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

Event-specific Method for the Quantification of Maize DP910521 using Real-time PCR

Validated Method

Method development:

Pioneer Hi-Bred International, Inc.

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 maize event DP910521 DNA to total maize 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 DP910521, a 108 bp fragment of the region spanning the 5' insert-to-plant junction in maize DP910521 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 MGBNFQ (Minor Groove Binding Non-Fluorescent Quencher) as non-fluorescent quencher dye at its 3' end.

For the relative quantification of GM event DP910521, a maize taxon-specific system amplifies a 79 bp fragment of a maize High Mobility Group (HMG) Protein A gene (*hmg*) endogenous gene (Accession number, GeneBank: AJ131373), using *hmg* gene-specific primers and a *hmg* gene-specific probe labelled with FAM (6-carboxyfluorescein) as reporter dye at its 5' end and BHQ-1 (Black Hole Quencher® 1) as non-fluorescent quencher dye at its 3' end.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called "Cq" value. For quantification of the amount of DP910521 DNA in a test sample, Cq values for the DP910521 and the *hmg* systems are determined for the sample. Standard curves are then used to estimate the relative amount of DP910521 DNA to total maize DNA.

2 Validation and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from genetically modified and conventional maize seeds and grain. 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 October 2023.

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

2.3 Limit of detection (LOD)

According to the method developer, the relative LOD of the method is at least 0.0085 % (related to copies GM/total haploid genome copies) in 325 ng of total suitable maize 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 0.042 % (related to copies GM/total haploid genome copies) in 325 ng of total suitable maize 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 5' insert-to-plant junction in maize DP910521 and is therefore event-specific for the event DP910521. This was confirmed in the validation study.

3 Procedure

3.1 General instructions and precautions

- The procedures require experience of working under sterile conditions.
- Laboratory organization, 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 maize event DP910521

3.2.1 General

The real-time PCR set-up for the taxon (*hmg*) and the GMO (event DP910521) 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 25 µL per reaction mixture for the GM (event DP910521) and the taxon (*hmg*), 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 four samples. The first point of the calibration curve (S1) should be established for a sample containing 10 % maize DP910521 DNA in a total of 325 ng of maize DNA (corresponding to 119048 maize haploid genome copies with one haploid genome assumed to correspond to 2.73 pg of maize genomic DNA) (1). Standards S2 to S4 are to be prepared by serial dilutions (dilution factor 6 for samples S2-S3 and dilution factor 8 for standard S4) according to Table 1 below.

Table 1. Copy number values of the standard curve samples

Sample code	**S1**	**S2**	**S3**	**S4**
Total amount of maize DNA in reaction (ng) (*)	325	54	9.0	1.13
Maize haploid genome copies	119048	19841	3307	413
DP910521 copies	11905	1984	331	41

* Total nanograms are rounded to the integral value

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 the DP910521 maize specific system (Table 2) and the *hmg* reference gene system (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 DP910521.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix No UNG (2x)	1x	12.5
PHN201324 (10 µM)	300 nM	0.75
PHN165631 (10 µM)	300 nM	0.75
PHN201325 (*) (10 µM)	150 nM	0.375
Nuclease free water	-	5.625
DNA	-	5.0
Total reaction volume:		25 µL

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

Table 3. Amplification reaction mixture in the final volume/concentration per reaction well for the maize *hmg*.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix No UNG (2x)	1x	12.5
MaiJ-F2 (10 µM)	300 nM	0.75
mhmg-rev (10 µM)	300 nM	0.75
mhmg-probe (*) (10 µM)	180 nM	0.45
Nuclease free water	-	5.55
DNA	-	5.0
Total reaction volume:		25 µL

*TaqMan® probe is labelled with 6-FAM at its 5'-end and BHQ1 at its 3'-end

3. Mix well and centrifuge briefly.
4. Prepare two 0.5 mL reaction tubes (one for the maize DP910521 and one for the *hmg* system) 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 (70 µL for the DP910521 maize system and 70 µL for the *hmg* system). 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 25 µL for DP910521 and for *hmg* 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 both the DP910521 and for the *hmg* reference systems. Define MGBNFQ or non-fluorescent as quencher dye for DP910521 and BHQ or non-fluorescent for *hmg*. Select ROX as the passive reference dye. Enter the correct reaction volume (25 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 DP910521/*hmg*.

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	

*UNG: Uracil-N-glycosylase

** 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:

a) Set the threshold following the automatic or the manual mode. In the manual mode, display the amplification curves of the event specific target in logarithmic mode. Locate the threshold line in the area where the amplification profiles are parallel (exponential phase of PCR) and where there is no "fork effect" between repetitions of the same sample. Press the "update" button to ensure changes affect Cq values (only needed for some analysis software). Switch to the linear view mode by clicking on the Y axis of the amplification plot and check that the threshold previously set falls within the exponential phase of the curves.

b) Set the baseline following the automatic or the manual mode. In the manual mode: determine the cycle number at which the threshold line crosses the first amplification curve and set the baseline three cycles before that value (e.g. earliest Cq = 25, set the baseline crossing at Cq = 25 - 3 = 22).

c) Save the settings.

d) Repeat the procedure described in a), b) and c) on the amplification plots of the taxon specific system.

e) Save the settings and export all the data for further calculations.

3.4 Calculation of results

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

The standard curves are generated both for the *hmg* and the DP910521 by plotting the Cq values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a linear regression line into these data.

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

To obtain the percentage value of event DP910521 DNA in the unknown sample, the DP910521 copy number is divided by the copy number of the maize endogenous gene *hmg* and multiplied by 100 (GM% = DP910521/*hmg* 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

- TaqMan® Universal PCR Master Mix No UNG. Applied Biosystems Part No 4324020.

4.3 Primers and Probes

Table 5. Primers and probes for DP910521 and *hmg*

	DP910521	DNA Sequence (5' to 3')	Length (nt)
Forward primer	PHN201324	CTC TTG ACA CTT TGT ATT GGT GCT C	25
Reverse primer	PHN165631	CAT AGT AAC CGT GAG CGC TTC A	22
Probe	PHN201325	6-FAM-TTG GGC TCA AGA GGG TA-MGBNFQ	17
Forward primer	MaiJ-F2	TTG GAC TAG AAA TCT CGT GCT GA	23
Reverse primer	mhmg-rev	GCT ACA TAG GGA GCC TTG TCC T	22
Probe	mhmg-probe	6-FAM-CAA TCC ACA CAA ACG CAC GCG TA-BHQ1	23

FAM: 6-carboxyfluorescein; MGBNFQ: Minor Groove Binding Non-Fluorescent Quencher; BHQ1: Black Hole Quencher.

5 References

1. Plant DNA C-values Database. Royal Botanic Gardens, Kew, https://cvalues.science.kew.org/

List of abbreviations and definitions

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

List of tables