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COMMUNITY REFERENCE LABORATORY FOR GM FOOD AND FEED

CRL assessment on the validation of an event specific method for the relative quantitation of maize line MON 810 DNA using real-time PCR as carried out by Federal Institute for Risk Assessment (BfR)

Biotechnology & GMOs Unit
Institute for Health and Consumer Protection
DG Joint Research Centre

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Executive Summary

An event-specific method for the quantitation of maize MON 810 by means of real-time PCR has been validated in a collaborative trial by the Federal Institute for Risk Assessment (BfR) in collaboration with the American Association of Cereal Chemists (AACC), Joint Research Centre (JRC) of the European Commission (EC), Institute for Reference Material and Measurement (IRMM), the Institute for Health and Consumer Protection (IHCP) and GeneScan, Berlin.

The trial involved fifteen laboratories and was conducted according to internationally accepted guidelines.

The method is annexed to the standard ISO 21570:2005, "Foodstuffs -- Methods of analysis for the detection of genetically modified organisms and derived products -- Quantitative nucleic acid based methods".

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1. General information

This protocol describes an event-specific detection and quantitative TaqMan® PCR procedure for the relative determination of event MON 810 maize in total maize. The real time PCR was optimized for block thermal cycler. Template DNA extracted should be tested for quality and quantity prior to PCR assay.

For specific detection of event MON 810 maize a 92 bp fragment of the single copy DNA integration-border region of the genomic sequence and the inserted sequence element originating from CaMV (35S promoter) as a result of *in vitro* recombination present in the genetically modified insect-protected MON 810 ("YieldGuard") maize (Monsanto) is amplified in TaqMan® PCR.

For relative quantitation of MON 810 maize, a 79 bp fragment of the taxon specific maize (*Zea mays*) high mobility group protein gene (hmg) gene using a gene specific combination of primers and probe is amplified.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called "Ct-value". For quantitation of the amount of event MON 810 maize in a test sample, event MON 810 and hmg Ct values are determined for the sample. A standard curve procedure is then used to calculate the relative number of MON 810 specific genome copies to total maize genome copies.

2. Validation status

The method was optimized for ground maize seeds (certified reference materials [CRM IRMM-413]), containing mixtures of genetically modified MON 810 and conventional maize.

The reproducibility and accuracy of the method was tested through a collaborative study using samples at different GMO contents.

The method was originally developed for the ABI PRISM® 7700 Sequence Detection System (SDS).

This method has been validated in a collaborative study conducted by the Federal Institute for Risk assessment (BfR) in collaboration with The American Association of Cereal Chemists (AACC), Joint Research Centre (JRC) of the European Commission (EC), Institute for Reference Material and Measurement (IRMM) and Institute for Health and Consumer Protection (IHCP) and GeneScan.

The operational procedure of the collaborative study comprised the following modules:

- DNA extraction: GENESpin DNA extraction system (GeneScan)

- Quantitative real-time PCR (Polymerase Chain Reaction): for detection of event MON 810 maize a 92 bp fragment of the single copy DNA integration-border region of the genomic sequence and the inserted sequence element originating from CaMV (35S promoter) as a result of *in vitro* recombination present in the genetically modified insect-protected MON 810 ("YieldGuard") maize (Monsanto) was amplified in TaqMan® PCR

The ring-trial 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).

The study was undertaken with 15 laboratories using either the ABI PRISM® 7700, ABI PRISM® 7900 (Applied Biosystems Inc) or the iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories).

Fourteen laboratories from countries all over the world reported results.

For each unknown sample one DNA extraction has been carried out. Each test sample was analyzed by real time PCR in 3 repetitions.
Each participant received 12 unknown samples. The samples consisted of 6 certified reference materials (CRM IRMM-413) between <0,02 % and 5 % GM MON 810 in conventional maize (w/w).

Each laboratory received each level of GM MON 810 CRM in two separate unknown samples.
Details of the results of the collaborative study performed in 2003/2004 are shown in table 1.

Table 1: Statistics of the collaborative study for the real time PCR procedure to quantify MON 810 specific material.

Sample	Sample 1 <0.02 %	Sample 2 0.1 %	Sample 3 0.5 %	Sample 4 1 %	Sample 5 2%	Sample 6 5 %
Number of laboratories reported	11	14	14	14	14	14
Number of outliers	1	1	0	2	0	0
Number of laboratories retained after eliminating outliers	10	13	14	12	14	14
Mean value (%)	0.028	0.1023	0.4613	0.8327	1.7814	4.5154
Repeatability standard deviation s_r	0.00736	0.03641	0.9606	0.13744	0.28385	1.29374
Repeatability relative standard deviation RSD_r (%)	26.27	35.60	20.82	16.51	15.93	28.65
Repeatability limit r (r = 2,8 x s_r)	0.0206	0.1019	0.269	0.3848	0.7948	3.6225
Reproducibility standard deviation s_R	0.02326	0.04646	0.20068	0.26534	0.56609	1.65451
Reproducibility relative standard deviation RSD_R (%)	83.03	45.43	43.5	31.86	31.78	36.64
Reproducibility limit R (R=2,8 x s_R)	0.0651	0.1301	0.5619	0.743	1.5851	4.6326
Bias (%)	-	2.3	- 7.74	- 16.73	- 10.93	- 9.69

a Outliers were identified with the Grubbs and Cochran tests

These results are evaluated with respect to the method acceptance criteria and to the method performance requirements, as established by ENGL and adopted by CRL. In table 1, estimates of both repeatability and reproducibility for each GM level are reported, after identification and removal of outliers through Cochran and Grubbs tests, according to ISO 5725-2.

Method bias, which allows estimating trueness, is reported for each GM level in table 1. Bias is estimated according to ISO 5725 data analysis protocol. According to ENGL method performance requirements, trueness should be □25% throughout the whole dynamic range. The method satisfies such requirement for all GM values tested.

The relative reproducibility standard deviation (RSDR), that describes the inter-laboratory variation, should be below 33% at the target concentration and over the majority of the dynamic range, while it should be below 50% at the lower end of the dynamic range. As it can be observed in table 1, the method satisfies this requirement at the target concentration (1%) and at GM level of 0.1% and 2%; a minor deviation can be seen at 5% (36.64), while the RSDR at GM level 0.5% is 43.5

3. Specificity

Specificity tests prior to the study showed no cross reactivity of the detection systems to the following non-target species/samples: soybean DNA.

No cross reactivity has been occurred with the following genetically modified maize: Event176, Bt11, T25, GA21 and GTS 40-3-2 soybean.

4. Limit of detection (LOD)

According to the method developer, the absolute LOD has been determined to be 5 copies of the target sequence

According to the method developer, the relative LOD has been demonstrated to be at least 0.1 % (w/w).

5. Limit of quantitation (LOQ)

According to the method developer, the absolute limit of quantitation has been determined to be 10 copies of the target sequence.

According to the method developer the relative limit of quantitation has been determined to be at least 0.1% (equal to the lowest concentration point of the calibration curve used; [w/w]).

6. Procedure

All handling of reagents and controls should occur in an ISO 17025 environment or equivalent.

Further appropriate ISO/EN Norms dealing with the detection and quantitation of GMO derived material should be taken into consideration.

7. Primer/probe systems

The following primers and TaqMan® probes were used in the collaborative study (table 2).

Table 2 : Primer and probe sequences

Name	Oligonucleotide DNA sequence	Final conc. in PCR
Reference gene target sequence		
ZM1-F	5´-TTg gAC TAg AAA TCT CgT gCT gA-3‘	300 nmol/l
ZM1-R	5´-gCT ACA TAg ggA gCC TTg TCC T-3‘	300 nmol/l
Probe ZM1	5´-FAM—CAA TCC ACA CAA ACg CAC gCg TA-TAMRA-3‘	160 nmol/l
GMO target sequence		
Mail-F1	5´-TCg AAg gAC gAA ggA CTC TAA CgT-3´	300 nmol/l
Mail-R1	5´-gCC ACC TTC CTT TTC CAC TAT CTT-3´	300 nmol/l
Probe Mail-S2	5´-FAM-AAC ATC CTT TgC CAT TgC CCA gC-TAMRA P-3´	180 nmol/l
FAM: 6-carboxylfluorecein, TAMRA: 6-carboxytetramethylrhodamine		

8. Sample extraction

For DNA extraction the GENESpin DNA extraction system (GeneScan) was used according to the manufacturer’s instruction.

9. PCR set-up

The PCR set-up for the taxon specific target sequence and for the GMO target sequence should be carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.

The method is described for a total volume of 25 µl per reaction mixture with the reagents listed in Table 3.

Table 3: Amplification reaction mixture in the final volume/concentration per reaction vial

Total reaction volume		25 µl
Template DNA added (2,3 ng to 150 ng maize DNA)		5 µl
DNA polymerase	AmpliTaq Gold® (Applied Biosystems Inc)	1.25 U
Decontaminati on system	dUTP AmpErase uracil N-glycosylase	400 µmol/l 0.5 U
Reaction buffer	TaqMan™ buffer A	1 fold
	$MgCl_2$	6.5 mmol/l
Primers	see Table D.6	see Table 2
DNTP	dATP. dCTP. dGTP	200 µmol/l each
Probe	see Table 2	see Table 2

As a positive control and as calibrant reference material, certified reference materials of MON810 (material containing <0.02 % to 5 % of genetically modified maize) produced by IRMM, Geel, Belgium (IRMM-413 series) may be used.

A series of 1:4 dilution steps of DNA from 5 % CRM is used to establish the standard curves for the MON 810 specific and hmg specific PCR, respectively.

10. Temperature-time-programme

The temperature-time-programme as outlined in Table 4 was optimised for the ABI PRISM® 7700 Sequence Detection System (Applied Biosystems Inc). In the validation study it was used in combination with the AmpliTaq Gold® DNA polymerase. Table 4 describes the reaction conditions.

Table 4: Procedure - Reaction conditions

		Time (s)	**Temperature (°C)**
Pre-PCR – decontamination (optional)		120	50
Pre-PCR – activation of DNA polymerase and denaturation of template DNA		600	95
PCR (45 cycles)			
	Denaturation	15	95
	Annealing Elongation	60	60

11. Data analysis

The baseline range is usually set to cycles 3 to 15. If amplifications do not appear before cycle 20, the baseline stop can be extended to cycle 20.

After defining a threshold value within the logarithmic phase of amplification (e.g. 0.01 to 0.1 normalized reporter dye fluorescence [Rn]) the instruments software calculates the Ct values for each reaction. The Ct values measured for the calibration points in the taxon specific maize or MON 810 specific PCR system, respectively, are plotted against the natural logarithm

of the DNA copy numbers introduced into PCR. The copy numbers measured for the unknown sample DNA are obtained by interpolation from the standard curves.

A calibration curve is produced by plotting Ct values against the logarithm of the target copy number for the calibration points.

For the determination of the amount of MON 810 DNA in the test sample, the MON 810 copy number is divided by the number of maize genome equivalents and multiplied by 100 to get the percentage value.

12. Conclusions

The overall method performance has been evaluated with respect to the method acceptance criteria and method performance requirements recommended by the ENGL (available at http://gmo-crl.jrc.it).

The results obtained during the collaborative trial indicate that the method can be considered as fit for enforcement purposes with respect to its trueness and inter-laboratory variability, taking into account the observations on RSD_R reported above.

13. References

ISO 21570:2005, "Foodstuffs -- Methods of analysis for the detection of genetically modified organisms and derived products -- Quantitative nucleic acid based methods".