EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Health, Consumers & Reference Materials
Food & Feed Compliance Unit

Event-specific Method for the Quantification of maize DP-ØØ4114-3 by Real-time PCR

Validated Method

Method development:

Pioneer Overseas Corporation

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 DP4114 (unique identifier DP-ØØ4114-3) genomic DNA to total maize genomic 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 DP4114, an 80 bp fragment spanning the junction between the 5' genomic region and the transgenic insert DP4114 is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled FAM (6-carboxyfluorescein) as reporter dye at its 5' end and BHQ (Black Hole quencher) at its 3' end.

For the relative quantification of maize GM event DP4114 DNA, a maize taxon-specific reference system amplifies a 79 bp fragment of a maize *high mobility group* (*hmg*) endogenous gene (GeneBank accession number AJ131373.1), using *hmg* gene-specific primers and a *hmg* gene-specific probe labelled with FAM as reporter dye at its 5' end and Minor Groove Binding (MGB) 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 DP4114 genomic DNA in a test sample, Cq values for the DP4114 and the *hmg* systems are determined for the sample. Standard curves are then used to estimate the relative amount of DP4114 genomic DNA to total maize genomic DNA.

2. Validation and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from genetically modified and conventional maize 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 October 2016.

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 % (related to mass fraction of GM material) in 300 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 0.09 % (related to mass fraction of GM material) in 300 ng of total suitable maize DNA. The lowest relative GM content of the target sequence included in the collaborative trial was 0.058 % calculated in terms of GM copy number ratio, corresponding to 0.1 % mass fraction of GM-material.

2.5 Molecular specificity

The method exploits a unique DNA sequence in the region spanning the junction between the 5' genomic region and the transgenic insert DP4114 and is therefore event-specific for the event DP4114. This was confirmed by bioinformatics analyses.

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).
- 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 DP4114

3.2.1 General

The real-time PCR set-up for the taxon (*hmg*) and the GMO (event DP4114) 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 DP4114) and the taxon (*hmg*) assay with the reagents as listed in Table 2 and Table 3.

3.2.2 Calibration

The calibration curves are established on at least five samples. The first point of the calibration curve (S1) should be established for a sample containing 10 % (relative to copy number fractions) maize DP4114 genomic DNA in a total of 325 ng of maize genomic DNA (corresponding to 119048 maize 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 5.0) 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) *	325	65	13	2.6	0.52
hmg copies	119048	23810	4762	952	190
DP4114 copies	11905	2381	476	95	19

* 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 DP4114 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 the DP4114 event-specific assay.

Component	**Final concentration**	**µL/reaction**
SsoAdvanced Universal Probe Supermix (2x)	1x	10
PHN164689 (10 µM)	600 nM	1.20
PHN1641690 (10 µM)	600 nM	1.20
PHN164691 (10 µM)	140 nM	0.28
BSA (Bovine Serum Albumin)	0.081 %	0.324
HPLC Molecular Biology Grade Water	/	1.996
(DNA)	/	(5.0)
Total volume		20 µL

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

Component	**Final concentration**	**µL/reaction**
SsoAdvanced Universal Probe Supermix (2x)	1x	10
ZM1-F (10 µM)	600 nM	1.20
ZM1-R (10 µM)	600 nM	1.20
PHN149436 (10 µM)	120 nM	0.24
BSA	0.081 %	0.324
HPLC Molecular Biology Grade Water	/	2.036
(DNA)	/	(5.0)
Total volume		20 µL

3. Mix well and centrifuge briefly.
4. Label two 0.5 mL reaction tubes (one for the DP4114 and one for the *hmg* system) for each DNA sample to be tested (standard curve samples, blind samples and the C0 amplification reagent control).
5. Add to each reaction tube the amount of reaction mix for 3.5 PCR repetitions (52.5 µL for the *hmg* system and 52.5 µL for the DP4114 maize system). Add to each tube the correct amount of DNA for 3.5 PCR repetitions (17.5 µL DNA). The additional 0.5 repetition included will ensure adequate volume when loading the samples.
6. Vortex each tube for approx. 10 seconds. This step is mandatory to reduce to a minimum the variability among the repetitions of each sample.
7. Spin down the tubes. Aliquot 20 µL for the DP4114 system and for the *hmg* reference system in each well.
8. Place an optical cover on the reaction plate and briefly centrifuge the plate.
9. 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.
10. Select FAM as reporter dye for the DP4114 and FAM for the *hmg* reference system. Define BHQ as quencher dye for DP4114 specific system and MGB for *hmg* reference system. Select ROX as the passive reference dye. Enter the correct reaction volume (20 µL).
11. Run the PCR with the cycling program described in Table 4. Users of Roche LC480 real-time PCR instrument should set the instrument to 45 cycles, instead of 40, and use the settings of the ramp rate as indicated at the important note below.

Table 4. Cycling program for DP4114/*hmg* assays.

Step	Stage		T (°C)	Time (s)	Acquisition	Cycles
1	Initial Enzyme Activation		95°C	180	No	1
3	Amplification	Denaturation	95°C	15	No	40x*
		Annealing & Extension	60°C	30	Yes	

** see point 11 above for Roche LC480 instruments*

Note:
Applied Biosystems 7900HT users: uncheck the 9600 emulation mode.
Roche LC480 users: ramp rates for the Roche LC480 are: 2.6°C/second Initial Enzyme Activation and Denaturation, and 1.6°C/second for Annealing and Extension.
According to the applicant users of ViiA7™ should select the default ramp rate; the EURL GMFF did not test the method on this instrument

3.3 Data analysis

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

a) Analyse data: automatic baseline and threshold settings have given the best results at the EURL GMFF. For users of Roche LC480 instruments, it is recommended that 45 cycles are run and the analysis is carried out with the Absolute Quantification/Second Derivative Maximum method with High Confidence algorithm.

b) 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, the instrument's software calculates the Cq values for each reaction.

The standard curves are generated both for the *hmg* and the DP4114 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 DP4114 DNA in the unknown sample, the DP4114 copy number is divided by the copy number of the maize endogenous gene *hmg* and multiplied by 100 % (GM % = DP4114 / *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
- Micropipettes
- Standard bench top centrifuge with rotor or standard microfuge fit for 0.5 mL, 1.5mL and 2.0 mL reaction tubes, centrifuge for 96-Well reaction plates
- Vortex
- Racks for reaction tubes, also cooled
- 0.5, 1.5 mL or 2 mL DNAse free reaction tubes

4.2 Reagents

- SsoAdvanced™ Universal Probes Supermix, Biorad Catalogue No 1725281.
- Bovine Serum Albumin (BSA), Ambion Catalogue No AM2618
- Nuclease free water

4.3 Primers and Probes

Table 5. Primers and probes for the DP4114 and *hmg* methods

	Name	DNA Sequence (5' to 3')	Length (nt)
DP4114			
Forward primer	PHN164689	gCT TTg gAg CCT CTC gTT TgT A	22
Reverse primer	PHN1641690	gCg TTT AAA CTA TCA gAT CTg TgT TgA A	28
Probe	PHN164691	6'FAM-CAC TTg CAC gTA gTT ACC Cgg ACC gAA-BHQ*	27
hmg			
Forward primer	ZM1-F	TTg gAC TAg AAA TCT CgT gCT gA	23
Reverse primer	ZM1-R	gCT ACA TAg ggA gCC TTg TCC T	22
Probe	PHN149436	6'FAM-CAA TCC ACA CAA ACg C-MGB**	16

* Black Hole Quencher
** Minor Groove Binding (MGB) quencher

5. References

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