content of standards S1 to S5 are reported in Table 1 below.

Table 1. Total amount of DNA in PCR reaction and GM% content of the standard curve samples.

Sample code	**S1**	**S2**	**S3**	**S4**	**S5**
Total amount of DNA in reaction (ng)	200	200	200	200	200
VCO-01981-5 maize GM copies	5.6%	2.0%	1.0%	0.50%	0.015%

A calibration curve is produced by plotting the ΔCq 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 normalized ΔCq values.

3.3 Real-time PCR set-up

1. Thaw, mix and centrifuge the components needed for the run. Keep thawed reagents on ice.

2. To prepare the amplification reaction mixtures add the following components (Table 2 and 3) in two reaction tubes (one for the VCO-01981-5 assay and one for the aldolase assay) on ice in the order mentioned below (except DNA).

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the VCO-01981-5 assay.

Component	Final concentration	µL/reaction
Universal Master Mix (2x)	1x	12.5
VCO-01981-5 primer F (10 µM)	300 nM	0.75
VCO-01981-5 primer R (10 µM)	300 nM	0.75
VCO-01981-5 probe (10 µM)	200 nM	0.5
Nuclease free water	/	8
(DNA)	/	(2.5)
Total reaction volume:		25 µL

Table 3. Amplification reaction mixture in the final volume/concentration per reaction well for the maize aldolase assay.

Component	Final concentration	µL/reaction
Universal Master Mix (2x)	1x	12.5
Aldolase primer F (10 µM)	300 nM	0.75
Aldolase primer R (10 µM)	300 nM	0.75
Aldolase probe (10 µM)	200 nM	0.5
Nuclease free water	/	8
(DNA)	/	(2.5)
Total reaction volume:		25 µL

3. Mix well and centrifuge briefly.

4. Prepare two reaction tubes (one for the maize VCO-01981-5 and one for the aldolase system) 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 reaction mix for 3.5 PCR repetitions (e.g. (78.75 µL for the maize aldolase reference system and 78.75 µL for the GM VCO-01981-5 system). Add to each tube the correct amount of DNA for 3.5 PCR repetitions (e.g. 8.75 µL DNA). The additional 0.5 repetition included will ensure adequate volume when loading the

samples. Vortex each tube for approx. 10 sec. This step is mandatory to minimise the variability among the repetitions of each sample.

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 spin down the reaction mixture.
7. Place the plate into the instrument.
8. Run the PCR with the cycling program described in Table 4.

Table 4. Cycling program for VCO-01981-5/aldolase assays.

Step	Stage		T (°C)	Time (s)	Acquisition	Cycles
1	UNG		50	120	No	1x
2	Initial denaturation		95	600	No	1x
3	Amplification	Denaturation	95	15	No	40x
		Annealing & Extension	60	60	Yes	

3.4 Data analysis

After the real-time PCR, analyse the run following the procedure below:

a) Set the threshold: display the amplification curves of one assay (e.g. VCO-01981-5) 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 Cq values (only needed for some analysis software). 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 exponential 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 Cq = 25, set the baseline crossing at Cq = 25 – 3 = 22).

c) Save the settings.

d) Repeat the procedure described in a), b) and c) on the amplification plots of the other system (e.g. aldolase).

e) Save the settings and export all the data to a text file for further calculations.

3.1 Calculation of results

After having defined a threshold value within the logarithmic phase of amplification as described above, the instrument's software calculates the Cq-values for each reaction.

The standard ΔCq curve is generated by plotting the ΔCq values measured for the calibration points (Cq_{GM}-Cq_{Ref}) against the logarithm of the GM % content, and by fitting a linear regression line into these data.

Thereafter, the standard ΔCq curve regression formula is used to estimate the relative amount (%) of VCO-01981-5 event in the unknown samples of DNA.

4. Equipment and 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 instruments (enabling undisturbed fluorescence detection)
- Software for run analysis (mostly integrated in the software of the real-time PCR instrument)
- Microcentrifuge
- Micropipettes
- Centrifuge for PCR-plates
- Vortex
- Rack for reaction tubes
- 0.2/1.5/2.0 mL reaction tubes

4.2 Reagents

TaqMan® Universal PCR Master Mix. Applied Biosystems Part No 4304437.

4.3 Primers and Probes

Oligonucleotides	Name	DNA Sequence (5' to 3')	Length (nt)
VCO-01981-5			
Forward primer	VCO-01981-5 primer F	CCA CTg AAC gTC ACC AAg AAg A	22
Reverse primer	VCO-01981-5 primer R	gCC gCT ACT CgA ggg ATT TA	20
Probe	VCO-01981-5 probe	6-FAM - CAg TAC TCA AAC ACT gAT Ag - MGB	20
aldolase			
Forward primer	Aldolase primer F	Agg gAg gAC gCC TCC CT	17
Reverse primer	Aldolase primer R	ACC CTg TAC CAg AAg ACC AAg g	22

Probe	Aldolase probe	VIC – TgA ggA CAT CAA CAA AAg gCT TgC CA - TAMRA	26

FAM: 6-carboxyfluorescein; TAMRA: carboxytetramethylrhodamine; MGB: Minor Groove Binding

5. References

1. Plant DNA C-values Database, http://data.kew.org/cvalues/

Annex 2: Identification of an outlier replicate at the 0.02% level in copy/copy corresponding to 0.1% in mass fractions of GM-DNA

Background

Further to data analysis of the ring-trial concerning the event VCO-01981-5 (EURL-VL-07/12), it appeared that the repeatability standard deviation RSD_r at the GM-level 0.02% copy/copy, corresponding to 0.1% mass/mass, was above the 25% ENGL acceptance limit. The resulting investigation into the individual values traced the cause of the inflated RSD_r back to results of one laboratory and, specifically, to the reactions (three wells) with the taxon-specific reference system for one of the four replicates in the second PCR plate for that GM-level (validation code U18). The question arises if there are indeed sufficient statistical grounds to exclude the corresponding reactions from the global analysis.

Methods

Real time PCR analysis was carried out using the Full Process Kinetics approach (Lievens *et al.* 2011). For each reaction a Cq value and efficiency estimate was obtained. The latter two parameters were used for k-means clustering (k=2) of the taxon-specific reference data using the algorithm of Hartigan and Wong (1979) as implemented in R (v2.15.3).

The median (med) and median average deviation (mad) were used as robust estimators for the parameters' population mean (μ) and standard deviation (σ) respectively. The former were used to calculate z-scores for individual observations.

Results

The observations for U18 are remote in Cq value (average z-score: 3.7) and fall at the edge of the efficiency distribution, as is confirmed by clustering (see Figure 1). In addition, the amplification curves are dissimilar from the other taxon-specific amplification curves (see figure 2) in both Cq value and plateau position (average plateau z-score: -4.67). Especially the latter is unexpected, as the position of the plateau is mainly influenced by the amount of primer/probe available and not directly by the amount of target sequence nor the reaction kinetics (Lievens et al., 2012b). The U18 plateaus can be shown to be significantly different from the rest of the taxon-specific reactions (t-test p value: 5.775e-14).

Conclusion

Taking into consideration the above, it is concluded that the results of U18 can be regarded as outliers beyond reasonable doubt and the values can be removed from the global analysis.

References

Lievens, A.; Van Aelst, S.; Van den Bulcke, M. & Goetghebeur, E. (2012) Enhanced analysis of real-time PCR data by using a variable efficiency model: FPK-PCR Nucleic Acids Res, 40, e10

Lievens, A.; Van Aelst, S.; Van den Bulcke, M. & Goetghebeur, E. (2012) Simulation of between Repeat Variability in Real Time PCR Reactions. PLoS ONE, Public Library of Science, 7, e47112

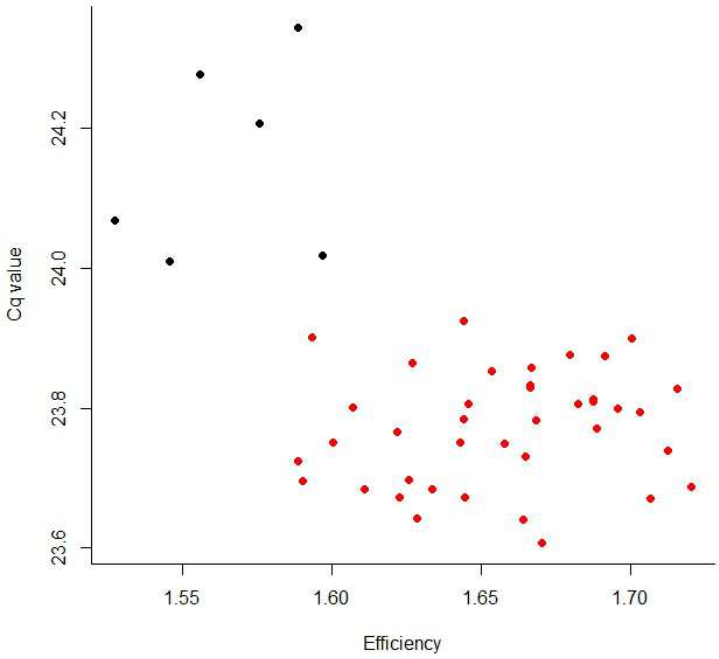

Figure 1: clustering analysis. Different clusters are represented by different colours. In total, six reactions cluster differently from the main group. Three of those belong to U18 (i.e. all U18 reactions).

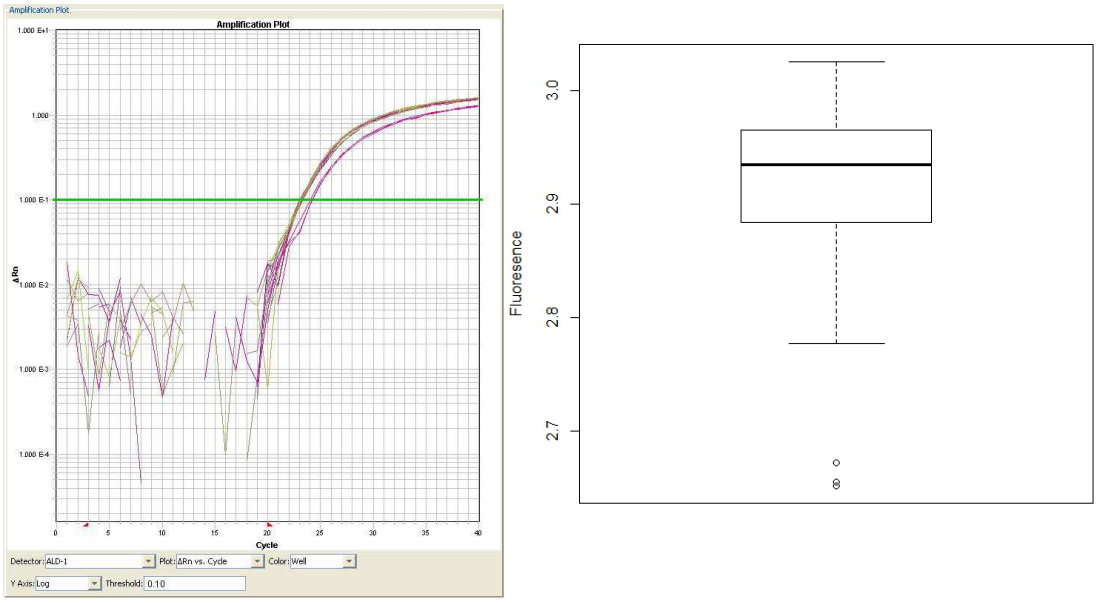

Figure 2: The left panel shows the amplification curves for the taxon-specific reactions of run B for the concerned laboratory. The curves for U18 cross the threshold later and attain a lower plateau. The Right panes shows a boxplot