EUROPEAN COMMISSION
JOINT RESEARCH CENTRE
Directorate F - Health and Food
Food and Feed Compliance

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

Validated Method

Method development:

KWS SAAT SE & Co. KGaA

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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 systems. 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 systems. 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

1. 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

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