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Event-specific Method for the Quantification of Soybean SYHTOH2 by Real-time PCR

Validation report

European Union Reference Laboratory for Genetically Modified Food and Feed

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EURL European Union Reference Laboratory for GM Food & Feed

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Molecular Biology and Genomics Unit

Event-specific Method for the Quantification of Soybean SYHT0H2 by Real-time PCR

Validation Report

08 July 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 soybean event SYHT0H2 (unique identifier SYN-ØØØH2-5). 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], Syngenta Crop Protection AG has provided the detection method and the positive and negative control samples (genomic DNA extracted from seeds harbouring the SYN-ØØØH2-5 event as positive control DNA, genomic DNA extracted from seeds of conventional soybean as negative control DNA). The EURL GMFF prepared the validation samples (calibration samples and blind samples at different GM percentage [GM-DNA/total DNA]), organised an international collaborative study and analysed the results.

The EURL GMFF in-house verification and the collaborative study confirmed that the method, in line with the provisions of Annex I-2.C.2 to Regulation (EC) No 641/2004, meets most of the method performance requirements, as established by the EURL GMFF and the ENGL, and it fulfils the analytical requirements of Regulation (EU) No 619/2013[c]. In agreement with the Steering Committee of the ENGL, the EURL GMFF concludes that the method is by all means fit for purpose and can be used for regulatory control of food and feed. Laboratories using the method for the purposes of Regulation (EU) No 619/2011 are asked to provide the EURL GMFF with their

[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".
[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.

experimental data and results in order to allow further verification of the performance of the method in that part of the dynamic range.

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

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.

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Content

1. Introduction

In line with Regulation (EC) No 1829/2003, Syngenta Crop Protection AG provided the EURL GMFF with a copy of the official application for authorisation of an event-specific method for the detection and quantification of soybean (*Glycine max*) event SYHT0H2 (unique identifier SYN-ØØØH2-5) together with genomic DNA as negative and positive control samples.

In response to an early submission of the method, the EURL GMFF started its step-wise validation procedure (step 1: dossier reception) already in advance to the official dossier, before EFSA declared the dossier as complete and valid.

The scientific dossier evaluation (step 2) verified the reported method performance characteristics against the ENGL method acceptance criteria[d] (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).

In step 3 of its validation procedure (experimental testing), the EURL GMFF verified the purity of the control samples and conducted an in-house testing of the method provided. The positive control DNA sample - submitted in accordance with Art 5(3)(j) and Article 17(3)(j) of Regulation (EC) No 1829/2003 - was found of good quality. The negative control DNA sample was contaminated with soybean event A2704-12 and it was replaced, after a request from the EURL GMFF, with a suitable control sample.

The method performance parameter were verified in-house by quantifying five blinded GM levels within the range of 0.10%-5.0% GM on a copy number basis. The experiments were performed under repeatability conditions and demonstrated that the PCR efficiency, linearity, accuracy 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 EURL GMFF also verified *i)* the zygosity ratio of the positive control sample submitted, by investigating the GM- to reference- target ratio, 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 0.10% related to mass fraction of GM-material on fifteen replicates. Step 3 was finished with the conclusion to enter into a collaborative trial (step 4).

The international collaborative trial (step 4) demonstrated that the method is well suited for analysing genomic DNA, appropriately extracted from food or feed, and for identifying and quantifying the presence of GM event SYHT0H2.

[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)

The preparation of the report (step 5) was aligned with the timelines communicated by EFSA for its risk assessment.

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

Documentation and data provided by the applicant were evaluated by the EURL GMFF for completeness (step 1) and compliance with the ENGL method acceptance criteria (step 2).

Specificity of the method was verified by the applicant and confirmed by the EURL GMFF by means of bioinformatics analysis, on the basis of the sequence data provided by the applicant.

In particular, the specificity of the event-specific assay had been assessed by the applicant with genomic DNA extracted from SYHT0H2 as positive control sample and from soybean GM events in the applicaint's pipeline SYHT0H1, SYHT0H7, SYHT0E9, SYHT0G6, SYHT07I, SYHT07C, SYHT06W, SYHT04R, from 10 authorized GM soybean events (A2704-12, A5547-127, 305423, 356043, GTS 40-3-2, MON89788, MON87701, FP72, DAS-68416-4, CV127), 15 authorized GM maize events (MON810, GA21, Bt-176, Bt-11, NK603, MON 863, 1507, MIR604, MON88017, 59122, 3272, T25, MON89034, 98140, MIR162) 8 GM oilseed rape events (Ms8, Rf3, T45, GT73/RT73, MS1, RF1, RF2), H71 sugar beet, 8 GM cotton events (MON531, MON15985-7, MON1445, GBH614, GBH119, T304-40, LL Cotton 25, 281-24-236 x 3006-210-23), EH925271 potato, LL601 rice and LLRice62 and conventional soybean, oilseed rape, maize, cotton, wheat, canola, rice, potato, sugar beet.
According to the method developer the SYHT0H2 method did not react with any sample except the positive control.

A previously validated soybean-specific PCR method (http://gmo-crl.jrc.ec.europa.eu/summaries/DP-356043_validated_Method_correctedversion1.pdf), which amplifies a 74 base pair (bp) fragment of the lectin gene (*Le1*) of *Glycine max*, was used as a reference method.

Bioinformatics specificity was assessed by the EURL GMFF. The detection method spans the 3' insert-to-plant junction in SYHT0H2 soybean. The forward primer "FE08316-F" binds to the insert. The reverse primer "FE08317-R" binding site was found in the 3' soybean (*Glycine max*) genomic border adjacent to the insertion. The probe "FE08318-P" binds to the edge of the insert close to the junction at the 3' genomic region of Glycine max. The amplicon size is expected to be 88 bp, consistent to what has been reported by the applicant.

The sequences of the amplicon has been analysed by BLAST (NCBI)[(1)] against local copies of the "nt" and "patents" databases, and no significant similarity was found with any other published sequence apart from related patent sequences. In addition, the primers were tested against the sequences of the other GMO events present in the Central Core Sequence Information System of

the JRC, as well as the whole genomes of more than 100 plants (including *Brassica rapa, Glycine max, Oryza sativa, Solanum lycopersicum* and *Zea mays*) using the e-PCR prediction tool (NCBI)[2, 3], and no potential amplicon was identified.

Concerning the verification of the ENGL method acceptance criteria the parameters of the calibration curves (slope, R^2 coefficient) were appropriately determined by the applicant by quantifying four test samples at different GM levels (0.080%, 0.5%, 0.9%, and 5%) expressed in copy numbers of GM material. The assays were performed on Applied Biosystems® 7500 Fast Real-Time PCR system (Standard 7500 run mode) (see Table 1).

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

	SYHT0H2		***Le1***	
	Slope	**R^2**	**Slope**	**R^2**
Run 1	-3.41	1.00	-3.43	1.00
Run 2	-3.29	1.00	-3.42	1.00
Run 3	-3.36	1.00	-3.45	1.00
Run 4	-3.36	1.00	-3.39	1.00
Run 5	-3.42	1.00	-3.42	1.00
Run 6	-3.45	1.00	-3.44	1.00
Run 7	-3.38	1.00	-3.41	1.00
Run 8	-3.38	1.00	-3.45	1.00
Mean	**-3.38**	**1.00**	**-3.43**	**1.00**

According to the ENGL method acceptance criteria, the average value of the slope of the standard curve shall be within the range of – 3.1 to – 3.6, and the R^2 shall be ≥ 0.98.

Table 1 indicates that the mean slope of the standard curves is – 3.38 and – 3.43 for the SYHT0H2 and *Le1* method, respectively, and that the R^2 coefficient for the SYHT0H2 and for the soybean specific reference system (*Le1*) is 1.00; therefore all values are within the ENGL acceptance criteria.

Table 2 reports precision and trueness for the four GM-levels tested by the applicant. Sixteen values for each GM-level were provided which is fully in line with common practice. Both parameters were within the ENGL acceptance criteria (trueness ± 25%, RSDr ≤ 25% across the entire dynamic range).

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

Expected GMO %*	**0.08**	**0.50**	**0.90**	**5.0**
Measured mean GMO%	0.08	0.48	0.78	5.2
Precision (RSDr %)	11	5.5	6.1	3.2
Trueness (bias %)	-2.5	-4.2	-13	3.8

* GM levels expressed in copy numbers of GM material

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

3.1 DNA extraction

Genomic DNA was isolated by the applicant from GM and conventional soybean seeds using a "CTAB-based" protocol previously submitted for detection of event DP-356043-5. This DNA extraction method was assessed earlier by the EURL GMFF in order to confirm its performance characteristics. The protocol for DNA extraction and a report on testing were published in 2009 at http://gmo-crl.jrc.ec.europa.eu/summaries/356043-5_DNAExtr_report.pdf.

In agreement with the ENGL position, which endorses the modularity principle (see also Annex I to Reg. (EC) No 641/2004), and given the similarity in the matrix, the EURL GMFF considers the above mentioned DNA extraction protocol applicable for the validation of the method for soybean event SYHT0H2.

3.2 Method protocol for the PCR analysis

The PCR 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 SYHT0H2 DNA to total soybean DNA. The procedure is a simplex system, in which a soybean *Le1* (*lectin*) specific assay, and the target assay (SYHT0H2) are performed in separate wells.

This method, as validated by the EURL GMFF, is fully described as "validated method" in Annex 1 and can also be found under http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx.

For the detection of GM event SYHT0H2, an 88-bp fragment of the region spanning the 3' plant-to-insert junction in soybean SYHT0H2 is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with FAM (6-carboxyfluorescein) as reporter dye at its 5' end and BHQ-1 (Black Hole Quencher®-1) as quencher dye at its 3' end.

For the relative quantification of GM event SYHT0H2, a soybean specific reference system previously validated is used (http://gmo-crl.jrc.ec.europa.eu/summaries/DP-356043_validated_Method_correctedversion1.pdf). It amplifies a 74-bp fragment of *lectin (Le1)*, using *Le1* gene-specific primers and a *Le1* gene-specific probe labelled with FAM as reporter dye at its 5' end, and TAMRA as quencher at its 3' end.

Standard curves are to be generated for both the SYHT0H2 and the *Le1* systems by plotting the Ct values measured for the calibration points against the logarithm of the DNA copy numbers and 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 SYHT0H2 DNA in a test sample, the SYHT0H2 copy number is divided by the copy number of the soybean reference gene (*Le1*) and multiplied by 100 to obtain the percentage value (GM% = SYHT0H2/*Le1* 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 soybean genome (1.13 pg) [(6)]. The copy number values used in the quantification, the GMO contents of the calibration samples and total DNA quantity used in PCR are listed in Table 3.

Note: Numerical values presented in the tables of this report were rounded keeping two digits for values ≤ 1, one digit for values between 1 and 10 and no digit for values ≥ 10. The calculations in the MS Excel files however were done over not rounded data. This approach might generate small inconsistencies in the numerical values reported in the tables.

Table 3. Copy number values of the standard curve samples

Sample code	S1	S2	S3	S4	S5
Total amount of DNA in reaction (ng)	100	30	9.2	2.8	0.80
Target taxon *Le1* copies	88496	26817	8126	2463	704
SYHT0H2 soybean GM copies	8850	2682	813	246	70

3.3 EURL 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 EURL." 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.

To this end, the copy number of the SYHT0H2 and of the *Le1* targets in the positive control sample were determined by digital PCR (dPCR), performed 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 as indicated in the Validated Method in Annex 1 and at (http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx): 1 µL of DNA at a concentration of 0.75

ng/µL, suitable to avoid panel saturation after analysis (optimal between 200<positive partitions<700).

The 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 chambers (or partitions) constituting one panel. The analysis was repeated three times; five replicates in five panels were run each time for both the GM- and reference-assay, with a total number of fifteen data sets for both targets. No template controls were included. Amplification conditions were as proposed by the applicant and reported in the Validated Method (see Annex 1). Data analysis and copy number calculation was performed using the BioMark digital PCR Analysis software, the range of Ct retention was from 20 to 35.

Calculations of means and variances were carried out according to the procedure outlined for random variables in 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 by quantifying on a copy number basis five blinded test samples containing a range of 0.10%-5.0% GM levels. The experiments were performed on an ABI 7300 real-time platform and on ABI 7900 under repeatability conditions and followed the protocol provided by the applicant. Test samples with GM-levels 5.0%, 2.0%, 0.90%, 0.35% and 0.10% 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 0.10% (corresponding to 0.10% in mass fractions of GM-material) was tested in 15 replicates in an additional run. Average values of the slope and of the R^2 coefficient of the standard curves and method trueness and precision over the dynamic range were evaluated against the ENGL method acceptance criteria.

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 (RSDr) at 0.10% GM level in mass fraction (m/m).

3.4 International collaborative study (step 4)

The international collaborative study (step 4) involved twelve laboratories, all being "National reference laboratories, 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:

[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

1. The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" (Horwitz, 1995) [4]
2. ISO 5725 (1994) [5]

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

3.4.1 List of participating laboratories

The 12 participants in the SYHT0H2 validation study (see Table 4) were randomly selected from the 31 national reference laboratories (NRL) that offered to participate.

Clear guidance was given to the selected laboratories to strictly follow the standard operational procedures that were provided for the execution of the protocol provided by the applicant and verified in the EURL GMFF laboratory (the protocol is detailed in Annex 1 and at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx). The participating laboratories are listed in Table 4.

Table 4. Laboratories participating in the international collaborative validation study of the detection method for soybean SYHT0H2

Laboratory	Country
Agricultural Institute of Slovenia	SI
State Sanitary and Epidemiological Station, Regional Laboratory of Genetically Modified Food	PL
ANSES - Laboratoire de la santé des végétaux	FR
Institute for National Investigation for the Health and Veterinarian Nature Saxonia	DE
INRAN - Seed Testing Station	IT
"BioGEVES laboratory GEVES"	FR
The Netherlands Food and Consumer Product Safety Authority	NL
Department of Gene Technology, Tallinn University of Technology	EE
State Institute of Chemical and Veterinarian Analysis - Freiburg	DE
National Institute of Biology	SI
National Center of Public Health and Analyses - Bulgarian National Laboratory for Genetically Modified Food	BG
Laboratory Agroalimentary of the Spanish Ministry of Agriculture, Food and Environment	ES

3.4.2 Real-time PCR equipment used in the study

Laboratories involved in the collaborative study used a range of real-time PCR equipment: four laboratories used the ABI 7900 HT, four used the ABI 7500, one used the ABI 7500 FAST, one used the Roche LC480 and two used the ABI 7300.

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

3.4.3 Material used in the international collaborative study

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

i) genomic DNA extracted by the applicant from homozygous seeds of soybean (*Glycine max*) harbouring the event SYHT0H2, and

ii) genomic DNA extracted by the applicant from conventional seeds of soybean genetically similar to those harbouring the SYHT0H2 event.

The control samples were prepared by the EURL GMFF from the genomic DNA provided by the applicant in accordance to Regulation (EC) No 1829/2003, Art 2.11[f].

These positive and negative control samples were also used by the EURL GMFF to prepare standards (of known GM-content) and test samples (of unknown GM-content), containing mixtures of SYHT0H2 soybean DNA and non-GM soybean DNA, as GM-DNA copy numbers in relation to target taxon-specific DNA copy numbers calculated in terms of haploid genomes.

The calibration sample S1 was prepared by mixing the appropriate amount of genomic SYHT0H2 DNA with non-GM soybean genomic DNA to obtain a 10% GM solution. Calibration samples S2-S4 were prepared by serial dilutions (3.3-fold) from the S1 sample and the S5 sample was prepared by 3.5-fold dilution from the S4 sample.

The twelve 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 18 ng/uL) labelled from U1 to U20, representing five GM levels (Table 5).

[f] Control sample defined as 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).

Table 5. SYHT0H2 GM contents in genome copy number

SYHT0H2 GM% GM copy number/soybean genome copy number x 100
5.0
2.0
0.90
0.35
0.10

- ✓ Reaction reagents:
  - TaqMan® Universal PCR Master Mix, no UNG (2x), one vial: 8 mL
  - distilled water, one vial: 3.8 mL

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

Le1 taxon-specific assay
- Lec for 2 (10 µM): 520 µL
- GMO3-126Rev (10 µM): 520 µL
- Lec probe (10 µM): 145 µL

SYHT0H2 assay
- FE08316-F (10 µM): 480 µL
- FE08317-R (10 µM): 480 µL
- FE08318-P (10 µM): 160 µL

3.4.4 Design of the collaborative study

Participant 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 SYHT0H2 specific system and for the *Le1* taxon-specific system. In total, two plates were run per each participating laboratory.

The laboratories prepared the master-mixes for the SYHT0H2 and *Le1* assays in accordance with the description provided in the validation protocol. Calibration and test samples were loaded on the PCR plates as per determined plate layout.

The amplification reactions followed the cycling program specified in the validation protocol. Participants determined the GM% in the test samples according to the instructions and reported the raw data to the EURL GMFF on an Excel sheet that was designed, validated and distributed by the EURL GMFF. 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

Nine laboratories reported no deviations from the validation protocol.

- One laboratory added a 2 min step at 50 ^{o}C before the initial denaturation.
- One laboratory reported a low PCR efficiency (lower than 90%) for plate B and deleted one replicate in unknown sample 8 as it resulted as outlier. Moreover, in plate A, the negative control for the *Le1* system gave a low signal (mean Ct of 33.83 corresponding to more or less 50 copies of endogenous target genome) and in plate B, the negative control for the *Le1* system gave no signal.
- One laboratory used 50 cycles instead of 45 for the SYHT0H2/*Le1* system.

The deviations were considered acceptable and the experimental results were analysed.

4. Results

4.1 EURL GMFF experimental testing

4.1.1 Zygosity ratio in the positive control sample

The results of the tests to determine the zygosity ratio in the positive control samples are shown in Table 6.

Table 6. Summary of dPCR analysis conducted on the SYHT0H2 and *Le1* targets in the positive control sample.

Mean ratio (SYHT0H2/*Le1*)	1.00
Standard deviation	0.076*
RSD_r (%)	7.6
Standard error of the mean	0.020
Upper 95% CI of the mean	1.04
Lower 95% CI of the mean	0.96

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

In conclusion, the 95% confidence interval (CI) spans around 1.0 and therefore the mean ratio is not significantly different from an expected ratio of 1.0, assuming a homozygous GM target and one copy reference target, for an alpha = 0.05 (see 3.2).

Hence:

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

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

Test samples with GM-levels 5.0%, 2.0%, 0.90%, 0.35% and 0.10% were tested by the EURL GMFF in two real-time PCR runs conducted on two instruments (run A and B on ABI 7300 and run D and E on ABI 7900) with two replicates for each GM-level on each plate (total of four replicates per GM-level). The sample with a GM-level 0.10% (corresponding to 0.10% in mass fractions of GM-material) was tested in 15 replicates in one run (run C on ABI 7300). The corresponding standard curve parameters are shown in Tables 7a and 7b and in Tables 8a and 8b.

Table 7a. Standard curve parameters of the real-time PCR testing carried out on ABI 7300

	SYHT0H2 method			***Le1* reference method**		
	Slope	PCR efficiency*	R^2	Slope	PCR efficiency*	R^2
Run A	-3.55	91	1.00	-3.59	90	1.00
Run B	-3.63	88	1.00	-3.58	90	1.00
Run C	-3.60	90	1.00	-3.52	92	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 ABI7900

	SYHT0H2 method			***Le1* reference method**		
	Slope	PCR efficiency*	R^2	Slope	PCR efficiency*	R^2
Run D	-3.52	92	1.00	-3.44	95	0.99
Run E	-3.52	92	1.00	-3.57	91	1.00

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

According to the ENGL method performance criteria, the average value of the slope of the standard curve shall fall into the range of -3.1 to -3.6 and the R^2 coefficient shall be ≥ 0.98.

Tables 7a and 7b document that the slopes of the standard curves and the R^2 coefficients were within the limits established by the ENGL.

Table 8a. Outcome of the in-house tests, with regards to the quantification of the five test samples. Testing carried out on ABI 7300.

Target GM-level %	**Measured GM %**	**Bias %**	**Precision (RSDr %)**
5.0	4.9	-2.5	1.4
2.0	2.0	-0.70	5.5
0.90	0.91	0.95	8.8
0.35	0.39	10	5.0
0.10	0.12	21	9.9

Table 8b. Outcome of the in-house tests, with regards to the quantification of the five test samples. Testing carried out on ABI7900.

Target GM-level %	Measured GM %	Bias %	Precision (RSDr %)
5.0	4.7	-6.2	4.6
2.0	1.9	-4.4	3.1
0.90	0.93	3.2	4.4
0.35	0.35	0.25	3.6
0.10	0.10	0.43	16

According to the ENGL method acceptance criteria the method's trueness, measured as bias %, should be within ±25% of the target value over the entire dynamic range. The method's precision, estimated as RSDr % (relative standard deviation of repeatability) should be ≤25% over the entire dynamic range. Tables 8a and 8b document that trueness and precision of quantification were within the limits established by the ENGL.

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 indicates that the efficiency of amplification for the SYHT0H2 system ranges from 83 to 92 and the linearity from 0.98 to 1.00; the amplification efficiency for the soybean-specific system ranges from 86% to 94% and the linearity is again about 1.00.

The mean PCR efficiency was 88% for the SYHT0H2 assay and 89% for the *Le1* assay.

These values are slightly outside the ENGL acceptance criteria.

The average R^2 of the methods was 0.99 and 1.00 for the SYHT0H2 and *Le1* assays, respectively.

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

		SYHT0H2			*Le1*		
Lab	**Plate**	**Slope**	**PCR Efficiency (%)**	**R^2**	**Slope**	**PCR Efficiency (%)**	**R^2**
1	A	-3.70	86	1.00	-3.72	86	1.00
	B	-3.66	88	1.00	-3.69	87	1.00
2	A	-3.73	85	1.00	-3.58	90	1.00
	B	-3.72	86	1.00	-3.61	89	1.00
3	A	-3.57	91	1.00	-3.63	89	1.00
	B	-3.67	87	1.00	-3.70	86	1.00
4	A	-3.79	83	1.00	-3.63	88	0.99
	B	-3.73	85	1.00	-3.68	87	0.99
5	A	-3.52	92	1.00	-3.48	94	1.00
	B	-3.59	90	1.00	-3.56	91	1.00
6	A	-3.70	86	0.99	-3.68	87	1.00
	B	-3.62	89	1.00	-3.64	88	1.00
7	A	-3.50	93	1.00	-3.55	91	1.00
	B	-3.68	87	1.00	-3.55	91	1.00
8	A	-3.71	86	1.00	-3.63	88	1.00
	B	-3.65	88	1.00	-3.57	91	1.00
9	A	-3.72	86	0.99	-3.68	87	1.00
	B	-3.66	88	1.00	-3.60	90	1.00
10	A	-3.69	87	1.00	-3.67	87	1.00
	B	-3.60	89	1.00	-3.65	88	1.00
11	A	-3.59	90	1.00	-3.50	93	0.99
	B	-3.52	92	1.00	-3.69	87	1.00
12	A	-3.58	90	1.00	-3.52	92	1.00
	B	-3.65	88	1.00	-3.56	91	0.99
	Mean	-3.65	88	1.00	-3.62	89	1.00

4.2.2 GMO quantification

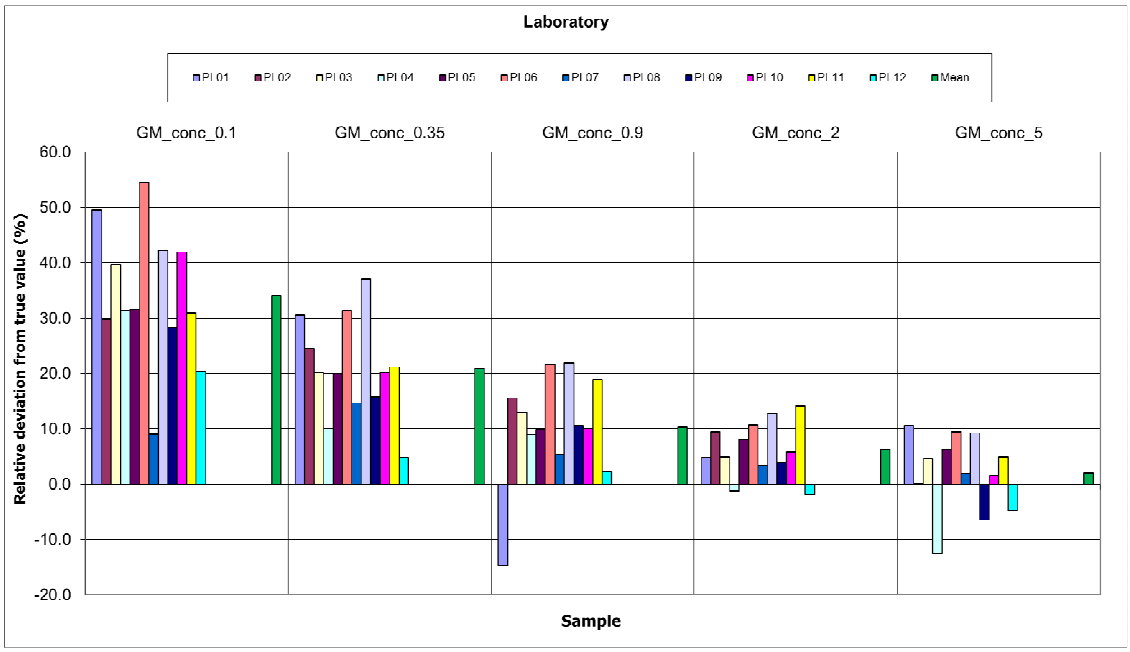
Table 10 reports the values of the four replicates for each GM level as provided by all laboratories. The % GM content is expressed in terms of GM DNA copy numbers in relation to target taxon-specific DNA copy numbers (copy/copy). The GM level at 0.10% in copy number ratio (copy/copy) corresponds to a GM level of 0.10% expressed in terms of mass fractions of GM material (mass/mass).

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

	GMO content (%)																			
LAB	0.10				0.35				0.9				2.0				5.0			
	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	0.16	0.18	0.10	0.16	0.45	0.45	0.47	0.45	0.64	0.63	0.78	1.02	2.23	2.25	1.65	2.26	6.48	4.05	5.66	5.94
2	0.12	0.11	0.14	0.14	0.47	0.42	0.44	0.42	1.03	1.00	1.12	1.02	2.16	2.12	2.24	2.23	4.84	5.12	5.20	4.84
3	0.14	0.14	0.14	0.13	0.42	0.43	0.38	0.45	1.00	0.97	1.04	1.06	1.91	2.06	2.15	2.27	5.53	5.02	4.97	5.40
4	0.14	0.12	0.12	0.15	0.42	0.37	0.37	0.38	0.96	0.92	1.07	0.97	1.99	1.98	1.99	1.94	4.27	4.63	4.15	4.44
5	0.13	0.12	0.14	0.13	0.43	0.44	0.42	0.39	1.06	1.07	0.88	0.94	2.37	2.23	2.00	2.04	5.60	5.65	4.72	5.27
6	0.17	0.13	0.15	0.18	0.40	0.45	0.47	0.51	1.02	1.06	1.14	1.16	2.15	2.14	2.30	2.27	5.32	5.65	5.40	5.51
7	0.11	0.11	0.10	0.11	0.42	0.43	0.41	0.35	0.97	0.96	0.95	0.91	2.17	2.12	2.12	1.86	4.69	5.18	5.33	5.18
8	0.16	0.14	0.13	0.14	0.45	0.46	0.50	0.52	1.20	1.06	1.05	1.08	2.33	2.17	2.32	2.21	5.59	5.48	4.83	5.94
9	0.13	0.11	0.12	0.15	0.39	0.40	0.43	0.39	0.95	0.96	1.08	0.99	2.05	2.08	2.18	2.01	4.25	4.70	4.86	4.89
10	0.14	0.14	0.13	0.16	0.42	0.41	0.41	0.44	0.99	0.98	0.96	1.02	2.06	2.22	2.08	2.10	5.12	5.09	5.04	5.05
11	0.13	0.13	0.15	0.12	0.45	0.45	0.39	0.40	1.12	1.10	1.01	1.04	2.60	2.29	2.23	2.01	5.70	5.53	4.58	5.17
12	0.11	0.12	0.14	0.11	0.39	0.37	0.34	0.36	0.95	0.95	0.90	0.89	2.03	2.02	1.95	1.87	4.80	5.18	4.39	4.67

A graphical representation of the data reported in Table 10 is provided in Figure 1 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 green bar on the right represents the mean relative deviation over all twelve participating laboratories for each true GM level.

Figure 1. Relative deviation (%) from the true value of SYHT0H2 for all laboratories*



*PL2 at GM level 5% had very small relative deviation from the true value and the corresponding histogram does not show up in Figure 1. PL: participating laboratory.

Overall a trend can be observed to overestimate the GM content at the lowest levels. Ten laboratories overestimated the GM-content of sample 0.10% by more than 25%.
All data were retained for the statistical analysis and for tests of outliers (Cochran and Grubbs) whose results are reported in Table 11.

4.2.3 Method performance requirements

Among the method performance requirements established by ENGL and adopted by the EURL 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 study.

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. Indeed, the highest value of RSD_R is 14% at the 0.10% GM level, thus well within the acceptance criterion.

Table 11. Summary of validation results for the SYHT0H2 detection and quantification method, expressed as GM-DNA copy numbers in relation to target taxon specific-DNA copy numbers

	Test Sample Expected GMO %				
	0.10	**0.35**	**0.90**	**2.0**	**5.0**
Laboratories having returned valid results	12	12	12	12	12
Samples per laboratory	4	4	4	4	4
Number of outliers	-	-	1	-	1
Reason for exclusion	-	-	C	-	C
Mean value of measured GM-content	0.13	0.42	1.0	2.1	5.1
Relative repeatability standard deviation, RSD_r (%)	12	6.5	5.6	6.9	6.2
Repeatability standard deviation	0.016	0.028	0.056	0.145	0.315
Relative reproducibility standard deviation, RSD_R (%)	14	9.5	7.4	7.6	8.6
Reproducibility standard deviation	0.018	0.040	0.075	0.161	0.437
Bias (absolute value)	0.034	0.073	0.113	0.125	0.063
Bias (%)	34	21	13	6.2	1.3

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.
* Standard deviation and bias (absolute value) are reported with three digits.

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 (see "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 repeatability standard deviation is below 25% at all GM levels, with the highest value of 12% at the 0.10% GM level.

The trueness of the method is estimated using the measures of the method bias for each GM level. According to the ENGL method performance requirements, trueness should be ± 25% across the entire dynamic range. In this case, the method satisfies this requirement across down to the 0.35% level, thus including the 0.90% GM level (legal threshold for labelling of adventitious presence of GM material, as per Regulation (EC) No 1829/2003); the method overestimates the true GM content at the 0.10% level, with a bias of 34%.

5. Compliance of the method of detection of event SYHT0H2 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 was carried out:

- at step 2 of the validation process (scientific assessment of the dossier), the EURL GMFF concluded that it could accept the applicant's data on method performance. Indeed, the RSDr at the level of 0.080% expressed in mass fraction of GM-material, corresponding to a GM-level of 0.10% related to copy numbers of GM-material (Paragraph 4.1.1), resulted to be 11% on 15 replicates (Table 2), hence below 25%;

- at step 3 of the validation process (in-house testing of the method), the EURL GMFF determined the RSDr% at the level of 0.10% in terms of GM-DNA copy numbers in relation to target taxon-specific DNA copy numbers. This value corresponds to a GM-level of 0.10% related to mass fraction of GM-material (Paragraph 4.1.1). The experimental testing was carried out under repeatability conditions on fifteen replicates. The RSDr (%) resulted to be 9.9% when the method was tested on ABI 7300 real-time PCR equipment (Tables 8a), hence below 25%;

- further to the conclusion of step 4 of the validation process (international collaborative study), the EURL GMFF analysed the data generated by the twelve participating laboratories for determining the method performance parameters. The RSDr (%) of the method at the level of 0.10% in copy number corresponding to a GM-level of 0.10% related to mass fraction of GM-material, was 12%, therefore below the limit of 25%.

Table 12. Precision of the event-specific method for quantitative detection of SYHT0H2 at or around 0.10% level related to mass fractions of GM material.

Source	**RSDr %**	**GM %**
Applicant's method optimisation	11 %	0.080 %
EURL GMFF in-house verification	9.9 %	0.10 %
International Collaborative Study	12 %	0.10 %

Based on the results of the EURL GMFF in-house verification and of the international collaborative study, it is concluded that the method RSDr is equal or less than 25% at the level of 0.10% related to mass fraction of GM-material, hence the method meets the requirement laid down in Regulation (EU) No 619/2011.

6. Conclusions

A method for detection, identification and quantification of GM event SYHT0H2 was provided by the applicant. The method has been fully validated in accordance to the EURL GMFF validation scheme, respecting all requirements of the relevant EU legislation and international standards for method validation.

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 Regulation (EC) No 641/2004 and Regulation (EU) No 619/2011 and meets all method performance requirements established by the ENGL down to the 0.35% GM level.

At 0.10% GM level the method shows a trueness value slightly outside the required range but at that low levels this is deemed to be acceptable for the following reasons: a) the precision is within the limits b) the positive bias would determine an overestimation of the GM content and not an underestimation which would instead cause a problem c) the true value falls within the 95% CI around the mean (0.13%± 0.036).

The method is therefore valid to be used for regulatory purposes, including the quantification of low level presence [0.10% (mass/mass)] of the GM event. It can be assumed that it is applicable to any appropriately extracted soybean genomic DNA.

Regarding the method performance requirements that were not met by the results of the collaborative study, i.e. trueness at the 0.10% level and mean slopes of the standard curves, the EURL GMFF, in collaboration with the European Network of GMO Laboratories (ENGL), will conduct a monitoring by asking laboratories using the method for the purpose of Regulation (EU) No 619/2011 to provide their experimental data to the EURL GMFF in order to clarify if the found minor deviations are confirmed in practice and of any significance.

7. References

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2. Schuler GD. Sequence mapping by electronic PCR. Genome Res. 1997 May;7(5):541-50. PMID: 9149949
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5. International Standard (ISO) 5725, 1994. Accuracy (trueness and precision) of measurement methods and results. International Organization for Standardization, Genève, Switzerland.
6. Plant DNA C-values Database, http://data.kew.org/cvalues/.

Annex 1: Event-specific Method for the Quantification of Soybean SYHT0H2 by Real-time PCR

Validated Method

Method development:

Syngenta Crop Protection AG

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 soybean event SYHT0H2 (unique identifier SYN-ØØØH2-5) genomic DNA to total soybean genomic DNA in a sample.

Template genomic DNA extracted by means of suitable methods should be tested for quality and quantity prior to use in PCR assay, in particular in case of complex and difficult matrices. 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 soybean event SYHT0H2, an 88-bp fragment of the region spanning the 3′ insert-to-plant junction in soybean SYHT0H2 event 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 BHQ-1 (Black Hole Quencher-1) as a quencher dye at its 3′ end.

For the relative quantification of soybean event SYHT0H2 DNA, a soybean-specific reference system amplifies a 74-bp fragment of soybean endogenous genes *lectin (Le1)*, (GenBank accession number K00821.1), using *Le1* gene-specific primers and a *Le1* gene-specific probe labelled with FAM as reporter dye at its 5′ end, and TAMRA (carboxytetramethylrhodamine) as 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 "Cq" value. For quantification of the amount of SYHT0H2 genomic DNA in a test sample, Cq values for the SYHT0H2 and *Le1* systems are determined for the sample. Standard curves are then used to estimate the relative amount of SYHT0H2 genomic DNA to total soybean genomic DNA.

2. Validation and performance characteristics

2.1 General

The method was optimised for suitable genomic DNA extracted from mixtures of genetically modified and conventional soybean seeds. The method was fully validated by the EURL GMFF and its precision and trueness were tested through an international collaborative trial, using genomic DNA samples at different GM contents.

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

2.3 Limit of detection (LOD)

The relative LOD of the method is at least 0.04% (related to mass fraction of GM-material) in 100 ng of total soybean genomic DNA. The relative LOD was not assessed in the international collaborative study.

2.4 Limit of quantification (LOQ)

The relative LOQ of the method is at least 0.080% (related to mass fraction of GM-material) in 100 ng of total soybean genomic DNA. The lowest relative GM content of the target sequence included in the international collaborative study was 0.10% (mass fraction of GM-material).

2.5 Molecular specificity

The method exploits a unique DNA sequence in the region spanning the 3' insert-to-plant junction in soybean SYHT0H2; the sequence is specific to event SYHT0H2 and thus imparts event-specificity to the event specific assay, which was confirmed in the EURL GMFF validation study (page 6 of the validation report).

3. Procedure

3.1 General instructions and precautions

- The procedures require 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 require dedicated equipment, especially pipettes.
- All the equipment 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 hypochlorite 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 soybean event SYHT0H2

3.2.1 General

The PCR set-up for the taxon-specific target sequence (*Le1*) and for the GMO (event SYHT0H2) target sequence is to be carried out in separate vials. 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 study, as given in table 1.

Table 1. Copy number values of the standard curve samples.

Sample	**S1**	**S2**	**S3**	**S4**	**S5**
Total amount of DNA in reaction (ng)	100	30	9.2	2.8	0.80
% GM (DNA/DNA)	10	10	10	10	10
Target taxon Le1 copies	88496	26817	8126	2463	704
SYHT0H2 soybean GM copies	8850	2682	813	246	70

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

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 event specific assay and one for the *taxon specific* assay) on ice and in the order mentioned below (except DNA).

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the SYHT0H2 event specific assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix 2x (no UNG)	1x	12,5
FE08316-F (10 µM)	600 nM	1.5
FE08317-R (10 µM)	600 nM	1.5
FE08318-P (10 µM)	200 nM	0.5
Nuclease free water Nuclease free water	#	4.0
DNA	#	5.0
Total reaction volume:		25 µL

Table 3. Amplification reaction mixture in the final volume/concentration per reaction well for the soybean *Le1* taxon specific assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix 2X (no UNG)	1x	12.5
Lec for 2 (10 µM)	650 nM	1.625
GMO3-126 Rev (10 µM)	650 nM	1.625
Lec probe (10 µM)	180 nM	0.45
Nuclease free water	#	3.8
DNA	#	5.0
Total reaction volume:		25 µL

3. Mix well and centrifuge briefly.

4. Prepare two reaction tubes (one for the event specific and one for the taxon specific 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 (70 µL for the *Le1* reference system and 70 µL for the SYHT0H2 soybean system). Add to each tube the correct amount of DNA for 3.5 PCR repetitions (e.g. 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 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) 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 SYHT0H2/*Le1* assays.

Step	Stage		T (°C)	Time (s)	Acquisition	Cycles
1	Initial denaturation		95	600	No	1X
2	Amplification	Denaturation	95	15	No	45X
		Annealing & Extension	60	60	Yes	

3.3 Data analysis

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

a) Set the threshold following the automatic or the manual mode. In the manual mode display the amplification curves of the event specific assay 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 following the automatic or the manual mode. In the manual mode: 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 taxon specific system.

e) Save the settings and export all the data 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 Cq-values for each reaction.

The standard curves are generated for both assays by plotting the Cq 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 number in the unknown sample.

To obtain the percentage value of event DNA in the unknown sample, the event copy number is divided by the copy number of the reference gene and multiplied by 100 (GM% = number of event copies/number of reference gene copies 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 (no UNG), Applied Biosystems Cat. No. 4326614.
- Nuclease free water

4.3 Primers and Probes

Oligonucleotides	**Name**	**DNA Sequence (5' to 3')**	**Length (nt)**
SYHT0H2			
Forward primer	FE08316-F	5' GGG AAT TGG GTA CCA TGC C 3'	19
Reverse primer	FE08317-R	5' TGT GTG CCA TTG GTT TAG GGT 3'	21
Probe	FE08318-P	5'-6FAM™- CCA GCA TGG CCG TAT CCG CAA –BHQ™-1-3'	21
Le1			
Forward primer	Lec for 2	5' CCA GCT TCG CCG CTT CCT TC 3'	20
Reverse primer	GMO3-126 Rev	5' GAA GGC AAG CCC ATC TGC AAG CC 3'	23
Probe	Lec probe	5' 6-FAM™- CTT CAC CTT CTA TGC CCC TGA CAC -TAMRA™-3'	24

FAM: 6-carboxyfluorescein; TAMRA: carboxytetramethylrhodamine; BHQ™-1:Black Hole Quencher 1

5. References

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

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