FOOD COMPOSITION AND ADDITIVES

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

THEODORE R. ALLNUTT
Food and Environmental Research Agency, Sand Hutton, York, YO411LZ, United Kingdom
MIRA AYADI
French National Institute for Agricultural Research (INRA), RD 10-78 026 Versailles, France
GILBERT BERBEN
Walloon Agricultural Research Centre (CRA-W), B-5030 Gembloux, Belgium
PETER BRODMANN
Biolytix AG, Benkenstrasse 254, Witterswil, CH-4108, Switzerland
DAVID LEE
National Institute of Agricultural Botany, Huntington Rd, Cambridge CB30LE, United Kingdom

Quantification of genetic modification (GM) is often undertaken to test for compliance with the European Union GM labeling threshold in food. Different control laboratories will often use common validated methods, but with different models of real-time PCR machines. We performed two separate ring trials to evaluate the relative precision and accuracy of different types of real-time PCR machines used to quantify the concentration of GM maize. Both trials used dual-labeled fluorogenic probes for quantification. The first ring trial used separate GM and reference assays (a single fluorescence channel), and the second used a combined duplex assay (two simultaneous fluorescence channels). Five manufacturers and seven models—including a 96-well microtiter-plate, rotary, and portable machines—were examined. In one trial, the machine used had a significant effect on precision, but in the other it did not. Overall, the degree of variation due to the machine model was lower than other factors. No significant repeatable difference in accuracy was observed between machine models. It was not possible to use sufficient replication of machine type in each laboratory to examine all sources of variation in this study, but the results strongly indicate that factors other than machine type or manufacturer (e.g., method or laboratory) contribute more to variation in a GM quantification result.

Since the introduction of a threshold (0.9% GM DNA) for adventitious presence of genetic modification (GM) in food and feed European Union (EU) regulations 1830/2003, 1829/2003, and recommendation 2004/787, European GM testing laboratories have increased efforts to produce validated GM quantification methods (*see* http: // bgmo.jrc.ec.europa.eu/home/ict/methodsdatabase.htm). These methods predominantly use real-time PCR requiring special types of PCR machines that provide thermocycling for DNA amplification and a fluorescence quantification system (using excitation lasers) to monitor and quantify DNA targets as they are amplified. The EU GM Community Reference Laboratory (CRL) at the Joint Research Center has organized several validation exercises of real-time PCR methods for specific GM events, now required under legislation. These validations usually use several laboratories with similar or identical real-time PCR machines, most commonly Applied Biosystems (AB) models. An increasing variety of real-time PCR machines is becoming available, with many cheaper and more portable than the AB types. No data are available comparing the performance of different machines for GM quantification. Currently, therefore, it would be difficult for competent EU authorities to attach confidence to a test result that used a machine other than those used in CRL and similar validation exercises.

In this study we have conducted two separate ring trials using a variety of real-time PCR machines. The aim was to obtain comparable performance data (precision and accuracy) from a wide selection of machines using uniform methods and test materials. Unfortunately, because individual laboratories do not generally possess more than two or three types of machines, it was not possible to eliminate variation due to laboratory from this study by replicating all machines within each laboratory. Also, due to practical cost considerations, the level of replication for each machine type was low, so it was not possible to test the significance of the effect of any particular machine. We therefore aimed to obtain an overall picture of the variation obtained among laboratories and different machines, and assess the scale of this variation in

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Corresponding author's e-mail: theodore.allnutt@fera.gsi.gov.uk

Table 1. Real-time PCR machines included in this study[a]

Machine	Lab	Sample bed format	Excitation source	Multiplex channels	Producer
AB7500[b]	P	96-well plate	Tungsten halogen	5	Applied Biosystems
AB7700	P, Q, S	96-well plate	Argon laser	5	Applied Biosystems
AB7900 × 2	P, Q	96- or 384-well plate	Argon laser	6	Applied Biosystems
CSII	P	16 tubes	LED	4	Cepheid
CHR4	Q	96-well plate	LED	4	Bio-Rad
AB7000[c]	6	96-well plate	Tungsten halogen	4	Applied Biosystems
RG3000	S	36/72 tubes rotary	LED	6	Corbett
RLC[c]	T	32 capillaries	LED	1	Roche

[a] AB = Applied Biosystems; CSII = Cepheid Smartcycler II; CHR4 = Bio-Rad Chromo4; RG3000 = Corbett Research Rotor-Gene 3000; RLC = Roche Light Cycler; LED = light-emitting diode.

[b] Not used in Trial 1.

[c] Not used in Trial 2.

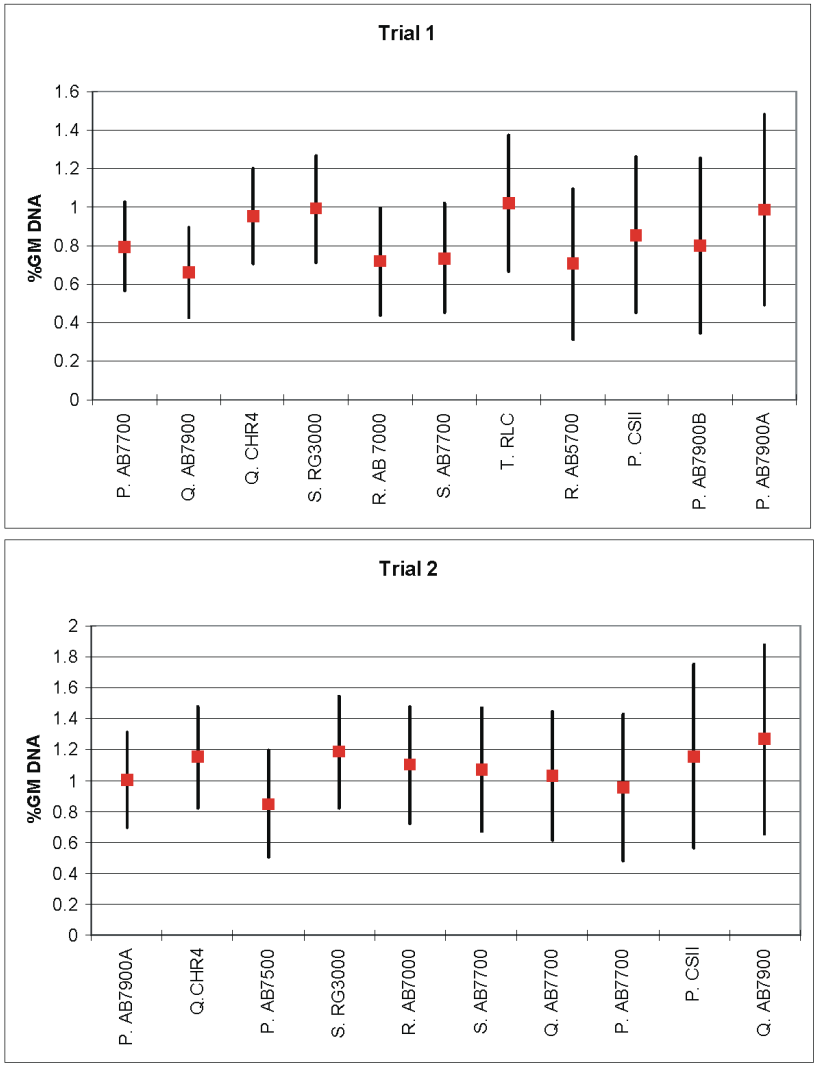

Figure 1. Mean (box) and variance (black bars) normalized values for GM quantifications in Trials 1 and 2. Results are ranked from most to least precise, from left to right.

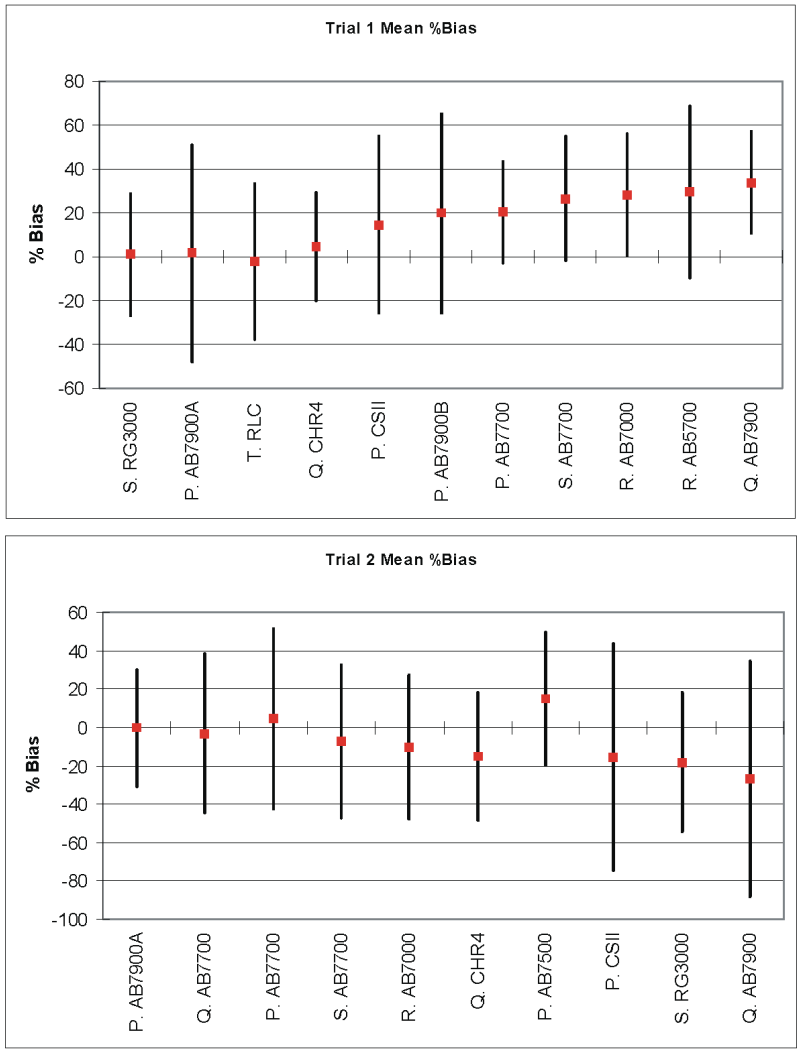

Figure 2. Mean bias (box) and variance of bias (black bars) in Trials 1 and 2. Results are ranked from lowest to highest absolute bias, from left to right.

comparison to CRL validation studies, which use a much narrower range of real-time machine types.

METHOD

Both trials used a real-time PCR quantification method for GM event, Mon810. Trial 1 used separate reactions for GM and reference assays (simplex reactions), and Trial 2 used a duplex reaction with GM and reference assays in the same tube with different reporter fluorophores. The GM assay part of the method was based on that by Hernandez et al. (1). Trial 1 was expected to give more intrinsically variable results due to the extra factor of two separate assays (GM and maize reference). Trial 2 was expected to be more precise in this respect, but was also expected to highlight any differences in machine when required to measure two relatively similar wavelength reporter dyes in the same reaction (FAM and TET, 520 and 539 nm, respectively). Standards and test materials were prepared separately for each trial. Detailed information is available free of charge at http://www.gm-inspectorate.gov.uk/gm_rand.cfm.

A consortium of five laboratories performed the trials using 12 machines, including eight different models (Table 1). The main design differences between machines were sample bed format, laser source and bandwidth, and number of detection channels. Six machines had microtiter plate-based sample beds (96 or 384 format); the Cepheid (Sunnyvale, CA) Smartcycler II used specialized individual static tubes; and the Roche (Burgess Hill, UK) Light Cycler and Corbett Research (Sydney, Australia) Rotor-Gene 3000 both used specialist tubes in a rotary format. All machines except the Roche Light Cycler were capable of measuring two or more fluorescence wavelength channels. Before this work, it was expected that the fluorescence filter bandwidths would be a critical factor in machine accuracy, and this may have been variable among machines due to the quality of optics and the source, power, and bandwidth of excitation lasers (argon or light-emitting diode).

Detailed real-time PCR protocols for both trials are available as supplementary data (unknown sample E in these methods was a blank and was not included in this analysis). As

far as possible, the protocols allowed for reagents, volumes, and other conditions to be identical among different machines. Both trials contained a set of DNA standards and a set of unknown DNA samples. A large master DNA solution of each was prepared, and aliquots were distributed to partners. In Trial 1, the standards used were extracted from Institute for Reference Materials and Measurements % w/w Mon810-certified reference material. These standards are made by mass from mixtures of flour. However, the genotypes of the tissues of maize grain (and their proportions) are variable (2) and % w/w standards can, therefore, provide useful relative comparisons of accuracy but cannot provide absolute standards. In Trial 2, % GM DNA standards were made by extracting DNA from GM and non-GM F1 seedlings, minus any kernel tissue. The DNAs were quantified relatively by fluorescence (pico green) and specific TaqMan assay (*adh*1 gene). Note that the less variable *adh*1 sequence was used, which does not contain previously described polymorphisms (3). Thus, 50% GM (F1 heterozygous somatic tissue) and 100% non-GM DNA of known relative concentrations (estimated from absolute pico green fluorescence and relative *adh*1 TaqMan assay) were mixed to provide known %GM DNA standards.

Within each laboratory, four quantification replicates were performed, each containing six standards, four unknown samples (1, 0.5, 0.1, and 0.05% for Trial 1; and 1, 0.5, 0.15, and 0.05% for Trial 2), and one blank. For Trial 1, this entailed eight machine runs (Mon810 and *adh*1 separately for each quantification) and for Trial 2, four machine runs. All data were normalized to 1.0%, and analysis of variance (ANOVA) was performed with the following structure: a) among replicate runs within each machine; b) among the same machine model in different laboratories; c) among different machine models in the same laboratory; d) among different machines among all laboratories; and e) between Trial 1 and Trial 2. Given the cost of real-time PCR machines, it was not possible to replicate sufficiently for statistical significance of all interactions in the data (e.g., each laboratory has a large quantity of each machine). Given these limitations, the analysis was expected to give a good comparison of using a large variety of machines (in these trials) and a restricted variety (e.g., AB machines only), in terms of the observed precision and accuracy. Also, any gross reliability differences in results between machine types should be exposed.

Results and Discussion

In both trials, four unknown sample levels of % GM DNA were examined. Figure 1 shows the range of the data in replications when data were adjusted to a mean of 1 for each machine. Between Trials 1 and 2, there was no clear repeatable ranking of machines in terms of precision, except that the Chromo4 machine performed well (top three) in both. Figure 2 shows the % bias for each machine in the two trials. Note that Trial 2 biases were smaller than those in Trial 1. Trial 2 biases were also more symmetrical, while Trial 1 tending to generally underestimate % GM DNA. This is likely due to a systematic error between the standards (% w/w GM) and the unknown samples which were made as % GM DNA. The relative bias among machines in the two trials again showed no clear repeatable rank.

The RSD and bias for both trials are shown in Table 2. Two levels of RSD were calculated: mean RSD repeatability within runs, the root mean square (RMS) of the SDs derived from the replications of runs on the same machine in the same laboratory; and RSD reproducibility, the RMS SD between machine and laboratory. These statistics are equivalent to RSD_r and RSD_R, respectively, as described in CRL method validations. The results are favorable compared to the European Network of GMO Laboratories validation method acceptance criteria for within-laboratory reliability (25% RSD_r, 50% bias; *see* http://gmo-crl.jrc.ec.europa.eu/doc/

Table 2. Summary statistics for Trials 1 and 2

	Level (% GM DNA)				
Statistic	1	0.5	0.1	0.05	RMS[a]
			Trial 1		
Mean RSD repeatability within runs	32.35	29.20	30.37	42.74	34.09
RSD reproducibility all runs	38.86	34.30	32.06	50.50	39.58
%Bias	–19.55	–29.89	11.91	–27.85	23.41
			Trial 2		
Mean RSD repeatability within runs	29.87	29.50	31.48	46.49	35.05
RSD reproducibility all runs	37.16	42.47	35.4	60.34	38.46
%Bias	16.41	–4.62	13.93	4.98	11.29

[a] RMS = Root mean square.

Table 3. ANOVA results of Trials 1 and 2

ANOVA	df[a]	F[b]	F_{crit}	MS[c]	*P*
Among replicate runs within each machine					
Trial 1	15	1.12	1.68	0.13	0.327
Trial 2	3	0.42	2.67	0.20	0.739
Among same machine model in different laboratories					
Trial 1					
ABI7700	1	1.65	3.92	0.07	0.20
ABI7900	1	13.07	3.89	0.07	3 10^{-4}[d]
Trial 2					
ABI7700	2	0.30	3.20	0.18	0.74
ABI7900	1	2.40	4.17	0.23	0.13
Among different machine models in the same laboratory					
Trial 1[e]					
P	3	2.81	2.64	0.08	0.04[d]
Q	1	47.37	3.9	0.17	2.45 10^{-10}[d]
R	1	0.05	3.91	0.12	0.82
S	1	26.65	3.92	0.06	9.23 10^{-7}[d]
Trial 2					
P	2	0.68	3.20	0.15	0.51
Q	1	1.65	4.17	0.14	0.21
S	1	0.68	4.17	0.28	0.41
Among different machines among all laboratories					
Trial 1	9	10.83	1.89	0.11	1 10^{-15}[d]
Trial 2	9	1.26	1.94	0.19	0.26
Between Trial 1 and Trial 2					
	1	38.62	3.85	0.15	7.96 10^{-10}

[a] df = Degrees of freedom. Trial 1 shows 15 df because each GM assay run is independent of the reference assay runs and can therefore be paired in any combination.

[b] F = *F*-ratio.

[c] MS = Mean sum of squares between groups.

[d] P = Significant P values 0.05.

Min_Perf_Requir_Analyt_methods_131008.pdf), considering that in this study a wide variety of machines was used between different laboratories. This would suggest that the type of machine used is not critical in GM quantification, at least for the methods examined here.

ANOVAs were carried out on normalized data from both trials (Table 3). Some significant differences were observed. Where machines were replicated among laboratories, the ABI7700 machine results were significantly different among laboratories in Trial 1, but they were not significant in Trial 2. Within laboratories that had different machines, there were significant differences among machines in three out of four laboratories for Trial 1, but again, these differences were not present in Trial 2. When all machines in all laboratories were compared by ANOVA, only Trial 1 showed significant differences. These results indicate that significant differences in accuracy among different machines may be linked to the method used. However, the magnitude of variance (mean squares between groups; Table 3) did not vary greatly in any of the comparisons and the mean variation due to different machine models was not greater than that within machine models [mean between group mean squares a) and b) versus c) and d) = 0.15 and 0.14, respectively]. Therefore, we did not find that machine model has a significant effect on the GM quantification result over both trials.

The hypothesis of this study was that a large component of the variation of real-time GM estimates was due to the type of hardware used. The results of both trials showed that the effect of the machine used can be significant for some methods, but the effect is not larger than run-to-run variation. Therefore,

given proper conditions, e.g., consistent calibrants (as used here, supplied by one central laboratory) the type of real-time PCR machine does not have a large effect on results. There was also no obvious systematic error or bias associated with any individual machine. The largest source of variation was between trials in the method used.

Although the limited number of laboratories and machines meant that replication was low in this experiment, in both trials, the variance due to machine was not significantly different from run-to-run replication variance within a single machine (ANOVA P range = 0.27–0.74 for all different comparisons). The future reduction in cost of real-time PCR machines and the increasing number of manufacturers, is likely to cause an increase in the variety of machines in regular use for quantifying GM. Based on this study and the quality of machines contained in it, this increasing variety should not be a source of concern in interpretation of GM quantifications, provided that sufficiently robust methods are used.

Acknowledgments

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References

(1) Hernandez, M., Pla, M., Esteve, T., Prat, S., Puigdomenech, P., & Ferrando, A. (2003) *Transgenic Res*. **12**, 179–189

(2) Trifa, Y., & Zhang, D. (2004) *J. Agric. Food Chem.* **52**, 1044–1048

(3) Broothaerts, W., Schimmel, H., Trapmann, S., Emons, H., & Vincent, S. (2008) *J. Agric. Food. Chem.* **56**, 8825–8831