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Event Specific Method for the Quantification of Maize 98140 by Real-time PCR

Validation Report and Validated Method

C. Savini, M. Mazzara, A. Bevilacqua, G. Van den Eede

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Event-specific Method for the Quantification of Maize 98140 by Real-time PCR

Validation Report

7 January 2011

Joint Research Centre
Institute for Health and Consumer Protection
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Executive Summary

The European Union Reference Laboratory for GM Food and Feed (EURL-GMFF), established by Regulation (EC) No 1829/2003, in collaboration with the European Network of GMO Laboratories (ENGL), has carried out a collaborative study to assess the performance of a quantitative event-specific method to detect and quantify the 98140 transformation event in maize DNA (unique identifier DP- Ø9814Ø-6). The collaborative study was conducted according to internationally accepted guidelines [1, 2].
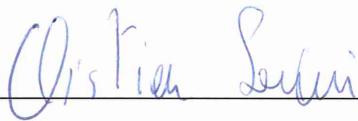
In accordance to Regulation (EC) No 1829/2003 of 22 September 2003 on genetically modified food and feed and with Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003, Pioneer Overseas Corporation provided the detection method and the samples (genomic DNA extracted from homogenised seeds containing the transformation event and from conventional homogenised seeds). The EURL-GMFF prepared the validation samples (calibration samples and blind samples at unknown GM percentage [DNA/DNA]). The collaborative study involved twelve laboratories from seven European countries.

The results of the international collaborative study met the ENGL performance requirements. The method is therefore considered applicable to the control samples provided, in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004.

The results of the collaborative study are made publicly available at http://gmo-crl.jrc.ec.europa.eu/.

Drafted by:
C. Savini (scientific officer)

Report review:
1) L. Bonfini
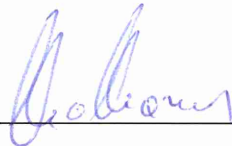
2) M. Querci

Scientific and technical approval:
M. Mazzara (scientific officer)

Compliance with EURL Quality System:
S. Cordeil (quality manager)

Authorisation to publish:
G. Van den Eede (head of MBG Unit)

Address of contact laboratory:

European Commission, Joint Research Centre
Institute for Health and Consumer Protection (IHCP)
Molecular Biology and Genomics Unit
European Union Reference Laboratory for GM Food and Feed
Via Fermi 2749, 21027 Ispra (VA) - Italy

Report on Steps 1-3 of the Validation Process

Pioneer Overseas Corporation provided the detection method and control samples for maize event 98140 (unique identifier DP- Ø9814Ø -6) according to Articles 5 and 17 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".
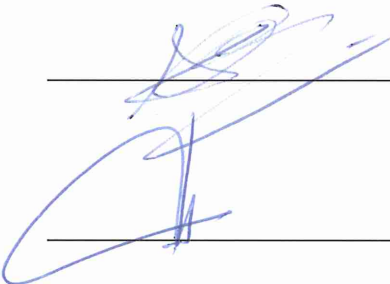
The European Union Reference Laboratory for GM Food and Feed (EURL-GMFF), following reception of the documentation and material, including control samples, (step 1 of the validation process) carried out the scientific assessment of documentation and data (step 2) in accordance with Commission Regulation (EC) No 641/2004 "on detailed rules for the implementation of Regulation (EC) No 1829/2003 of the European Parliament and of the Council as regards the application for the authorisation of new genetically modified food and feed, the notification of existing products and adventitious or technically unavoidable presence of genetically modified material which has benefited from a favourable risk evaluation" and according to internal procedures ("Description of the EURL-GMFF Validation Process", http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm).

The scientific assessment focused on the method performance characteristics assessed against the method acceptance criteria set out by the European Network of GMO Laboratories and listed in the "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm) (see Annex 1 for a summary of method acceptance criteria and method performance requirements). During step 2, two scientific assessments were performed and requests for complementary information addressed to the applicant. Upon reception of complementary information, the scientific evaluation of the detection method for event 98140 was positively concluded in August 2008.

In April-September 2008, the EURL-GMFF verified experimentally the method characteristics (step 3, experimental testing of samples and methods) by quantifying five GM levels within the range 0.09%-7.0% on a copy number basis. The experiments were performed under repeatability conditions and demonstrated that the PCR efficiency, linearity, trueness and precision were within the limits established by the ENGL. The DNA extraction method was previously tested on samples of food and feed and a report was published on the EURL-GMFF website (http://gmo-crl.jrc.ec.europa.eu/summaries/TC1507-DNAextrc.pdf).

A Technical Report summarising the results of tests carried out by the EURL-GMFF (step 3) is available on request.

Content

1.   Introduction

Pioneer Overseas Corporation submitted the detection method and control samples for maize event 98140 (unique identifier DP-Ø9814Ø-6) according to Articles 5 and 17 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The European Union Reference Laboratory for GM Food and Feed, established by Regulation (EC) No 1829/2003, organised the international collaborative study for the validation of the method of detection and quantification of maize 98140. The study involved twelve laboratories from seven European countries among those listed in Annex II ("National reference laboratories assisting the CRL for testing and validation of methods for detection") of Commission Regulation (EC) No 1981/2006 of 22 December 2006.

Upon reception of method, samples and related data (step 1), the EURL-GMFF carried out the assessment of the documentation (step 2) and the in-house evaluation of the method (step 3) according to the requirements of Regulation (EC) No 641/2004.

The internal experimental evaluation of the method was carried out in April-September 2008.

Following the evaluation of the data and the results of the internal tests, the international collaborative study was organised (step 4) and took place in September-October 2008.

The collaborative study aimed at validating a quantitative real-time PCR (Polymerase Chain Reaction) method. The methodology is an event-specific real-time quantitative TaqMan® PCR procedure for the determination of the relative content of 98140 DNA to total maize DNA. The procedure is a simplex system, in which a maize *hmg* (high mobility group) endogenous assay (targeting the taxon-specific *hmg* gene) and the target assay (98140) are performed in separate wells.

The international collaborative study was carried out in accordance with the following internationally accepted guidelines:

- ✓ ISO 5725 (1994).
- ✓ The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" (Horwitz, 1995).

2.   Selection of participating laboratories

As part of the international collaborative study the method was tested in twelve laboratories to determine its performance.

On 12th September 2008 the EURL-GMFF invited all National Reference Laboratories nominated under Commission Regulation (EC) No 1981/2006 of 22 December 2006 and listed in Annex II ("National reference laboratories assisting the CRL for testing and validation of methods for

detection") of that Regulation to express the availability to participate in the validation study of the quantitative real-time PCR method for the detection and quantification of maize GM event 98140.

Forty laboratories expressed in writing their willingness to participate, while thirty-two did not answer. The EURL-GMFF carried out a random selection of twelve laboratories out of those that responded positively to the invitation, making use of a validated software application.

Clear guidance was given to the selected laboratories with regards to standard operational procedures to follow for the execution of the protocol. The participating laboratories are listed in Table 1.

Table 1. Laboratories participating in the validation of the detection method for maize 98140.

Laboratory	Country
Federal State Agency of Analysis and Diagnosis for Rhineland-Palatinate – Institute of Food Chemistry Trier	DE
Genetically Modified Organism Controlling Laboratory	PL
Institute of Chemical Technology Prague	CZ
Institute of Molecular Biology of the Slovak Academy of Sciences	SK
Laboratory for the Detection of GMO in Food	DE
Lower Saxony Federal State Office for Consumer Protection and Food Safety, State Food Laboratory Braunschweig	DE
National Diagnostic Centre of Food and Veterinary Service	LV
National Veterinary Laboratory, GMO Department	LT
National Veterinary Research Institute in Pulawy, Department of Feed Hygiene	PL
Scientific Institute of Public Health (IPH)	BE
State Institute of Chemical and Veterinarian Analysis	DE
State Office for Agriculture, Food Safety and Fisheries - Mecklenburg Western Pomerania	DE

3.   Materials

For the validation of the quantitative event-specific method, samples consisted of:

i)   genomic DNA extracted from maize seeds harbouring the event 98140
ii)   genomic DNA extracted from conventional maize seeds.

Samples were provided by the applicant in accordance to the provisions of Regulation (EC) No 1829/2003, Art 2.11 [control sample defined as the GMO or its genetic material (positive sample) and the parental organism or its genetic material that has been used for the purpose of the genetic modification (negative sample)].

Samples containing mixtures of maize 98140 and non-GM maize genomic DNA at different GMO percentages were prepared by the EURL-GMFF, using the control samples provided, in a constant amount of total maize DNA.

Participants received the following materials:

- ✓ Four calibration samples (200 µL of DNA solution each) for the preparation of the standard curve, labelled from S1 to S4.
- ✓ Twenty unknown DNA samples (100 µL of DNA solution each), labelled from U1 to U20.
- ✓ Reaction reagents:
    - ❑ TaqMan® Universal PCR Master Mix 2x No Amperase UNG,
      1 tube: 8.0 mL
    - ❑ Sterile distilled water: 3.1 mL

- ✓ Primers and probes (1 tube each) as follows:

    hmg taxon-specific system
    - ❑ MaiJ-F2, one tube   (10 µM):   320 µL
    - ❑ mHMG-rev, one tube   (10 µM):   320 µL
    - ❑ mHMG-probe, one tube   (10 µM):   120 µL

    98140 system
    - ❑ DP098-f6, one tube   (10 µM):   400 µL
    - ❑ DP098-r2, one tube   (10 µM):   400 µL
    - ❑ DP098-p5, one tube   (10 µM):   160 µL

4.   Experimental design

Twenty unknown samples (labelled from U1 to U20), representing five GM levels, were used in the validation study (Table 2). On each PCR plate, the samples were analysed either for the 98140 specific system or the *hmg* taxon-specific system. In total, two plates were run per laboratory and four replicates for each GM level were analysed. PCR was performed in triplicate for all samples. Participating laboratories carried out the estimation of the GM% according to the instructions provided in the protocol and using the application provided.

Table 2. 98140 GM contents

98140 GM% [GMO copy number/maize genome copy number (x 100)]
0.09
0.40
0.90
2.50
7.00

4.   Method

For the detection of event 98140, an 80 bp fragment of the region spanning the 5' insert-to-plant junction is amplified. PCR products are measured at each cycle (real-time) by means of a specific oligonucleotide probe labelled with FAM dye (6-carboxyfluorescein) and TAMRA (tetramethylrhodamine) as quencher dye.

For the relative quantification of GM event 98140, a maize specific reference system amplifies a 79 bp fragment of the maize endogenous gene *hmg* (High Mobility Group, GenBank accession number AJ131373), using two *hmg* gene-specific primers and an *hmg* probe labelled with FAM and TAMRA.

Standard curves are generated for both the 98140 and the *hmg* systems by plotting the Ct values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a regression line into these data. Thereafter, the standard curves are used to estimate the copy numbers in the unknown sample DNA by interpolation from the standard curves.

For relative quantification of event 98140 DNA in a test sample, the 98140 copy number is divided by the copy number of the maize reference gene (*hmg*) and multiplied by 100 to obtain the percentage value (GM% = 98140 / *hmg* x 100).

Calibration sample S1 was prepared by mixing the appropriate amount of 98140 DNA in control non-GM maize DNA to obtain a 10% 98140 in a total of 200 ng maize DNA. Calibration samples S2-S4 were prepared by 1:5 serial dilutions from the S1 sample.

The absolute copy numbers of the calibration curve samples are calculated by dividing the sample DNA mass (nanograms) by the published average 1C value for maize genome (2.5 pg) [3]. The copy number values used in the quantification, the GMO contents of the calibration samples and the total DNA quantity used in PCR are listed in Table 3.

Table 3. Copy number values of the standard curve samples.

Sample code	S1	S2	S3	S4
Total amount of DNA in reaction (ng/5 µL)	200	40	8	1.6
Maize genome copies	80000	16000	3200	640
98140 copies	8000	1600	320	64

6. Deviations reported

Eleven laboratories reported no deviations from the protocol.

One laboratory inverted the positions of the event-specific and taxon-specific assays on both plates. The analysis was performed taking into account the change in plate layout.

7.   Results

PCR efficiency and linearity

The values of the slopes [from which the PCR efficiency is calculated using the formula [(10^(-1/slope))-1)*100] of the standard curve and of the R^2 (expressing the linearity of the regression) reported by the laboratories for the 98140 and the *hmg* assays are reported in Table 4.

Table 4. Values of slope, PCR efficiency and R^2

| LAB | 98140 | | | hmg | | |
	Slope	PCR Efficiency (%)	R^2	Slope	PCR Efficiency (%)	R^2
1	-3.55	91	1.00	-3.34	99	1.00
	-3.52	92	1.00	-3.38	98	1.00
2	-3.41	97	1.00	-3.34	99	1.00
	-3.52	92	1.00	-3.34	99	1.00
3	-3.31	101	1.00	-3.33	100	1.00
	-3.44	95	1.00	-3.36	99	1.00
4	-3.47	94	1.00	-3.32	100	1.00
	-3.37	98	0.99	-3.34	99	1.00
5	-3.28	102	1.00	-3.37	98	1.00
	-3.37	98	0.99	-3.39	97	1.00
6	-3.46	95	1.00	-3.61	89	1.00
	-3.50	92	1.00	-3.38	98	1.00
7	-3.36	98	1.00	-3.35	99	1.00
	-3.55	91	1.00	-3.43	96	1.00
8	-3.49	94	1.00	-3.44	95	1.00
	-3.44	95	1.00	-3.37	98	1.00
9	-3.37	98	1.00	-3.26	103	1.00
	-3.43	96	1.00	-3.41	96	1.00
10	-3.75	85	0.98	-3.40	97	1.00
	-3.50	93	1.00	-3.43	96	1.00
11	-3.47	94	1.00	-3.38	97	1.00
	-3.44	95	0.99	-3.34	99	1.00
12	-3.55	91	1.00	-3.32	100	1.00
	-3.30	101	0.98	-3.38	98	0.99
Mean	-3.45	95	1.00	-3.38	98	1.00

The mean PCR efficiency was 98% for the *hmg* reference system and 95% for the 98140. The R^2 of the method was 1.00 for both assays. The data confirm the appropriate performance characteristics of the method tested in terms of PCR efficiency and linearity.

GMO quantification

Table 5 reports the mean values of the four replicates for each GM level as estimated by all laboratories. Each mean value is the average of three PCR repetitions.

Table 5. GM% mean values determined by laboratories for unknown samples.

LAB	GMO content (GM% = GMO copy number/maize genome copy number x 100)																			
	0.09				0.40				0.90				2.50				7.00			
	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4
1	0.12	0.10	0.13	0.11	0.44	0.48	0.46	0.57	1.31	1.10	1.19	1.04	2.71	2.69	3.42	2.86	7.21	7.98	7.08	8.63
2	0.12	0.11	0.10	0.09	0.44	0.46	0.46	0.40	0.95	1.06	1.04	1.09	2.79	2.69	2.82	2.63	7.38	7.11	7.26	7.13
3	0.11	0.08	0.08	0.10	0.44	0.35	0.49	0.39	0.94	0.98	0.98	0.97	2.72	2.29	3.22	3.22	6.37	7.14	8.92	8.07
4	0.10	0.11	0.10	0.10	0.44	0.49	0.46	0.55	1.19	1.13	1.08	0.95	2.75	3.08	2.72	2.49	7.69	7.58	6.61	7.00
5	0.09	0.08	0.07	0.09	0.46	0.38	0.48	0.38	0.72	1.06	0.83	0.83	2.39	2.47	2.06	1.97	6.45	6.75	7.75	6.51
6	0.10	0.09	0.11	0.14	0.45	0.28	0.45	0.41	0.99	0.95	1.09	1.03	2.73	2.84	2.63	2.75	7.12	7.15	7.60	5.74
7	0.09	0.11	0.07	0.08	0.43	0.44	0.41	0.40	0.86	1.02	1.19	0.90	2.45	2.93	2.81	2.65	6.48	7.90	8.47	6.44
8	0.08	0.06	0.11	0.09	0.30	0.29	0.44	0.31	1.14	0.79	0.48	0.83	2.81	1.69	2.56	2.86	5.71	7.33	6.74	7.79
9	0.11	0.12	0.09	0.08	0.44	0.49	0.33	0.36	1.13	1.14	1.12	0.86	2.39	3.14	3.01	2.80	7.94	9.66	6.85	7.85
10	0.09	0.10	0.11	0.11	0.42	0.42	0.45	0.39	0.80	1.00	1.01	0.90	2.31	2.57	2.38	2.27	7.41	7.40	5.59	5.72
11	0.08	0.09	0.12	0.12	0.37	0.82	0.41	0.46	1.07	1.02	1.04	0.93	2.68	2.84	2.70	2.30	6.42	6.77	7.81	7.08
12	0.15	0.12	0.20	0.13	0.52	0.46	0.63	0.76	1.75	1.41	1.15	1.35	2.90	3.26	3.25	2.98	11.51	9.69	8.18	9.74

In Figure 1 the relative deviation from the true value for each GM level tested is shown for each laboratory, following removal of statistical outliers. The coloured bars represent the relative GM quantification obtained by the participating laboratories; the green bar represents the overall mean value for each GM level.

Figure 1. Relative deviation (%) from the true value of 98140 for all laboratories
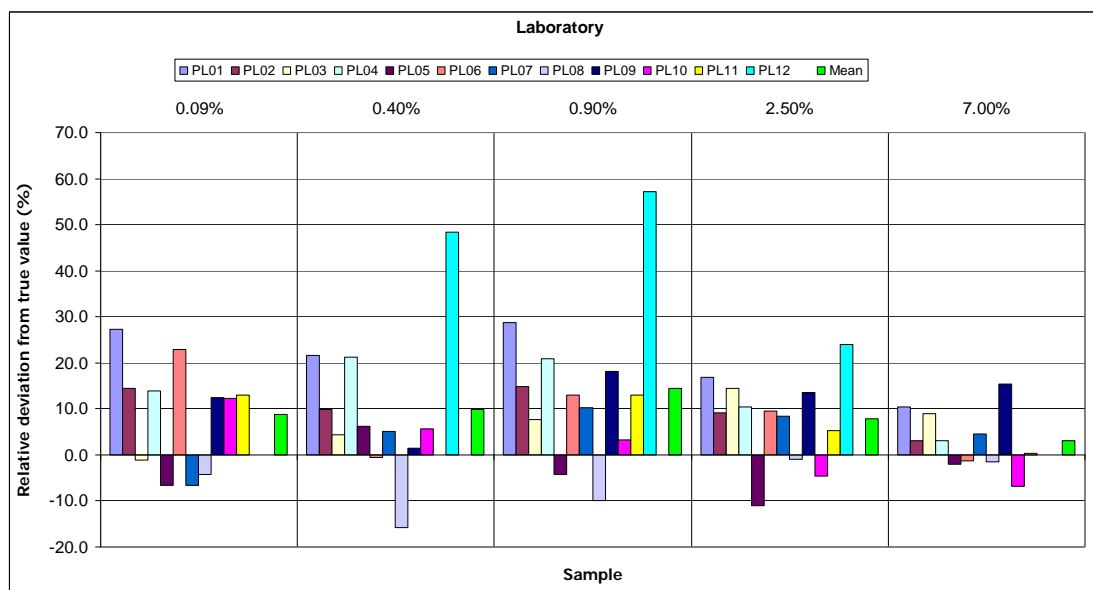
The mean relative deviations from the true values are positive for all GM levels, being well within the acceptance criterion of maximum 25%. One laboratory overestimated the DNA content of samples 0.40% and 0.90%, though not falling in the requisites for outlying values.

The average bias generated by all laboratories is modest, being equal or below 14% at all GM levels tested, indicating a satisfactory trueness of the method.

8.   Method performance requirements

Among the performance criteria established by the ENGL and adopted by the EURL-GMFF (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm, see also Annex 1), repeatability and reproducibility are assessed through an international collaborative trial, carried out with the support of twelve European laboratories (see Table 1). Table 6 illustrates the estimation of repeatability and reproducibility at various GM levels, according to the range of GM percentages tested during the collaborative trial.

The relative reproducibility standard deviation (RSD_R), describing the inter-laboratory variation, should be below 33% over the majority of the dynamic range, while it should be below 50% at the lower end of the dynamic range.

As can be observed in Table 6, the method satisfies this requirement at all GM levels tested. In fact, the highest value of RSD_R (%) is 20% at the 0.4% GM level.

Table 6. 98140: summary of validation results.

Sample	Target value (GMO%)				
	0.09	0.4	0.9	2.5	7.0
Laboratories having returned results	12	12	12	12	12
Samples per laboratory	4	4	4	4	4
Number of outliers	1	1	-	-	1
Reason for exclusion	G	C	-	-	G
Mean value	0.10	0.44	1.03	2.70	7.22
Relative repeatability standard deviation, RSD_r (%)	16	15	14	11	12
Repeatability standard deviation	0.016	0.066	0.141	0.289	0.832
Relative reproducibility standard deviation, RSD_R (%)	18	20	19	13	12
Reproducibility standard deviation	0.017	0.087	0.197	0.347	0.849
Bias (absolute value)	0.008	0.039	0.130	0.198	0.217
Bias (%)	9	10	14	8	3

C = Cochran's test; G= Grubbs' test; identification and removal of outliers through Cochran and Grubbs tests, according to ISO 5725-2.

Bias is estimated according to ISO 5725.

Table 6 further documents the relative repeatability standard deviation (RSD_r), as estimated for each GM level. In order to accept methods for collaborative study, the EURL-GMFF requires that RSD_r value is below 25%, as indicated by ENGL (Definition of Minimum Performance

Requirements for Analytical Methods of GMO Testing" (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm).

As it can be observed from the values reported in Table 6, the method shows a relative repeatability standard deviation below 25% over the dynamic range with a maximum of 16% at 0.09%.

The trueness of the method is estimated using the measures of the method bias for each GM level. According to ENGL method performance requirements, trueness should be ± 25% across the entire dynamic range. The method fully satisfies this requirement; in fact, the highest value of bias (%) is 14% at the 0.9% level, thus within the acceptance criterion.

9.   Conclusions

The method performance has been evaluated with respect to the method acceptance criteria and method performance requirements recommended by the ENGL (as detailed at http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm). The method acceptance criteria were reported by the applicant and were used to evaluate the method prior to the international collaborative study (see Annex 1 for a summary of method acceptance criteria and method performance requirements).

The results obtained during the collaborative study indicate that the analytical module of the method submitted by the applicant complies with ENGL performance criteria.

The method is therefore applicable to the control samples provided (see paragraph 3 "Materials"), in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004.

10.   Quality assurance

The EURL-GMFF operates according to ISO 9001:2000 (certificate number: 32231) and ISO 17025:2005 (certificate number: DAC-PL-0459-06-00) [DNA extraction, qualitative and quantitative PCR in the area of Biology (DNA extraction and PCR method validation for the detection and identification of GMOs in food and feed materials)].

11.   References

1. Horwitz, W. (1995) Protocol for the design, conduct and interpretation of method performance studies, *Pure and Appl. Chem*, 67, 331-343.
2. International Standard (ISO) 5725. 1994. Accuracy (trueness and precision) of measurement methods and results. International Organization for Standardization, Genève, Swizerland.

3. Arumuganathan K, Earle ED. 1991. Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter* 9: 208-218

12. Annex 1: method acceptance criteria and method performance requirements as set by the European Network of GMO Laboratories (ENGL)

Method Acceptance Criteria should be fulfilled at the moment of submission of a method (Phase 1: acceptance for the collaborative study).

Method Performance Requirements should be fulfilled in a collaborative study in order to consider the method as fit for its purpose (Phase 2: evaluation of the collaborative study results).

Method Acceptance Criteria

Applicability

Definition: The description of analytes, matrices, and concentrations to which a method can be applied.

Acceptance Criterion: The applicability statement should provide information on the scope of the method and include data for the indices listed below for the product/s for which the application is submitted. The description should also include warnings to known interferences by other analytes, or inapplicability to certain matrices and situations.

Practicability

Definition: The ease of operations, the feasibility and efficiency of implementation, the associated unitary costs (e.g. Euro/sample) of the method.

Acceptance Criterion: The practicability statement should provide indication on the required equipment for the application of the method with regards to the analysis *per se* and the sample preparation. An indication of costs, timing, practical difficulties and any other factor that could be of importance for the operators should be indicated.

Specificity

Definition: Property of a method to respond exclusively to the characteristic or analyte of interest.

Acceptance Criterion: The method should be event-specific and be functional only with the GMO or GM based product for which it was developed. This should be demonstrated by empirical results from testing the method with non-target transgenic events and non-transgenic material. This testing should include closely related events and cases where the limit of the detection is tested.

Dynamic Range

Definition: The range of concentrations over which the method performs in a linear manner with an acceptable level of accuracy and precision.

Acceptance Criterion: The dynamic range of the method should include the 1/10 and at least 5 times the target concentration. Target concentration is intended as the threshold relevant for legislative

requirements. The acceptable level of accuracy and precision are described below. The range of the standard curve(s) should allow testing of blind samples throughout the entire dynamic range, including the lower (10%) and upper (500%) end.

Accuracy

Definition: The closeness of agreement between a test result and the accepted reference value.

Acceptance Criterion: The accuracy should be within $\pm$ 25% of the accepted reference value over the whole dynamic range.

Amplification Efficiency

Definition: The rate of amplification that leads to a theoretical slope of −3.32 with an efficiency of 100% in each cycle. The efficiency of the reaction can be calculated by the following equation: Efficiency = $[10^{(-1/slope)}] - 1$.

Acceptance Criterion: The average value of the slope of the standard curve should be in the range of (-3.1 $\geq$ slope $\geq$ - 3.6).

R^2 Coefficient

Definition: The R^2 coefficient is the correlation coefficient of a standard curve obtained by linear regression analysis.

Acceptance Criterion: The average value of R^2 should be $\geq$ 0.98.

Repeatability Standard Deviation (RSD$_r$)

Definition: The standard deviation of test results obtained under repeatability conditions. Repeatability conditions are conditions where test results are obtained with the same method, on identical test items, in the same laboratory, by the same operator, using the same equipment within short intervals of time.

Acceptance Criterion: The relative repeatability standard deviation should be below 25% over the whole dynamic range of the method.

Note: Estimates of repeatability submitted by the applicant should be obtained on a sufficient number of test results, at least 15, as indicated in ISO 5725-3 (1994).

Limit of Quantitation (LOQ)

Definition: The limit of quantitation is the lowest amount or concentration of analyte in a sample that can be reliably quantified with an acceptable level of precision and accuracy.

Acceptance Criterion: LOQ should be less than 1/10th of the value of the target concentration with an RSD$_r$ $\leq$ 25%. Target concentration should be intended as the threshold relevant for legislative requirements. The acceptable level of accuracy and precision are described below.

Limit of Detection (LOD)

Definition: The limit of detection is the lowest amount or concentration of analyte in a sample, which can be reliably detected, but not necessarily quantified, as demonstrated by single laboratory validation.

Acceptance Criterion: LOD should be less than $1/20^{th}$ of the target concentration. Experimentally, quantitative methods should detect the presence of the analyte at least 95% of the time at the LOD, ensuring $\leq$ 5% false negative results. Target concentration should be intended as the threshold relevant for legislative requirements.

Robustness

Definition: The robustness of a method is a measure of its capacity to remain unaffected by small, but deliberate deviations from the experimental conditions described in the procedure.

Acceptance Criterion: The response of an assay with respect to these small variations should not deviate more than $\pm$ 30%. Examples of factors that a robustness test could address are: use of different instrument type, operator, brand of reagents, concentration of reagents, and temperature of reaction.

Method Performance Requirements

Dynamic Range

Definition: In the collaborative trial the dynamic range is the range of concentrations over which the reproducibility and the trueness of the method are evaluated with respect to the requirements specified below.

Acceptance Criterion: The dynamic range of the method should include the 1/10 and at least five times the target concentration. Target concentration should be intended as the threshold relevant for legislative requirements.

Reproducibility Standard Deviation (RSD$_R$)

Definition: The standard deviation of test results obtained under reproducibility conditions. Reproducibility conditions are conditions where test results are obtained with the same method, on identical test items, in different laboratories, with different operators, using different equipment. Reproducibility standard deviation describes the inter-laboratory variation.

Acceptance Criterion: The relative reproducibility standard deviation should be below 35% at the target concentration and over the entire dynamic range. An RSD$_R$ < 50 % is acceptable for concentrations below 0.2%.

Trueness

Definition: The closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. The measure of trueness is usually expressed in terms of bias.

Acceptance Criterion: The trueness should be within $\pm$ 25% of the accepted reference value over the whole dynamic range.

Event-specific Method for the Quantification of Maize 98140 Using Real-time PCR

Protocol

7 January 2011

Joint Research Centre
Institute for Health and Consumer Protection
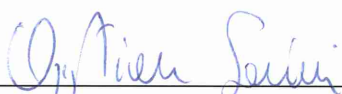**Molecular Biology and Genomics Unit**

Method development:

Pioneer Overseas Corporation

Method validation:

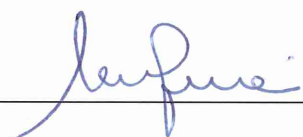European Union Reference Laboratory for GM Food and Feed (EURL-GMFF)

Drafted by
C. Savini

Report review
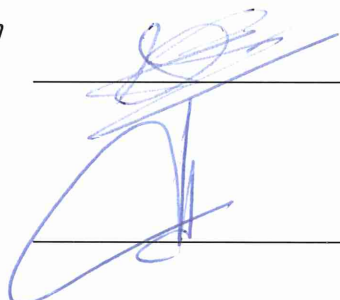1) L. Bonfini

2) M. Querci

Scientific and technical approval
M. Mazzara

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S. Cordeil

Authorisation to publish
G. Van den Eede

Contact:

European Commission, Joint Research Centre
Institute for Health and Consumer Protection (IHCP)
Molecular Biology and Genomics Unit
European Union Reference Laboratory for GM Food and Feed
Via Fermi 2749, 21027 Ispra (VA) - Italy

Content

1. General information and summary of the methodology

This protocol describes an event-specific real-time quantitative PCR (polymerase chain reaction) procedure for the determination of the relative content of maize event 98140 DNA to total maize DNA in a sample.

The PCR assay was optimised for use in real-time PCR instruments for plastic reaction plates.

DNA extracted by means of suitable methods should be tested for quality and quantity prior to use in PCR assay. Tests for the presence of PCR inhibitors (e.g. monitor run of diluted series, use of DNA spikes) are recommended.

For the detection of maize event 98140, a 80 bp fragment of the integration region of the construct inserted into the plant genome (5′ insert-to-plant junction) is amplified using specific primers. PCR products are measured at each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with FAM dye and TAMRA as quencher dye.

For the relative quantification of maize event 98140, a maize-specific reference system amplifies a 79 bp fragment of the maize *high mobility group* gene (*hmg*), using specific primers and an *hmg* specific probe labelled with FAM and TAMRA as described above.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Ct" value. For quantification of the amount of 98140 DNA in a test sample, Ct values for the 98140 and *hmg* system are determined for the sample. Standard curves are then used to estimate the relative amount of 98140 DNA to total maize DNA.

2. Validation and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from homogenised maize grains.
The reproducibility and trueness of the method were tested through an international collaborative ring trial using DNA samples at different GMO contents.

2.2 Collaborative trial

The method was validated in a collaborative study coordinated by the EURL-GMFF. The study was undertaken with twelve participating laboratories in September-October 2008.

Each participant received twenty blind samples containing 98140 genomic DNA at five GM contents, ranging from 0.09 % to 7.0 %.

Each test sample was analysed by PCR in three repetitions. The study was designed as a blind quadruplicate collaborative trial; each laboratory received each GM level in four unknown samples. Two replicates of each GM level were analysed on the same PCR plate.

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

2.3 Limit of detection (LOD)

According to the method developer, the relative LOD of the method is at least 0.04% in 200 ng of total maize DNA. The relative LOD was not assessed in a collaborative study.

2.4 Limit of quantification (LOQ)

According to the method developer, the relative LOQ of the method is at least 0.08% in 200 ng of total maize DNA. The lowest relative GM content of the target sequence included in collaborative trial was 0.09 %.

2.5 Molecular specificity

According to the method developer, the method exploits a unique DNA sequence in the region of recombination between the insert and the plant genome; the sequence is specific to maize event 98140 and thus imparts event-specificity to the method.

The specificity of the event-specific assay was tested by the method developer in real-time PCR against 20 ng of genomic DNA of 98140 and DP-005431-6, E7111.6.6.3 (sister events); against 100 ng of genomic DNA at 1% GM of maize events Bt11, Bt176, CBH351, GA21, NK603, MON810, MON863, MON88017, MIR604, TC1507, Herculex Rootworm, 3272, T25, soybean RoundUp Ready; and against 20 ng genomic DNA of non-GM sugar beet, rapeseed, soybean, cotton, rice, potato, wheat, maize.
According to the method developer, none of the materials tested, except the positive control maize event 98140 yielded detectable amplification.

The specificity of the maize reference assay *hmg* was tested by the method developer in real-time PCR against DNA extracted from plant materials containing 20 ng genomic DNA of maize, Hulless oat, sugar beet, rapeseed, soybean, barley, rice, rye, tomato, potato, wheat, spelt.

According to the method developer, none of the materials tested, except the positive control, yielded detectable amplification.

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 the guidelines given by relevant authorities, e.g. ISO 24276

- 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 has to be 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 frequently.

- 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 0 - 4°C.

- In order to avoid repeated freeze/thaw cycles, aliquots should be prepared.

3.2   Real-time PCR for quantitative analysis of maize event 98140

3.2.1 General

The PCR set-ups for the taxon-specific target sequence (*hmg*) and for the GMO (event 98140) are carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.

The use of maximum 200 ng of template DNA per reaction well is recommended.

The method is developed for a total volume of 25 µL per reaction mixture with the reagents as listed in Table 1 and Table 2.

3.2.2 Calibration

The standard curves consist of four samples. The first point of the calibration curves is a 10% 98140 maize DNA in a total of 200 ng of maize DNA (corresponding to approximately 80,000 maize genome copies with one genome assumed to correspond to 2.5 pg of haploid maize genomic DNA). The other three standard samples are prepared by serial dilution.

A calibration curve is produced by plotting the Ct values against the logarithm of the target copy number for the calibration points. This can be done e.g. by means of spreadsheet software, e.g. Microsoft Excel, or directly using the options available with the sequence detection system software.

The copy number measured for the unknown sample DNA is obtained by interpolation from the standard curves.

3.2.3 Real-time PCR set-up

1. Thaw, mix gently and centrifuge the required amount of components needed for the run. Keep thawed reagents at 1-4 °C on ice.

2. To prepare the amplification reaction mixtures add the following components (Tables 1 and 2) In two reaction tubes (one for the 98140 assay and one for the *hmg* assay) on ice in the order mentioned below (except DNA).

Table 1. Amplification reaction mixture in the final volume/concentration per reaction well for the 98140 assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.50
DP098-f6 primer (10 µM)	500 nM	1.25
DP098-r2 primer (10 µM)	500 nM	1.25
DP098-p5 probe (10 µM)	200 nM	0.50
Nuclease free water	#	4.50
Template DNA (max 200 ng)	#	5.0
Total reaction volume:		25

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the maize *hmg* reference assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	12.5
MaiJ-F2 primer (10 µM)	400 nM	1.0
mHMG-rev primer (10 µM)	400 nM	1.0
mHMG-probe (10 µM)	150 nM	0.375
Nuclease free water	#	5.125
Template DNA (max 100 ng)	#	5.0
Total reaction volume:		25

3. Mix gently and centrifuge briefly.

4. Prepare two reaction tubes (one for the 98140 and one for the *hmg* reaction mixes) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).

5. Add to each reaction tube the correct amount of master mix (e.g. 20 x 3 = 60 µL master mix for three PCR repetitions). Add to each tube the correct amount of DNA (e.g. 5 x 3 = 15 µL DNA for three PCR repetitions). Vortex each tubes for approx. 10 sec. This step is mandatory to reduce to a minimum the variability among the repetitions of each sample.

6. Spin down the tubes in a micro-centrifuge. Aliquot 25 µL in each well. Seal the reaction plate with optical cover or optical caps. Centrifuge the plate at low speed (e.g. approximately 250x *g* for 1 minute at 4 °C) to spin down the reaction mixture.

7. Place the plate in the instrument.

8. Run the PCR with cycling conditions described in Table 3:

Table 3. Cycling program for 98140/*hmg* assays

Step	Stage		T°C	Time (sec)	Acquisition	Cycles
1	Initial denaturation		95 °C	600	No	1
2	Amplification	Denaturation	95 °C	15	No	40
		Annealing & Extension	60 °C	60	Yes	

3.3   Data analysis

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

a) Set the threshold: display the amplification curves of one system (e.g. 98140) 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. 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 geometric phase of the curves.

b) Set the baseline: 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 Ct = 25, set the baseline crossing at Ct = 25 − 3 = 22).

c) Save the settings.

d) Repeat the procedure described in a) and b) on the amplification plots of the other system (e.g. *hmg* system).

e) Save the settings and export all the data to a text file 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 Ct-values for each reaction.

The standard curves are generated both for the *hmg* and the 98140 specific systems by plotting the Ct 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 copy numbers in the unknown sample DNA.

To obtain the percentage value of event 98140 DNA in the unknown sample, the 98140 copy number is divided by the copy number of the maize reference gene (*hmg*) and multiplied by 100 (GM% = 98140/hmg x 100).

4. Materials

4.1   Equipment

- Real-time PCR instrument for plastic reaction plates (glass capillaries are not recommended for the described buffer composition)
- Plastic reaction plates suitable for real-time PCR instrument (enabling undisturbed fluorescence detection)

- Software for run analysis (mostly integrated in the software of the real-time PCR instrument)
- Microcentrifuge
- Centrifuge for plates
- Micropipettes
- Vortex
- Rack for reaction tubes
- 0.5/1.5/2.0 mL reaction tubes

4.2 Reagents

- TaqMan® Universal PCR Master Mix No AmpErase UNG. Applied Biosystems Part No 4324018

4.3 Primers and Probes

Table 4. Primers and probes sequences

Name	Oligonucleotide DNA Sequence (5′ to 3′)
98140 target sequence	
DP098-f6 (forward)	5′ – GTG TGT ATG TCT CTT TGC TTG GTC TT – 3′
DP098-r2 (reverse)	5′ – GAT TGT CGT TTC CCG CCT TC – 3′
DP098-p5	6 - FAM 5' –CTC TAT CGA TCC CCC TCT TTG ATA GTT TAA ACT – 3' TAMRA
Taxon specific *hmg* target sequence	
MaiJ-F2 (forward)	5′ – TTG GAC TAG AAA TCT CGT GCT GA – 3′
mHMG-rev (reverse)	5′ – GCT ACA TAG GGA GCC TTG TCC T – 3′
mHMG-probe	6-FAM 5′ – CAA TCC ACA CAA ACG CAC GCG TA – 3′ TAMRA

FAM: 6-carboxyfluorescein; TAMRA: tetramethylrhodamine

Abstract
The European Union Reference Laboratory for GM Food and Feed (EURL-GMFF), established by Regulation (EC) No 1829/2003, in collaboration with the European Network of GMO Laboratories (ENGL), has carried out a collaborative study to assess the performance of a quantitative event-specific method to detect and quantify the 98140 transformation event in maize DNA (unique identifier DP- Ø9814Ø-6). The collaborative study was conducted according to internationally accepted guidelines (1, 2).
In accordance to Regulation (EC) No 1829/2003 of 22 September 2003 on genetically modified food and feed and with Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003, Pioneer Overseas Corporation provided the detection method and the samples (genomic DNA extracted from homogenised seeds containing the transformation event and from conventional homogenised seeds). The EURL-GMFF prepared the validation samples (calibration samples and blind samples at unknown GM percentage [DNA/DNA]). The collaborative study involved twelve laboratories from seven European countries.
The results of the international collaborative study met the ENGL performance requirements. The method is therefore considered applicable to the control samples provided, in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004.
The results of the collaborative study are made publicly available at http://gmo-crl.jrc.ec.europa.eu/.

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