

Validation of a Real-time PCR On-Site Quantification Method for MON810 Maize

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Abstract

A rapid on-, or near-site, quantitative method for use as a pre-harvest predictive decision tool for adventitious genetically modified (GM) presence (AGMP) has been developed. Based on a laboratory-based protocol for real-time (RT) quantification of the MON810 GM event in maize kernels, the duplex RT polymerase chain reaction (PCR) method was constructed around the portable Cepheid SmartCyclerII platform, requiring only modest support infrastructure for field application. Pre-validation through an international ring trial showed good compliance with minimum assay performance requirements as defined by the European Network of GMO Laboratories ($RSD_r = 18.5\%$; $RSD_R = 32.8$; $Bias = 26.7\%$).

Introduction

In the European Union (EU), co-existence is within the competence of individual Member States, with Commission Recommendation 2003/556/EC setting out recommended guidelines and best practices to assist individual Member States in the development of their national strategies or other approaches to co-existence (some of which have already developed specific legislation). Further, at the behest of the European Agriculture Council, non-binding crop-specific measures will be developed to help facilitate this recommendation aimed at assisting farm-level production at or below the arbitrary EU adventitious GM presence (AGMP) threshold of 0.9% (Commission Regulation 1829/2003). Accidental breach of co-existence measures may result in AGMP above 0.9% or at a level which consequently could result in a level above 0.9% further down the production stream. Assuming that, as in the current climate, non-GM products will have a price premium, this would incur a cost for the non-GM producer. The most common methods for GM quantification are based on real-time PCR. In the past, this has required complex and dedicated laboratory-based instrumentation unsuitable for on-site use. However, recent developments in technology and analytical biochemistry, fuelled by the need for rapid, specific, on-site detection of pathogens have made on-site real-time PCR possible. It is not envisaged that routine on-site real-time PCR would be practical or desirable, but on-site real-time PCR testing could become cost-effective in some GM co-existence scenarios. Current on-site testing is performed using lateral flow immunoassays, which are relatively cheap (a few Euros per test) and quick (~20 minutes) (Grothaus *et al.*, 2006). However, although these methods are not quantitative, quantitation can be achieved statistically by using seed pools (Redmund *et al.*, 2001), but to achieve accuracy equivalent to real-time PCR, the cost advantage is lost. Aside to co-existence regimes, on-site quantitative testing may be especially useful in monitoring of pre-approval GM field trials (where risk impacts may be higher) and monitoring/management of unauthorised GM releases, including malicious releases.

The Cepheid SmartCyclerII instrument is a rugged, portable real-time PCR machine capable of quantification accuracy and repeatability equivalent to non-portable laboratory-based systems. It has successfully been used for on-site detection of human pathogens (Kowalski *et al.*, 2006; Thompson *et al.*, 2008) and plant pathogens (Tomlinson *et al.*, 2005). To our knowledge it has not been previously used quantitatively for on-site plant applications. This paper describes a maize kernel or flour DNA extraction and purification process and event-specific real-time quantitative TaqMan[®] PCR method for determination of the relative content of GM event MON810 DNA to total maize DNA (%GM DNA) in a sample optimised for use on the portable Cepheid SmartCyclerII instrument.

Materials & Methods

The main drawback of the Cepheid SmartCyclerII instrument compared to most laboratory-based real-time PCR systems is the limited sample capacity, which is 16 (although up to 6 machines can be run simultaneously from a single computer this limits portability). However, similar to other systems, the SmartCyclerII can multiplex assays using multiple fluorescence channels (four available). Unfortunately, quantitative multiplexing is severely affected by competition between assays in the same tube. Therefore to ensure robustness, we limited the assay to a duplex between the endogenous plant reference and a single event specific GM assay.

For the specific detection of GM event MON810, a 98 bp fragment of the region that spans the 5 insert-to-plant junction (P-35S/plant junction) in maize event MON810 was amplified using two specific primers. PCR products were measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labeled with two fluorescent dyes: tetrachlorofluorescein (TET) as a reporter dye at its 5' end and Black Hole Quencher (BHQ1) as a quencher dye at its 3' end. The 5'-nuclease activity of the Taq DNA polymerase is exploited, which results in the specific cleavage of the probe, leading to increased fluorescence, which was monitored.

For the relative quantification of event MON810 DNA, a maize-specific reference system amplified a 134 bp fragment of *Adh1-1F* (*adh1*), an allele of a maize endogenous gene, using a pair of *adh1* gene-specific primers and an *adh1* specific probe labeled with 6-carboxyfluorescein (FAM) and tetramethylrhodamine (TAMRA) as described above (Hernandez *et al.*, 2004).

The emission peaks of the reporter dyes of the MON810 and *adh1* Taqman assay allow their simultaneous measurement in the same single reaction (a duplex Taqman assay), i.e. both primer and probe sets were used in the same reaction.

The method was calibrated with DNA solutions prepared in the laboratory and used *on site* as solutions. DNA for this purpose was extracted from GM (F1 hybrid) and non-GM (independently tested with qualitative PCR and protein-based methods) maize kernel seedlings after four day's germination. The seedlings were dissected from the endosperm to ensure contamination with endosperm was avoided (whose presence would have complicated %GM DNA measurement). Note, the F1 heterozygote GM seedling DNA contained 50% GM DNA. The DNA was accurately quantified using Pico Green fluorescence (Molecular Probes Inc.) and mixtures were made containing 2, 0.5, 0.1 and 0 % GM DNA at 20 ng/ μ l total DNA). A standard curve was constructed of Δ Ct (MON810 assay Ct value minus *adh1* maize endogenous DNA assay Ct value) versus Log_{10} of MON810 concentration (as % GM DNA).

This protocol has been tested using maize kernels, giving a detection range from 50% to 0.05% MON810 GM DNA. The method has been validated for quantification down to 0.1% GM DNA using 100ng total DNA per reaction with the accuracy and precision shown below. The method is intended for use in non-laboratory conditions using portable equipment, however, the calibration standards' DNA preparation is best performed in the laboratory.

Equipment critical to the operation of the method is the Cepheid Smartcycler II real-time PCR system, model SC1000-2 (US patent 5589.136). For DNA purification, the method has been optimised using the Qiagen (GmbH, D-40724 Hilden) DNeasy Plant Mini Kit (part 69104). All other equipment, centrifuges, water baths, blenders etc. have been interchanged between several models without affecting the DNA extraction and purification method.

The DNA extraction and purification are given below for maize seedlings (GM DNA standards) and ground kernels (on site samples). In the field, several small-scale aliquots of ground flour will be required to be extracted, purified and tested. It is not in the current scope of this method to specify the size of maize kernel samples from fields or the number of aliquots to be tested (see Allnutt *et al.*, 2008). This method is designed to accurately quantify one 1000 kernel sample, using four replicate DNA extractions from the analytical sample.

Extraction and Purification Protocols.

Reagents: Qiagen DNeasy Plant Mini Kit (part 69104). 100 × TE buffer: 1 M Tris base, 100mM EDTA, pH 8. Pico Green DNA quantification kit (Molecular Probes Inc. part: P7589).

Maize seedling DNA extraction for %GM DNA standards preparation

Use only independently certified/tested GM and non-GM maize kernels. Perform the method below for both GM and non-GM kernels separately to obtain two stock solutions.

1. Germinate approximately 50 of both GM and non-GM maize kernels (on damp tissue in a covered container for two days). As soon as the seedlings have emerged, dissect the kernels, removing the seedling tissue from the endosperm until 1 g each of seedlings is obtained.
2. Grind the seedlings to a fine powder with a mortar and pestle using liquid nitrogen.
3. Follow the Qiagen DNeasy instructions to make up 10 ml lysis solution (enough for the GM and non-GM extraction).
4. Add 4 ml of lysis solution to the powder and mix into a paste. Label two 15 ml Falcon centrifuge tubes, 'GM' and 'non-GM', and transfer the paste to the corresponding tube. Incubate at 65 °C for 30 minutes, shake vigorously every ten minutes.
5. Centrifuge the lysate for 10 mins, 5000 × g.
6. Remove the supernatant to a new 15 ml Falcon tube.
7. Follow the Qiagen DNeasy instructions for purification using the mini-spin columns, using four columns for the entire supernatant from each Falcon tube (binding 400 µl aliquots of supernatant to each of four columns until all

supernatant has been passed through the columns). Eight columns in total are therefore used four for GM DNA and four for non-GM DNA. It is not necessary to use the optional 'shredder column' steps of the Qiagen procedure. Column binding and washing will be much quicker if a vacuum manifold can be used instead of the microfuge method.

8. Continue with the Qiagen DNeasy instructions for purification using the mini-spin columns until the DNA elution step, use $2 \times 200 \mu\text{l}$ of EB rather than the recommended $2 \times 100 \mu\text{l}$ for each of the four columns (total eluted volume over four columns = 1.6 ml).
9. Add $16 \mu\text{l}$ of $100 \times$ TE to the DNA solution and store at 4°C .

N.B. If a 50 ml tube volume capacity centrifuge is available, the Qiagen DNeasy Plant Maxi kit can be used instead, using one column for the entire lysate.

GM DNA standards quantification and preparation

Using the Pico Green DNA quantification kit, follow the manufacturers instructions to obtain an estimate ($\pm 5\%$) of DNA concentration ($\text{ng}/\mu\text{l}$) for the GM and non-GM maize seedling DNA. Calculate the volume required of GM, non-GM and $1 \times$ TE buffer required to make 2, 0.5, 0.1 and 0% GM DNA solutions each containing $20 \text{ ng}/\mu\text{l}$ total maize DNA. Mix each solution thoroughly and store at 4°C .

Therefore, as an example, for 1 ml of 2% GM DNA, $20 \text{ ng}/\mu\text{l}$ total maize DNA standard:

$$\text{volume of GM DNA } (\mu\text{l}), A = \frac{p \times t}{c} \times V$$

$$\text{volume of non-GM DNA } (\mu\text{l}), B = \frac{(1-p) \times t}{c} \times V$$

$$\text{volume of TE } (\mu\text{l}), C = 1000 - A - B$$

where p = target proportion of GM DNA (target percentage divided by 100); t = total DNA target concentration $20 \text{ (ng}/\mu\text{l)}$, V = total volume (μl); c = working DNA solution concentration ($\text{ng}/\mu\text{l}$), typically the GM stock must be diluted to a 1:10 working solution for the range of concentrations recommended here. Other dilutions may be necessary to achieve the total volume if the GM DNA is more concentrated. To ensure accurate dilutions, The volume of GM DNA working solution used should not be below $10 \mu\text{l}$. Note that for F1 seedlings (heterozygous) the GM DNA concentration will be half the total DNA concentration as estimated by the Pico Green method. Note there is a real but negligible error from an excess contribution of non-GM DNA from the heterozygous GM maize genome which does not need to be included in calculations.

Qiagen DNeasy Plant mini Kit DNA isolation method from sample kernels (for on-site samples)

The following method is designed for ~200 mg aliquots of ground maize kernels from 1000 kernel samples. Each 1000 kernel sample may be the sole analytical sample or a sub-sample of the analytical sample (which usually consist of at least 3000 kernels total). Prepare sufficient Qiagen DNeasy lysis buffer for all samples beforehand, according to the kit instructions.

1. Place the 1000 kernel sample in a clean kitchen blender or coffee grinder. Grind at full power for two minutes. Stop the blender/grinder occasionally to mix with a spatula any un-blended lumps and further homogenise the sample. Ensure there are no visible kernels - if there are then disturb them with a spatula and continue blending for another minute. Required grinding time will be affected by several factors such as kernel water content and the model of blender used. *N.B. As fine a powder as practical is required from the kernels but it is not possible to quantify this in on-site conditions. However, longer grinding may result in increased DNA degradation. Kernels may vary greatly in their toughness and moisture content and some grinding may result in a paste rather than a powder. Pastes will be more susceptible to DNA degradation than powders and should not be allowed to become warm through excessive grinding. The use of four replicate samples of the ground sample is intended to negate the effect of heterogeneity in the ground sample which may occur on-site.*
2. Remove four test samples of approximately 200 mg each from the ground analytical sample. Take the four test samples from different layers of the analytical sample (e.g. top, two middle, bottom), suffix label 1, 2, 3, 4. i.e. for analytical sample 'A', the four replicates are; A1, A2, A3, A4.
3. Transfer the remaining flour to a suitable sealed plastic container and store at 4°C.
4. Add 800 µl Qiagen DNA easy lysis buffer to 1 - 4, mix thoroughly and incubate at 65 °C for 30 minutes. Mix every ten minutes.
5. Centrifuge at full speed in a microcentrifuge for five minutes. Transfer 400 µl of supernatant to a new tube and follow Qiagen DNA easy instructions for purification, omitting the optional 'shredder column' steps. Elute DNA twice using 2 × 100 µl EB so that the total volume eluted is 200 µl. Add 2 µl 100 × TE to the eluate in a 1.5 ml Eppendorf microcentrifuge tube.

Smart Cycler Protocol

Reagents: ABsolute QPCR 2 × mix (no ROX), part number: AB-1132 (ABgene, www.ABgene.com); 10 × dNTP mix solution = 2 mM each TTP, dATP, dCTP, dGTP in water; ADH forward primer: ADHF3-4 CGTCGTTTCCCATCTCTTCC; ADH reverse primer: ADHR4-4 CCACTCCGAGACCCTC; ADH probe: ADH1-MDO FAM-AATCAGGGCTCATTTTCTCGCTCCTCA-TAMRA; GM forward primer: MON810X AAGGACGAAGGACTCTAACGT; GM reverse primer: MON810Y

ATTTGTAGGAGCCACCTTCCT; GM probe: MON810Ztet TET-CGACCTGAACGAGGACTTTCGGTAGCC-BHQ1. Oligonucleotides were obtained from Sigma Genosys, (http://www.sigmaaldrich.com/Brands/Sigma_Genosys.html). All solutions were stored at -20°C long-term, 4°C medium-term.

Each reaction had a 25 µl volume. A master mix was prepared containing for each reaction: 12.5 µl ABsolute QPCR 2 × mix; 1 µl of 5 µM each primer stock (for all four primers = 4 µl); 0.5 µl of 5 µM each probe stock (for two probes = 1 µl); 2.5 µl 10 × dNTP mix stock (2 mM each TTP, dATP, dGTP, dCTP).

Assay calibration and analysis

The MON810 assay was calibrated using DNA extracted from independently tested GM and non-GM maize seedlings. MON810 and *adh1* assays were performed on the same standard series in a duplex reaction. Where a single standard Δ Ct value was judged to be anomalous, i.e. significantly deviating from the linear relationship to Log_{10} GM DNA concentration of the other standards, it was excluded from the analysis. If more than one standard value significantly deviated, then the entire run was repeated. Similarly, if one replicate sample's Ct value deviated significantly from the others, then it could be excluded. A standard curve was constructed of Δ Ct versus Log_{10} % GM DNA where Δ Ct is the MON810 assay Ct value minus the *adh1* Ct value. A linear regression of the data points provided the equation for calculation of % GM DNA from sample Δ Ct values.

Ring Trial

%GM DNA standards were prepared at the organising laboratory (FERA) and distributed to three others with taqman reagents. Four whole maize kernel samples were also distributed (1.47, 0.589, 0.176 and 0.056 % GM DNA, 1000 kernels total). These were made by mass of F1 kernels, assuming 58 % GM DNA per GM kernel (Trifa and Zhang, 2004). DNA extractions and Smartcycler analyses were duplicated in each laboratory.

Results and Discussion

The duplex *adh1* / MON810 real time PCR assay performed well. Linearity of standard curves was generally good and consistent: 0.992 - 0.998 (means for each laboratory). Only one 'unknown' sample replicate in one lab was required to be excluded under the analysis rules. Table 1 shows the mean values obtained for 'unknowns' by each lab and the relative standard deviation within runs (RSD_r) and among laboratories (RSD_R). The RSD_r , RSD_R and bias estimates at the 0.06 % GM DNA level were above the ENGL minimum performance requirements (25%, 50% and 25% respectively), but this level is lower than the specified dynamic range of the method (50%-0.1%).

Table 1. Ring trial results. Values in bold exceed thresholds as defined under the ENGL Minimum Performance Requirements for Analytical Methods of GMO Testing. Underlined results were excluded as outliers, or having poor RSD_r.

Lab	%GM DNA Level							
	1.47		0.59		0.18		0.06	
	rep1	rep2	rep1	rep2	rep1	rep2	rep1	rep2
A	0.90	1.46	0.47	0.43	0.16	<u>0.26</u>	0.16	0.15
B	1.25	1.16	0.43	0.40	0.15	0.11	0.05	0.04
C	0.95	0.91	0.53	0.33	0.11	0.12	0.05	0.03
D	2.12	1.25	0.53	0.53	0.24	0.20	0.12	0.14
Mean	1.25		0.46		0.16		0.09	
RSD _r	23.18		20.68		23.93		32.56	
RSD _R	25.24		11.23		31.95		63.42	
%Bias	-14.97		-22.67		-6.25		54.17	

The ring trial demonstrated that under current EU acceptance criteria, this on-site method of GM DNA quantification would be acceptable. The variability and accuracy of the results were found to be within the range seen in laboratory-only real-time systems (e.g. Applied Biosystems). The relative simplicity of the Cepheid Smartcycler II system was therefore not a disadvantage.

This on-site method has been designed to be as simple and quick as possible, and also to minimise costs. Despite this, it would not be viable for routine monitoring or analysis. Currently, only high risk co-existence incidences are likely to require this high degree of accuracy, precision and rapid response. The method takes approximately 2 hours from sample grinding to result. It is expected that the sampling of a single field could take an additional half day. Only one operator is required for the analysis, but several (depending on size of field and sampling intensity) would be required for sampling. Currently, we estimate costs per field as follows: two persons, half day collecting 50 samples = 350 Euros; one person sample processing, DNA extraction and real-time PCR, 3 hours = 120 Euros; consumables = 140 Euros; total = 610 Euros. Sampling carried out by the producer would significantly reduce costs.

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