EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Directorate F – Health, Consumers and Reference Materials

Food & Feed Compliance (F.5)

Event-specific Method for the Quantification of maize MZHG0JG by Real-time PCR

Validated Method

Method development:

Syngenta Crop Protection nv/sa

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 MZHG0JG (unique identifier SYN-ØØØJG-2) 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 assays. 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 MZHG0JG, an 81 bp fragment of the region spanning the 3' insert-to-plant junction in maize MZHG0JG 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 BHQ-1plus (Black Hole Quencher® 1plus) as non-fluorescent quencher dye at its 3' end.

For the relative quantification of GM event MZHG0JG, a maize taxon-specific system amplifies a 135 bp fragment of an Alcohol dehydrogenase (Adh1) endogenous gene, using *adh1* gene-specific primers and a *adh1* gene-specific probe labelled with VIC® as reporter dye at its 5' end and TAMRA™ (5-Carboxytetramethylrhodamine) as 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 the "Cq" value. For quantification of the amount of MZHG0JG DNA in a test sample, Cq values for the MZHG0JG and the *Adh1* systems are determined for the sample. Standard curves are then used to estimate the relative amount of MZHG0JG 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 April 2018.

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 0.01 % (copy number ratio to haploid genome copy number of GM-material) in 250 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 0.08 % (copy number ratio to haploid genome copy number of GM-material) in 250 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 3' insert-to-plant junction in maize MZHG0JG and is therefore event-specific for the event MZHG0JG. 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 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 maize event MZHG0JG

3.2.1 General

The real-time PCR set-up for the taxon (*Adh1*) and the GMO (event MZHG0JG) 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 MZHG0JG) and the taxon (*Adh1*) assay 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 % maize MZHG0JG DNA in a total of 250 ng of maize DNA (corresponding to 91575 maize haploid genome copies with one haploid genome assumed to correspond to 2.73 pg of maize genomic DNA) [(1)]. Standards S2 to S5 are to be prepared by serial dilutions (dilution factor 4.0) 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 maize DNA in reaction (ng)*	250	62.5	15.6	3.9	1.0
maize haploid genome copies	91575	22894	5723	1431	358
MZHG0JG copies	9158	2289	572	143	36

* 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 MZHG0JG maize specific system (Table 2) and the *Adh1* 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 the MZHG0JG assay.

Component	Final concentration	µL/reaction
Supplemented Jumpstart™ *Taq* ReadyMix™ (2x)	1x	12.5
MZHG0JG forward primer (10 µM)	300 nM	0.75
MZHG0JG reverse primer (10 µM)	300 nM	0.75
MZHG0JG probe* (10 µM)	100 nM	0.25
Nuclease free water	-	5.75
DNA	-	5.0
Total reaction volume:		25 µL

* TaqMan® probe labelled with 6-FAM™ at its 5'-end and BHQ®-1plus at its 3'-end

Table 3. Amplification reaction mixture in the final volume/concentration per reaction well for the maize *Adh1* assay.

Component	Final concentration	µL/reaction
Supplemented Jumpstart™ Taq ReadyMix™ (2x)	1x	12.5
Zm adh1 primer F (10 µM)	300 nM	0.75
Zm adh1 primer R (10 µM)	300 nM	0.75
Zm adh1 probe * (10 µM)	200 nM	0.50
Nuclease free water	-	5.5
DNA	-	5.0
Total reaction volume:		25 µL

*TaqMan® probe is labelled with VIC® at its 5'-end and TAMRA™ at its 3'-end

3. Mix well and centrifuge briefly.
4. Prepare two 0.5 mL reaction tubes (one for the maize MZHG0JG and one for the *Adh1* 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 MZHG0JG maize system and 70 µL for the *Adh1* 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 MZHG0JG system and for the *Adh1* reference system 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 6-FAM™ as reporter dye for the MZHG0JG and VIC® for the *Adh1* reference system. Define BHQ® or non-fluorescent as quencher dye for MZHG0JG specific system and TAMRA™ for *Adh1* reference system. Select Sulforhodamine 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 MZHG0JG/*Adh1* assays.

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 in point 10 for users of second derivative maximum analysis method

Note: According to the applicant instructions, users of Applied Biosystems 7900HT should select 9600 emulation mode

3.3 Data analysis

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

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

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

c) Save the settings.

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

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

3.4 Calculation of results

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

The standard curves are generated both for the *Adh1* and the MZHG0JG specific assays by plotting the Cq values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a linear regression line into these data.
Thereafter, the standard curves are used to estimate the DNA copy number in the unknown samples.

To obtain the percentage value of event MZHG0JG DNA in the unknown sample, the MZHG0JG copy number is divided by the copy number of the maize endogenous gene *Adh1* and multiplied by 100 (GM% = MZHG0JG/*Adh1* 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™ (requires supplement see below) Sigma-Aldrich® Catalog Number P2893
- 1X TE buffer pH 8.0 Sigma-Aldrich® Catalog Number 93283
- Nuclease-free Water (e.g., HPLC Gradient Grade) Ambion® Product Number AM9937
- 1 M $MgCl_2$ Sigma-Aldrich® Catalog Number M1028
- Sulforhodamine 101 Sigma-Aldrich® Catalog Number S7635

10.000X Sulforhodamine 101 stock:

Resuspend 227.5 mg of Sulforhodamine 101 in 250 mL nuclease-free water to make a 1.5 mM stock solution.
Vortex well and store at -20 °C.

Supplemented JumpStart™ Taq ReadyMix™ (2X)

For 50 mL: To 2X JumpStart™ Taq ReadyMix™, add:

- 550 µL of 1 M MgCl2 (11mM final concentration)
- 20 µL 10000X Sulforhodamine 101 stock (600nM final concentration)

Vortex well and store at 4°C for up to 1 year.

4.3 Primers and Probes

Table 5. Primers and probes for the MZHG0JG and *Adh1* methods

	Name	DNA Sequence (5' to 3')	Length (nt)
MZHG0JG			
Forward primer	MZHG0JG forward primer	CAA CTA GCT AGA TTA ATT AAC GCA ATC TG	29
Reverse primer	MZHG0JG reverse primer	ATT TGT TTG CAA GGT GTG GGA	21
Probe	MZHG0JG probe	FAM–TTA AGT TGT CTA AGC GTC AAT TTG-BHQ	24
Adh1			
Forward primer	Zm adh1 primer F	CGT CGT TTC CCA TCT CTT CCT CC	23
Reverse primer	Zm adh1 primer R	CCA CTC CGA GAC CCT CAG TC	20
Probe	Zm adh1 probe	VIC-AAT CAG GGC TCA TTT TCT CGC TCC TCA-TAMRA	27

FAM: 6-FAM™, 6-carboxyfluorescein; TAMRA: TAMRA™, 5-Carboxytetramethylrhodamine; VIC: VIC®; BHQ: Black Hole Quencher® 1plus.

5. References

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