Title

Real-time PCR method for specific quantitation of GHB811 (BCS-GHB811-4) in *Gossypium hirsutum* (cotton)

Issue date

March 28, 2024

Contact information

CLImethods@basf.com

The use of this method is subject to terms and conditions as specified at http://www.detection-methods.com

LOTR-412

TABLE OF CONTENTS

TABLE OF CONTENTS 2
LIST OF TABLES 2
1. General information on the method 3
1.1. Purpose and scope 3
1.2. Principle of the method 3
2. Certified reference Materials 3
3. Procedures 3
3.1. General instructions 3
3.2. Preparation of the standard curve samples 4
3.3. Preparation of the test samples 4
3.4. Details on composition and preparation of reaction mixes 4
3.4.1. Preparation of the reaction mix 4
3.4.2. PCR mix for the GHB811 target system reaction 4
3.4.3. PCR mix for the *AdhC* reference system reaction 4
3.5. Thermal cycling parameters 5
4. Data Analysis 5
4.1. Primary data analysis 5
4.2. Calculation of percent GHB811 DNA to total cotton gDNA 5
5. Performance characteristics 5
6. References 7
7. Equipment, Materials and Reagents 7
7.1. Equipment 7
7.2. Materials 7
7.3. Reagents 8
8. Tables 9

LIST OF TABLES

Table 1: Primers and probes for the real-time PCR methods for GHB811 9
Table 2: Dilution scheme and corresponding GHB811 cotton and total genomic DNA content used for the standard curve 9
Table 3: PCR mix for the GHB811 cotton system reaction 9
Table 4: PCR mix for *AdhC* reference system reaction 10
Table 5: Thermal cycling parameters for GHB811 + *AdhC* reactions 10

The use of this method is subject to terms and conditions as specified at http://www.detection-methods.com

1. GENERAL INFORMATION ON THE METHOD

1.1. Purpose and scope

BASF Corporation developed a real-time PCR method for identification and GHB811-specific quantitation of GHB811 in cotton relative to total cotton DNA in a test sample.

The method was optimized for suitable DNA extracted from cotton leaves, seeds and grain.

Template DNA extracted by means of suitable methods should be tested for quality, quantity and for the presence of PCR inhibitors prior to use in PCR assays.

The real-time PCR method should be successfully verified according to applicable standards (Hougs et al., 2017) prior to its application on samples with unknown contents.

1.2. Principle of the method

In a real-time PCR, the PCR amplification occurs in a thermal cycler requiring a fluorescently-labeled oligonucleotide homologous to the internal part of the amplified sequence in addition to a PCR primer pair. The fluorescence increases if the probe binds to the target sequence during amplification due to the hydrolysis of quenching molecules by *Taq* DNA polymerase.

The measured fluorescence is proportional to the DNA mass (amplified DNA target) and needs a standard curve for absolute quantitation purposes. The fluorescence can be detected in 'real-time' during the PCR process.

For specific detection of the GHB811 locus DNA, the GHB811 reaction amplifies a 144 bp fragment spanning the 5' insert-to-plant junction (QT-EVE-GH-013) using two specific primers and an oligonucleotide probe carrying FAM as a reporter dye at its 5' end and a non-fluorescent MGB (Minor Groove Binder) quencher dye at its 3' end.

For relative quantification of GHB811 cotton, a 73 bp fragment of the cotton D-subgenome alcohol dehydrogenase C gene , *AdhC* (QT-TAX-GH-020) is amplified using a pair of specific primers and a specific probe labeled with JOE as a reporter dye at its 5' end and a BHQ-1 (Black Hole Quencher®) as a non-fluorescent quencher dye at its 3' end.

Both PCR reaction modules are performed as simplex reaction, preferably on one real-time PCR run.

The sequences of primers and probes for the GHB811 method are summarized in Table 1.

2. CERTIFIED REFERENCE MATERIALS

Certified reference material for event GHB811 and for conventional cotton can be obtained from IRMM (https://crm.jrc.ec.europa.eu/)

3. PROCEDURES

3.1. General instructions

- The procedure requires personnel trained in PCR techniques.
- Laboratory organization and management should follow the guidelines given by relevant standards such as ISO 24276 and ISO 17025.
- PCR-reagents should be stored and handled in separate rooms and/or freezers and in equipment 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; this is especially true for pipettes.
- All material used (e.g. vials, containers, pipette tips, etc.) must be suitable for use in PCR and molecular biology applications. They must be DNase-free, DNA-free, and sterile.

The use of this method is subject to terms and conditions as specified at
http://www.detection-methods.com

- To avoid contamination, it is recommended to use filter pipette tips protected against aerosols.
- Pipettes should be regularly calibrated. .
- Use only powder-free gloves and change them frequently.
- Clean lab-benches and equipment periodically with appropriate cleaning reagents such as 10% sodium hypochloride solution (bleach) or equivalent.
- All handling steps - unless specified otherwise - should be carried out at 0 – 4 °C.
- Avoid repeated freeze/thaw cycles of frozen reaction components.

3.2. Preparation of the standard curve samples

The standard curves comprise a number of five standard curve samples (S1 to S5).

The first standard curve sample S1 is derived from a DNA sample containing 10 % homozygous GHB811 DNA in 300 ng total cotton DNA (corresponding to approximately 125,000 haploid cotton genome copies with one genome assumed to correspond to 2.4 pg of haploid cotton genomic DNA) (Hendrix & Stewart, 2005).

Standard curve samples S2 to S5 are obtained by serial dilution in water of standard S1. The dilution scheme and corresponding GHB811 and total genomic DNA content used in real-time PCR are described in Table 2.

3.3. Preparation of the test samples

The following approach is recommended:

- For each DNA extraction sample, at least two technical replicates are assessed by real-time PCR method.
- For each test sample, the following DNA input is recommended: the use of 200 ng of template DNA per reaction well is required with a recommended DNA concentration of 40 ng/µL.

3.4. Details on composition and preparation of reaction mixes

3.4.1. Preparation of the reaction mix

When appropriate, reagents are thawed by equilibration to room temperature. Once thawn, subsequent incubation either on wet ice or a cooling block is recommended while setting up the reaction. Ensure thorough mixing of each reagent before use.

Two separate PCR master mixes reaction are to be prepared, one for the GHB811 cotton system reaction and one for the *AdhC* reference system reaction, consisting of all components of the PCR, except gDNA template, in sufficient quantities for all reactions (including the standard curve samples). It is advised to prepare sufficient reaction mix to account for pipetting losses e.g. covering at least two additional reactions.

3.4.2. PCR mix for the GHB811 target system reaction

The composition of the PCR reaction mix is outlined in Table 3. For optimal performance of the PCR amplification, it is recommended to add the reagents in the specific order as listed.

3.4.3. PCR mix for the *AdhC* reference system reaction

The composition of the PCR reaction mix is outlined in Table 4. For optimal performance of the PCR amplification, it is recommended to add the reagents in the specific order as listed.

3.5. Thermal cycling parameters

Run the PCR with cycling conditions listed in Table 5 for both the GHB811 cotton system reaction and the *AdhC* reference system reaction.

4. DATA ANALYSIS

4.1. Primary data analysis

Subsequent to the real-time PCR, analyze the run on the instrument's software following the procedure below:

- Use the default baseline range from cycles 3 to 15.
  - If necessary, the baseline can be adjusted, ensuring that the upper limit differs a sufficient number of cycles with the lowest Ct-value determined.
- Set the threshold:
  - Display the amplification curves of one system (e.g. target) using the ΔRn-axis displayed in the logarithmic mode.
  - Set the threshold line in the centre of 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 or re-analyze button to ensure that changes affect the Ct values.
- Repeat the threshold setting procedure on the amplification plots of the other system (e.g. reference system).

Save the settings and export all the raw data into a new file (=export file). This file will be used further for data analysis.

After having defined a threshold value within the logarithmic phase of amplification as described above, the instrument's software calculates the Ct values for each reaction.

The standard curves are generated for both the *AdhC* reference and the GHB811 cotton specific systems by plotting the Ct values measured for the calibration points against the logarithm of the DNA amounts and by fitting a linear regression line into the data points.

Thereafter, the standard curves are used to calculate the percent GHB811 DNA to total cotton gDNA in the test sample.

4.2. Calculation of percent GHB811 DNA to total cotton gDNA

To obtain the percentage value of GHB811 cotton DNA to total cotton DNA in the test sample, the GHB811 DNA copy number (CN) result is divided by the copy number result of the cotton *AdhC* reference system reaction and then set to give a percentage value.

$$\%\, GHB811\ cotton\ DNA = \frac{GHB811\ cotton\ CN}{AdhC\ cotton\ CN} * 100$$

5. PERFORMANCE CHARACTERISTICS

The GHB811 real-time PCR method was validated according to the defined Minimum Performance Requirements for analytical methods for GMO testing (ENGL, 2015), including the evaluation of a series of method performance data in order to decide whether the method is acceptable to undergo full validation and the confirmation of the fitness for purpose of the method through a full validation study by collaborative trial involving twelve randomly selected national reference laboratories.

The use of this method is subject to terms and conditions as specified at
http://www.detection-methods.com

The event-specificity of the GHB811 reaction was experimentally confirmed against DNA extracted from a series of GMO events including cotton events LLCOTTON25, GHB614, GHB119, MON1445, MON531, MON 15985, MON88913, 281-24-236, 3006-210-23, and COT102.

The Limit Of Detection (LOD) of the method was found to be at least below 10 haploid genome copies for GHB811 event-specific and for the cotton reference system reaction. The LOD for the combined PCR modules was found to be at least 0.025 % GHB811 event DNA in a total of 200 ng cotton DNA.

The dynamic range was found to be at least 4.5 % to 0.1 % GHB811 event DNA in a total of 200 ng cotton DNA.

6. REFERENCES

European Network of GMO Laboratories (ENGL) – 2015 - Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing. doi 10.2760/63656.

European Union Reference Laboratory for GM Food and Feed (EURL GMFF), Joint Research Centre (JRC), European Commission – 2020 - QT-EVE-GH-013 and QT-TAX-GH-020 "Event-specific Method for the Quantification of Cotton GHB811 Using Real-time PCR - Validation Report". Online Publication.

Hendrix, B., Stewart, J.M. – 2005 - Estimation of the nuclear DNA content of Gossypium species. Annals of Botany 95: 789-797.

Horwitz, W. Protocol for the design, conduct and interpretation of method-performance studies: Revised 1994 (Technical Report). Pure Appl Chem. 1995;67(2):331-343

Hougs, L., Ovesna, J., Papazova, N., Lieske, K., Narendja, F, Grohmann, L., Goerlich, O., Grohmann, L., Scholtens, I., Mazzara, M., Zel, J., Gatto F. – 2017 - Verification of analytical methods for GMO testing when implementing interlaboratory validated methods. doi 10.2760/645114

International Organization for Standardization ISO 5725-1 - 1994 -. Accuracy (Trueness and Precision) of Measurement Methods and Results.

7. EQUIPMENT, MATERIALS AND REAGENTS

Below is a proposed outline of materials to perform the described real-time PCR method for GHB811-specific quantitation in cotton.

7.1. Equipment

Equipment	Proposed Specification (or equivalent)
Calibrated Micropipettes for volumes ranging 1-20 µL, 20-200 µL, and 200-1000 µL	Rainin
Bench top microcentrifuge for reaction tubes	Sigma
Plate centrifuge	Eppendorf
Vortex for reaction tubes	Genie-2 Scientific Industries
Software: CFX Maestro 1.0 version 4.0.2325.0418	Catalog number: 12013758
Real-time PCR system with thermal cycler	CFX96 real time system with C1000 touch thermal cycler

7.2. Materials

Materials	Proposed Specification (or equivalent)
MicroAmp® Fast Optical 96-well Reaction Plates or equivalent	Applied Biosystems Part No. 4346906
Polypropylene reaction tubes (1.5 or 2 mL)	Different brands
Micropipette tips for volumes 1-20 µL, 20-200 µL, and 200-1000 µL	Different brands, preferentially “low-adhesive” quality and with filter

7.3. Reagents

Reagents	Number/Specification (or equivalent)
Nuclease-free water	Invitrogen Cat N° 10320995 (or equivalent)
HPLC-purified PCR primers	Synthesized by Integrated DNA Technologies
Labeled probes	Synthesized by Integrated DNA Technologies
TaqMan®Universal PCR Master Mix 2X	Applied Biosystems Part No. 4304437

The use of this method is subject to terms and conditions as specified at
http://www.detection-methods.com

8. TABLES

Table 1: Primers and probes for the real-time PCR methods for GHB811

Name	Orientation	Sequence 5' → 3'
PRIM0638	Forward primer GHB811	5' - CgAATAgTTCCATCAATTTTATCATTTATg - 3'
PRIM1870	Reverse primer GHB811	5' - CgCTTTAACgTCCCTCAgATTT - 3'
TM2207	Probe GHB811	5'- FAM - AAgCCTTgAAACAgAACA - MGBNFQ - 3'
KVM157	Forward primer *AdhC*	5' - CACATgACTTAgCCCATCTTTgC - 3'
KVM158	Reverse primer *AdhC*	5' - CCCACCCTTTTTTggTTTAgC- 3'
TM1304	Probe *AdhC*	5' – JOE – TgCAggTTTTggTgCCACTgTgAATg – BHQ1 -3'

Table 2: Dilution scheme and corresponding GHB811 cotton and total genomic DNA content used for the standard curve

Sample	S1	S2	S3	S4	S5
DNA quantity in real-time PCR (ng)	300	100	20	5	0.93
Total cotton genome copy numbers*	125,000	41,667	8,333	2,083	400
GHB811 cotton quantity in real-time PCR (ng)	30	10	2	0.5	0.093
GHB811 cotton copy numbers	12,500	4,167	833	208	40

* Hendrix & Stewart, 2005

Table 3: PCR mix for the GHB811 cotton system reaction

GHB811 cotton system components	Final concentration in PCR	Volume per reaction
1. Nuclease-free water	-	5.0 µL
2. TaqMan® Universal PCR Master Mix No AmpErase® UNG 2X	1x	12.5 µL
3. PRIM0638 primer (10 µM)	400 nM	1.0 µL
4. PRIM1870 primer (10 µM)	400 nM	1.0 µL
5. TM2207 probe (10 µM)	200 nM	0.5 µL
PCR Mix volume	**20 µL**	
Standard curve/ Test samples*	5 µL	
Total volume	**25 µL**	

*** proposed concentration 40 ng/µL to reach the required DNA input of 200 ng**

Table 4: PCR mix for *AdhC* reference system reaction

***AdhC* reference system components**	**Final concentration in PCR**	**Volume per reaction**
1. Nuclease-free water	-	6.0 µL
2. TaqMan® Universal PCR Master Mix 2X	1x	12.5 µL
3. KVM157 primer(10 µM)	200 nM	0.5 µL
4. KVM158 primer (10 µM)	200 nM	0.5 µL
5. TM1304 probe (10 µM)	200 nM	0.5 µL
PCR Mix volume	**20 µL**	
Standard curve/ Test samples*	5 µL	
Total volume	**25 µL**	

*** proposed concentration 40 ng/µL to reach the required DNA input of 200 ng**

Table 5: Thermal cycling parameters for GHB811 + *AdhC* reactions

	Stage	**Temp**	**Time**	**Data Collection**	**Cycles**
1	UNG	50°C	2 min	No	1
2	Initial denaturation	95°C	10 min	No	1
3	Amplification	95°C	15 sec	No	40
		60°C	1 min	Yes	