Evaluation of Different Machines Used To Quantify Genetic Modification by Real-Time PCR

Supplementary Material

Ring Trial Methods

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TRIAL 1.

Background
The following method aims to provide performance statistics (RSD) for the TaqMan real-time PCR quantification of GM event Mon810 using IRMM CRMs as standards to quantify five 'unknown' %GM maize DNA samples provided by FERA. In order to minimize variation due to factors other than equipment used, primers, probes and DNA will be prepared at FERA and distributed as a kit to participating labs.

The method below is similar to that recently validated in the EU project QPCRGMOFOOD. Primer and probe sequences have been provided by Maria Pla, CSIC, Spain.

Quantification is achieved with two assays: a GM target (Mon810) and an endogenous control target (adh1). For the purposes of this study Mon810 probe is labelled with FAM and black-hole quencher 1 (BHQ1) and adh1 probe is labelled with FAM and TAMRA, although we are aware that some equipment may perform better with other fluorescent labels. Results of parallel WP5 work on assay chemistry comparisons may enable other labels to be assessed later. Primer and probe sequences are shown for reference in Table 1.

Table 1. Primer and probe sequences.

Name	Sequence
Adh-F3	5'-CGTCGTTTCCCATCTCTTCCTCC-3'
Adh-R4	5'-CCACTCCGAGACCCTCAGTC-3'
Adh1-MDO	FAM-5'-AATCAGGGCTCATTTTCTCGCTCCTCA-3'-TAMRA
MONF	5'-CAAGTGTGCCCACCACAGC-3'
MONR	5'-GCAAGCAAATTCGGAAATGAA-3'
MONP	FAM-5'-CGACCTGAACGAGGACTTTCGGTAGCC-3'-BHQ1

Some RT-PCR equipment has more limited capacity than others. In particular, the Cepheid Smart Cycler has only 16 sample slots per run. A maximum run size of *12 samples* is proposed in order to prevent larger capacity equipment giving apparently better results because they require fewer runs to complete the analysis. Although this issue is an important factor in equipment selection it is not a factor for investigation in this initial study. In order to ensure we can statistically compare results from each machine each experiment run will only have 12 samples and four runs in total will be performed in the initial phase of the study for the mon810 and adh1 assay **(8 runs in total per machine per lab)**.

Samples

Each participating lab will receive 10 samples as detailed in Table 2 in screw-cap tubes. In each run, the twelfth sample will be a reagent only control. More than sufficient sample will be provided. Each DNA solution will be an identical equivalent aliquot from an homogenous larger volume sample held at FERA. All DNA samples must be used without any further dilution or treatment. DNA samples must be stored at -20°C and defrosted for a minimum time at room temperature only, do not heat the samples to defrost.

Table 2. DNA solutions provided

Number	Name / description
1	CRM 5%
2	CRM 2%
3	CRM 1%
4	CRM 0.5%
5	CRM 0.1%
6	CRM 0 %
7	Unknown A
8	Unknown B
9	Unknown C
10	Unknown D
11	Unknown E

Reaction Set-Up

Reaction components provided by FERA are as shown in Table 3. When setting up reactions, use only pipettes with valid calibration certification. The same operator should set up and run all reactions in each lab. Your labs' normal contamination prevention procedures, e.g. UV cabinets, laminar flow hoods, separate preparation areas etc. should be used, but it is not possible to standardise these across all participating labs. Store all components at -20°C and defrost for minimal length of time at room temperature. **Primers and probes are provided vaccuum dried. To make the 10X stock in Table 3, add 200 µl water (molecular biology grade) to each tube. One tube of each primer and probe will be provided for each piece of equipment to be tested (e.g. if you are testing three machines you will get three tubes of each primer and each probe).**

Table 3. Reaction components provided by FERA.

Component	Concentration
Adh1 assay	
Primer Adh-F3	5 µM (10X)
Primer Adh-R4	5 µM (10X)
Probe Adh1-MDO	2 µM (10X)
Mon810 assay	
Primer MONF	5 µM (10X)
Primer MONR	5 µM (10X)
Probe MONP	2 µM (10X)

Labs must use Applied Biosystems Universal PCR Mastermix (no amperase UNG) 2X mix (Part number: 4324018), called **'ABI-mix'** below. This will not be supplied by FERA.

Complete the 8 required runs (12 reactions in each) for each machine tested in the following chronological order:

1. Mon810 assay replicate 1
2. Adh1 assay replicate 1
3. Mon810 assay replicate 2
4. Adh1 assay replicate 2
5. Mon810 assay replicate 3
6. Adh1 assay replicate 3
7. Mon810 assay replicate 4
8. Adh1 assay replicate 4

For each run, a single master-mix will be made-up as detailed in Table 4. Components should be added in the order shown to a 1.5 ml microcentrifuge tube then briefly centrifuge before use. **20 µl of Master-mix**, and **5 µl DNA sample** are then added to the reaction tubes as shown in Table 5. Use a 200 µl pippette for master-mix components, a 20 µl pippette for transfering master-mix to reaction tubes, and a 5 µl pippette for addition of sample DNA or water. For each machine, reaction tubes should be treated and loaded into the machine as per manufacturers' instructions. **For each run, the position of reaction tubes in the machine must be random. Use a suitable random number generator to do this**. Due to the varying plate / tube formats we cannot recommend a single supplier or type, but labs should use whichever they normally use.

Table 4. Reaction master-mix recipe (no water required).

Component	Volume (µl)
ABI-mix	163
Primer 1	33
Primer 2	33
Probe	33

Table 5. Reaction tube set-up

Tube / Well number	Master-mix (µl)	DNA sample	Water (ul)
1	20	5 µl CRM 5%	0
2	20	5 µl CRM 2%	0
3	20	5 µl CRM 1%	0
4	20	5 µl CRM 0.5%	0
5	20	5 µl CRM 0.1%	0
6	20	5 µl CRM 0 %	0
7	20	5 µl Unknown A	0
8	20	5 µl Unknown B	0
9	20	5 µl Unknown C	0
10	20	5 µl Unknown D	0
11	20	5 µl Unknown E	0
12	20	-	5

Thermal cycling parameters for both Mon810 and Adh1 assays for ABI and Cepheid machines are:

10 minutes 95ºC
15 seconds, 95ºC; 1 minute, 60°C × 45 cycles.

ABI acquisition settings: manual baseline = 3 - 15 cycles; threshold = 0.2 units.
The Cepheid Smartcycler variable analysis settings must be set as follows: 'Bkrd Min cycle' = 5; 'Bkrd Max cycle' = 40; 'Manual Thresh setting units' = 30.

We cannot recommend conditions for Light Cycler or other machines, but if possible they should be as recommended by the manufacturer for the type of primers and probes used in this study. **Labs using machines other than ABI or Cepheid above, and who cannot use these standard reaction conditions, must liase with other labs to ensure identical parameters are used.**

All runs should be carried out within as short a time-span as possible and convenient. We should aim to be able to collate all results by end of April, 2006.

Results

Use the accompanying Excel spreadsheet to record results. Only Ct values are required. FERA will collate the results and perform a statistical analysis. We will also circulate the entire dataset to all partners for their own analysis, should they wish, as soon as it is all received by us.

TRIAL 2: Using duplex TaqMan PCR assay.

Background

The following method aims to provide performance statistics precision and accuracy (%CV and %Bias respectively) for the TaqMan real-time PCR quantification of GM event Mon810 using genomic DNA as standards to quantify five 'unknown' %GM maize DNA samples provided by FERA. In order to minimize variation due to factors other than equipment used, primers, probes and DNA will be prepared at FERA and distributed as a kit to participating labs.

The sequence for adh1 primers and probes in this method was provided by Y. Berteau (Hernandez M. *et al.,*. 2004. Development and Comparison of Four Real-Time Polymerase Chain Reaction Systems for Specific Detection and Quantification of *Zea mays* L. *J. Agric. Food Chem.*, *52,* 4632-4637)

Quantification is achieved with two assays: a GM target (Mon810) and an endogenous control target (adh1). For the purposes of this study Mon810 probe is labelled with TET and black-hole quencher 1 (BHQ1) and adh1 probe is labelled with FAM and TAMRA. Both assays are performed simultaneously in the same reaction (a duplex reaction). Primers and probes and reaction conditions have been optimised to avoid competition between assays. Primer and probe sequences are shown for reference in Table 1.

Table 1. Primer and probe sequences.

Name	Sequence 5' - 3'
ADHF3-4	CGTCGTTTCCCATCTCTTCC
ADHR4-4	CCACTCCGAGACCCTC
Adh1-MDO	FAM-AATCAGGGCTCATTTTCTCGCTCCTCA-TAMRA
MON810X	AAGGACGAAGGACTCTAACGT
MON810Y	ATTTGTAGGAGCCACCTTCCT
MON810ZTET	TET-CGACCTGAACGAGGACTTTCGGTAGCC-BHQ1

The duplex reaction avoids several problems that were in Trial 1 of this study. In Trial 1, adh1 and Mon810 assays had to be performed in different runs due low capacity in the Cepheid Smartcycler. This meant that all possible pairs of adh1 and Mon810 results had to be assessed and delta C_t variability (%CV) was high. The duplex assay halves the number of runs required and provides matched results from adh1 and Mon810 from the same reaction tube. We would therefore expect this to significantly reduce uncertainty compared to Trial 1. It is the aim of this trial to test if this is the case, and to examine if the relative performance of real-time PCR machines found in Trial 1 is affected by using a duplex assay.

Samples

Each participating lab will receive 11 samples as detailed in Table 2 in screw-cap tubes. In each run, the twelfth sample will be a reagent only control, using molecular biology grade water instead of DNA sample. More than sufficient sample will be provided. Each DNA solution will be an identical equivalent aliquot from an homogenous larger volume sample held at FERA. All DNA samples must be used without any further dilution or treatment. DNA samples must be

stored at -20°C and defrosted for a minimum time at room temperature only, do not heat the samples to defrost.

Table 2. DNA solutions provided

Number	Name / description
1	5% GM DNA standard
2	2% GM DNA standard
3	1% GM DNA standard
4	0.5% GM DNA standard
5	0.1% GM DNA standard
6	0 % GM DNA standard
7	Unknown A
8	Unknown B
9	Unknown C
10	Unknown D
11	Unknown E

Reaction Set-Up

Reaction components provided by FERA are as shown in Table 3. When setting up reactions, use only pipettes with valid calibration certification. The same operator should set up and run all reactions in each lab. Your labs' normal contamination prevention procedures, e.g. UV cabinets, laminar flow hoods, separate preparation areas etc. should be used, but it is not possible to standardise these across all participating labs. Store all components at -20°C and defrost for minimal length of time at room temperature.

Primers, probes and dNTPs are provided vacuum dried. To make the stock solutions add the volume of water (molecular biology grade) shown in Table 3, and mix vigorously until dissolved. One tube of each primer and probe and dNTPs will be provided for each piece of equipment to be tested (e.g. if you are testing three machines you will get three tubes of each primer and each probe).

Table 3. Reaction components provided by FERA.

Component	Stock Concentration	Volume of water to add to dry aliquot
Adh1		
ADHF3-4	5 µM	100 µl
ADHR4-4	5 µM	100 µl
Adh1-MDO	5 µM	50 µl
Mon810		
MON810X	5 µM	100 µl
MON810Y	5 µM	100 µl
MON810ZTET	5 µM	50 µl
dNTPs	2 mM	200 µl

Labs must use ABgene Mastermix, ABsolute QPCR 2 × mix (plus ROX), part number: AB-1138 for those machines that require ROX (e.g. ABI). For machines that do not require ROX, use the no ROX mix, number AB-1132 (ABgene, www.ABgene.com), henceforth either is called **'AB-mix'**. This will not be supplied by FERA.

Complete 4 replicate runs (12 reactions in each) for each machine tested. Each replicate run will provide C_t for Mon810 and adh1, and ΔC_t (Mon810 C_t - adh1 C_t).

For each run, a single master-mix will be made-up as detailed in Table 4. Components should be added in the order shown in a 1.5 ml microcentrifuge tube then briefly centrifuged before use. **20 µl of Master-mix**, and **5 µl DNA sample** are then added to the reaction tubes as shown in Table 5. Use a 200 µl pipette for master-mix components AB-mix and dNTPs, a 20 µl pipette for primers, a 10 µl pipette for probe, a 20 µl pipette for transferring master-mix to reaction tubes, and a 10 µl pipette for addition of sample DNA or water. For each machine, reaction tubes should be treated and loaded into the machine as per manufacturers' instructions. **For each run, the position of reaction tubes in the machine must be random. Use a suitable random number generator to do this**. Due to the varying plate / tube formats we cannot recommend a single supplier or type, but labs should use whichever they normally use.

Table 4. Reaction master-mix recipe (for one run = 12 reactions).

Component	Volume (µl)
AB-mix	163
dNTPs	33
Adh-F3	13
Adh-R4	13
Adh1-MDO	7
MON810X	13
MON810Y	13
MON810ZTET	7

Table 5. Reaction tube set-up

Tube / Well number	Master-mix (µl)	DNA sample	Water (ul)
1	20	5 µl 5% GM DNA standard	0
2	20	5 µl 2% GM DNA standard	0
3	20	5 µl 1% GM DNA standard	0
4	20	5 µl 0.5% GM DNA standard	0
5	20	5 µl 0.1% GM DNA standard	0
6	20	5 µl 0 % GM DNA standard	0
7	20	5 µl Unknown A	0
8	20	5 µl Unknown B	0
9	20	5 µl Unknown C	0
10	20	5 µl Unknown D	0
11	20	5 µl Unknown E	0
12	20	-	5

Thermal cycling parameters for ABI and Cepheid machines are:

15 minutes 95ºC

15 seconds, 95°C; 90 seconds, 60°C × 45 cycles.
N.B 50°C step not required

ABI acquisition settings: manual baseline = 3 - 15 cycles; threshold = 0.2 units.
The Cepheid Smartcycler variable analysis settings must be set as follows: 'Bkrd Min cycle' = 5; 'Bkrd Max cycle' = 40; 'Manual Thresh setting units' = 30. Other settings can be used if unavoidable. Data collection is only required for the annealing step.

All machines used must be capable of measuring the fluorophores used. If TET is not specified by the manufacturer, then use the VIC channel for obtaining the Mon810 C_t.

We cannot recommend conditions for Light Cycler or other machines, but if possible they should be as recommended by the manufacturer for the type of primers and probes used in this study. **Labs using machines other than ABI or Cepheid above, and who cannot use these standard reaction conditions, must liase with other labs to ensure identical parameters are used. PLEASE NOTE THAT THE ANNEALING TIME IS LONGER THAN STANDARD (90 SEC VS 60 SEC), THIS WAS FOUND TO BE CRITICAL TO THE DUPLEX METHOD - 90 SECONDS MUST BE USED.**

All runs should be carried out within as short a time-span as possible and convenient. We should aim to be able to collate all results by end of October, 2006.

C_t values should be recorded in the accompanying Excel spreadsheet (trial2results.xls) with the name of the lab and the machine appended to the file name. As in Trial 1, all results will be collated and distributed to all partners.

Results

Use the accompanying Excel spreadsheet to record results. Only Ct values are required. FERA will collate the results and perform a statistical analysis. We will also circulate the entire dataset to all partners for their own analysis, should they wish, as soon as it is all received by us.