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Molecular Biology and Genomics Unit

EURL
European Union Reference Laboratory
for GM Food & Feed

Event-specific Method for the Quantification of Cotton T304-40 Using Real-time PCR

Validation Report

19 December 2012

European Union Reference Laboratory for GM Food and Feed

Executive Summary

In line with its mandate[1] the European Union Reference Laboratory for GM Food and Feed (EU-RL 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 cotton event T304-40 (unique identifier BCS-GHØØ4-7). The validation study was conducted according to the EU-RL GMFF validation procedure [http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm] and internationally accepted guidelines.

In accordance with current EU legislation[1], Bayer CropScience has provided the detection method and the samples (genomic DNA from cotton seeds harbouring the T304-40 event as positive control DNA, genomic DNA from conventional cotton seeds as negative control DNA). The EU-RL 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 EU-RL GMFF and the ENGL, in line with the provisions of Annex I-2.C.2 to Regulation (EC) No 641/2004[1] and it fulfils the analytical requirements of Regulation (EU) No 619/2011[2].
This validation report is published at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm.

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[1] Regulation (EC) No 1829/2003 of 22 September 2003 "on genetically modified food and feed" and Regulation (EC) No 641/2004 of 6 April 2004 "on detailed rules for the implementation of Regulation (EC) No 1829/2003".

[2] 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 EU-RL GMFF is ISO 9001:2008 certified (certificate number: CH-32232) and ISO 17025:2005 accredited [certificate number: ACCREDIA 1172, (Flexible Scope for DNA extraction and qualitative/quantitative PCR) - Accredited tests are available at http://www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7].

The original version of the document containing evidence of internal checks and authorisation for publication is archived within the EU-RL GMFF quality system.

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1. Timeline

In line with Regulation (EC) No 1829/2003, Bayer CropScience provided the EU-RL GMFF with a copy of the official application for authorisation of an event-specific method for the detection and quantification of cotton event T304-40 (unique identifier BCS-GHØØ4-7) together with negative and positive control samples (February 2011).

In response to an earlier submission of the method, the EU-RL GMFF started its step-wise validation procedure (step 1: dossier reception) before the formal approval by EFSA of the official dossier (October 2011).

The scientific dossier assessment (step 2) focused on the reported method performance characteristics assessed against the ENGL method acceptance criteria[3] (see http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf for a summary of method acceptance criteria and method performance requirements) and it was positively concluded already in March 2011.

In step 3 of the validation procedure (experimental testing), the EU-RL GMFF verified the purity of the control samples provided and conducted the in-house testing of samples and methods. The positive and negative control DNA, submitted in accordance with Art 5(3)(j) and Article 17(3)(j) of Reg. (EC) No 1829/2003, were found of good quality.

The method characteristics were verified by quantifying five blind GM levels within the range 0.1%-4.5% on a genome copy number basis. The experiments were performed under repeatability conditions and demonstrated that the PCR efficiency, linearity, trueness and precision were within the limits established by the ENGL.

In addition, and in line with the requirements of Reg. (EU) No 619/2011, the EU-RL GMFF also verified *i)* the zygosity ratio of the submitted positive control sample by investigating the GM- to reference- target ratio by means of digital PCR, in order to determine the conversion factor between copy numbers and mass fractions, and *ii)* the method's precision (relative repeatability standard deviation, RSDr) at the 0.1% related to mass fraction of GM-material on fifteen replicates. Step 3 was completed in June 2012 and concluded that the method could be finally tested into a collaborative study (step 4).

The collaborative study (step 4) took place in July-August 2012. It demonstrated that the method is well suited for analysing DNA, appropriately extracted from food or feed, and identifying the presence of GM event T304-40. The method is therefore applicable for this purpose.

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

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

Documentation and data supplied by the applicant were evaluated in-house by the EU-RL GMFF for compliance with the ENGL method acceptance criteria. The parameters of the calibration curves (slope, R^2 coefficient) were determined by the applicant by quantifying in three runs three test samples at different GM levels (see Table 1).

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

	T304-40		*AdhC*	
	Slope	**R^2**	**Slope**	**R^2**
Run 1	-3.3	1.00	-3.3	0.99
Run 2	-3.3	1.00	-3.3	1.00
Run 3	-3.3	1.00	-3.4	1.00

According to the ENGL method acceptance criteria, the average value of the slope of the standard curve shall range from -3.1 to -3.6 and the R^2 coefficient shall be ≥ 0.98.
Table 1 indicates that the slope and the R^2 coefficient of the standard curves for the GM-system (T304-40) and the cotton–specific reference system (*AdhC*) are between -3.3 to -3.4 and ≥ 0.99 and therefore within the ENGL acceptance criteria.

Table 2 reports precision and trueness for the three GM-levels tested by the applicant. Eighteen values for each GM-level were provided. The mean values of trueness and precision were within the ENGL acceptance criteria (trueness ± 25%, RSDr ≤ 25% across the entire dynamic range).

Table 2. Mean %, precision and trueness (measured at three GM-levels by the applicant)

	Test results		
Expected GMO%	**0.08**	**0.9**	**4.5**
Measured mean %	0.07	0.82	4.27
Precision (RSDr)	11	7.4	7.2
Trueness (bias %)	13	9.3	5.4

3. Materials and methods

3.1 DNA extraction

The applicant made reference to an EU-RL GMFF in-house verified method to extract genomic DNA from ground cotton seeds and grains for the quantitative analysis of T304-40 cotton event[4].

In agreement with the ENGL position, which endorses the modularity principle (see also Annex I to Reg. (EC) No 641/2004), the EU-RL GMFF considers the above mentioned DNA extraction method applicable in the context of the validation of the method for analysis of event T304-40 cotton, given the similarity of the cotton matrix.

3.2 Method protocol for the PCR analysis

The PCR method provided by the applicant (see the corresponding Validated Method at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm) and subsequently validated by the EU-RL GMFF is an event-specific, quantitative, real-time TaqMan® PCR procedure for the determination of the relative content of GM event T304-40 DNA to total cotton DNA. The procedure is a simplex system, in which a cotton alcohol dehydrogenase C (*AdhC)* specific assay and the GM target assay (T304-40) are performed in separate wells.

For the specific detection of cotton event T304-40, a 78-bp fragment of the region spanning the 3′ insert-to-plant junction in cotton event T304-40 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 TAMRA (carboxytetramethylrhodamine) as a quencher dye at its 3′ end.

For the relative quantification of GM event T304-40, a cotton-specific reference system amplifies a 73-bp fragment of the *AdhC* gene, a cotton endogenous gene (Accession number, GeneBank: AF036569 and AF403330), using *AdhC* gene-specific primers and a *AdhC* gene-specific probe, labelled with VIC as a reporter dye at its 5′ end and TAMRA as a quencher at its 3′ end.

Standard curves are generated for both the T304-40 and the *AdhC* systems by plotting the Ct values measured for the calibration points against the logarithm of the DNA copy numbers and

[4] "CTAB/Genomic-tip 20" method for DNA extraction suitable for the isolation of genomic DNA from ground cotton seeds and grains, submitted in support of the validation of the method for detection of event LLCotton25. The extraction procedure was evaluated and tested by the EU-RL GMFF in order to confirm its performance characteristics. The protocol for DNA extraction and a report on testing were published in 2007 at http://gmo-crl.jrc.ec.europa.eu/summaries/LLCotton25_DNAExtr_report.pdf

by fitting a regression line into these data. Thereafter, the standard curves are used to estimate the copy numbers in the test sample DNA by interpolation from the standard curves.

For relative quantification of event T304-40 DNA in a test sample, the T304-40 copy number is divided by the copy number of the cotton reference gene (*AdhC*) and multiplied by 100 to obtain the percentage value (GM% = T304-40/*AdhC* x 100).

The absolute copy numbers of the calibration curve samples are calculated by dividing the sample DNA mass (expressed in picograms) by the published average 1C value for the cotton genome (2.33 pg) (Arumuganathan & Earle, 1991) (1). The total DNA quantity used in PCR and the corresponding GM and *AdhC* copy number values of the calibration samples are listed in Table 3.

Table 3. DNA amount and copy number values of the standard curve samples, as determined by the EU-RL GMFF

Sample code	S1	S2	S3	S4	S5
Total amount of DNA in reaction (ng)	300	100	20	5	1
Target taxon *AdhC* copies	128755	42918	8584	2146	429
T304-40 Cotton GM copies	12876	4292	858	215	43

3.3 EU-RL GMFF experimental testing (step 3)

3.3.1 Determination of the zygosity ratio in the positive control sample

Annex II of Reg. (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 EU-RL GMFF." In order to satisfy this requirement, the EU-RL 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 T304-40 and *AdhC* targets was determined by digital PCR (dPCR) on the BioMark HD System using the 12.765 digital arrays (Fluidigm).

Four micrograms of control genomic DNA were digested overnight at 37 ºC with 60 units of restriction enzyme EcoRI that does not cleave within the annealing sites of the primers for the T304-40 or *AdhC* amplification systems.

Bioinformatics analysis found that EcoRI restriction sites are located outside the respective targeted sequences. Further to digestion, the DNA was precipitated with ammonium acetate (2.5 M final) and two volumes of absolute ethanol. The outcome of the enzymatic digestion was controlled by running approximately 200 ng of digested and undigested DNA alongside DNA molecular markers in 1% agarose-gel electrophoresis.

The digested DNA was then used as a template for the digital PCR experiments. Reaction mixes were prepared in a final volume of 9 µL and contained 1X TaqMan® Universal PCR Master Mix with UNG (Applied Biosystems), 1X GE sample loading reagent (Fluidigm), primers and probe at the reaction concentrations indicated in the corresponding Validated Method (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm), 1 µL of DNA at a concentration of 1.5 ng/µL, suitable to avoid panel saturation after analysis (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 approximately 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 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. Data analysis and copy number calculation was performed using the BioMark digital PCR Analysis software, the range of Ct retention was from 15 to 35.

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[5].

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

The method performance characteristics were verified (EU-RL GMFF step 3) by quantifying on a copy number basis five blinded test samples with known GM levels, within the range 0.1%-4.5%. The experiments were performed on an ABI 7900 real-time platform under repeatability conditions. Test samples with GM-levels, 0.1%, 0.4%, 0.9%, 2.0%, 4.5%, 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 0.1% was tested in 15 replicates in one 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.

[5] 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 EU-RL GMFF also determined the zygosity of the GM-insert in the positive control sample and estimated the method precision (RSDr) at 0.1% GM level in mass fraction on the basis on 15 replicates.

3.4 International collaborative study (step 4)

The international collaborative study (EU-RL 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)
- ISO 5725 (1994)

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

3.4.1 List of participating laboratories

The participants in the T304-40 validation study were randomly selected from the 17 national reference laboratories (NRLs) that offered to participate. They are listed in Table 4.

Clear guidance was given to the selected laboratories for strictly following the standard operational procedures provided for the execution of the protocol (the "Validated Method" is available in Annex 1 and at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

Table 4. Laboratories participating in the validation of the detection method for cotton T304-40

Laboratory	Country
Laboratory of DNA analysis - Department of Gene Technology - Tallinn University of Technology	EE
Laboratory for the Detection of GMO in Food - Bad Langensalza	DE
Bavarian Health and Food Safety Authority	DE
Food and Environment Research Agency	UK
INRAN - Seed Testing Station	IT
National Health Laboratory, Food Control Department	LU
Plant Health Laboratory	FR
National Centre for Food, Spanish Food Safety Agency and Nutrition	ES
Genetically Modified Organism Controlling Laboratory	PL
National Institute of Biology	SI
Veterinary Public Health Institute for Lazio and Toscana Regions; National Reference Centre for GMO Analysis	IT
Italian National Institute for Health - Department of Veterinary Public Health and Food Safety - Unit GMOs and Mycotoxins	IT

3.4.2 Real-time PCR equipment used in the study

Laboratories involved in the collaborative study used the following real-time PCR equipment: five laboratories used ABI 7900, four laboratories used ABI 7500, and three laboratories used respectively Roche LC480, ABI 7700 and Stratagene Mx3005p.

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

For the validation of the quantitative event-specific method, control samples were provided by the EU-RL GMFF to the participating laboratories. They were derived from:

i) genomic DNA extracted by the applicant from cotton seeds harbouring the event T304-40 homozygously, and
ii) genomic DNA extracted by the applicant from conventional cotton seeds genetically similar to those harbouring the T304-40 event.

The control samples were used by the EU-RL GMFF for preparing standard and test samples (of unknown GM-content), containing mixtures of T304-40 cotton and non-GM cotton. The content

was expressed in terms of haploid genomes ratios between the calculated GM-DNA and target taxon-specific DNA copy numbers.

The calibration sample S1 was prepared by mixing the appropriate amount of T304-40 DNA with control non-GM cotton DNA to obtain a 10% solution of GM T304-40 (12876 GM copies). Calibration samples S2 to S4 were prepared by serial dilutions of the 10% standard. S2 contains 4292 cotton GM copies (3-fold dilution), S3 contains 858 GM copies (five-fold dilution), S4 contains 215 GM copies (four-fold dilution) and S5 contains 43 GM copies (five-fold dilution) (see Table 3).

The 12 NRLs participating in the validation study received the following materials:

- ✓ Five calibration samples with known concentrations of GM-event (175 µL of DNA solution each) labelled from S1 to S5 (Table 3).
- ✓ Twenty blinded test DNA samples (87.5 µL of DNA solution each at 40ng/uL) labelled from U1 to U20, representing 5 GM levels (Table 5).

Table 5. T304-40 GMO contents

T304-40 GM% GM copy number/cotton genome copy number x 100
0.1
0.4
0.9
2.0
4.5

- ✓ Reaction reagents:
  - TaqMan® Universal PCR Master Mix (2x), two vials: 10 mL
  - distilled sterile water, one vial: 5 mL

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

  AdhC taxon-specific assay
  - KVM157 (10 µM): 160 µL
  - KVM158 (10 µM): 160 µL
  - TM012 (VIC) (10 µM): 160 µL

  T304-40 assay provided by the applicant
  - SHA 029 (10 µM): 320 µL
  - SHA 030 (10 µM): 320 µL
  - TM 089 (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 T304-40 specific system and for the *AdhC* taxon-specific system. In total, two plates were run per participating laboratory.

The laboratories prepared the master-mixes for the T304-40 and *AdhC* 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. The amplification reactions followed the cycling program specified in the protocol and the raw data were reported to the EU-RL GMFF on an excel sheet that was designed, validated and distributed by the EU-RL 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 EU-RL GMFF on a dedicated and protected server.

The EU-RL 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

One lab reported that plate A was repeated for an error in loading sample U8 and that the plates spin down step (point 7 of the protocol) were not carried out.

4. Results

4.1 EU-RL GMFF experimental testing

4.1.1 Determination of the zygosity ratio in the positive control sample

A summary of the dPCR analysis conducted on the positive control sample for both the T304-40 and the *AdhC* targets is shown in Table 6. The results were determined on a total of thirteen data sets.

Table 6. Results of the tests to determine the zygosity ratio in the positive control sample.

Mean ratio (T304-40/*AdhC*)	1.05*
Standard deviation	0.112
RSD_r %	11
Standard error of the mean	0.029
Upper 95% CI of the mean	1.11
Lower 95% CI of the mean	0.99

* Mean of thirteen datasets, two replicates were removed

In conclusion, the 95% confidence interval (CI) spans around 1 and therefore the mean ratio is significantly different from an expected ratio of 1, assuming a GM homozygous and a single-copy reference target, for an alpha = 0.05.

Hence:

GM % in DNA copy number ratio = GM % in mass fraction

4.1.2 In-house verification of method performance against ENGL method acceptance criteria

Test samples with GM-levels 0.4%, 0.9%, 2.0%, 4.5%, were tested in two real-time PCR runs (run A and B) with two replicates for each GM-level on each plate (total of four replicates per GM-level), using an ABI 7900 platform.
The test sample with GM-level 0.1% was tested in 15 replicates in one run (run C).

The corresponding standard curve parameters and the results of efficiency, linearity, trueness and precision are shown in Table 7 and 8.

Table 7. Standard curve parameters

	T304-40-system			*AdhC* reference system		
	Slope	PCR efficiency*	R^2	Slope	PCR efficiency*	R^2
Run A	-3.3	101	0.99	-3.2	104	0.99
Run B	-3.2	104	1.00	-3.2	106	1.00
Run C	-3.3	100	1.00	-3.2	104	0.99

* 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 range from -3.1 to -3.6 and the R^2 coefficient shall be ≥ 0.98.
Table 7 documents that the slope of the standard curve and the R^2 coefficient were within the limits established by the ENGL. The EU-RL in-house results confirmed the data provided by the applicant.

Table 8. Outcome of the *in-house* verification of trueness (bias %) and precision

Target GM-levels %	Measured GM level %	Bias % of the target GM-level	Precision (RSDr %)
0.1	0.10	-4	12
0.4	0.36	-11	4.8
0.9	0.87	-3.1	4.7
2.0	1.9	-7.5	4.6
4.5	4.5	-0.71	5.9

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 (RSDr) should be ≤25% over the dynamic range. Table 8 documents that trueness and precision of quantification were within the limits established by the ENGL. The EU-RL 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), reported by participating laboratories for the T304-40 and the *AdhC* assays are displayed in Table 9. The PCR efficiency (%) was calculated from the standard curve slopes using the formula Efficiency = (10 (-1/slope)) – 1) x 100.

Table 9. Values of slope, PCR efficiency and R^2 obtained during the validation study

		T304-40			***AdhC***		
Lab	**Plate**	**Slope**	**PCR Efficiency (%)**	**R^2**	**Slope**	**PCR Efficiency (%)**	**R^2**
1	A	-3.4	98	1.00	-3.4	99	0.98
	B	-3.4	99	1.00	-3.3	101	1.00
2	A	-3.4	98	1.00	-3.3	101	1.00
	B	-3.4	98	1.00	-3.3	100	1.00
3	A	-3.1	112	0.98	-3.1	109	0.98
	B	-3.1	111	0.98	-3.0	117	0.98
4	A	-3.3	101	1.00	-3.3	101	1.00
	B	-3.4	98	1.00	-3.3	99	1.00
5	A	-3.4	95	0.99	-3.4	97	1.00
	B	-3.3	100	1.00	-3.4	96	1.00
6	A	-3.4	96	1.00	-3.3	100	1.00
	B	-3.4	95	1.00	-3.3	102	1.00
7	A	-3.3	99	1.00	-3.3	100	1.00
	B	-3.4	96	0.99	-3.3	102	1.00
8	A	-3.3	99	1.00	-3.3	100	1.00
	B	-3.3	99	1.00	-3.3	101	1.00
9	A	-3.4	97	1.00	-3.4	96	1.00
	B	-3.3	101	1.00	-3.4	97	1.00
10	A	-3.3	99	1.00	-3.3	101	1.00
	B	-3.4	97	1.00	-3.2	105	1.00
11	A	-3.2	103	1.00	-3.3	102	1.00
	B	-3.1	108	1.00	-3.2	105	1.00
12	A	-3.2	105	1.00	-3.2	103	1.00
	B	-3.3	101	0.99	-3.2	103	0.99
	Mean	**-3.3**	**100**	**1.00**	**-3.3**	**102**	**0.99**

Table 9 indicates that the efficiency of amplification for the T304-40 system ranges from 95 to 112% and the linearity from 0.98 to 1.00 while the amplification efficiency for the cotton-specific reference system ranges from 96% to 117% and the linearity from 0.98 to 1.00. The mean PCR efficiency was 100% for the T304-40 assay and 102% for the *AdhC* assay, with both values within the ENGL acceptance criteria. The average R^2 of the methods was equal to 1.00 and 0.99 for the T304-40 and the *AdhC* assays respectively.

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 performed for identifying outlying values.

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

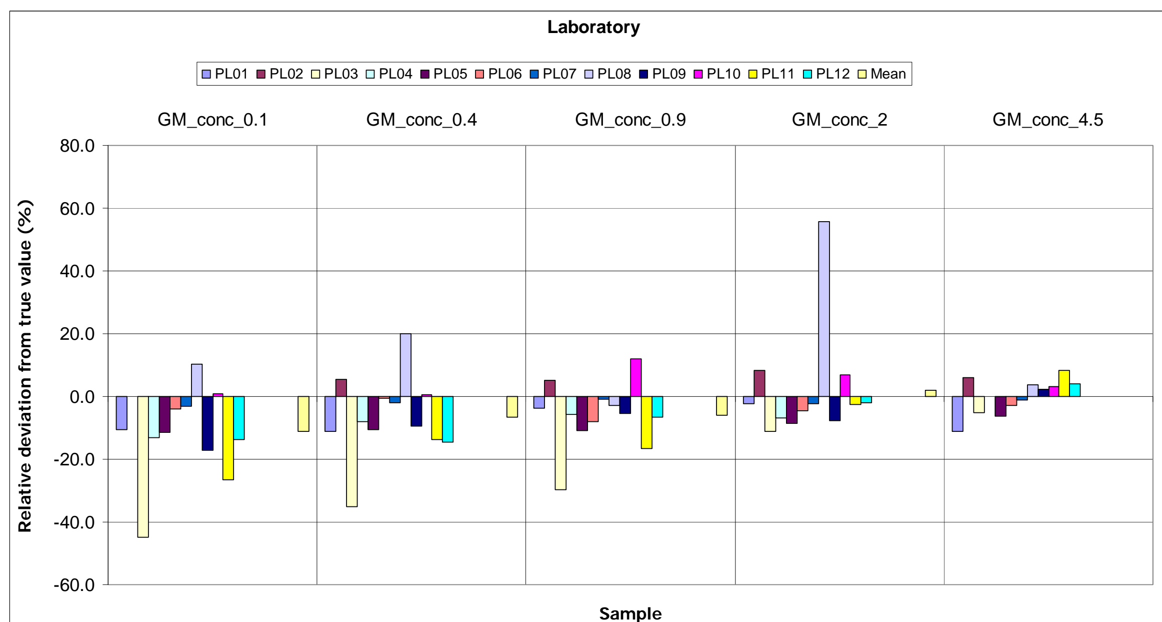
	GMO content (%) *																			
LAB	0.1				0.4				0.9				2.0				4.5			
	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4
1	0.07	0.08	0.09	0.11	0.31	0.36	0.42	0.33	0.71	0.83	0.99	0.95	2.20	1.65	1.67	2.31	3.69	3.84	3.93	4.52
2	0.09	0.09	0.11	0.11	0.39	0.43	0.41	0.46	1.00	0.88	0.91	0.99	2.27	2.25	2.06	2.08	4.55	4.70	4.86	4.95
3	0.05	0.06	0.05	0.06	0.26	0.18	0.33	0.27	0.58	0.58	0.67	0.70	1.79	1.70	1.85	1.79	4.74	3.85	3.79	4.71
4	0.09	0.11	0.07	0.08	0.39	0.40	0.36	0.33	0.91	0.88	0.83	0.78	1.68	1.99	1.95	1.82	4.43	4.51	4.60	4.45
5	0.08	0.08	0.10	0.09	0.36	0.37	0.35	0.35	0.79	0.79	0.84	0.78	1.81	1.87	1.98	1.66	4.43	4.03	4.39	4.04
6	0.10	0.09	0.09	0.11	0.38	0.35	0.42	0.43	0.78	0.82	0.82	0.88	1.94	1.79	1.81	2.09	4.63	4.43	4.05	4.39
7	0.09	0.10	0.10	0.10	0.39	0.40	0.41	0.37	0.86	0.89	0.91	0.90	1.95	1.83	2.00	2.04	4.12	4.49	4.57	4.64
8	0.08	0.17	0.09	0.09	0.70	0.39	0.41	0.42	0.82	0.88	0.88	0.92	5.84	2.04	2.33	2.25	4.85	4.49	4.88	4.46
9	0.07	0.07	0.09	0.10	0.31	0.35	0.41	0.37	0.79	0.80	0.89	0.92	1.92	1.90	1.76	1.79	4.74	4.38	4.62	4.65
10	0.09	0.10	0.10	0.11	0.42	0.41	0.42	0.37	0.89	0.88	1.22	1.04	2.07	2.06	2.16	2.27	4.78	4.92	4.17	4.70
11	0.08	0.06	0.08	0.08	0.33	0.33	0.39	0.33	0.72	0.73	0.72	0.83	1.78	2.22	1.88	1.92	4.66	5.53	4.67	4.66
12	0.08	0.09	0.10	0.08	0.29	0.37	0.37	0.34	0.74	0.75	0.88	1.00	2.21	1.92	1.74	1.97	4.79	4.84	4.67	4.43

* GMO% = (GMO copy number/cotton genome copy number) x 100 and (GM DNA mass/cotton DNA mass) x 100

A graphical representation of the data reported in Table 10 is provided in Figure 1

In Figure 1, the relative deviation from the true 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 yellow bar on the right represents the mean relative deviation over all 12 participating laboratories for each true GM level.

Figure 1. Relative deviation (%) from the true value of T304-40 for all laboratories*



*PL02 at GM level 0.1% and PL04 at GM level 4.5% had very small relative deviations from the true value and the corresponding histograms do not show up in Figure 1.

Overall, most mean relative deviations from the true values were within a maximum of 25%. At GM-level 0.1% ten laboratories were within the limits, at GM-level 0.4% eleven laboratories were within the limits, at GM-levels 0.9% 2.0% 11 laboratories were within the limits and at GM-level 4.5% 12 laboratories. Two laboratories underestimated GM-level 0.1% and one laboratory underestimated GM-level 0.4% and 0.9% by more than 25%, with a trend for underestimation for all laboratories at all GM levels. One laboratory overestimated by more than 50% the GM-content of sample 2%.

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

5. Method performance requirements

Among the method performance requirements established by the ENGL and adopted by the EU-RL GMFF (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm), repeatability and reproducibility are to be assessed through an international collaborative trial. Table 11 illustrates the estimation of repeatability and 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 satisfies this requirement at all GM levels tested. In fact, the highest value of RSD_R is 19% at the 0.1% GM level, thus within the acceptance criterion.

Table 11. Summary of validation results for the T304-40 method

	Test Sample GMO %				
	0.1	**0.4**	**0.9**	**2.0**	**4.5**
Laboratories having returned valid results	12	12	12	12	12
Samples per laboratory	4	4	4	4	4
Number of outliers	1	1	0	1	0
Reason for exclusion	C	C	-	C	-
Mean value of measured GM content (%)	**0.09**	**0.36**	**0.84**	**1.9**	**4.5**
Relative repeatability standard deviation, RSD_r (%)	12	9.9	9.6	8.4	6.4
Repeatability standard deviation	0.01	0.04	0.08	0.16	0.29
Relative reproducibility standard deviation, RSD_R (%)	19	15	14	9.6	7.9
Reproducibility standard deviation	0.02	0.05	0.12	0.19	0.36
Bias (absolute value)	-0.01	-0.04	-0.06	-0.06	0.005
Bias (%)	-13	-9	-6.1	-3	0.1

C= Cochran's test; identification and removal of outliers through Cochran and Grubbs tests, according to ISO 5725-2 (3).
Bias is estimated according to ISO 5725 data analysis protocol.

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 EU-RL GMFF requires the RSD_r value is 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 highest value of RSD_r of 12% at 0.1% GM level.

The trueness of the method is estimated 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. In this case, the method satisfies this requirement across the dynamic range tested, with the highest value of bias (%) of -13% at the 0.1% GM level.

6. Compliance of the method of detection of event T304-40 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 steps were carried out and their outcome is summarised in Table 12:

- at step 2 of the validation process (scientific assessment of the dossier), the EU-RL GMFF acknowledged the applicants data indicating that the RSDr at the level of 0.08%, expressed as ratio of GM- DNA copy numbers to target taxon-specific DNA copy numbers, was about 11% (Table 2), hence below 25%. The results were determined on 18 replicates. The EU-RL GMFF accepted therefore, the applicant's data on method performance.

- at step 3 of the validation process (*in-house* testing of the method), the EU-RL GMFF determined the RSDr at the level of 0.1% expressed as mass fraction of GM-material on the basis of fifteen replicates carried out under repeatability conditions. The RSDr resulted to be 12% (Table 8), hence similar to the value obtained by the applicant and below 25%.

- the collaborative study (step 4 of the validation process) established that over the twelve participating laboratories at the level of 0.1% the RSDr of the method related to mass fraction of GM-material was 12 %, therefore below 25%.

Table 12. Precision of the method for quantitative detection of T304-40

Source	**RSDr %**	**GM %**
Applicant' method optimisation*	11 %	0.08 %
EU-RL GMFF tests	12 %	0.1 %
Collaborative study	12 %	0.1%

* GM- DNA copy numbers in relation to target taxon specific DNA copy numbers

Based on the results of *in-house* verification and of the collaborative study, it is concluded that the method RSDr% is less than 25% at the level of 0.1% related to mass fraction of GM-material, hence the method for quantitative detection of event T304-40 cotton meets the requirement laid down in Regulation (EU) No 619/2011.

7. Conclusion

The method provided by the applicant and described in detail under 3.2 (and available as "Validated Method" at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm and in Annex 1) has been validated in accordance to the EU-RL GMFF validation scheme, respecting all requirements of the relevant EU legislation and international standards for method validation.

The dossier was found complete (step 1) and its scientific analysis (step 2) concluded that the method could meet the ENGL minimum performance criteria for entering into validation.

The subsequent *in-house* verification of the method (step 3) by the EU-RL GMFF confirmed this conclusion.

The data obtained in the international collaborative study (step 4) also indicated that the method meets all acceptance criteria and method performance requirements recommended by the ENGL (as detailed at http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm) for valid PCR detection and quantification of GM-events.

In conclusion, the 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 Commission Regulation (EC) No 641/2004 and (EU) No 619/2011. The method is also applicable to appropriately extracted cotton DNA.

8. References

1. Arumuganathan K. and Earle E. D., 1991. Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter* 9, 208-218.
2. Horwitz W., 1995. Protocol for the design, conduct and interpretation of method performance studies, *Pure and Appl. Chem.* 67, 331-343.
3. International Standard (ISO) 5725, 1994. Accuracy (trueness and precision) of measurement methods and results. International Organization for Standardization, Genève, Switzerland.

Annex 1: Event-specific Method for the Quantification of Cotton T304-40 using Real-time PCR

Validated Method

Method development:

Bayer CropScience

Method validation:

European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF)

Quality assurance

The EU-RL GMFF is ISO 9001:2008 certified (certificate number: CH-32232) and ISO 17025:2005 accredited [certificate number: ACCREDIA 1172, (Flexible Scope for DNA extraction and qualitative/quantitative PCR) - Accredited tests are available at http://www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7].

The original version of the document containing evidence of internal checks and authorisation for publication is archived within the EU-RL GMFF quality system.

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1. General information and summary of the methodology

This protocol describes an event-specific real-time quantitative TaqMan® polymerase chain reaction (PCR) procedure for the determination of the relative content of cotton event T304-40 DNA to total cotton 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 cotton event T304-40, a 78-bp fragment of the region spanning the 3′ insert-to-plant junction in cotton event T304-40 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 TAMRA (6-carboxytetramethylrhodamine) as a quencher dye at its 3′ end.

For the relative quantification of cotton event T304-40, a cotton-specific reference system amplifies a 73-bp fragment of the alcohol dehydrogenase C (*AdhC*) gene, a cotton endogenous gene (Accession number, GeneBank: AF036569 and AF403330), using *AdhC* gene-specific primers and a *AdhC* gene-specific probe labelled with VIC as a reporter dye at its 5′ end and TAMRA as a quencher at its 3′ end.

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 T304-40 DNA in a test sample, Ct values for the T304-40 and *AdhC* systems are determined for the sample. Standard curves are then used to estimate the relative amount of T304-40 DNA to total cotton DNA.

2. Validation and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from mixtures of genetically modified and conventional cotton seeds, grain and leaves. The trueness and precision 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 (EU-RL GMFF). The study was undertaken with twelve participating laboratories in July/ August 2012.

Each participant received twenty test samples containing cotton T304-40 genomic DNA at five GM contents, ranging from 0.1% to 4.5%.

Each laboratory received each GM level of event T304-40 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.

A detailed validation report can be found at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm.

2.3 Limit of detection (LOD)

According to the method developer, the relative LOD of the method is at least 0.023% in 200 ng of total cotton DNA. 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 0.08% in 200 ng of total cotton DNA. The lowest relative GM content of the target sequence included in the collaborative trial was 0.1%.

2.5 Molecular specificity

The method exploits a unique DNA sequence spanning the 3' region of recombination between the insert and the plant genome and is therefore event-specific for the event T304-40.

The specificity of the assay was assessed by the applicant in real-time PCR using genomic DNA (50 ng) extracted from event T304-40 as a positive control sample and from conventional cotton, rice LLRICE62, oilseed rape (OSR) MS1, MS8, RF1, RF2, RF3, Topas19-2, T45, OXY-235, RT73, soybean LL27, LL55, FG72, GTS 40-3-2, cotton LLCotton25, GHB614, GHB119, MON1445, and maize MON810, BT11, GA21, NK603, T25.

According to the method developer, apart from the positive control reaction, the forward and reverse oligonucleotide primers and the TaqMan® probe of the T304-40 system showed no amplification signals following quantitative PCR analysis.

The specificity of the cotton taxon-specific assay was assessed by the method developer in real-time PCR using 200 ng of conventional genomic DNA extracted from soybean, rice, cotton, oilseed rape and maize. According to the method developer the cotton-specific reference system did not react with any target DNA except the positive control.

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 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.
- 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 cotton event T304-40

3.2.1 General

The PCR set-up for the taxon-specific target sequence (*AdhC*) and for the GMO target sequence (event T304-40) is to be 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 1 and Table 2.

3.2.2 Calibration

The calibration curves has to be established on at lest five DNA samples. The first point of the calibration curve (S1) should be established for a sample containing 10% cotton event T304-40 DNA in a total of 300 ng of cotton DNA (corresponding to approximately 128755 cotton genome copies with one genome assumed to correspond to 2.33 pg of haploid cotton genomic DNA) [(1)].
Standards S2 to S4 are to be prepared by serial dilutions according to Table 1 below.

Table 1. Copy number values of the standard curve samples

Sample code	S1	S2	S3	S4	S5
Total amount of DNA in reaction (ng)	300	100	20	5	1
Target taxon *AdhC* copies	128755	42918	8584	2146	429
T304-40 Cotton GM copies	12876	4292	858	215	43

A calibration curve is to be produced by plotting the Ct values against the logarithm of the target genome copy number for the calibration points. This can be done by means of spreadsheet software, e.g. Microsoft Excel, or directly by options available within the sequence detection system software.

3.2.3 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 T304-40 assay and one for the *AdhC* assay) on ice and in the order mentioned below (except DNA).

Table 2. Amplification reaction mixture, final volume/concentration per reaction well for the T304-40 method.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
SHA 029 (10 µM)	400 nM	1
SHA 030 (10 µM)	400 nM	1
TM 089 (10 µM)	200 nM	0.5
Nuclease free water	#	5.0
DNA	#	5.0
Total reaction volume:		25 µL

Table 3. Amplification reaction mixture, final volume/concentration per reaction well for the cotton *AdhC* assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
KVM157 (10 µM)	200 nM	0.5
KVM158 (10 µM)	200 nM	0.5
TM012 (10 µM)	200 nM	0.5
Nuclease free water	#	6.0
Template DNA	#	5.0
Total reaction volume:		25 µL

3. Mix well and centrifuge briefly.
4. Prepare two reaction tubes (one for the T304-40 and one for the *AdhC* assay) 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. 70 µL for the *AdhC* cotton system and 70 µL for the event T304-40 system). Add to each tube the correct amount of DNA for 3.5 PCR repetitions (17.5 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 reduce to a minimum the variability among the repetitions of each PCR 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 room temperature) to spin down the reaction mixture.
7. Place the plate into the instrument.
8. Run the PCR with the cycling program described in Table 3.

Table 3. Cycling program for T304-40/*AdhC* methods.

<table>
<tr><th>Step</th><th colspan="2">Stage</th><th>T°C</th><th>Time (sec)</th><th>Acquisition</th><th>Cycles</th></tr>
<tr><td>1</td><td colspan="2">UNG*</td><td>50°C</td><td>120</td><td>No</td><td>1x</td></tr>
<tr><td>2</td><td colspan="2">Initial denaturation</td><td>95°C</td><td>600</td><td>No</td><td>1x</td></tr>
<tr><td rowspan="2">3</td><td rowspan="2">Amplification</td><td>Denaturation</td><td>95°C</td><td>15</td><td>No</td><td rowspan="2">45x</td></tr>
<tr><td>Annealing & Extension</td><td>60°C</td><td>60</td><td>Yes</td></tr>
</table>

*UNG: Uracil-N-glycosylase

3.3 Data analysis

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

a) <u>Set the threshold</u>: display the amplification curves of one assay (e.g. T304-40) 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 (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) <u>Set the baseline</u>: 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), b) and c) on the amplification plots of the other system (e.g. *AdhC*).

e) Save the settings and export all the data to a text 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 standard curves are generated both for the *AdhC* and the T304-40 specific assays by plotting the Ct values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a linear regression line into these data.

Thereafter, the standard curves are used to estimate the DNA copy numbers in the unknown samples.

To obtain the percentage value of event T304-40 DNA in the unknown sample, the T304-40 copy number is divided by the copy number of the cotton reference gene (*AdhC*) and multiplied by 100 (GM% = T304-40/*AdhC* x 100).

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
- 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 with UNG. Applied Biosystems (catalogue n. 4318157)

4.3 Primers and Probes

Oligonucleotides	Name	DNA Sequence (5′ to 3′)	Length (nt)
T304-40			
Forward primer	SHA029	5′- AGC GCG CAA ACT AGG ATA AAT T-3′	22
Reverse primer	SHA030	5′- CCT AGA TCT TGG GAT AAC TTG AAA AGA-3′	27
Probe	TM089	6-FAM 5′- TCG CGC GCG GTG TCA TCT ATC TC - TAMRA 3′	23
AdhC			
Forward primer	KVM157	5′-CAC ATG ACT TAG CCC ATC TTT GC-3′	23
Reverse primer	KVM158	5′-CCC ACC CTT TTT TGG TTT AGC-3′	21
Probe	TM012	VIC 5′-TGC AGG TTT TGG TGC CAC TGT GAA TG - TAMRA 3′	26

FAM: 6-carboxyfluorescein, TAMRA: 6-carboxytetramethylrhodamine

5. References

1. Arumuganathan K. and Earle E.D., 1991. Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter,* 9: 208-218.