European Commission

Event-specific Method for the Quantification of Sugar beet KWS20-1 Using Real-time PCR

Validation Report

Vasileva, V., Savini, C., Sacco, M., Mazzara, M., Vincent, U.

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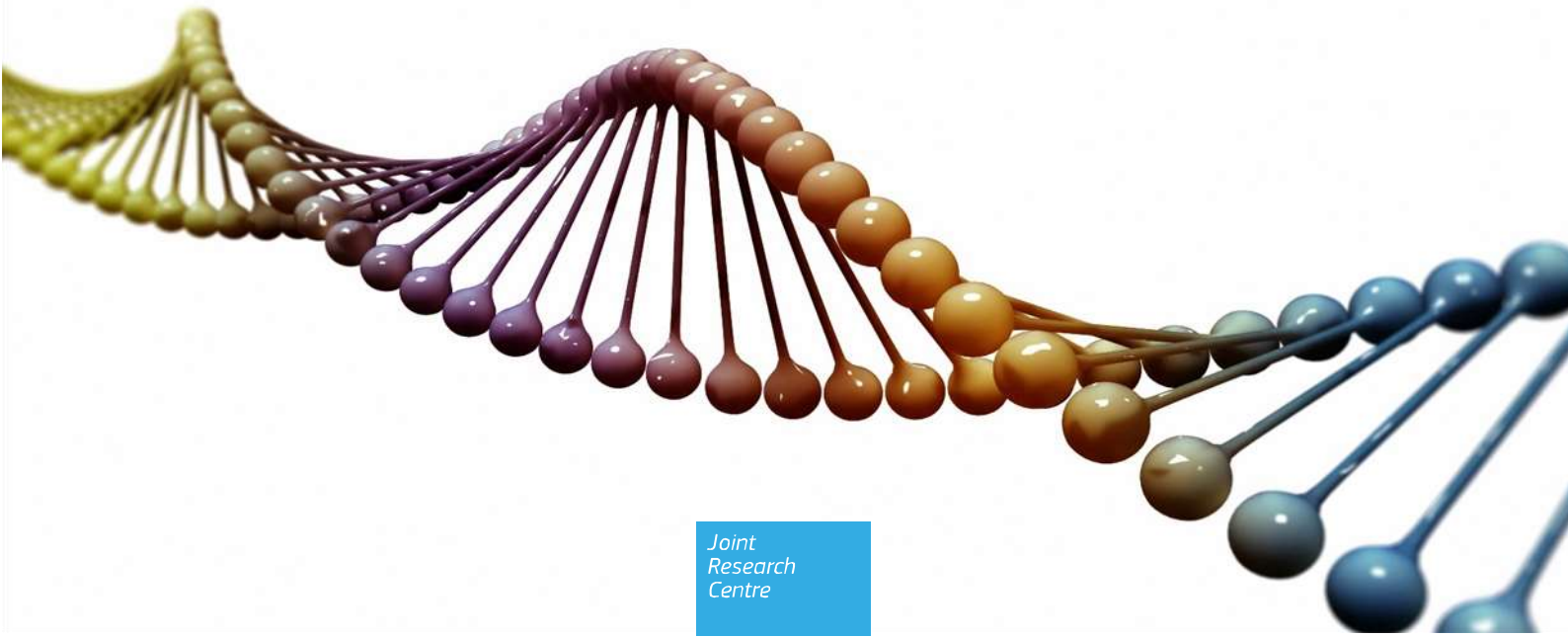

Joint Research Centre

Contact information
European Commission
Directorate General Joint Research Centre
Directorate F – Health and Food
European Union Reference Laboratory for GM Food and Feed
Food & Feed Compliance (F.5)
Via E. Fermi, 2749.
I-21027 Ispra (VA), Italy

Functional mailbox: JRC-EURL-GMFF@ec.europa.eu

EU Science Hub
https://joint-research-centre.ec.europa.eu

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Abstract

In line with its mandate (1) the European Union Reference Laboratory for GM Food and Feed (EURL GMFF), in collaboration with the European Network of GMO Laboratories (ENGL), validated an event-specific real-time polymerase chain reaction (qPCR) method for detecting and quantifying sugar beet KWS20-1 (unique identifier KWS20-1). The validation study was conducted according to the EURL GMFF validation procedure [http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm] and the relevant internationally accepted guidelines (2-6).

In accordance with current EU legislation (1), KWS SAAT SE & Co. KGaA and Bayer CropScience LP represented by Bayer Agriculture BV provided the detection method and the positive and negative control samples (genomic DNA from seeds of KWS20-1 sugar beet as positive control DNA, and genomic DNA from seeds of conventional sugar beet as negative control DNA). The EURL GMFF verified the method performance data provided by the applicant, where necessary experimentally, prepared the validation samples (calibration samples and blind samples at different GM percentage (copies GM/total sugar beet haploid genome copies), organised an international collaborative study and analysed the results.

The EURL GMFF in-house verification and the collaborative study confirmed that the method meets the method performance requirements as established by the EURL GMFF and the ENGL, in line with the provisions of Annex III-3.C.2 to Regulation (EU) No 503/2013 (5), and it fulfils the analytical requirements of Regulation (EU) No 619/2011 (6). This validation report is published at https://gmo-crl.jrc.ec.europa.eu/method-validations.

Quality assurance

The EURL GMFF is ISO/IEC 17025:2017 accredited [certificate number: BELAC 268 TEST (Flexible Scope for determination of Genetically Modified content in % (m/m) and % (cp/cp) in food and feed by DNA extraction, DNA identification and Real-time PCR and for determination of Genetically Modified content in % (cp/cp) in food and feed by DNA extraction and digital PCR)].

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

Event-specific Method for the Quantification of Sugar beet KWS20-1 Using Real-time PCR

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13 September 2024

European Union Reference Laboratory for GM Food and Feed

1 Introduction

In line with Regulation (EC) No 1829/2003 (1), KWS SAAT SE & Co. KGaA and Bayer Agriculture BV provided the EURL GMFF with an event-specific method for detection and quantification of sugar beet KWS20-1 (unique identifier KB-KWS2∅1-6) together with genomic DNA as positive and negative control samples.

The dossier was found complete (step 1 of the EURL GMFF validation procedure) and the scientific dossier assessment (step 2) concluded that the reported method performance characteristics, assessed against the ENGL method acceptance criteria (7), allowed moving the method forward to step 3 of the procedure (experimental testing), where the EURL GMFF verified the purity of the control samples provided and conducted an in-house testing of samples and method.

The positive and negative control DNA, submitted in accordance with Art 5(3)(j) and Article 17(3)(j) of Regulation (EC) No 1829/2003, were found of good quality.

Step 3 was completed with the conclusion that the method could be submitted to the collaborative study (step 4). This study confirmed that the method is suited for quantifying genomic DNA of GM sugar beet KWS20-1, appropriately extracted from food or feed, down to a GM content level of 0.1% m/m.

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

2 Dossier reception and acceptance (step 1)

KWS SAAT SE & Co. KGaA and Bayer Agriculture BV submitted the identification and quantification method, data demonstrating its adequate performance when applied to genomic DNA extracted from sugar beet KWS20-1 GM event and from non GM sugar beet, and the corresponding positive and negative control DNA samples.

The dossier was found to be complete and was thus moved to step 2.

3 Scientific assessment and bioinformatics analysis (step 2)

Documentation and data supplied by the applicant were evaluated by the EURL GMFF for compliance with the ENGL method acceptance criteria.

The specificity of the event-specific 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.

3.1 Specificity assessment conducted by the applicant

The specificity of the event-specific method was assessed by the applicant in triplicate real-time PCR reactions, according to the method described in Annex 1 (Tables 1, 2, 3, 4 and 5), using at least 250 copies/reaction of target genomic DNA and at least 2500 copies/reaction of non-target genomic DNA extracted from:

sugar beet KWS20-1, H7-1;

maize T25, TC1507, NK603, MIR162, MIR604, MON810, 3272, 98140, 59122, 5307, MON88017, MON89034, MON863, GA21, Bt11, Bt176, MON87427, MON87460, DAS-40278-9, DP4114, VCO-1981-5, MON87403, MON87411, MZHG0JG, MZIR098;

soybean DAS-44406-6, A2704-12, A5547-127, BPS-CV127-9, DAS-81419-2, MON87705, MON87751, FG72, MON87769, 356043, 305423, GTS-40-3-2, MON87701, DAS-68416-4, MON89788, MON87708, SYHT0H2, GMB151;

cotton LLCotton25, GHB614, MON531, MON15985-7, MON1445, 281-24-236 x 3006-210-23, GHB119 , T304-40, MON88913, MON88701, DAS-81910-7, COT102, GHB811;

canola Rf3, GT73/RT73, MON88302, Rf1, Ms1, T45, Rf2, Ms8, Topas 19/2, 73496, Ms11;

rice LLRice62;

potato EH92-527-1; AM04-1020, AV43-6-G7, PH05-026-0048;

and conventional sugar beet, fodder beet, maize, soybean, cotton, canola, rice, potato, wheat, brassica rapa subsp. rapa subvar. esculenta (Autumn Beet), brassica rapa subsp. rapa var. majalis (Turnip);

According to the method developer's data, the KWS20-1 method did not react with any sample except the positive control. Each sample reacted with the taxon-specific target.

In addition, the applicant performed an in-silico specificity analysis by using the amplicon sequence as a query for BLASTN algorithm search (December 2022) against the public sequence of National Center for Biotechnology Information (NCBI) nucleotide collection and GenBank® patent sequences. No sequence showed alignment of the entire KWS20-1 sugar beet amplicon.

A previously validated sugar beet-specific PCR method (https://gmo-crl.jrc.ec.europa.eu/summaries/H7-1-Protocol%20Validated%20-%20corrected%20version%201.pdf), amplifying a 118 base pair (bp) fragment of sugar beet glutamine synthetase (Genbank AY026353.1), was used as a reference method.

The specificity of the taxon-specific method was assessed by the applicant in triplicate real-time PCR reactions, according to the method described in Annex 1 (Tables 1, 2, 3, 4 and 5), with a minimum of 50 ng genomic DNA extracted from: conventional sugar beet, fodder beat, maize, canola, cotton, potato, wheat, rice, soybean, *Brassica rapa and KWS20-1*. According to the method developer the *GS* method did not react with any sample except conventional sugar beet, fodder beet and KWS20-1.

The *glutamine synthase* (*GS*) allelic variation was tested by the applicant on 21 sugar beet lines with 100 ng DNA per reaction in triplicate. The delta Cq (ΔCq) between the higher and the lower Cq value was 0.6.

Therefore, the range of variability of Cq values in amplification does not exceed 1 Cq within the tested lines, thus complying with the ENGL requirement.

3.2 Specificity assessment conducted by the EURL GMFF

The detection method spans the 3' insert-to-plant junction in sugar beet KWS20-1. The forward primer 2109_fwd1 binds to the insert. The reverse primer 2109_rev1 binding site was found in the sugar beet (*Beta vulgaris*) 3' flanking genomic border adjacent to the insertion. The probe 2109 probe 1 binds to the junction between the insert and the 3' genomic region of *Beta vulgaris*.

The amplicon size is expected to be 77 bp, consistent to what reported by the applicant. The sequence of the amplicon was analysed by BLAST (NCBI) against local copies of the "nt" and "patents" databases, and no significant similarity of both primer annealing sites was found with any published sequence. In addition, the primers were tested against the sequences of the other GMO events present in the Central Core Sequence Information System (CCSIS) of the JRC, as well as the whole genomes of more than 80 plants (including *Brassica rapa*, *Glycine max*, *Oryza sativa*, *Solanum lycopersicum* and *Zea mays*) using the e-PCR prediction tool (NCBI), and no potential amplicon was identified. A perfect match of the amplicon and of the primers was identified with the sequence deposited for KWS20-1. The tool "Perfect match GMO methods matrix" and "ePCR matrix" did not find any perfect match of the event-specific methods of the GMO Method database (https://gmo-crl.jrc.ec.europa.eu/gmomethods/) with the KWS20-1 insert

3.3 Verification of the ENGL acceptance parameters

The applicant prepared the calibration curve from a DNA solution (S1) of 10% sugar beet KWS20-1 genomic DNA (expressed as copy number ratio) which was serially diluted (1:8) in to obtain samples S2, S3, S4. The sample S5 was diluted 1:6 from sample S4 and only used as fifth calibration point for the sugar beet reference gene curve. The parameters (slope, R^2 coefficient) of eight runs of the calibration curve are reported as provided by the applicant (Table 1).

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

KWS20-1		*GS*	
Slope	R^2	Slope	R^2
-3.31	1.00	-3.35	1.00
-3.25	1.00	-3.33	1.00
-3.23	1.00	-3.30	1.00
-3.34	1.00	-3.30	1.00
-3.27	1.00	-3.39	1.00
-3.31	1.00	-3.29	1.00
-3.38	1.00	-3.38	1.00
-3.31	1.00	-3.38	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 R^2 coefficient of the standard curves for the GM (KWS20-1) and the sugar beet-specific glutamate synthetase (*GS*), as established by the applicant, were within the ENGL acceptance criteria.

Precision and trueness of the method were established by the applicant and 16 values for each of four GM levels (expressed as copy fraction of GM-material) were provided. Table 2A reports precision and trueness values for the four GM-levels as provided by the applicant. Both parameters were within the ENGL acceptance criteria (trueness ± 25%, RSD_r ≤ 25% across the entire dynamic range).

Table 2A. Mean %, precision and trueness values (*) provided by the applicant

	Test results			
Expected GM %	5.00	0.90	0.090	0.025**
Measured mean GM %	5.11	0.951	0.092	0.023
Precision (RSD_r %)	4.1	3.5	7.3	13.0
Trueness (bias %)	2.2	5.7	2.2	-8.0

* Numbers are not rounded but are presented as reported by the applicant expressed in copy number ratio

** equivalent to 0.10% in mass fraction, according to the applicant's determination of the zygosity factor of 0.25 for the DNA extracted from the KWS20-1 seeds,

The method met the ENGL acceptance criteria for trueness and precision at the lowest GM level i.e. 0.025 % (expressed as copy number ratio), which contains 33 copies of KWS20-1 in 100 ng of total DNA per reaction. The GM content of this sample is in line with the requirements for testing the Limit of Quantification (LOQ, below or equal to 0.09% or 50 copies).

The LOD of the KWS20-1 and *GS* methods was determined on 60 replicates. The LOD_{rel} was found to be below 0.015% (20 copies) in 100 ng of total sugar beet DNA per reaction for the KWS20-1 event-specific method and the LOD_{abs} was determined as below 20 haploid genome copies for *GS* reference method. The LOD_{abs} and LOD_{rel} are in line with the ENGL acceptance criteria (below 0.045% or 25 copies with a level of confidence of 95%).

The robustness of the method was assessed in 16 combinations on as ample at 0.025% GM-content of the following variations to the method: exact /+10%/-10% master mix concentration, exact +30%/-30% primer concentration, exact/+30%/-30% probe concentration, exact/+1 µL/-1 µL master mix volume, +/-1 °C in annealing temperature. The RSD_r and the trueness calculated for each combination of variations on a sample at the LOQ level (0.025%) did not exceed 30%, thus meeting the ENGL acceptance criteria.

Precision and trueness of the method were tested in a transferability study: two values for each of the four GM levels (expressed as copies GM/total haploid genome copies) were provided. Table 2B reports precision and trueness values for the four GM-levels as provided by a laboratory different from the method developer. Both parameters were within the ENGL acceptance criteria (trueness ≤ 25%, RSDr ≤ 25% across the entire dynamic range).

Table 2B. Mean %, precision and trueness values obtained in the transferability study of the applicant

Laboratory	Expected GM %	5.00	0.90	0.090	0.025
A	Measured mean GM %	5.38	1.036	0.092	0.024
	Precision (RSD_r %)	2.1	2.0	3.8	2.9
	Trueness (bias %)	7.6	15.1	2.2	-4.0
B	Measured mean GM %	5.03	1.021	0.106	0.024
	Precision (RSD_r %)	1.4	2.9	2.6	5.8
	Trueness (bias %)	0.6	13.4	17.8	-4.0

3.4 DNA extraction

A genomic DNA extraction method from ground sugar beet seeds was previously validated in-house by the EURL GMFF. The protocol for DNA extraction and a report on testing are published at https://gmo-crl.jrc.ec.europa.eu/summaries/H7-1-DNAExtr_sampl.pdf. The applicant used the validated method to extract the DNA from seeds of KWS20-1 and conventional sugar beet which yielded DNA of sufficient quality and quantity for its method validation (§ 3.3).

In agreement with the ENGL position, endorsing the modularity principle (see also Annex III to Regulation (EU) No 503/2013), and given the similarity in the matrix, the EURL GMFF considers the above mentioned DNA extraction protocol applicable in the context of the validation of the method for sugar beet KWS20-1.

Annex III to Reg. (EU) No 503/2013 (5) requires the applicant to discuss the validity and limitations of the detection methods in the various types of foods and feeds (matrices) that are expected to be placed on the market. To this regard the applicant stated that "*In determining the appropriate materials for KWS20-1 sugar beet to fulfil the requirements for the food/feed sample according to Regulation (EU) No 503/2013, the sugar beet and the fractions generated by sugar beet processing, sugar, molasses and pulp, were assessed. Seeds have been chosen as the appropriate samples rather than sugar, molasses or pulp since DNA and proteins are either not present or only in very low amounts in these products.*

The applicability of the Quantitative PCR Method developed for KWS20-1 sugar beet depends on the isolation of sufficient quantity and quality of purified DNA. This method has been tested on DNA extracted from ground seed material. Conceptually, the detection method for KWS20-1 sugar beet should work as far as good quality and intact DNA can be extracted from processed food and feed materials. The provided DNA extraction method is intended for extraction of genomic DNA from seed which results in primarily high molecular weight DNA, indicating that the DNA is intact with limited fragmentation.

The processing of the sugar beet root is a complicated, multi-step procedure involving heat treatment and high pH. Both of these conditions are known to significantly deplete DNA content and quality present in whole sugar beet roots[1]*".*

The applicant also explained that the sugar produced from sugar beet roots is not an appropriate material: Klein et al. (1998)[2] determined that DNA is not detectable in sugar and, furthermore, it is reduced "*during the processing procedures by a factor of >10*^{14}*, far exceeding the total amount of DNA present in sugar beet. The*

[1] OECD (2002). Consensus document on compositional considerations for new varieties of sugar beet: key food and feed nutrients and antinutrients, https://one.oecd.org/document/env/jm/mono(2002)4/en/pdf

[2] Klein J. *et al*. 1998. Nucleic acid and protein elimination during the sugar manufacturing process of conventional and transgenic sugar beets. J Biotech 60:145-153

results obtained by Klein et al. were confirmed by a Monsanto sponsored experiment that determined no DNA and no CP4 EPSPS protein were detected in sugar processed from glyphosate tolerant sugar beet (ASSBT, 2001)". Similarly, it expects that no DNA is present in sugar derived from KWS20-1 sugar beet, making it an unsuitable material for detection purposes.

Moreover, the applicant informed that also molasses are not considered an appropriate material for detection purposes since they are produced through the same procedure as sugar and that published data indicate that "*it is very difficult to routinely isolate DNA in dried pulp for reliable detection purposes, probably due to the heat (above 100° C) and pressure treatments during the processing procedures to remove the extractable sugars and to dry the pulp*[3,4]*. The applicants have also examined the possibility of providing wet pulp or homogenized sugar beet roots. Despite it has been reported that it is possible to extract intact DNA from wet pulp*[3,4]*, in-house data indicate that the quality of the resulting DNA is rather poor. Additionally, due to the presence of endogenous enzymes in the homogenized root, this matrix should be kept at –80°C, otherwise stability issues would occur during storage and shipment of the material to the laboratories involved in the method validation*[2]*.*

In conclusion, the sugar beet root, sugar, molasses and pulp are not considered to be appropriate control samples and samples of food and feed to be provided to meet the requirements of Regulation (EC) No. 503/2013.

Based upon the above considerations, the applicants are recommending that sugar beet seeds are utilized as the appropriate matrix for detection purposes. The applicants consider the seed to be the appropriate control samples (and samples of food and feed) based upon the reliable DNA extraction methodology and the stability during storage conditions available for this matrix, which is anticipated to meet the requirements under Regulation (EC) No. 503/2013".

Whenever DNA is extracted from more complex and difficult matrices, a thorough control of the quality of the DNA is recommended in order to ensure that it has the required quality for subsequent PCR analysis.

[3] Forbes J. M. *et al.* 2000. Effect of feed processing conditions on DNA fragmentation. UK MAFF report CS0116:4-26.
[4] Chiter A. *et al.* 2000. DNA stability in plant tissues: implications for the possible transfer of genes from genetically modified food. FEBS lett 481:164-168

4 Materials and method

4.1 Samples

The following positive and negative control samples were provided and described by the applicant to the EURL GMFF in accordance to Regulation (EC) No 1829/2003 Art 2.11 ([5]):

- genomic DNA extracted by the applicant from sugar beet seeds hemizygous for the KWS20-1 event, and
- genomic DNA extracted by the applicant from conventional sugar beet seeds with a comparable genetic background.

4.2 Method 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 KWS20-1 DNA to total sugar beet DNA. The procedure is a simplex system, in which a sugar beet specific method targeting the endogenous gene *glutamate synthetase* (*GS*), and the GM target method for KWS20-1 are performed in separate wells. The validated method protocol is published by the EURL GMFF at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx and can be found in Annex 1 to this report.

For the detection of KWS20-1, a 77 bp fragment of the region spanning the 3' insert-to-plant junction in sugar beet KWS20-1 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 MGB (minor groove binder) as non-fluorescent quencher dye at its 3' end.

For the relative quantification of GM KWS20-1, a sugar beet taxon-specific method amplifies a 118 bp fragment of a sugar beet *glutamate synthetase* (*GS*) endogenous gene, using *GS* gene-specific primers and a *GS* gene-specific probe labelled with FAM (6-carboxyfluorescein) as reporter dye at its 5' end and Black Hole Quencher (BHQ) at its 3' end.

Standard curves are generated for both KWS20-1 and *GS* by plotting the Cq 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 the relative quantification of KWS20-1 DNA in a test sample, the KWS20-1 copy number is divided by the copy number of the sugar beet haploid genome and multiplied by 100 to obtain the percentage value (GM % = KWS20-1/ sugar beet [haploid genome] x 100).

The sugar beet haploid genome weight[6] (0.75 pg) is estimated by the applicant based on the sugar beet genome size of 731 Mb (8) and on the estimated average weight of a base pair corresponding to 617.96 g/mol (9).

[5] 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).

[6] The weight of one genome of sugar beet is estimated considering its genome length of 731 Mb (731,000,000 bp) base pairs (bp) and assuming a weight of 617.96 g/mol per bp. Therefore, the molecular weight of the sugar beet haploid genome is 731,000,000 x 617.96 = 4.52 x 10^{11} g/mol. Given that a mole represents a substance containing the Avogadro's number (6.022 x 10^{23}) of molecules, one sugar beet genome weighs approximately 0.75 picograms (4.52 x 10^{11} / 6.022 x 10^{23}, expressed in pg).

The copy number values used in the quantification, the GMO contents of the calibration samples, and the total DNA quantity used in the PCR reactions are listed in Table 3.

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

Table 3. Copy number values of the standard curve samples

Sample code	S1	S2	S3	S4	S5
Total amount of sugar beet DNA in the reaction (ng)	100	12.5	1.6	0.2	0.03
Target taxon haploid genome copies	133 333	16 667	2 083	260	43
Target KWS20-1 copies	13333	1 667	208	26	-

4.3 EURL GMFF experimental testing (step 3)

4.3.1 Determination of the zygosity ratio in the positive control sample

The EURL GMFF experimentally verified the zygosity ratio (GM-target to reference target ratio) in the positive control sample to assess the method performance at 0.1% GM level -expressed as mass fraction of GM material- in relation to the provisions of Regulation (EU) No 619/2011 (6).

The copy number of the KWS20-1 and of the *GS* targets in the positive control sample were determined by digital PCR (dPCR) performed on the Bio Rad QX200 Droplet Reader.

Reaction mixes were prepared in order to test the zygosity in eigtheen replicates to a final volume of 22 µL and contained 1X ddPCR Super Mix no dUTP (Bio Rad, Cat. number 64299440), primers and probes at concentrations indicated in the corresponding validated method (2109_fwd1 and 21090_rev1 primers at 300 nM each, 2109_Probe 1 at 150 nM; GluA3-F and GluA3-R primers at 300 nM each, GluD1-probe at 150 nM]), and 2 µL of DNA at a concentration of 10 ng/µL.

Reaction mixes were loaded into a semi skirt 96-well plate. 'No template controls' were included. After sealing with a sealing aluminium foil using the PX1™ PCR Plate Sealer, the plate was briefly centrifuged (1 min at 1000 rpm) and placed on Bio Rad Automated Droplet Generator (AutoDG).

The instrument added the Automated Droplet Generation oil for Probes (Bio Rad, Cat. number 1864110), generated the droplets in a final volume of 40 µL of the emulsion containing droplets. The AutoDG then transferred all the emulsions into a new semi skirt 96-well plate. The new plate was sealed with a sealing foil with the PX1™ PCR Plate Sealer and run in a Bio Rad C1000 TouchThermal Cycler. The thermal cycling conditions in a final volume of 40 µL is described below.

Box 1: Thermal cycling conditions in a final volume of 40 µL.

Step	Temperature (°C)	Time (mm:ss)	Ramp rate	Number of cycles
Polymerase activation	95	10:00	2°C/sec	1
DNA denaturation	94	00:30		40
Annealing/extension	60	01:00		
Enzyme deactivation	98	10:00		1
Hold	4	infinite		1

The sealed 96-well plate was then placed in the QX200 Droplet Reader to determine through cytofluorimetry the fraction of fluorescent PCR-positive droplets with respect of the total number of droplets in the original sample by selecting the proper fluorescent dye used. Data analysis and copy number calculations were performed using the Bio-Rad QX200 Droplet Reader Analysis software (QuantaSoft version 1.7.4).

Calculations of means and variances were 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 interlaboratory validated methods - Version 2' (10).

4.3.2 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 blind test samples distributed over a range of GM levels (5% - 0.03%, see Table 4). The blind test samples were prepared by the EURL GMFF from the genomic DNA provided by the applicant (see 4.1 for details) by mixing KWS20-1 sugar beet DNA and non-GM sugar beet DNA.

Table 4. KWS20-1 blinded samples GM % contents

KWS20-1 GM % GM copy number/sugar beet [haploid genome copy number] x 100
5.00
0.90
0.270
0.090
0.030

The calibration sample S1 was prepared from the genomic DNA provided by the applicant by mixing the appropriate amount of KWS20-1 DNA with control non-GM sugar beet DNA to obtain a 10% (in copy number ratio related to haploid genome copies) GM sample. Calibration samples S2-S4 were prepared by 8-fold serial dilutions from the S1 sample and sample S5 by a 6-fold serial dilution from the S4 sample (see Table 3).

The experiments were performed on an ABI 7500, a QuantStudio 7 Flex System and a Roche LC480 II] real-time platform under repeatability conditions and followed the protocol provided by the applicant.

Test samples from GM level 5.00%, to 0.030%, 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 0.030% (in copy number ratio, equal to 0.10% mass ratio), containing 40 copies of KWS20-1 in 100 ng of total DNA per reaction, was tested in 15 replicates in an additional run for each platform. 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. On Roche LC480 II platform the method was run at 45 cycles as described in the validated method published at https://gmo-crl.jrc.ec.europa.eu/method-validations and in Annex 1 below, and analysed with the second derivative maximum method.

4.4 International collaborative study (step 4)

The international collaborative trial involved twelve randomly selected laboratories, all being "national reference laboratories, assisting the EURL GMFF for testing and validation of methods for detection", as listed in annex to Regulation (EC) No 120/2014 (11) who had expressed their interest in participation. 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) (2)

- 5725-1: 2023 "Accuracy (trueness and precision) of measurement methods and results. General principles and definitions." (3)

- 5725-2: 2019. "Accuracy (trueness and precision) of measurement methods and results — Basic method for the determination of repeatability and reproducibility of a standard measurement method" (4)

The objective of the international collaborative study was to verify in experienced laboratories the trueness and precision of the PCR analytical method provided by the applicant and verified in-house by the EURL GMFF.

4.4.1 List of participating laboratories

The twelve laboratories participating in KWS20-1 international collaborative study were randomly selected from 20 national reference laboratories (NRL) that offered to participate.

Clear guidance was given to the selected laboratories for strictly following the validation protocol that was provided to them. The participating laboratories are listed in Table 5.

Table 5. Laboratories participating in the validation of the detection method for sugar beet KWS20-1

Laboratory	Country
Center for Agricultural Technology Augustenberg	DE
CREA-DC Sede di Tavazzabo - Laboratorio	IT
Danish Veterinary and Food Administration	DK
Federal Office of Consumer Protection and Food Safety - BVL	DE
Institute for Agricultural, Fisheries and Food Research	BE
Institute for Diagnosis and Animal Health, Molecular Biology and GMOs Unit-National	RO
Laboratory for Detection of GMO in Food – Bad Langensalza	DE
Plant Health Laboratory	FR
Sciensano	BE
State Institute of Chemical and Veterinarian Analysis – Freiburg	DE
Swedish Food Agency, Science Department	SE
Walloon Agricultural Research Centre – Knowledge and valorization of agricultural products Department	BE

4.4.2 Real-time PCR equipment used in the study

Laboratories involved in the collaborative study used the following real-time PCR equipment: three laboratories used Biorad CFX96, three QuantStudio 5, two QuantStudio 3, one laboratory used QuantStudio 7 Flex, one used ABI 7500, one used Stratagene Mx3000 and one Roche LC 480 II.

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

4.4.3 Materials used in the international collaborative study

For the validation of the quantitative event-specific method, calibration samples (of known GMO content) and blind test samples (of undisclosed GM content = blind samples) were provided by the EURL GMFF to the participating laboratories (for test samples preparation see 4.3.2).

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 (87.5µL of DNA solution, each at 20 ng/µL) labelled from U1 to U20, representing five GM levels, each in four replicates (Table 4)

- ✓ Reaction reagents:
  - Sigma JumpStart® Taq Ready Mix (2x), one vial]: 6.4 mL
  - 50X ROX reference dye 30uL
  - 100 mM MgCl2 700 uL
  - distilled sterile water, one vial 1.6 mL

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

  GS taxon-specific
  - GluA3-F primer (10 µM): 192 µL
  - GluA3-R primer (10 µM): 192 µL
  - GluD1 probe (10 µM): 96 µL

  KWS20-1
  - 2109_fwd1 primer (10 µM): 192 µL
  - 2109_rev1 primer (10 µM): 192 µL
  - 2109_probe1 (10 µM): 96 µL

4.4.4 Design of the collaborative study

Participating laboratories received a detailed validation protocol that included the exact design of the PCR plates, ensuring that on each PCR plate the samples were analysed for the KWS20-1 event-specific method and for the *GS* taxon-specific method. In total, two plates were run by each participating laboratory.

The laboratories prepared the PCR master-mixes for the sugar beet KWS20-1 and the *GS* 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 layout.

The amplification reaction followed the cycling program specified in the protocol. Participants determined the GM % in the test samples according to the instructions and also 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.

4.4.5 Deviations reported from the protocol

Eight laboratories reported no deviations from the validation protocol. One laboratory reported a mistake on plate a in well E12, which was consequently excluded from the results. Another laboratory reported, for sample U9 VL0223, that the third replicate (well H6) of the reference gene method was not pipetted; thus, only two measurements for this sample were taken. One laboratory shifted of three wells the sample loading in plate B. In one laboratory, plate B was repeated due to an evident amplification deviation of the reference gene method in one of the test samples; however, the re-run exhibited a large deviation in Cq values for the S2 sample of the GM method, resulting in a value of 0.94 for the coefficient of determination (R^2) of the calibration curve, which is well below the ENGL acceptance criteria (R2 ≥ 0.98). This led to the misquantification of most of the test samples. Since there were no technical explanations for the occurrences in the two runs of plate B, the EURL GMFF decided to consider only the results of the first plate, as a conservative measure to retain the laboratory data for analysis.

5 Results

5.1 EURL GMFF experimental testing

5.1.1 Zygosity ratio in the positive control sample

The results of the digital PCR analysis conducted by the EURL GMFF on the KWS20-1 and *GS* targets to determine the zygosity ratio in the positive control samples are shown in Table 6. For reference, the zygosity ratio reported by the applicant was 0.25.

Table 6. Zygosity ratio of the KWS20-1 and *GS* targets in the positive control sample.

Mean ratio (KWS20-1/*GS*)	0.30
Standard deviation	0.01175
RSD_r (%)	3.93
Standard error of the mean	0.0028
Upper 95% CI of the mean	0.30
Lower 95% CI of the mean	0.29

The mean ratio (KWS20-1/*GS*) in the conducted experiments equals 0.3.

Hence, the 0.03 GM % in DNA copy number ratio corresponds to a 0.10 GM% in mass fraction

Box 2: Note on reporting of analytical results

The zygosity ratio herein reported is valid for the positive control sample DNA in the context of the present validation study. It is used to assess the method performance at 0.1% GM level -expressed as mass fraction of GM material- in relation to the provisions of Regulation (EU) No 619/2011.

When analytical results of official laboratories are primarily expressed as ratio of GM- DNA copy numbers, they shall be translated into mass fraction results by means of the specific conversion factor published in the document "Conversion factors (CF) for certified references materials (CRM)" (https://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm).

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

Test samples with GM levels from 0.03% to 5% (expressed in copy number) 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 sample at 0.03% GM-level (copy number, corresponding to 0.1% mass fraction) was tested for its precision in quantification in 15 replicates in separate runs.

Tests were conducted on ABI 7500, a QS7 (QuantStudio 7 Flex System) and a Roche LC480II for robustness.

The standard curve parameters and the results of efficiency, linearity, trueness and precision obtained in the three real-time PCR runs with the test samples are shown in Tables 7A, 7B, 8, 9 and 10.

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 $\geq$ 0.98. Table 7A and 7B document that the slopes of the standard curves and the R^2 coefficients were within the limits established by the ENGL. The EURL GMFF in-house results confirm the data provided by the applicant.

Table 7A. Standard curve parameters of the real-time PCR tests, carried out on ABI 7500, Q7 and Roche LC480 II to quantify GM-levels in the range 0.03% to 5% in four replicates each. Slope and R^2 coefficient values were rounded to two digits.

	KWS20-1			*GS*		
	Slope	PCR efficiency (*)	R^2	Slope	PCR efficiency (*)	R^2
Run A	-3.45	95	1.00	-3.27	102	1.00
Run B	-3.30	101	1.00	-3.29	101	1.00
Run C	-3.42	96	1.00	-3.28	102	1.00
Run D	-3.38	98	1.00	-3.28	102	1.00
Run E	-3.38	97	1.00	-3.35	99	1.00
Run F	-3.39	97	1.00	-3.31	100	1.00

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

Runs A-B were carried out on ABI 7500; runs C-D were carried out on QS7; runs E and F were carried out on Roche LC480II II.

Table 7B. Standard curve parameters of the real-time PCR tests, carried out on ABI 7500, QS7, and Roche LC480 II to quantify the GM-level 0.03% in 15 replicates. Slope and R^2 coefficient values were rounded to two digits.

	KWS20-1			*GS*		
	Slope	PCR efficiency (*)	R^2	Slope	PCR efficiency (*)	R^2
Run G	-3.34	99	1.00	-3.36	98	1.00
Run H	-3.37	98	1.00	-3.30	101	1.00
Run I	-3.42	96	1.00	-3.37	98	1.00

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

Run G was carried out on ABI 7500; run H was carried out on QS7; run I was carried out on Roche LC480 II.

According to the ENGL method acceptance criteria the method trueness (measured as bias in % of the target GM level) should be within ± 25% of the accepted reference value over the entire dynamic range and the precision, expressed as RSD_r % (relative standard deviation of repeatability), should be ≤ 25 %, also over the entire dynamic range.

Tables 8, 9 and 10 show that trueness and precision of quantification were within the limits established by the ENGL for the PCR machines used.

Table 8. Values of trueness and precision as established by the EURL GMFF in its in-house verification using an ABI 7500. GM % in copy/copy haploid genomes.

Target GM-levels %	Measured GM-level %	Bias % of the target GM-level	Precision (RSD_r %)
5.0	4.5	-9.1	0.65
0.90	0.89	-0.85	0.90
0.27	0.25	-8.1	8.3
0.09	0.09	-1.9	7.1
0.03	0.033	8.7	10

Table 9. Values of trueness and precision as established by the EURL GMFF in its in-house verification using a QS7. GM % in [copy/copy haploid genomes].

Target GM-levels %	Measured GM-level %	Bias % of the target GM-level	Precision (RSD_r %)
5.0	4.7	-6.4	1.2
0.90	0.89	-1.1	3.5
0.27	0.26	-2.4	5.8
0.09	0.09	-3.3	4.2
0.03	0.033	10	17

Table 10. Values of trueness and precision as established by the EURL GMFF in its in-house verification using a Roche LC480 II. GM % in copy/copy haploid genomes.

Target GM-levels %	Measured GM-level %	Bias % of the target GM-level	Precision (RSD_r %)
5.0	4.8	-4.0	0.95
2.0	0.87	-3.0	3.0
0.27	0.27	0.48	1.8
0.09	0.09	-1.5	5.6
0.03	0.035	15	13

5.2 Results of the international collaborative study

5.2.1 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 11. The PCR efficiency (%) was calculated from the standard curve slopes using the formula:

$$\text{Efficiency (\%)} = \left(10^{\frac{-1}{slope}} - 1\right) \times 100$$

Table 11 indicates that the efficiency of amplification for KWS20-1 ranges from 92 to 104 and the linearity is 1.00; the amplification efficiency for the sugar beet-specific method ranges from 98% to 104% and the linearity is 1.00. The mean PCR efficiency was 98% for KWS20-1 and 101% for *GS*. The average R^2 of the methods was 1.00 for KWS20-1 and *GS*, respectively. Both PCR efficiency and linearity values were within the ENGL acceptance criteria.

Table 11. Values of slope, PCR efficiency and R^2 obtained during the international collaborative trial. Slope and R^2 coefficient values were rounded to two digits.

		KWS20-1			*GS*		
Lab	Plate	Slope	PCR Efficiency (%)	R^2	Slope	PCR Efficiency (%)	R^2
1	A	-3.31	100	1.00	-3.29	101	1.00
	B	-3.32	100	1.00	-3.29	101	1.00
2	A	-3.22	104	1.00	-3.28	102	1.00
	B	-3.39	97	1.00	-3.33	100	1.00
3	A	-3.37	98	1.00	-3.33	99	1.00
	B	-3.37	98	1.00	-3.36	98	1.00
4	A	-3.39	97	1.00	-3.31	100	1.00
	B	-3.38	98	1.00	-3.30	101	1.00
5	A	-3.38	97	1.00	-3.29	101	1.00
	B	-3.53	92	1.00	-3.32	100	1.00
6	A	-3.39	97	1.00	-3.33	100	1.00
	B	-3.46	95	1.00	-3.33	100	1.00
7	A	-3.44	95	1.00	-3.15	108	1.00
	B	-3.38	98	1.00	-3.30	101	1.00
8	A	-3.43	96	1.00	-3.37	98	1.00
	B	-3.38	98	1.00	-3.36	98	1.00
9	A	-3.37	98	1.00	-3.26	103	1.00
	B	-3.35	99	1.00	-3.31	101	1.00
10	A	-3.29	101	1.00	-3.31	100	1.00
	B	-3.33	100	1.00	-3.30	101	1.00
11	A	-3.44	95	1.00	-3.36	99	1.00
	B	-3.35	99	1.00	-3.23	104	1.00
12	A	-3.44	95	1.00	-3.29	101	1.00
	B	-3.42	96	1.00	-3.31	101	1.00
	Mean	**-3.38**	**98**	**1.00**	**-3.30**	**101**	**1.00**

These results confirm the appropriate performance characteristics of the methods tested in terms of efficiency and linearity.

5.2.2 GMO quantification

Table 12 reports the values of quantification for the four replicates of each GM level as reported by each of the twelve participating laboratories.

Table 12. GM % values determined by laboratories for test samples

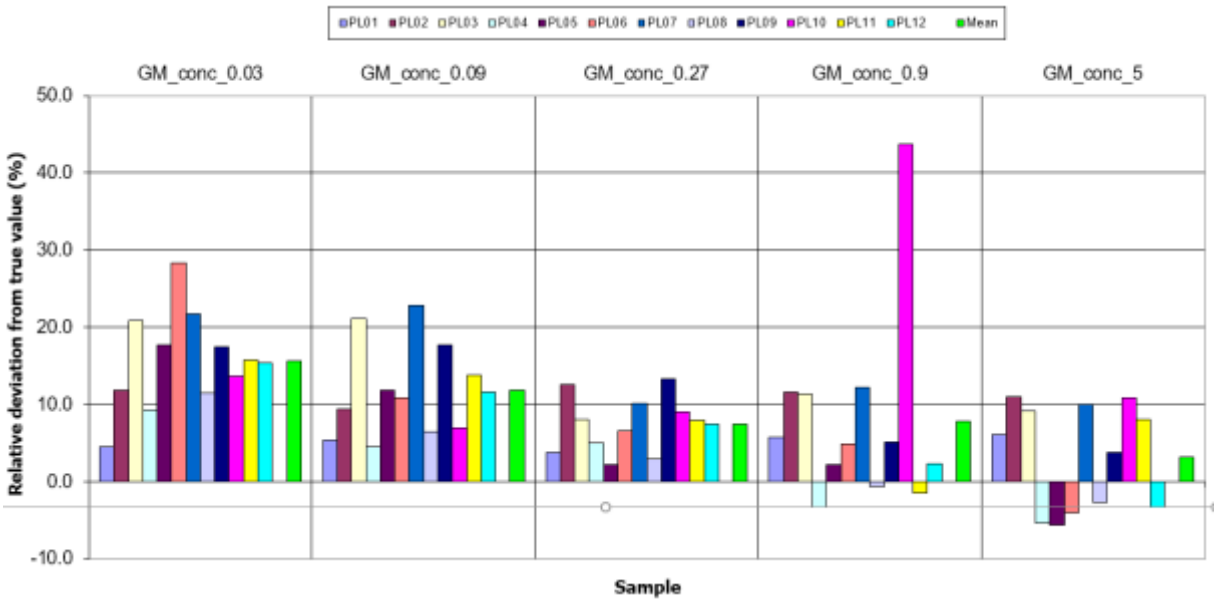
	GMO content (%) (*)																			
LAB	0.03				0.09				0.027				0.9				5.0			
	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4
1	0.03	0.03	0.03	0.04	0.09	0.09	0.10	0.10	0.29	0.28	0.27	0.28	0.90	1.0	0.93	0.96	5.4	4.9	5.5	5.4
2	0.04	0.04	0.03	0.03	0.10	0.09	0.11	0.09	0.31	0.30	0.29	0.32	0.93	1.0	0.99	1.1	5.3	5.5	5.2	6.2
3	0.04	0.03	0.04	0.03	0.10	0.13	0.12	0.09	0.35	0.31	0.23	0.27	1.1	1.1	0.91	0.86	6.2	4.5	6.2	4.9
4	0.03	0.03	0.04	0.03	0.08	0.10	0.09	0.10	0.30	0.25	0.32	0.26	0.89	0.81	0.84	0.94	5.1	4.6	4.4	4.8
5	0.04	0.04	0.03	0.03	0.09	0.11	0.11	0.10	0.29	0.30	0.24	0.27	0.98	0.89	0.89	0.92	4.6	4.8	4.5	4.9
6	0.04	0.04	0.04	0.04	0.10	0.10	0.10	0.10	0.27	0.30	0.29	0.29	0.95	0.96	0.96	0.91	4.9	4.9	4.9	4.6
7	0.03	0.03	0.04	0.04	0.12	0.11	0.11	0.11	0.27	0.27	0.33	0.31	0.95	0.93	1.0	1.1	5.3	5.9	5.3	5.5
8	0.03	0.03	0.04	0.03	0.10	0.09	0.09	0.10	0.27	0.28	0.27	0.29	0.88	0.89	0.86	0.95	4.9	5.0	4.8	4.9
9	0.03	0.04	0.04	0.03	0.10	0.11	0.10	0.10	0.28	0.31	0.31	0.32	0.98	0.95	0.94	0.92	5.2	5.1	5.1	5.4
10	0.03	0.03	0.03	0.04	0.10	0.10	0.10	0.08	0.29	0.31	0.29	0.28	0.99	2.2	1.0	0.92	5.6	5.5	5.5	5.5
11	0.04	0.03	0.04	0.03	0.12	0.10	0.09	0.10	0.24	0.27	0.32	0.34	0.78	0.84	0.94	0.98	4.5	6.8	4.8	5.5
12	0.03	0.04	0.03	0.03	0.10	0.10	0.11	0.10	0.30	0.28	0.30	0.29	0.90	0.94	0.91	0.94	5.0	4.8	4.7	4.8

* GMO % = (GMO copy number/sugar beet [haploid genome copy number]) x 100

n.a. not available

A graphical representation of the data reported in Table 12 is provided in Figure 1 that shows the relative deviation from the true value for each GM level tested by the participating laboratory. The coloured bars represent the deviation of the GM level measured in % of the true GM level; the green bar on the right represents the mean relative deviation over all data before eliminating outliers for each GM level.

Figure 1. Relative deviation (%) from the true value of GM level (*)



PL = participating laboratory.

Overall, most laboratories' mean relative deviations from the true values were within a maximum of ± 25%. At GM-level 0.09%, 0.27% and 5.0% all the laboratories were within the limit; at GM-level 0.03% and 0.9% eleven laboratories were within the limit and one laboratory (PL06) overestimated the two GM-levels by more than 25%,

5.2.3 Method performance requirements

Among the 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 13 illustrates the estimation of repeatability and reproducibility at the various GM levels tested during the study (see Table 5 for a list of the participant laboratories).

According to the ENGL method performance requirements the relative reproducibility standard deviation (RSD_R), that describes the inter-laboratory variation, should be below 35% at the target concentration and 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 13, the method satisfies this requirement at all GM levels tested. Indeed, the highest value of RSD_R % is 11% at the 0.03% GM level, thus within the acceptance criterion.

Table 13. Summary of validation results for the KWS20-1 method, expressed as GM copy numbers in relation to target taxon haploid genome copy numbers.

	Test Sample Expected GMO %				
	0.03	0.09	0.27	0.90	5.0
Laboratories having returned valid results	12	12	12	12	12
Samples per laboratory	20	20	20	20	20
Number of outliers	0	0	0	1	2
Reason for exclusion (*)	-	-	-	C	2C
Mean value	0.03	0.10	0.29	0.94	5.1
Relative repeatability standard deviation, RSD_r (%)	11	8.9	9.1	7.1	4.6
Repeatability standard deviation	0.004	0.009	0.026	0.067	0.235
Relative reproducibility standard deviation, RSD_R (%)	11	9.4	9.1	8.1	8.0
Reproducibility standard deviation	0.004	0.009	0.026	0.076	0.406
Bias (**) (absolute value)	0.005	0.011	0.020	0.041	0.101
Bias (%)	16	12	7.4	4.5	2.0

* 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 (mean of the measured results from all laboratories – accepted reference value).

Table 13 also documents the relative repeatability standard deviation (RSD_r) estimated for each GM level. In order to accept methods for collaborative study, the EURL GMFF and ENGL require that the RSD_r value indicated by the applicant and confirmed by the EURL GMFF through in-house experiments, is below 25% (see ENGL document "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 11% at the 0.03% 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. The method satisfies this requirement across the dynamic range tested, with the highest value of bias (%) of 16% at the 0.03% GM level.

6 Compliance of the method for detection and quantification of KWS20-1 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 14:

- at step 2 of the validation process (scientific assessment of the dossier), the EURL GMFF acknowledged that the RSD_r value at the 0.10% level shown by the applicant's dossier (expressed as mass fraction of GM-material (0.025% in copy number ratio according to the zygosity ratio estimated by the applicant) was 13.0%, based on 16 replicates (Table 2A), and 2.9% - 5.8% based on 2 replicates in the transferability study (Table 2B), hence below the maximum value of 25% required by the ENGL. The EURL GMFF therefore concluded that it could accept the applicant's data on method performance;

- at step 3 of the validation process (experimental testing of samples and methods), the EURL GMFF determined the RSD_r % value at the level of 0.1% in mass fraction of GM-material (corresponding to 0.03% expressed in terms of copy number ratio to haploid genome copy numbers, according to the zygosity ratio determined at the EURL GMFF). The experiments were carried out under repeatability conditions on fifteen replicates. The RSD_r resulted to range between 10% and 17% (Table 8, 9 and 10) depending on the qPCR platform applied, hence also below 25%;

- the collaborative study (step 4 of the validation process) established that over the twelve participating laboratories at the level of 0.1% related to mass fraction of GM-material the RSD_r of the method was 11%, therefore also below 25% and well in line with the previous data.

The outcome of the different steps is summarised in Table 14.

Table 14. Precision of the event-specific method for quantitative detection of KWS20-1 at or around 0.1% level related to mass fractions of GM material.

Source	RSD_r %	GM %
Applicant's method optimisation	13%	0.1%
Applicant's transferability study	2.9%-5.8%	0.1%
EURL GMFF tests	10 - 17%	0.1%
Collaborative study	11%	0.1%

Based on the results of the EURL GMFF in-house verification and of the international collaborative study, it is concluded that the method RSD_r % is lower than 25% at the level of 0.1% related to mass fraction of GM material, hence the method meets the requirement laid down in Regulation (EU) No 619/2011.

7 Conclusion

The method provided by the applicant has been validated in accordance to the EURL GMFF validation process, respecting all requirements of the relevant EU legislation and international standards for method validation.

This validation study confirmed that the method is applicable to the control samples provided by the applicant (see paragraph 4.1), in accordance with the requirements of Annex I-3.C.2 to Commission Regulation (EU) No 503/2013 and (EU) No 619/2011 and meets all method performance requirements established by the ENGL and the EURL GMFF. The method is therefore valid to be used for regulatory purposes, including the quantification of low level presence of 0.1% (m/m) of the GM event. It can be assumed that it is applicable to any appropriately extracted sugar beet genomic DNA.

In any case the user of the method is advised to verify the quality of the extracted genomic DNA in order to ensure that it is suitable for the subsequent PCR analysis. This is particularly relevant for more complex matrices of samples from food and feed products.

The validated method is described in detail as "Validated Method" at https://gmo-crl.jrc.ec.europa.eu/method-validations and in Annex 1.

8 References

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11. Commission Implementing Regulation (EU) No 120/2014 of 7 February 2014 amending Regulation (EC) No 1981/2006 on detailed rules for the implementation of Article 32 of Regulation (EC) No 1829/2003 of the European Parliament and the Council as regards the Community reference laboratory for genetically modified organisms.

List of abbreviations and definitions

EURL GMFF	European Union Reference Laboratory for GM Food and Feed
PCR	Polymerase chain reaction
dPCR	digital Polymerase chain reaction
ENGL	European Network of GMO Laboratories
LOD	Limit of Detection
LOQ	Limit of Quantification

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Annex 1. Event-specific Method for the Quantification of Sugar beet KWS20-1 using Real-time PCR

Validated Method

Method development:

KWS SAAT SE & Co. KGaA

Bayer Agriculture BV

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 sugar beet event KWS20-1 DNA to total sugar beet DNA in a sample.

Template DNA extracted by means of suitable methods should be tested for quality and quantity prior to use in PCR. Tests for the presence of PCR inhibitors (e.g. monitor run of diluted series, use of DNA spikes) are also recommended to ensure suitability of the extracted DNA.

For the detection of GM event KWS20-1, a 77 bp fragment of the region spanning the 3' insert-to-plant junction in sugar beet KWS20-1 is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with 6-FAM (6-carboxyfluorescein) as reporter dye at its 5' end and MGB-NFQ (non-fluorescent quencher) as non-fluorescent quencher dye at its 3' end.

For the relative quantification of GM event KWS20-1, a sugar beet taxon-specific method amplifies a 118 bp fragment of a sugar beet *glutamate synthetase* (*GS*) endogenous gene (Accession number, GeneBank: AY026353.1), using *GS* gene-specific primers and a *GS* gene-specific probe labelled with FAM as reporter dye at its 5' end and BHQ1 (black hole 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 KWS20-1 DNA in a test sample, Cq values for the KWS20-1 and the *GS* methods are determined for the sample. Standard curves are then used to estimate the relative amount of KWS20-1 DNA to total sugar beet DNA.

2. Validation and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from genetically modified and conventional sugar beet seeds. Precision and trueness of the method were tested through an international collaborative ring trial using DNA samples at different GM contents.

2.2 Collaborative trial

The method was validated in an international collaborative study by the European Union Reference Laboratory for GM Food and Feed (EURL GMFF). The study was undertaken with twelve participating laboratories in April-May 2024.

A detailed validation report can be found at https://gmo-crl.jrc.ec.europa.eu/method-validations.

2.3 Limit of detection (LOD)

According to the method developer, the relative LOD of the method is at least 0.015% (expressed in copy ratio) in 100 ng of total suitable sugar beet 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 0.025% in copy number (corresponding to 0.1% mass fraction of GM material) in 100 ng of total suitable sugar beet DNA. The lowest relative GM content of the target sequence included in the collaborative trial was 0.1% (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 sugar beetKWS20-1 and is therefore event-specific for the event KWS20-1 (§ 3.1 and 3.2 in 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 requires dedicated equipment, especially pipettes.
- All the equipment should be sterilised prior to use and any residue of DNA should have been 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 regularly
- 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 room temperature.
- In order to avoid repeated freeze/thaw cycles aliquots should be prepared.

3.2 Real-time PCR for quantitative analysis of sugar beet event KWS20-1

3.2.1 General

The real-time PCR set-up for the taxon (*GS*) and the GMO (event KWS20-1) target sequences are carried out in separate vials. Multiplex qPCR (using differential fluorescent labels for the probes) has not been tested or validated by the EURL GMFF.

The method is developed for a total volume of 20 µL per reaction mixture for the GM (event KWS20-1) and the taxon (*GS*) targets with the reagents as listed in Table 2 and Table 3.

3.2.2 Calibration

The calibration curves have to be established on at least five samples. The first point of the calibration curve (S1) should be established for a sample containing 10% sugar beet KWS20-1 DNA in a total of 100 ng of sugar beet DNA (corresponding to 13333 sugar beet KWS20-1 haploid genome copies with one haploid genome assumed to correspond to 0.75 pg of sugar beet genomic DNA) (1). Standards S2 to S5 are to be prepared by serial dilutions (dilution factor 8 for samples S2-S4 and dilution factor 6 for standard S5) 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 sugar beet DNA in reaction (ng) (*)	100	12.5	1.55	0.20	0.05
Sugar beet haploid genome copies	133333	16667	2083	260	43
KWS20-1 copies	13333	1667	208	26	-**

* Total nanograms are rounded to the integral value

** The GM curve has only four calibration points

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 by means of spreadsheet software, e.g. Microsoft Excel, or directly by options available with the software.

The copy number measured for each unknown sample DNA is obtained by interpolation from the standard curves.

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. In two tubes on ice, add the components in the order mentioned below (except DNA) to prepare the reaction mixes for KWS20-1 (Table 2) and for *GS* (Table 3). Please note that additional volume is included in the total to cover pipetting variability due to the viscosity of the solution.

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for KWS20-1.

Component	Final concentration	µL/reaction
JumpStart® Taq ReadyMix (2x)	1x	10
ROX Reference Dye (50X)	0.1x**	0.04
$MgCl_2$ (100mM)	5.5mM	1.10
2109_fwd1 (10 µM)]	300 nM	0.60
2109_rev1 (10 µM)]	300 nM	0.60
2109_Probe 1 (10 µM)]	150 nM	0.30
Nuclease free water	-	2.36
DNA	-	5.00
Total reaction volume:		20.00 µL

* TaqMan® probe labelled with 6-FAM at its 5'-end and MGB at its 3'-end

** ROX concentration optimised for the use of low ROX real-time PCR. The applicant used low ROX concentration on ABI 7500, CFX96, and QuantStudio 5. The EURL GMFF used 0.1x ROX with QuantStudio 7, ABI 7500 and Roche LC480 II. According to the applicant, when using real-time PCR platforms requiring a high ROX concentration as passive reference, such as the ABI 7900HT Real-Time PCR System, the final ROX concentration needs adjusting to 1x.

Table 3. Amplification reaction mixture in the final volume/concentration per reaction well for sugar beet *GS*.

Component	Final concentration	µL/reaction
JumpStart® Taq ReadyMix (2x)	1x	10
ROX Reference Dye (50X)**	0.1x	0.04
$MgCl_2$ (100mM)	5.5mM	1.10
GluA3-F (10 µM)]	300 nM	0.60
GluA3-R (10 µM)]	300 nM	0.60
GluD1 probe (10 µM)]	150 nM	0.30
Nuclease free water	-	2.36
DNA	-	5.00
Total reaction volume:		20.00 µL

* TaqMan® probe is labelled with FAM at its 5'-end and BHQ at its 3'-end

** ROX concentration optimised for the use of low ROX real-time PCR. The applicant used low ROX concentration on ABI 7500, CFX96, and QuantStudio 5. The EURL GMFF used 0.1x ROX with QuantStudio 7, ABI 7500 and Roche LC480 II. According to the applicant, when using real-time PCR platforms requiring a high ROX concentration as passive reference, such as the ABI 7900HT Real-Time PCR System, the final ROX concentration needs adjusting to 1x.

3. Mix well and centrifuge briefly.
4. Prepare two 0.5 mL reaction tubes (one for the sugar beet KWS20-1 and one for *GS*) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
5. Add into each reaction tube the amount of reaction mix for 3.5 PCR repetitions (52.5 µL for KWS20-1 and 52.5 µL for *GS*). Add to each tube the correct amount of DNA for 3.5 PCR repetitions (17.5 µL DNA). The volume for the additional 0.5 repetition will ensure adequate volume when loading the samples.

Vortex each tube for approx. 10 seconds. This step is mandatory to reduce to a minimum the variability among the repetitions of each sample.

6. Spin down the tubes. Aliquot 20 µL for KWS20-1 and for *GS* reference in each well.
7. Place an optical cover on the reaction plate and briefly centrifuge the plate.
8. Place the reaction plate in the real-time PCR apparatus (possibly apply a compression pad, depending on the model), according to the manufacturer's instructions and your Standard Operating Procedures and start the run.
9. Select FAM as reporter dye for KWS20-1 and FAM for *GS* reference. Define MGB NFQ as quencher dye for KWS20-1 and BHQ1 for *GS* reference. Select ROX as the passive reference dye if needed. Enter the correct reaction volume (20 µL).
10. Run the PCR with the cycling program described in Table 4. Users who plan to use the second derivative maximum analysis method (an option e.g. on Roche LC480 instruments) are advised to program 45 cycles instead of 40, in order to be able to quantify down to Cq 40.

Table 4. Cycling program for KWS20-1/*GS*.

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

** see comment above for users of second derivative maximum analysis method

3.3 Data analysis

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

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 method 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 method.

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 both for the *GS* and the KWS20-1 by plotting the Cq values measured for the calibration points against the logarithm of the DNA copy number 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 samples.

To obtain the percentage value of event KWS20-1 DNA in the unknown sample, the KWS20-1 copy number is divided by the copy number of the sugar beet endogenous gene *GS* and multiplied by 100 (GM% = KWS20-1/*GS* x 100).

4. Equipment and Materials

4.1 Equipment

- Real-time PCR instrument for plastic reaction vessels (glass capillaries are not recommended for the described buffer composition) and appropriate analysis software
- 96-well reaction plates
- Optical caps/adhesion covers
- Microcentrifuge
- Micropipettes
- Standard bench top centrifuge with rotor or standard microfuge fit for 0.5 mL reaction tubes, centrifuge for 96-Well reaction plates
- Vortex
- Racks for reaction tubes, also cooled
- 0.5, 1.5 mL and 5 or 15 mL DNAse free reaction tubes

4.2 Reagents

- Jumpstart™ Taq ReadyMix™ (2x) Sigma P2893
- ROX Reference Dye (100x) Sigma-Aldrich R4526, diluted 1:2 in water (Ambion, #AM997)
- MgCl2 Sigma-Aldrich M1028

4.3 Primers and Probes

Table 5. Primers and probes for the KWS20-1 and *GS* methods

	KWS20-1	**DNA Sequence (5' to 3')**		Length (nt)
	KWS20-1			
Forward primer	2109_fwd1	TGTCGTTTCCCGCCTTC		17
Reverse primer	2109_rev1	TCCTACCAATTCTTGAACTTCGTG		24
Probe	2109_Probe 1	ACTATCAGTGTTTCAT	6-FAM; MGBNFQ	16
	GS			
Forward primer	GluA3-F	GACCTCCATATTACTGAAAGGAAG		24
Reverse primer	GluA3-R	GAGTAATTGCTCCATCCTGTTCA		23
Probe	GluD1-probe BHQ	CTACGAAGTTTAAAGTATGTGCCGCTC	FAM; BHQ-1	27

[FAM: 6-carboxyfluorescein; MGB: minor groove binder; MGB minor groove binder; BHQ1: black hole quencher]

5. References

Dohm *et al.* The genome of the recently domesticated crop plant sugar beet (*Beta vulgaris*). Nature, 2014: 505: 546-549

List of abbreviations and definitions

EURL GMFF	European Union Reference Laboratory for GM Food and Feed
PCR	Polymerase chain reaction
ENGL	European Network of GMO Laboratories
LOD	Limit of Detection
LOQ	Limit of Quantification

List of tables

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