CORESTA

Task Force Genetically Modified Tobacco – Collaborative Study on Quantitation Methods

PROFICIENCY TESTING TRIALS FOR QUANTIFICATION OF GM TOBACCO USING REAL-TIME PCR

Report to Participants

July 2003

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Both rounds 1 and 2 were conducted in accordance with the project Protocol (October 2002). The cured tobacco leaf samples for round 1, together with calibration standards, primers and probes were despatched on March 27, 2002 with a result deadline of April 28, 2003. Out of the 12 possible participants all 12 submitted results. 35S primers and probes were included with each sample set, together with instructions for use, and a suggested extraction protocol. Participants were encouraged, however, to use their own procedures as the objective of a proficiency test is to study routine procedures used in participating laboratories.

A further set of cured tobacco leaf samples, together with calibration standards for round 2 were despatched on 17 June 2003 with a result deadline of 17 July 2003. Out of the 12 possible participants all 12 submitted results.

Samples were labelled A to G. The details of each sample are given below.

1.2 Sample Information

Sample Identifier	Description Round 1	Description Round 2
A	1.5% GM Tobacco	0.5% GM Tobacco
B	4.0% GM Tobacco	1.5% GM Tobacco
C	0.15% GM Tobacco	0.8% GM Tobacco
D	1.0% GM Tobacco	2.0% GM Tobacco
E	0.8% GM Tobacco	4.0% GM Tobacco
F	0.5% GM Tobacco	1.0% GM Tobacco
G	2.0% GM Tobacco	0.15% GM Tobacco

Participants were not given these sample descriptions; all samples were supplied "blind".

Homogeneity Data

After homogenisation, samples were analysed by DNA extraction followed by real-time PCR amplification using primers designed to detect the 35S promoter. Two extractions were made on eight replicates of each sample, and two PCR analyses of each extract carried out.

The delta-CT values for each analysis were recorded, and the mean values for each extraction were subjected to the Analysis of Variance (ANOVA) to determine homogeneity. A summary of this data is given below:

Test Sample (Round 1 designation)	Mean	Sample Standard Deviation (SSD	Sampling Deviation (SD)	F-Ratio	F_{crit} for 8 replicates	Status
A	8.14	0.647	0.254	1.3613	3.581	OK
B	3.20	0.320	0.150	1.5474	3.581	OK
C	8.26	0.594	0.113	0.9295	3.581	OK
D	5.31	0.283	0.078	1.1612	3.581	OK
E	6.47	0.570	0.225	1.3669	3.581	OK
D	7.04	1.428	0.247	0.9416	3.581	OK
G	4.37	0.169	0.047	1.1698	3.581	OK
0.1% GM standard	8.38	0.532	0.398	3.3242	3.581	OK
5.0% GM standard	3.41	0.408	0.408	2.2039	3.581	OK

All values for means and standard deviations are in delta-CT units.

All samples are sufficiently homogeneous for use in a proficiency testing trial.

Results and Evaluation Round 1

SAMPLE A

Lab ID	GM Tobacco		GM Tobacco		GM Tobacco		GM Tobacco	
	35S Primers (supplied)	%	35S Primers (In-house)	%	Nos Primers	%	35S (In-house)/LGC Extraction	%
	Result	Z Score	Result	Z Score	Result	Z Score	Result	Z Score
1	1.2	-1.14						
2			1.5	0.57	1.5	0.57		
3			0.7	-4.00				
4	2.32	>4						
5	2.0	3.43	1.6	1.14	1.1	-1.71	1.4	0.00
6	1.8	2.29	1.2	-1.14				
7	0.875	-3.00						
8	1.42	0.11	2.89	>4	1.10	-1.71		
9	1.3	-0.57	1.3	-0.57	1.5	0.57		
10	1.77	2.11	1.62	1.26	1.36	-0.23		
11	1.8	2.29	1.1	-1.71	1.4	0.00		
12	>0.5		0.5	<-4				
# Results	9		9		6		1	
Median	1.77		1.3		1.38		1.4	
Mean	1.61		1.38		1.33		1.4	
Robust SD	0.52		0.44		0.18		0	
SD	0.45		0.69		0.18		0	
Min Value	0.88		0.5		1.1		1.4	
Max Value	2.32		2.89		1.5		1.4	
Median (all methods)	1.4							
Acceptability criteria	0.175							
Target value	1.5							

SAMPLE B

Lab ID	GM Tobacco 35S Primers (supplied) Result	% Z Score	GM Tobacco 35S Primers (In-house) Result	% Z Score	GM Tobacco Nos Primers Result	% Z Score	GM Tobacco 35S (In-house)/LGC Extraction Result	% Z Score
1	>5							
2			5.0	2.00	5.0	2.00		
3			2.4	-3.20				
4	4.85	1.70						
5	5.4	2.80	4.6	1.20	4.8	1.60	4.4	0.80
6	3.0	-2.00	3.2	-1.60				
7	3.389	-1.22						
8	3.98	-0.04	4.39	0.78	3.33	-1.34		
9	4.0	0.00	4.0	0.00	4.2	0.40		
10	3.81	-0.38	3.48	-1.04	3.34	-1.32		
11	4.2	0.40	1.5	**<-4**	2.5	-3.00		
12	>5		10	**>4**				
# Results	8		9		6		1	
Median	3.99		4		3.77		4.4	
Mean	4.08		4.29		3.86		4.4	
Robust SD	0.6		1.19		1.09		0	
SD	0.76		2.41		0.97		0	
Min Value	3		1.5		2.5		4.4	
Max Value	5.4		10		5		4.4	
Median (all methods)	4							
Acceptability criteria	0.5							
Target value	4							

SAMPLE C

Lab ID	GM Tobacco 35S Primers (supplied) Result	% Z Score	GM Tobacco 35S Primers (In-house) Result	% Z Score	GM Tobacco Nos Primers Result	% Z Score	GM Tobacco 35S (In-house)/LGC Extraction Result	% Z Score
1	<0.1							
2			0.1	-4.00	0.1	-4.00		
3			0.1	-4.00				
4	0.00	<-4						
5	0.2	0.00	0.1	-4.00	0.2	0.00	0.3	4.00
6	0.2	0.00	0.2	0.00				
7	0.156	-1.76						
8	0.12	-3.20	0.27	2.80	0.32	>4		
9	0.2	0.00	0.2	0.00	0.2	0.00		
10	0.32	>4	0.29	3.60	0.26	2.40		
11	0.3	4.00	0.3	4.00	0.2	0.00		
12	0.2	0.00	0.1	-4.00				
# Results	9		9		6		1	
Median	0.2		0.2		0.2		0.3	
Mean	0.19		0.18		0.21		0.3	
Robust SD	0.07		0.15		0.04		0	
SD	0.09		0.09		0.07		0	
Min Value	0		0.1		0.1		0.3	
Max Value	0.32		0.3		0.32		0.3	
Median (all methods)	0.2							
Acceptability criteria	0.025							
Target value	0.15							

SAMPLE D

Lab ID	GM Tobacco 35S Primers (supplied) Result	% Z Score	GM Tobacco 35S Primers (In-house) Result	% Z Score	GM Tobacco Nos Primers Result	% Z Score	GM Tobacco 35S (In-house)/LGC Extraction Result	% Z Score
1	1.6	>4						
2			0.5	-4.00	0.5	-4.00		
3			0.8	-1.60				
4	1.98	>4						
5	1.2	1.60	0.8	-1.60	1.1	0.80	1.0	0.00
6	1.20	1.60	0.80	-1.60				
7	0.465	<-4						
8	0.91	-0.72	2.05	>4	1.24	1.92		
9	0.7	-2.40	0.7	-2.40	1.0	0.00		
10	1.66	>4	1.43	3.44	1.15	1.20		
11	1.5	4.00	1.2	1.60	0.9	-0.80		
12	1	0.00	1	0.00				
# Results	10		9		6		1	
Median	1.2		0.8		1.05		1.0	
Mean	1.22		1.03		0.98		1.0	
Robust SD	0.52		0.3		0.19		0	
SD	0.47		0.47		0.26		0	
Min Value	0.47		0.5		0.5		1.0	
Max Value	1.98		2.05		1.24		1.0	
Median (all methods)	1.0							
Acceptability criteria	0.125							
Target value	1.0							

SAMPLE E

Lab ID	GM Tobacco 35S Primers (supplied) Result	% Z Score	GM Tobacco 35S Primers (In-house) Result	% Z Score	GM Tobacco Nos Primers Result	% Z Score	GM Tobacco 35S (In-house)/LGC Extraction Result	% Z Score
1	1.8	>4						
2			1	0.70	1	0.70		
3			0.9	-0.17				
4	0.95	0.26						
5	1.1	1.57	0.7	-1.91	1.1	1.57	1.1	1.57
6	1.0	0.70	1.0	0.70				
7	0.399	<-4						
8	0.89	-0.26	0.55	-3.22	0.90	-0.17		
9	0.6	-2.78	0.6	-2.78	0.7	-1.91		
10	0.95	0.26	0.94	0.17	0.59	-2.87		
11	0.8	-1.04	0.8	-1.04	0.6	-2.78		
12	2	>4	2	>4				
# Results	10		9		6		1	
Median	0.95		0.9		0.8		1.1	
Mean	1.05		0.94		0.82		1.1	
Robust SD	0.22		0.15		0.3		0	
SD	0.49		0.43		0.22		0	
Min Value	0.4		0.55		0.59		1.1	
Max Value	2		2		1.1		1.1	
Median (all methods)	0.92							
Acceptability criteria	0.115							
Target value	0.8							

SAMPLE F

Lab ID	GM Tobacco 35S Primers (supplied) Result	% Z Score	GM Tobacco 35S Primers (In-house) Result	% Z Score	GM Tobacco Nos Primers Result	% Z Score	GM Tobacco 35S (In-house)/LGC Extraction Result	% Z Score
1	0.48	-1.60						
2			0.1	**<-4**	0.1	**<-4**		
3			0.3	-4.00				
4	0.52	-1.07						
5	0.9	4.00	0.5	-1.33	0.7	1.33	0.9	4.00
6	0.60	0.00	0.60	0.00				
7	0.068	**<-4**						
8	0.57	-0.40	0.74	1.87	0.60	0.00		
9	0.4	-2.67	0.4	-2.67	0.5	-1.33		
10	0.77	2.27	1.21	**>4**	0.48	-1.60		
11	0.6	0.00	0.7	1.33	0.6	0.00		
12	5	**>4**	5	**>4**				
# Results	10		9		6		1	
Median	0.59		0.6		0.55		0.9	
Mean	0.99		1.06		0.5		0.9	
Robust SD	0.22		0.3		0.09		0	
SD	1.43		1.51		0.21		0	
Min Value	0.07		0.1		0.1		0.9	
Max Value	5		5		0.7		0.9	
Median (all methods)	**0.6**							
Acceptability criteria	**0.075**							
Target value	**0.5**							

SAMPLE G

Lab ID	GM Tobacco		GM Tobacco		GM Tobacco		GM Tobacco	
	35S Primers (supplied)	%	35S Primers (In-house)	%	Nos Primers	%	35S (In-house)/LGC Extraction	%
	Result	Z Score	Result	Z Score	Result	Z Score	Result	Z Score
1	>5							
2			2	-0.06	2	-0.06		
3			1.2	-3.24				
4	3.96	>4						
5	2.6	2.32	2.0	-0.06	2.5	1.93	2.6	2.32
6	2.30	1.13	3.0	3.91				
7	0.472	<-4						
8	2.03	0.06	3.05	>4	1.49	-2.08		
9	2.0	-0.06	2.0	-0.06	2.2	0.73		
10	2.37	1.41	2.25	0.93	2.07	0.22		
11	1.3	-2.84	1.6	-1.65	0.9	<-4		
12	1	<-4	>1					
# Results	9		8		6		1	
Median	2.03		2		2.04		2.6	
Mean	2		2.14		1.86		2.6	
Robust SD	0.85		0.48		0.47		0	
SD	1.02		0.63		0.57		0	
Min Value	0.47		1.2		0.9		2.6	
Max Value	3.96		3.05		2.5		2.6	
Median (all methods)	2.015							
Acceptability criteria	0.252							
Target value	2.0							

Comments on Results: Round 1

Overall, the quality of results reported were very good. No statistically significant differences were observed between the primers used by participants. A few trends were noted in the results submitted by individual laboratories:

Laboratory 1 reported high results for the samples containing higher levels of GM Tobacco.

Laboratory 7 reported low results for most samples.

Summary of Method Details

Lab ID	Sample Weight (g)	Extraction methods used	Quantity of DNA used	Dilution factor	PCR cycles	In-house Primer details	Other Control Samples
1	0.1-0.49	In-house	~100ng	8%	-	-	Negative as supplied
2	0.1-0.49	In-house	50ng		45	35S/NOS: Light cycler GMO Screening kit	Extraction blank negative control
3	1.0-1.49	In-house	n.a.	Undiluted	50	-	Negative control
4	<0.1	Modified CTAB	100ng	OD value of 260nm	-	-	-
5	1.0-1.49	LGC & In-house	100ng	~1:10	40	-	-
6	Not given						
7	<0.1	QIAgen DNA extraction kit	100-200ng	1:5	40	None	None
8	0.1	DNeasy Plant Kit Qiagen	20-22ng	4	50	Multiplex systems	Extraction blanks OK
9	0.1-0.49	LGC supplied	50ng		45		

10	<0.1	LGC supplied	100ng	TE buffer	40	See below	TE buffer
11	0.1-0.49	Dneasy Plant Mini Kit Qiagen	80ng		50		
12	-	PR5700	-	-	-	-	-

ID 10: 35S

      FW: 5'-CAGTGGTCCCAAAGATGGAC -3'

      RV: 5'-GATAGTGGGATTGTGCGTCAT-3'

NOS   FW: 5' GAATCCTGTTGCCGGTCTT- -3'

      RV: 5'-TTGCGCGCTATATTTTGTTTT -3'

Results and Evaluation
Round 2

Lab ID	GM Tobacco 35S Primers (supplied) Result	% Z Score	GM Tobacco 35S Primers (In-house) Result	% Z Score	GM Tobacco Nos Primers Result	% Z Score
1	0.28	**<-4**	0.32	-3.62		
2			0.40	-2.53	0.40	-2.53
3			0.9	**>4**		
4	0.601	0.22				
5	0.4	-2.53	0.4	-2.53	0.4	-2.53
6	0.60	0.21	0.50	-1.16		
7	0.12	**<-4**				
8	0.57	-0.21	0.35	-3.21	0.43	-2.12
9	0.6	0.21	0.6	0.21	0.6	0.21
10	0.71	1.71	0.55	-0.48	0.67	1.16
11	1.1	**>4**	0.95	**>4**	0.91	**>4**
12	1.8	**>4**	2	**>4**		
# Results	10		10		6	
Median	0.6		0.53		0.52	
Mean	0.68		0.7		0.57	
Robust SD	0.23		0.22		0.17	
SD	0.47		0.51		0.2	
Min Value	0.12		0.32		0.4	
Max Value	1.8		2		0.91	
Median (all methods)	**0.59**					
Acceptability criteria	**0.073**					
Target Value	**0.5**					

Lab ID	GM Tobacco		GM Tobacco		GM Tobacco	
	35S Primers (supplied)	%	35S Primers (In-house)	%	Nos Primers	%
	Result	Z Score	Result	Z Score	Result	Z Score
1	1.39	-0.03	1.57	1.00		
2			1.20	-1.12	1.20	-1.12
3			1.2	-1.12		
4	1.249	-0.84				
5	0.9	-2.84	1.0	-2.27	1.1	-1.69
6	1.30	-0.54	1.40	0.03		
7	0.28	**<-4**				
8	1.10	-1.69	1.75	2.04	1.27	-0.72
9	1.6	1.18	1.6	1.18	1.5	0.60
10	2.22	**>4**	1.87	2.72	1.65	1.46
11	2.3	**>4**	1.85	2.61	2.2	**>4**
12	1.5	0.60	0.98	-2.38		
# Results	10		10		6	
Median	1.35		1.49		1.39	
Mean	1.38		1.44		1.49	
Robust SD	0.37		0.42		0.33	
SD	0.59		0.34		0.4	
Min Value	0.28		0.98		1.1	
Max Value	2.3		1.87		2.2	
Median (all methods)	**1.4**					
Acceptability criteria	**0.174**					
Target value	**1.5**					

SAMPLE C (Round 2)

Lab ID	GM Tobacco 35S Primers (supplied) Result	% Z Score	GM Tobacco 35S Primers (In-house) Result	% Z Score	GM Tobacco Nos Primers Result	% Z Score
1	0.67	-1.26	0.69	-1.06		
2			0.62	-1.76	0.62	-1.76
3			2.9	**>4**		
4	0.652	-1.44				
5	0.6	-1.96	0.6	-1.96	0.6	-1.96
6	0.50	-2.97	0.70	-0.96		
7	0.19	**<-4**				
8	0.61	-1.86	1.45	**>4**	1.06	2.67
9	1	2.06	1	2.06	0.9	1.06
10	1.32	**>4**	1.14	3.47	0.89	0.96
11	1.2	**>4**	0.95	1.56	1.0	2.06
12	0.08	**<-4**	1.0	2.06		
# Results	10		10		6	
Median	0.63		0.98		0.9	
Mean	0.68		1.11		0.85	
Robust SD	0.37		0.42		0.2	
SD	0.4		0.68		0.19	
Min Value	0.08		0.6		0.6	
Max Value	1.32		2.9		1.06	
Median (all methods)	**0.8**					
Acceptability criteria	**0.099**					
Target value	**0.8**					

SAMPLE D (Round 2)

Lab ID	GM Tobacco 35S Primers (supplied) Result	% Z Score	GM Tobacco 35S Primers (In-house) Result	% Z Score	GM Tobacco Nos Primers Result	% Z Score
1	2.03	0.88	1.92	0.40		
2			0.77	**<-4**	0.77	**<-4**
3			0.3	**<-4**		
4	1.856	0.12				
5	1.7	-0.56	1.5	-1.44	1.7	-0.56
6	1.30	-2.31	1.50	-1.44		
7	0.38	**<-4**				
8	2.60	3.38	3.10	**>4**	1.80	-0.12
9	2.3	2.07	2.3	2.07	2.3	2.07
10	3.51	**>4**	2.41	2.55	2.09	1.15
11	0.9	**<-4**	0.9	**<-4**	0.8	**<-4**
12	2	0.75	2.25	1.85		
# Results	10		10		6	
Median	1.93		1.71		1.75	
Mean	1.86		1.7		1.58	
Robust SD	0.74		0.96		0.66	
SD	0.88		0.86		0.65	
Min Value	0.38		0.3		0.77	
Max Value	3.51		3.1		2.3	
Median (all methods)	**1.83**					
Acceptability criteria	**0.229**					
Target value	**2**					

SAMPLE E (Round 2)

Lab ID	GM Tobacco 35S Primers (supplied) Result	% Z Score	GM Tobacco 35S Primers (In-house) Result	% Z Score	GM Tobacco Nos Primers Result	% Z Score
1	4.65	1.79	3.79	-0.02		
2			1.50	<-4	1.50	<-4
3			1.2	<-4		
4	3.508	-0.61				
5	3.8	0.00	3.8	0.00	3.9	0.21
6	3.80	0.00	3.80	0.00		
7	0.62	<-4				
8	3.80	0.00	3.26	-1.14	2.75	-2.21
9	4	0.42	4	0.42	3.8	0.00
10	6.50	>4	3.98	0.38	3.3	-1.05
11	6.1	>4	5.9	>4	6.1	>4
12	4.21	0.86	4.8	2.11		
# Results	10		10		6	
Median	3.9		3.8		3.55	
Mean	4.1		3.6		3.56	
Robust SD	0.52		0.55		0.85	
SD	1.59		1.39		1.52	
Min Value	0.62		1.2		1.5	
Max Value	6.5		5.9		6.1	
Median (all methods)	3.8					
Acceptability criteria	0.475					
Target value	4.0					

SAMPLE F (Round 2)

Lab ID	GM Tobacco 35S Primers (supplied) Result	% Z Score	GM Tobacco 35S Primers (In-house) Result	% Z Score	GM Tobacco Nos Primers Result	% Z Score
1	0.16	<-4	0.16	<-4		
2			0.75	-2.00	0.75	-2.00
3			3.0	>4		
4	1.305	2.44				
5	1.0	0.00	0.9	-0.80	0.7	-2.40
6	0.70	-2.40	1.10	0.80		
7	0.22	<-4				
8	1.30	2.40	1.10	0.80	1.30	2.40
9	1.2	1.60	1.2	1.60	1	0.00
10	1.93	>4	1.12	0.96	1.13	1.04
11	1.0	0.00	0.8	-1.60	1.04	0.32
12	0.4	<-4	0.5	-4.00		
# Results	10		10		6	
Median	1		1		1.02	
Mean	0.92		1.06		0.99	
Robust SD	0.45		0.3		0.28	
SD	0.56		0.75		0.23	
Min Value	0.16		0.16		0.7	
Max Value	1.93		3		1.3	
Median (all methods)	1.0					
Acceptability criteria	0.125					
Target value	1.0					

SAMPLE G (Round 2)

Lab ID	GM Tobacco 35S Primers (supplied) Result	% Z Score	GM Tobacco 35S Primers (In-house) Result	% Z Score	GM Tobacco Nos Primers Result	% Z Score
1	0.99	>4	0.91	>4		
2			0.38	>4	0.38	>4
3			0.0	<-4		
4	0.129	-2.42				
5	0.1	-3.68	0.1	-3.68	0.1	-3.68
6	0.12	-2.81	0.14	-1.95		
7	0.067	<-4				
8	0.23	1.95	0.21	1.08	0.15	-1.51
9	0.2	0.65	0.2	0.65	0.2	0.65
10	0.32	>4	0.18	-0.22	0.19	0.22
11	0.13	-2.38	0.09	<-4	0.1	-3.68
12	0.7	>4	0.8	>4		
# Results	10		10		6	
Median	0.17		0.19		0.17	
Mean	0.3		0.3		0.19	
Robust SD	0.1		0.14		0.07	
SD	0.3		0.31		0.1	
Min Value	0.07		0		0.1	
Max Value	0.99		0.91		0.38	
Median (all methods)	0.19					
Acceptability criteria	0.023					
Target value	0.15					

Comments on Results: Round 2

Overall, the quality of results reported were very good, but with a few unexpected results. Some laboratories had improved their results from the first round. The overall quality of results was not statistically significantly different from the first round. No statistically significant differences were observed between the primers used by participants. A few trends were noted in the results submitted by individual laboratories, and some of the unexpected results may have been reporting (i.e. non-analytical) errors.

Summary of Method Details

Lab ID	Sample Weight (g)	Extraction methods used	Quantity of DNA used	Dilution factor	PCR cycles	In-house Primer details	Other Control Samples
1	<0.1	In-house	~100ng	8%	40	Not stated	Negative as supplied and in-house negative
2	0.1-0.49	In-house	50ng		45	35S/NOS: Light cycler GMO Screening kit	Extraction blank negative control
3	0.1-0.49	-	Unknown	1:8	50	Not given	No samplecontrol
4	<0.1	Modified CTAB	300ng/25µl	OD value of 260nm	40	-	-
5	1.0-1.49	In-house	100ng	~1:10	40	-	-
6	0.5-0.99	In-house	-	-	-	-	-
7	<1.0	QIAgen DNA extraction kit	100-200ng	5x	40	None	None
8	0.1	DNeasy Plant Kit Qiagen	20-22ng	4	50	Multiplex systems	Extraction blanks OK
9	0.1-0.49	LGC supplied	50ng		45		
10	<0.1	LGC supplied	100ng	260OD	40	See below	TE buffer
11	0.1-0.49	Dneasy Plant Mini	80ng		50		

		Kit Qiagen					
12	0.1-0.3	ABI PE5700	100-200ng	-	40	See below	-

ID 10: 35S

     FW: 5'-CAGTGGTCCCAAAGATGGAC -3'

     RV: 5'-GATAGTGGGATTGTGCGTCAT-3'

NOS   FW: 5' GAATCCTGTTGCCGGTCTT- -3'

      RV: 5'-TTGCGCGCTATATTTTGTTTT -3'

ID 12:   35s208.SEQ-64F: sequence GACAGTGGTCCCAAAGATGGA

        35s208.SEQ-139R: sequence TTGAAGACGTGGTTGGAACGT