European Commission

JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

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

Validation report

European Union Reference Laboratory for Genetically Modified Food and Feed

2016

Joint Research Centre

EURL European Union Reference Laboratory for GM Food & Feed

This publication is a Validated Methods, Reference Methods and Measurements report by the Joint Research Centre (JRC), the European Commission's science and knowledge service. It aims to provide evidence-based scientific support to the European policy-making process. The scientific output expressed does not imply a policy position of the European Commission. Neither the European Commission nor any person acting on behalf of the Commission is responsible for the use which might be made of this publication.

Contact information
Molecular Biology and Genomics Unit
Address: Joint Research Centre, Via Enrico Fermi 2749, TP 201, 21027 Ispra (VA), Italy
E-mail: eurl-gmff@jrc.ec.europa.eu
Tel.: +39 0332 78 5165
Fax: +39 0332 78 9333

JRC Science Hub
https://ec.europa.eu/jrc

JRC102144

Ispra, Italy: European Commission, 2016

How to cite: European Union Reference Laboratory for GM Food and Feed; Event-specific Method for the Quantification of Maize VCO-01981-5 Using Real-time PCR; JRC102144

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE
Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit
EUROPEAN COMMISSION
JOINT RESEARCH CENTRE
Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit

European Union Reference Laboratory for GM Food & Feed

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

Validation Report

24 June 2016

European Union Reference Laboratory for GM Food and Feed

Executive Summary

In line with its mandate[a], the European Union Reference Laboratory for GM Food and Feed (EURL GMFF), in collaboration with the European Network of GMO Laboratories (ENGL), has validated an event-specific polymerase chain reaction (PCR) method for detecting and quantifying maize event VCO-01981-5 (unique identifier VCO-Ø1981-5). The validation study was conducted according to the EURL GMFF validation procedure [http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm] and internationally accepted guidelines [1, 2].

In accordance with current EU legislation[b], Genective SA provided the detection method and the samples (genomic DNA extracted from maize seeds harbouring the VCO-01981-5 event as positive control DNA and genomic DNA extracted from conventional near isogenic maize counterpart as negative control DNA). The EURL GMFF prepared the validation samples (calibration samples and blind samples at test GM percentage [DNA/DNA]), organised an international collaborative study and analysed the results.

The study confirms that the method meets the method performance requirements as established by the EURL GMFF and the ENGL, in line with the provisions of Annex I-2.C.2 to Regulation (EC) No 641/2004 and fulfils the analytical requirements of Regulation (EU) No 619/2011[c].

This validation report is published at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx.

[a] Regulation (EC) No 1829/2003 of 22 September 2003 "on genetically modified food and feed".

[b] Regulation (EC) No 641/2004 of 6 April 2004 "on detailed rules for the implementation of Regulation (EC) No 1829/2003 of the European Parliament and of the Council as regards the application for the authorisation of new genetically modified food and feed, the notification of existing products and adventitious or technically unavoidable presence of genetically modified material which has benefited from a favourable risk evaluation".

[c] Regulation (EU) No 619/2011 of 24 June 2011 laying down the methods of sampling and analysis for the official control of feed as regards presence of genetically modified material for which an authorisation procedure is pending or the authorisation of which has expired.

Quality assurance

The EURL GMFF is ISO 17025:2005 accredited [certificate number: ACCREDIA 1172 (Flexible Scope for DNA extraction and qualitative/quantitative PCR).
The original version of the document containing evidence of internal checks and authorisation for publication is archived within the EURL GMFF quality system.

The EURL GMFF is also ISO 17043:2010 accredited (proficiency test provider) and applies the corresponding procedures and processes for the management of ring trials during the method validation.

The EURL GMFF conducts its activities under the certification ISO 9001:2008 of the Institute for Health and Consumer Protection (IHCP) provided by SGS.

Address of contact laboratory:

European Commission, Joint Research Centre (JRC)
Institute for Health and Consumer Protection (IHCP)
Molecular Biology and Genomics Unit (MBG)
European Union Reference Laboratory for GM Food and Feed
Via E. Fermi 2749, 21027 Ispra (VA) – Italy
Functional mailbox: eurl-gmff@jrc.ec.europa.eu

Content

1. INTRODUCTION

In line with Regulation (EC) No 1829/2003, Genective SA provided the EURL GMFF with an event-specific method for the detection, identification and quantification of maize event VCO-01981-5 (unique identifier VCO-Ø1981-5) together with genomic DNA as positive and positive control samples.

The dossier was found to be complete (step 1) and the scientific dossier assessment (step 2) found that the reported method performance characteristics met the ENGL method acceptance criteria[d], allowing to move the method into step 3, experimental testing.

In step 3, the EURL GMFF verified the purity of the control samples provided and conducted an *in-house* testing of samples and method. The positive and negative control DNA, submitted in accordance with Art 5(3) (j) and Article 17(3)(j) of Regulation (EC) No 1829/2003, were found to be of good quality and the performance of the method allowed accepting it for step 4, the international collaborative study.

The international collaborative study (step 4) confirmed that the method also performed adequately in the hands of 12 experienced laboratories and the final reporting (step 5) was finalised in time for the end of the EFSA assessment and confirmed that the method is fit-for-purpose.

2. Step 1 (dossier acceptance) and step 2 (scientific dossier assessment)

Documentation and data supplied by the applicant were found by the EURL GMFF to be complete (step 1) and compliance with the ENGL method acceptance criteria[d] was confirmed in step 2, which also included a bioinformatics analysis of the specificity of the method.

2.1. Specificity

The specificity of the event-specific assay was assessed by the method developer in real-time PCR using genomic DNA (200 ng) extracted from conventional maize and maize VCO-01981-5 and a selection of DNAs extracted from reference materials: GTS 40-3-2, 356043, 305423, MON89788, A5547-127 soybean; Bt176, Bt11, MON810, GA21, NK603, MON863, TC1507, 3272, MIR604, 59122, 98140, T25, MON88017, MON89034, MIR162 maize; H7-1 sugar beet; EH-92-527-1 potato; GHB119, 281-24-236 x 3006-210-23, MON1445, MON531, MON15985, LLCotton25, GHB614 cotton; GT73, T45, Ms8, Rf3, oilseed rape, LLRICE62.

[d] EURL/ENGL guidance doc "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm)

According to the method developer, apart from reacting with DNA extracted from VCO-01981-5, the forward and reverse oligonucleotide primers and the TaqMan® probe of the VCO-01981-5 event showed no amplification signals following quantitative PCR analysis. Each extracted DNA was also tested with the respective taxon-specific reference system to prove amplifiability of DNA.

The aldolase maize reference system was also tested against the same selection of extracted DNAs and two wheat and two barley conventional varieties. It reacted with all the maize extracts and with none of the DNA extracted from other species. The absence of allelic and copy number variation across a globally representative sample of diverse maize varieties was shown in stability tests: two-hundred nanograms of DNA from 20 different *Zea mays* cultivars originating from four different geographical regions reacted with the aldolase reference system and resulted in a mean Cq of 24.49 (lowest value: 24.08; highest value: 24.90).

Bioinformatics analyses of specificity

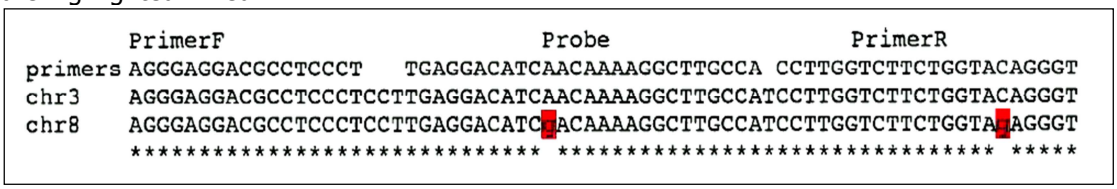
The specificity of the event-specific and the taxon specific assay was verified by the applicant and confirmed by the EURL GMFF by means of a bioinformatics analysis on the basis of the sequence data provided by the applicant. The target sequence (primers and probe), on which the method is based, spans the junction between the transgenic insert and the 5' genomic region. The binding site of the forward primer "VCO-01981-5 primer F" was found to be located in the genomic sequences adjacent to the insertion. The binding site of the probe "VCO-01981-5 probe" spans the junction region between the transgenic insert and the 5' genomic region, while the binding site of the reverse primer "VCO-01981-5 primer R" is located within the insert. According to the bioinformatics analysis, the amplicon size is expected to be of 85 bp, consistent with what is reported by the applicant. The sequence of the amplicon was analysed by BLAST[3] against local copies of the "nt" and "patents" databases, and no similarity was found with any other published sequence. In addition, the primers were tested against the sequences of the other GM events present in the Central Core Sequence Information System of the JRC, as well as the whole genomes of more than 80 plants (including *Brassica rapa*, *Glycine max*, *Oryza sativa*, *Triticum aestivum* and *Zea mays*) using the e-PCR prediction tool[4,5], and no potential amplicon was identified. The GM-assay therefore is expected to be event specific as requested.

For quantification it is important to have a reference gene with a known number of copies, ideally one, per genome. The amplification system (primers and probe) chosen by the applicant is the aldolase reference gene *(ald1)* with a stated amplicons length of 69 bp. The bioinformatics analysis confirmed that it anneals with 100% identity on chromosome 3 of maize (*Zea mays*). However, an additional sequence with high similarity was found on chromosome 8 of the maize genome. The latter locus shows only two SNPs (single strand polymorphisms), one in the probe and one in the reverse primer (Figure 1).

According to the applicant, there is no evidence that this second locus influences the PCR amplification of the target aldolase reference gene *(ald1)* and that therefore the taxon aldolase reference assay proposed by the applicant reacts as a one copy system within the TaqMan® assay.

However, the two observed SNPs would normally not suffice to reliably prevent the annealing of primers and probe of the aldolase amplification system to the additional binding sites on chromosome 8 in the conditions described in the method protocol.

Figure 1. Alignment of the two binding sites for the aldolase reference system of maize. SNPs are highlighted in red.



2.2. Method performance

The mean parameters of the calibration curve (slope, R^2 coefficient, Table 1) were determined by the applicant by quantifying in eight runs five test samples (sixteen replicates in total) at different GM levels. Tests were carried out on ABI Prism® 7900HT.

Table 1. Values of slope and R^2 obtained by the applicant

	ΔCq curve	
	Slope	**R^2**
Run1	-3.41	1.00
Run2	-3.46	0.99
Run3	-3.48	0.99
Run4	-3.39	0.99
Run5	-3.52	0.99
Run6	-3.54	0.99
Run7	-3.22	0.98
Run8	-3.47	0.99
Mean	**-3.43**	**0.99**

According to the ENGL method acceptance criteria, the mean value of the slope of the standard curve shall be within the range of -3.1 to -3.6, and the R^2 coefficient shall be ≥ 0.98.
Table 1 indicates that the mean slope of the standard curve is -3.43 and the mean R^2 coefficient is 0.99 and therefore both values are within the ENGL acceptance criteria.

Table 2 reports precision (measured as relative repeatability standard deviation RSD_r) and trueness (bias) for five GM levels as established by the applicant. Sixteen values for each GM level were provided (not shown). The mean values of trueness and precision were within the ENGL acceptance criteria (trueness ± 25%, RSD_r ≤ 25% across the entire dynamic range).

Table 2. Mean % (copy/copy), precision and trueness (measured across the dynamic range by the applicant over 16 values per GM level)

	Test results				
Expected GMO%	**0.09**	**0.45**	**0.90**	**1.80**	**4.50**
Measured mean GMO%	0.09	0.41	0.88	1.67	4.30
Precision (RSD_r %)	15.50	8.63	8.68	10.03	9.07
Trueness (bias %)	-3.49	-7.84	-2.02	-7.07	-4.34

* Numbers are not rounded but are presented as reported by the applicant

Acceptable performance was also observed by the applicant when the method was tested on ABI Prism® 7500 in the context of assessing robustness (data not shown) by altering the concentration of the primers and probe (±3%); by testing a different master mix (TaqMan fast advanced master mix), and by changing annealing/extension temperature (±1°C). In all cases, the bias (%) and the RSD_r (%) remained within the ENGL limits throughout the dynamic range.

3. Step 3 (experimental testing of the samples and method)

3.1 DNA extraction

A DNA extraction method from maize seeds was used by the method developer for extracting genomic DNA from VCO-01981-5 and non-GM maize seeds. This DNA extraction method was in-house verified by the EURL GMFF and found to provide DNA of acceptable quality. The verification report is published at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx.

3.2 Method protocol for the PCR analysis

The method provided by the applicant is an event-specific, quantitative, real-time TaqMan® PCR procedure for the determination of the relative content of GM event VCO-01981-5 DNA to total maize DNA. The procedure is a simplex system, in which a maize aldolase (*ald1*) taxon specific assay and the GM target assay (VCO-01981-5) are performed in separate wells.

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 (see also above, bioinformatics analysis). 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 an MGB (Minor Groove Binding) quencher at its 3′ end. The event specificity of this assay was verified and confirmed by the applicant against 35 closely related events and species. It was also confirmed by the bioinformatics analysis carried out by the EURL GMFF on the basis of the sequence information provided by the applicant.

As taxon specific reference method a maize (*Zea mays* L.) specific system is used that amplifies a fragment of the aldolase endogenous gene (Entrez® Database Accession No. NM_001111866.1), using gene-specific primers and a 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.

For relative quantification of event VCO-01981-5 in a test sample, the ΔCq values of calibration samples 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.

Note: While this procedure is scientifically correct and functions, the official GMO control laboratories do not use normally the ΔCq-approach but two standard curves to calibrate the GM- and the taxon-specific reference system. Hence they might see a need to carry out bridging studies that confirm that currently used approaches can equally be used.

According to the method provided by the applicant, the GM% content of the calibration samples is calculated considering the 1C value for maize genomes as equivalent to 2.73 pg (Plant DNA C-values Database[6]). The total DNA amount used in the PCR reactions and the corresponding % GM content of the calibration samples are listed in Table 3.

Note: Numerical values presented in the following tables were rounded keeping two digits for values ≤ 1, one digit for values between 1 and 10 and no digit for values ≥ 10, unless otherwise stated. The calculations in the MS Excel files however were done over not rounded data. This approach might create small inconsistencies in the numerical values reported in the tables but it allows a higher precision in the final results.

Table 3. Copy number values of the standard curve samples. The GM% content for the S5 sample is reported with three digits.

Sample code	S1	S2	S3	S4	S5
Total amount of DNA in reaction (ng)	200	200	200	200	200
GM% content (copy/copy)	5.6	2.0	1.0	0.5	0.015

3.3 EURL GMFF experimental testing (step 3)

3.3.1 Determination of the zygosity ratio in the positive control sample

Annex II of Regulation (EU) No 619/2011 requires that "when results are primarily expressed as GM- DNA copy numbers in relation to target taxon-specific DNA copy numbers calculated in terms of haploid genomes, they shall be translated into mass fraction in accordance with the information provided in each validation report of the EURL GMFF." In order to satisfy this

requirement, the EURL GMFF conducted an assessment of the zygosity (GM-target to reference-target ratio) in the positive control sample submitted by the applicant.

The copy number of the VCO-01981-5 and aldolase *(ald1)* targets were determined by digital PCR (dPCR) on the BioMark HD System using the 12.765 digital arrays (Fluidigm).
Reaction mixes were prepared in a final volume of 9 µL and contained 1X TaqMan® Universal PCR Master Mix (Applied Biosystems), 1X GE sample loading reagent (Fluidigm), primers and probe at the reaction concentrations indicated in the corresponding Validated Method document (http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx) and 1.5 ng or 3 ng of positive control sample DNA for the aldolase and VCO-01981-5 assays, respectively, in order to avoid panel saturation (optimal between 200<positive partitions<700).
Loading of the digital chip was performed according to the manufacturer's instructions by using the IFC controller (Fluidigm). A volume of 9 µL of reaction mix was loaded into each well of which only 4.6 µL were distributed into the 765 partitions (or chambers) constituting one panel. Five replicates of the same dilution were loaded in five panels for both the GM- and the reference assay. The experiments were repeated three times for a total number of fifteen data sets for both targets. No-template controls were included. Amplification conditions were as reported in the Validated Method document. Data analysis and copy number calculation were performed using the BioMark digital PCR Analysis software using a range of Cq retention from 15 to 35 or to 40.

Calculations of mean and variances where carried out according to the procedure outlined for random variables in the Annex 4 of the ENGL guidance document 'Verification of analytical methods for GMO testing when implementing inter-laboratory validated methods'[e].

3.3.2 In-house verification of the method performance against ENGL method acceptance criteria

The method performance characteristics were verified (EURL GMFF step 3) by quantifying on a copy number basis five blinded test samples with known GM levels, within the range 0.02%-4.5% copy/copy (equivalent to 0.1%-21.43% mass/mass). The experiments were performed on an ABI 7900, on Roche LC480 and at 0.02% (copy/copy) on ABI 7500 real-time platforms under repeatability conditions. Test samples with GM levels 4.5%, 1.8%, 0.9% and 0.45% were tested in two real-time PCR runs with two replicates for each GM-level on each plate (total of four replicates per GM level). The test sample with GM level 0.02% copy/copy (corresponding to 0.1% in mass fractions of GM material) was tested in 15 replicates in an additional real-time PCR run. Average values of the slope and of the R^2 coefficient of the standard curves and method trueness and precision of quantification over the dynamic range were evaluated for compliance against the ENGL method acceptance criteria.

[e] Verification of analytical methods for GMO testing when implementing interlaboratory validated methods. European Network of GMO laboratories (ENGL), 2011.
http://gmo-crl.jrc.ec.europa.eu/doc/ENGL%20MV%20WG%20Report%20July%202011.pdf

In order to assess the method compliance with Regulation (EU) No 619/2011, the EURL GMFF estimated, based on 15 replicates, also the method precision (RSD_r) at 0.1% GM level in mass fraction (m/m).

3.4 International collaborative study (step 4)

The international collaborative study (EURL GMFF step 4) involved twelve laboratories, all being "National Reference Laboratories (NRL), assisting the CRL for testing and validation of methods for detection", as listed in annex to Regulation (EC) No 1981/2006. The study was carried out in accordance with the following internationally accepted guidelines:

- The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" (Horwitz, 1995) (1)
- ISO 5725 (1994) (2)

The objective of the international collaborative study was to verify in experienced laboratories the trueness and reproducibility of the PCR analytical method provided by the applicant and described under 3.2 above and in the "Validated method" (Annex 1).

3.4.1 List of participating laboratories

The 12 participants in the VCO-01981-5 validation study (see Table 4) were randomly selected from the 25 NRLs that offered to participate.
Clear guidance was given to the selected laboratories for strictly following the validation protocol that was provided for the execution to them. The participating laboratories are listed in Table 4.

Table 4. Laboratories participating in the international validation study of the detection method for maize VCO-01981-5

Laboratory	Country
Center for Agricultural Technology Augustenberg	DE
Crop Research Institute - Reference Laboratory for GMO Detection and DNA fingerprinting	CZ
Laboratorio Arbitral Agroalimentario	ES
Laboratory of DNA analysis - Department of Gene Technology - Tallinn University of Technology	EE
Landeslabor Schleswig-Holstein - Food, Veterinary and Environmental Diagnostic Institute	DE
LGC Limited	UK
National Centre for Food, Spanish Food Safety Agency and Nutrition	ES
National Food and Veterinary Risk Assessment Institute Molecular Biology and GMO Department	LT
National Research Institute of Animal Production, National Feed Laboratory	PL
National Veterinary Research Institute in Pulawy, Department of Hygiene of Animal Feedingstuff	PL
State Institute of Chemical and Veterinarian Analysis - Freiburg	DE
State Office for Agriculture, Foodsafety and Fisheries - Mecklenburg Western Pomerania- Rostock	DE

3.4.2 Real-time PCR equipment used in the study

Laboratories involved in the collaborative study used the following real-time PCR equipment: ABI 7900 (five laboratories), ABI 7500 (four laboratories), Mx 3000P Agilent (one laboratory), Mx 3005P Agilent (one laboratory), Roche LC480 (one laboratory).

This variability of equipment, with its known potential influence on the PCR results, reflects the real-life situation in the control laboratories and provides additional assurance that the method is robust and useable under real conditions.

3.4.3 Materials used in the international collaborative study

The control samples for the validation of the quantitative event-specific method were prepared by the EURL GMFF from the genomic DNA provided by the applicant in accordance to Regulation (EC) No 1829/2003, Art 5.3.(j)[f]. They were derived from:

[f] Control sample means the GMO or its genetic material (positive sample) and the parental organism or its genetic material that has been used for the purpose of the genetic modification (negative sample).

i) genomic DNA extracted by the applicant from maize seeds harbouring the VCO-01981-5 event in hemizygous status and the GM-contribution derives from the male parent of the crossing (positive control);

ii) genomic DNA extracted by the applicant from conventional maize seeds genetically similar to those harbouring the VCO-01981-5 event (negative control).

The positive and negative control samples were used by the EURL GMFF for preparing standards (of known GM-content) and test samples (of unknown GM-content), containing mixtures of VCO-01981-5 maize genomic DNA and non-GM maize genomic DNA as GM-DNA copy numbers in relation to target taxon-specific genomic DNA copy numbers calculated in terms of haploid genomes.

The calibration samples S1-S5 were prepared by mixing the appropriate amount of VCO-01981-5 genomic DNA with control non-GM maize genomic DNA to obtain from 5.6% to 0.015% (copy/copy) solutions of GM VCO-01981-5. The total amount of genomic DNA/reaction and the GM% content of standards S1 to S5 are reported in Table 3.

The twelve NRLs participating in the validation study received the following materials:

- ✓ Five calibration samples with known concentrations of GM-event (90 µL of DNA solution each at 80 ng/µL) labelled from S1 to S5 (Table 3),
- ✓ Twenty blinded genomic DNA test-items (45 µL of DNA solution each at 75 ng/µL) labelled from U1 to U20, representing five GM levels (Table 5).

Table 5. VCO-01981-5 GM contents in blinded DNA samples

VCO-01981-5 GM% GM copy number/maize genome copy number x 100
4.50
1.80
0.90
0.45
0.020

- ✓ Reaction reagents:
  - 2x TaqMan Universal Master Mix™ Taq Ready Mix™, one vial: 8 mL
  - Sterile distilled sterile water, one vial: 6 mL

- ✓ Primers and probes (1 tube each) as follows:

Aldolase taxon-specific assay

- Aldolase-F (10 µM): 250 µL
- Aldolase-R (10 µM): 250 µL
- Aldolase-P (10 µM): 160 µL

VCO-01981-5 assay

- VCO-01981-5-F (10 µM): 250 µL
- VCO-01981-5-R (10 µM): 250 µL
- VCO-01981-5-P (10 µM): 160 µL

3.4.4 Design of the collaborative study

Participating laboratories received a detailed validation protocol that included, *inter alia*, the exact design of the PCR plates, ensuring that on each PCR plate the samples were analysed for the VCO-01981-5 event-specific system and for the aldolase taxon-specific system. In total, two plates were run per participating laboratory. Two replicates of each GM level were analysed on the same PCR plate and each test sample was analysed by PCR in three repetitions.

The laboratories prepared the amplification reaction mixes for the VCO-01981-5 and the aldolase assay in accordance with the description provided in the validation protocol. Calibration and test samples were loaded on the PCR plates as per pre-determined plate lay-out and amplified following the cycling program specified in the protocol. The raw data were reported to the EURL GMFF on an excel sheet that was designed, validated and distributed by the EURL GMFF. Participants determined the GM% in the test samples according to the instructions and using the excel sheet provided. All data are stored by the EURL GMFF on a dedicated and protected server.

The EURL GMFF analysed the data against the parameters and the limits set by the ENGL, i.e. trueness, precision, amplification efficiency and linearity.

3.4.5 Deviations reported from the protocol

Laboratories reported that they had followed the protocol with no deviations. One laboratory submitted data analysed with automatic or manual adjustment of baseline and thresholds. The latter were retained in line with the procedure recommended in the validation protocol for processing the data.

4. Results

4.1 EURL GMFF experimental testing

4.1.1 Zygosity ratio in the positive control sample

A summary of the dPCR analysis conducted on the positive control sample for both the VCO-01981-5 and the aldolase targets is shown in Table 6. The results were determined on a total of 15 data sets.

Table 6. Summary of dPCR analysis conducted on the VCO-01981-5 and aldolase targets in the positive control sample.

Mean ratio (VCO-01981-5/aldolase)	0.21
Standard deviation	0.019
RSD_r %	9.2
Standard error of the mean	0.005
Upper 95% CI of the mean	0.221
Lower 95% CI of the mean	0.199

Standard deviation, standard error of the mean, upper and lower confidence interval of the mean are reported with three digits.

The 95% confidence interval (CI) spans around 0.21. This value is about half of what is expected for a male parent contribution for the transgenic event VCO-01981-5 in the seeds used for the extraction of the positive control DNA[7-8] (3.4.3) under the assumption of a single copy reference target per haploid genome; however, these data are in line with the findings of the bioinformatics analysis (see 2.1) indicating the presence of two maize genomic loci reacting with the aldolase reference system, for an alpha = 0.05. From the Table 6 it can be deduced that a GM-level at 0.21% GM in copy number corresponds to a GM-level of 1.0% in mass fractions of GM DNA; a GM-level at 0.02% in copy number corresponds to a GM-level of 0.1% in mass fractions of GM DNA.

This is consistent with a GM-target from male parental contribution and with the presence of two aldolase copies per maize haploid genome capable to react with the aldolase-specific reference system. As a consequence, a GM level was designed at 0.02%, expressed in terms of GM DNA copy numbers in relation to target taxon-specific DNA copy numbers. This corresponds to a GM level of 0.1% related to mass fraction of GM material used for validation of the quantitative identification of maize event VCO-01981-5 with aldolase as maize reference system.

Further investigations carried out by the EURL GMFF by digital PCR showed that the ratio between the aldolase and the *hmg* reference system in maize was about 1.7 (from 1.6 to 1.8, 95% CI, number of replicates = 5, not shown). Maize *hmg* is accepted as a single copy reference system, therefore the measured ratio aldolase/*hmg* would confirm a deviation from the expected single copy number locus for the aldolase annealing sites of the respective amplification system in the maize haploid genome.

4.1.2 Result of the in-house verification of method performance against ENGL method acceptance criteria

Test samples with GM levels 0.45%, 0.9%, 1.8%, 4.5% (copy/copy), were tested in two real-time PCR runs (run A and B) on ABI 7900 and in two real-time PCR runs (run D and E) on Roche LC 480) with two replicates for each GM level on each plate (total of four replicates per GM-level).

The test sample with GM level 0.1% mass/mass (equivalent to 0.02% copy/copy) was tested in 15 replicates in one run (run C on ABI 7900, run F on Roche LC 480 and run G on ABI 7500). The corresponding standard curve parameters and the results of efficiency and linearity are shown in Table 7a, 7b and 7c. Slope and R^2 coefficient values were rounded to two digits.

Table 7a. Standard curve parameters of the real-time PCR testing carried out on ABI 7900

	VCO-01981-5		
	Slope	PCR efficiency*	R^2
Run A	-3.40	97	1.00
Run B	-3.52	92	1.00
Run C	-3.36	99	1.00

* PCR efficiency (%) is calculated using the formula Efficiency = $(10^{(-1/slope)}) - 1) \times 100$

Table 7b. Standard curve parameters of the real-time PCR testing carried out on Roche LC 480

	VCO-01981-5		
	Slope	PCR efficiency*	R2
Run D	-3.40	97	1.00
Run E	-3.50	93	1.00
Run F	-3.51	93	1.00

* PCR efficiency (%) is calculated using the formula Efficiency = $(10^{(-1/slope)}) - 1) \times 100$

Table 7c. Standard curve parameters of the real-time PCR testing carried out on ABI 7500

	VCO-01981-5		
	Slope	PCR efficiency*	R^2
Run G	-3.45	95	1.00

* PCR efficiency (%) is calculated using the formula Efficiency = $(10^{(-1/slope)}) - 1) \times 100$

According to the ENGL method acceptance criteria, the average value of the slope of the standard curve shall fall into the range -3.1 to -3.6 and the R^2 coefficient shall be ≥ 0.98. Tables 7a, 7b and 7c document that the slope of the standard curve and the R^2 coefficient were within the limits established by the ENGL.

The relative values of trueness and precision are reported in Tables 8a, 8b and 8c.

Table 8a. Values of trueness and precision as established by the EURL GMFF in its in-house verification using ABI 7900. GM% in copy/copy.

Target GM-levels % (copy/copy)	Measured GM level %	Bias % of the target GM-level	Precision (RSD_r %)
4.50	4.01	-11	9.0
1.80	1.93	7.2	20
0.90	0.82	-8.8	9.9
0.45	0.43	-4.4	17
0.02	0.016	-21	21

Table 8b. Values of trueness and precision as established by the EURL GMFF in its in-house verification using Roche LC 480. GM% in copy/copy.

Target GM-levels % (copy/copy)	Measured GM level %	Bias % of the target GM-level	Precision (RSD_r %)
4.50	4.70	4.4	2.2
1.80	1.70	-5.4	4.3
0.90	0.94	4.3	6.2
0.45	0.40	-10	6.8
0.02	0.019	-3.6	24

Table 8c. Values of trueness and precision as established by the EURL GMFF in its in-house verification using ABI 7500 at the 0.02% GM level. GM% in copy/copy.

Target GM-levels %	Measured GM level %	Bias % of the target GM-level	Precision (RSD_r %)
0.02	0.021	2.8	25

According to the ENGL method acceptance criteria, the method trueness (measured as bias %) should be within ±25% of the target value over the entire dynamic range. The method's precision estimated through relative standard deviation of repeatability (RSD_r) should be ≤25% over the entire dynamic range. Tables 8a, 8b and 8c document that trueness and precision of quantification were within the limits established by the ENGL. The EURL *in-house* results confirmed the data provided by the applicant.

4.2 Results of the international collaborative study

4.2.1 PCR efficiency and linearity

The PCR efficiency (%) and R^2 values (expressing the linearity of the regression) for the standard curve, reported by participating laboratories are displayed in Table 9.
The PCR efficiency (%) was calculated from the standard curve slopes using the formula:

$$\text{Efficiency} = (10 * (-1/\text{slope})) - 1) \times 100.$$

Table 9. Values of slope, PCR efficiency and R^2 obtained during the international collaborative trial. Slope and R^2 coefficient values were rounded to two digits.

Lab	Plate	Slope	PCR Efficiency (%)	R^2
1	A	-3.38	98	1.00
	B	-3.53	92	1.00
2	A	-3.26	102	1.00
	B	-3.56	91	1.00
3	A	-3.41	97	1.00
	B	-3.53	92	1.00
4	A	-3.17	107	1.00
	B	-3.27	102	1.00
5	A	-3.49	93	1.00
	B	-3.33	100	1.00
6	A	-3.32	100	1.00
	B	-3.31	100	1.00
7	A	-3.20	105	1.00
	B	-3.19	106	1.00
8	A	-3.30	101	1.00
	B	-3.34	99	1.00
9	A	-3.39	97	1.00
	B	-3.58	90	1.00
10	A	-3.31	101	0.99
	B	-3.23	104	1.00
11	A	-3.49	94	0.99
	B	-3.32	100	0.99
12	A	-3.51	93	0.98
	B	-3.34	99	0.99
	Mean	-3.36	98	1.00

Table 9 indicates that the efficiency of amplification for the standard curve ranges from 90 to 107%. The mean PCR efficiency is 98%, a value within the ENGL acceptance criteria. The average R^2 of the method is equal to 1.00.
These results confirm the appropriate performance of the method tested in terms of efficiency and linearity.

4.2.2 GMO quantification

Table 10 reports the values of quantification for the four replicates of each GM level as generated by each of the twelve participating laboratories, before application of the Cochran and Grubbs tests, which according to ISO 5725 are to be performed for identifying outlying values.

Table 10. GM% values determined by laboratories for test samples

	GMO content (%) *																			
LAB	**0.02**				**0.45**				**0.9**				**1.80**				**4.5**			
	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4
1	0.01	0.03	0.02	0.02	0.51	0.60	0.38	0.38	0.97	0.96	0.91	0.88	1.76	1.97	2.26	1.43	4.99	4.92	4.49	3.91
2	0.02	0.03	0.02	0.02	0.44	0.47	0.50	0.51	0.92	1.14	1.13	1.29	2.27	2.54	1.64	2.73	5.31	4.37	5.65	4.72
3	0.02	0.03	0.02	0.02	0.39	0.42	0.40	0.40	0.97	0.82	0.85	0.81	1.60	1.56	1.72	1.68	4.38	3.90	3.81	3.80
4	0.01	0.03	0.02	0.02	0.54	0.59	0.46	0.38	0.87	0.93	0.80	0.87	2.06	1.49	2.47	1.73	7.58	5.82	4.33	5.47
5	0.02	0.02	0.02	0.02	0.39	0.31	0.44	0.44	0.83	0.84	1.04	0.99	1.89	1.70	1.41	1.85	4.47	4.11	4.29	3.67
6	0.02	0.01	0.04	0.02	0.57	0.41	0.44	0.42	0.88	0.95	0.89	0.83	1.47	1.87	1.71	1.71	4.32	4.20	4.93	4.77
7	0.02	0.02	0.01	0.02	0.40	0.42	0.38	0.34	0.72	0.94	0.85	0.96	1.87	1.66	1.68	1.92	4.48	5.32	4.79	5.20
8	0.02	0.01	0.02	0.02	0.38	0.47	0.40	0.46	0.84	1.23	0.92	0.93	2.21	1.48	1.61	2.11	4.52	4.58	4.01	4.09
9	0.02	0.03	0.03	0.02	0.49	0.71	0.42	0.48	0.95	0.98	0.88	0.86	1.65	5.42	2.41	1.77	5.40	5.25	1.72	6.27
10	0.02	0.02	0.02	0.02	0.50	0.56	0.48	0.46	0.80	1.23	1.20	0.90	1.89	1.73	1.88	1.66	5.78	5.06	3.95	4.80
11	0.02	0.02	0.01	0.03	0.57	0.43	0.44	0.41	0.92	1.28	0.87	0.92	2.78	1.82	2.00	2.26	5.66	4.56	4.67	4.58
12	0.04	0.02	0.03	0.03	0.28	0.42	0.39	0.61	1.50	0.65	0.96	0.83	2.82	1.32	1.77	1.74	4.39	3.75	3.10	5.31

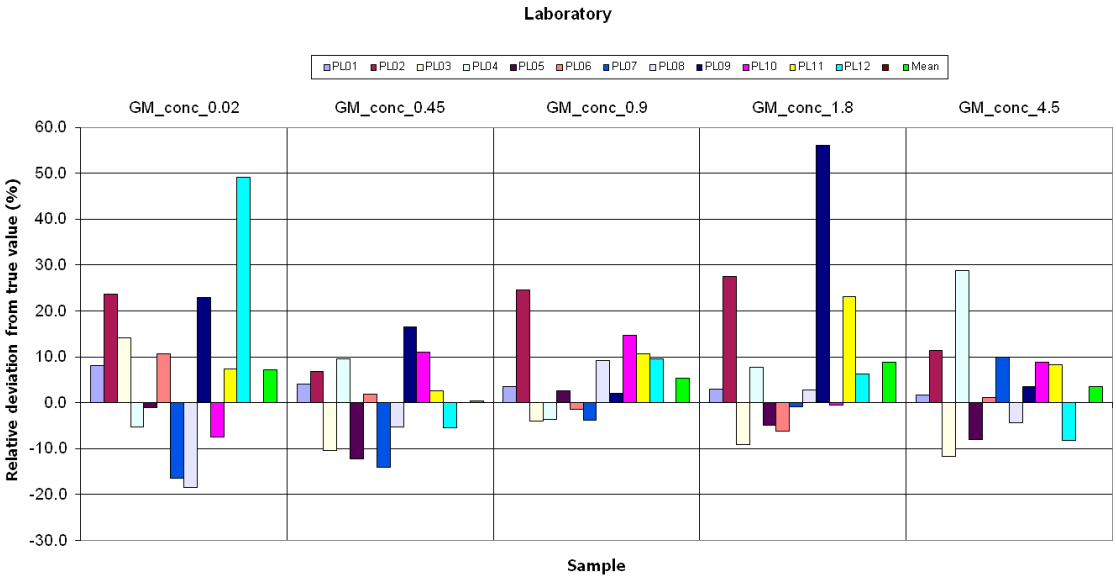
* GMO% = (GMO copy number/maize genome copy number) x 100

A graphical representation of the data reported in Table 10 is provided in Figure 2 where the relative deviation from the target value for each GM level tested is shown for each laboratory. The coloured bars represent the deviation of the GM level measured by the respective laboratory in % of the true GM level; the light green bar on the right represents the mean relative deviation over all 12 participating laboratories for each true GM level.

One laboratory greatly overestimated the GM-content at the 0.02% GM-level and another laboratory at the 1.8% level. Overall, the mean relative deviations from the true values were within a maximum of ±25% at all GM levels for all laboratories, with no trend towards overestimation or underestimation over the dynamic range. The mean relative deviations from the true values (light green bar) were within a maximum of 10%.

All data were retained for the statistical analysis and for tests of outliers (Cochran and Grubbs) whose results are reported in Table 11.

Figure 2. Relative deviation (%) from the true value of concentration (% copy/copy,) of VCO-01981-5 for all laboratories



4.2.3 Method performance requirements

According to the method performance requirements established by the ENGL and adopted by the EURL GMFF (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm), reproducibility is assessed through an international collaborative trial. Table 11 illustrates the estimation of reproducibility at the various GM levels tested during the collaborative trial.

According to the ENGL method performance requirements, the relative reproducibility standard deviation (RSD_R), that describes the inter-laboratory variation, should be below 35% over the majority of the dynamic range, while it should be below 50% at the lower end of the dynamic range.

As it can be observed in Table 11, the method fully satisfies this requirement at all GM levels tested. In fact, the highest value of RSD_R is 30% at the 0.02% GM level, thus well within the acceptance criterion.

The trueness of the method is estimated in the collaborative trial using the measures of the method bias for each GM level. According to ENGL method performance requirements, trueness should be ± 25% across the entire dynamic range. The method satisfies this requirement across the dynamic range tested, with the highest value of bias (%) being 7.2% at the 0.02% GM level.

Table 11 also documents the relative repeatability standard deviation (RSD_r) estimated for each GM level. In order to accept methods for a collaborative study, the EURL GMFF requires the RSD_r value to be below 25%, as indicated by the ENGL (Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm). As it can be observed from the values reported, the method showed a relative repeatability standard deviation below 25% at all GM levels, with the exception of an RSD_r at 27% at the lowest GM-level (0.02% in copy numbers corresponding to 0.1% in mass fractions of GM-DNA).

Table 11. Summary of validation results for the VCO-01981-5 method

	Test Sample GMO % (*)				
	0.02	**0.45**	**0.9**	**1.8**	**4.5**
Laboratories having returned valid results	12	12	12	12	12
Samples per laboratory	4	4	4	4	4
Number of outliers	0	0	1	1	1
Reason for exclusion	-	-	C	C	C
Mean value of measured GM content (%)	0.02	0.45	0.94	1.9	4.7
Relative repeatability standard deviation, RSD_r (%)	27(24**)	17	13	19	14
Repeatability standard deviation	0.006	0.079	0.126	0.351	0.653
Relative reproducibility standard deviation, RSD_R (%)	30	18	14	20	17
Reproducibility standard deviation	0.006	0.082	0.136	0.369	0.773
Bias (absolute value)	0.001	0.002	0.044	0.080	0.155
Bias (%)	7.2	0.42	4.9	4.4	3.4

* GMO % expressed as copy/copy.
** Further to removal of one outlying replicate out of the four replicates for level 0.02% in one laboratory, the RSD_r corresponded to 24% (see discussion in the text).
C= Cochran's test; identification and removal of outliers through Cochran and Grubbs tests, according to ISO 5725-2; bias is estimated according to ISO 5725 data analysis protocol [2].

However, the EURL GMFF performed in depth analyses to understand the apparent underperformance of the method at the lower end of the dynamic range (i.e. at the 0.02% level in copy ratio corresponding to 0.1% in mass fractions of GM-DNA). These analyses showed that the deviating value is due to one of the four replicates performed by one laboratory. Each replicate is the result of the mean of three PCR reactions run in three wells (§ 3.4.4). The observation is that the three individual wells averaged in that replicate are remote in Cq value (average *z*-score: 3.7) and fall at the edge of the efficiency distribution, as confirmed by k-means clustering[9]. In addition, the amplification curves are dissimilar from the other amplification curves of the maize reference system in both Cq value and plateau position (average plateau *z*-score: -4.67). Taking these observations into consideration, the EURL GMFF concluded that the results for this replicate (the three repeated wells) can be regarded as outlier beyond reasonable doubt (see Annex 2 for further details).

When the outlying replicate is removed the performance parameters at the 0.02% in copy/copy (corresponding to 0.1% in mass fractions of GM-DNA) are those reported in Table 12.

Table 12. Summary of validation results for the VCO-01981-5 method at the lowest end of the dynamic range

Mean value of measured GM content (%)	Relative repeatability standard deviation, RSD_r (%)	Repeatability standard deviation	Relative reproducibility standard deviation, RSD_R (%)	Reproducibility standard deviation	Bias (absolute value)	Bias (%)
0.02	24	0.05	28	0.06	0.001	5.1

Thus, it can be observed that the method fully satisfies the performance requirements at all GM levels tested. In fact, further to removal of the outlier replicate, the highest value of RSD_R, RSDr and trueness are respectively 28%, 24% and 5.1% at the 0.02% GM level, thus within the acceptance criteria.

5. Compliance of the method for detection of event VCO-01981-5 with the requirements of Regulation (EU) No 619/2011

To verify the compliance of the method under validation with the requirements of Regulation (EU) No 619/2011, the following evaluations were carried out:

- at step 2 of the validation process (scientific assessment of the dossier), the applicant's data (Table 2) indicated that the RSD_r at the level of 0.09%, expressed as ratio of GM-DNA copy numbers to target taxon-specific DNA copy numbers, was 15.5%, hence below 25%. The results had been determined on 16 replicates. The EURL GMFF accepted the applicant's data and moved into step 3;

- at step 3 of the validation process (*in-house* testing of the method), the EURL GMFF determined the RSD_r at the level of 0.1% m/m (expressed as mass fraction of GM-material and corresponding to a level of 0.02% expressed in terms of copy number ratio). The measurements were carried out under repeatability conditions on 15 replicates. The RSD_r was found to be about 21% on real-time PCR platform ABI 7900, 24% on real-time PCR platform Roche LC480 and close to 25% on real-time PCR platform ABI 7500, respectively (Table 8a, 8b and 8c), hence less than or equal to 25%;

- the collaborative study (step 4 of the validation process) established that (over the twelve participating laboratories) the mean RSD_r of the method at the level of 0.1% (m/m, corresponding to a level of 0.02% expressed in terms of copy number ratio) was 24% after removal of the outlying replicate, therefore below the limit of 25%.

The outcome of these tests is summarised in Table 13.

Table 13. Precision of the method for quantitative detection of event VCO-01981-5 around 0.1%

Source	Real-time PCR	**RSD_r %**	**GM %**
Applicant's method optimisation	ABI Prism 7900HT	15.5	0.09*
EURL GMFF in-house verification	ABI 7900	21	0.1**
	Roche LC480	24	
	ABI 7500	25	
Collaborative study	§ 4.2.3	24	0.1**

* copy/copy: GM- DNA copy numbers in relation to target taxon specific DNA copy numbers
** m/m: mass fraction of GM material in relation to non-GM material

Based on the results of the *in-house* verification and of the collaborative study, it is concluded that the method RSD_r% is lower than 25% at the level of 0.1% related to mass fraction of GM-material, hence the method for quantitative detection of maize event VCO-01981-5 meets the requirement laid down in Regulation (EU) No 619/2011.

6. Conclusion

A method for detection, identification and quantification of GM event VCO-01981-5 was provided by the applicant. This method has been fully validated in accordance to the EURL GMFF validation scheme (step 1, 2, 3 and 4), respecting all requirements of the relevant EU legislation and international standards for method validation.

This validation study confirmed that the method is applicable to the control samples provided by the applicant (see paragraph 3.4.3), in accordance with the requirements of Annex I-2.C.2 to Regulation (EC) No 641/2004 and (EU) No 619/2011. The method is therefore valid to be used for control purposes, including the quantification of low level presence of 0.1% (m/m). It can be assumed that it is applicable to any appropriately extracted maize DNA.

7. References

1. Horwitz W., 1995. Protocol for the design, conduct and interpretation of method-performance studies, *Pure and Appl. Chem.* 67, 331-343.
2. International Standard (ISO) 5725, 1994. Accuracy (trueness and precision) of measurement methods and results. International Organization for Standardization, Genève, Switzerland.
3. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990 Oct 5;215(3):403-10. PMID:2231712
4. Schuler GD. Sequence mapping by electronic PCR. Genome Res. 1997 May;7(5):541-50
5. Rotmistrovsky K, Jang W, Schuler GD. A web server for performing electronic PCR. Nucleic Acids Res. 2004 Jul 1;32(Web Server issue):W108-12
6. Plant DNA C-values Database, http://data.kew.org/cvalues/.
7. Trifa Y. and Zhang D. DNA content in Embryo and endosperm of maize kernel (*Zea mays* L.): impact on GMO quantification. *J. Agric. Food Chem.* 2004; 52: 1044-1048

8. Zhang D., Corlet A., Fouilloux S. Impact of genetic structure on haploid genome-based quantification of genetically modified DNA: theoretical considerations, experimental data in MON 810 maize kernels (*Zea mays* L.) and some practical applications. *Transgenic* Res. 2008: 17; 393-402.1
9. Hartigan, J. A. and Wong, M. A. (1979). A K-means clustering algorithm. Applied Statistics 28, 100–108

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE
Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit

Annex 1: 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.

3.5 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

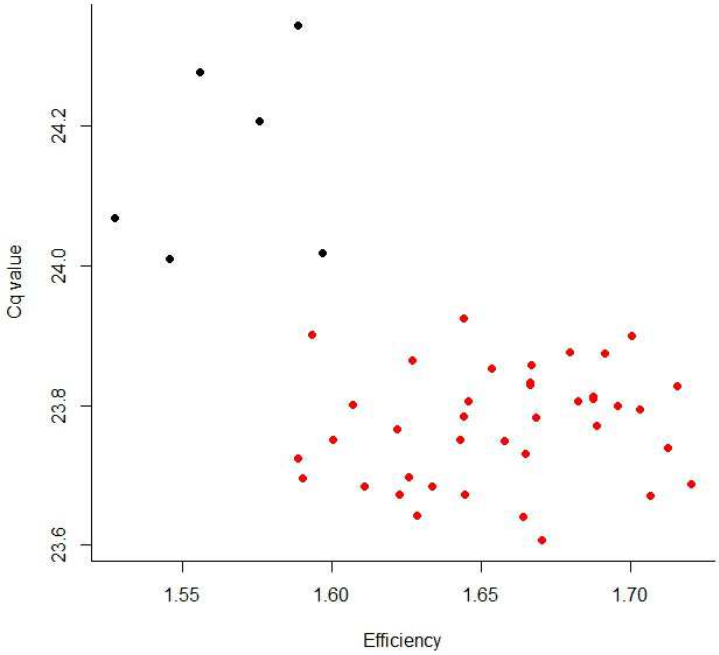


Figure 1: clustering analysis. Different clusters are represented by different colours. In total, six reactions cluster differently from the main group. Three of those belong to U18 (i.e. all U18 reactions).

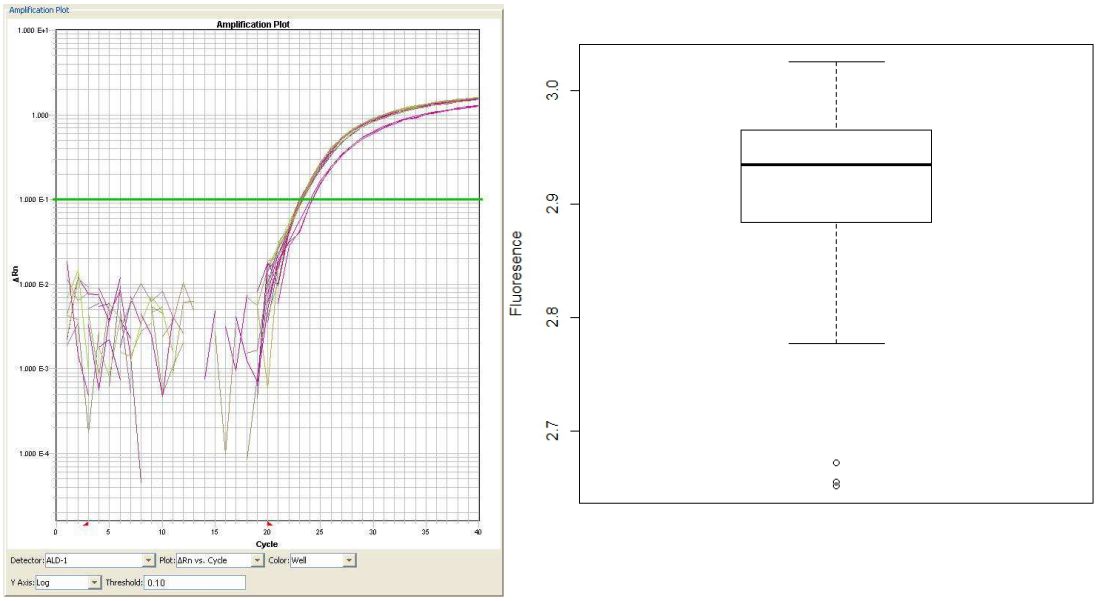


Figure 2: The left panel shows the amplification curves for the taxon-specific reactions of run B for the concerned laboratory. The curves for U18 cross the threshold later and attain a lower plateau. The Right panes shows a boxplot

---------- Page intentionally left blank ----------

JRC102144

JRC Mission

As the science and knowledge service of the European Commission, the Joint Research Centre's mission is to support EU policies with independent evidence throughout the whole policy cycle.

EU Science Hub
ec.europa.eu/jrc

@EU_ScienceHub

EU Science Hub - Joint Research Centre

Joint Research Centre

EU Science Hub