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COMMUNITY REFERENCE LABORATORY FOR GM FOOD AND FEED



Event-specific methods for the quantitation of the hybrid cotton line 281-24-236/3006-210-23 using real-time PCR

Validation Report

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

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Executive Summary

The JRC as Community Reference Laboratory (CRL) for the GM Food and Feed (see Regulation EC 1829/2003), in collaboration with the European Network of GMO Laboratories (ENGL), has carried out a collaborative study to assess the performance of two quantitative event-specific methods to detect and quantify the Cry1F 281-24-236 (referred as 281-24-236 cotton) and Cry1Ac 3006-210-23 (referred as 3006-210-23) transformation events in cotton DNA (unique identifier DAS-24236-5 x DAS-21023-5). The collaborative trial was conducted according to internationally accepted guidelines.

Dow AgroSciences LLC provided the method-specific samples (genomic DNA extracted from the 0% and 100% cotton line 281-24-236 x 3006-210-23) whereas the JRC prepared the validation samples (calibration samples and blind samples at unknown GM percentage). The trial involved 12 laboratories from nine European countries.

The results of the collaborative trial met ENGL's performance requirements and the scientific understanding about satisfactory method performance. Therefore, the JRC as Community Reference Laboratory considers the methods validated as fit for the purpose of regulatory compliance.

The results of the collaborative study are publicly available under <http://gmo-crl.jrc.it/>. The methods will also be submitted to ISO for consideration as international standards.

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1. Introduction

The Joint Research Centre (JRC, Biotechnology and GMOs Unit of the Institute of Health and Consumer Protection) as Community Reference Laboratory for the GM Food and Feed (see Regulation EC 1829/2003) organised the collaborative trial of the event-specific methods for the detection and quantification of 281-24-236 and 3006-210-23 cotton lines in hybrid 281-24-236 x 3006-210-23. The study involved twelve laboratories, members of the European Network of GMO Laboratories (ENGL).

Upon reception of methods, samples and related data, the JRC carried out the scientific evaluation of documentation and the in-house testing of the methods, according to the requirements of Regulation (EC) 641/2004 and following its operational procedures.

The internal tests were carried out in September-October 2005.

Following the evaluation of the data and the results of the laboratory tests, the ring trial was organized and took place in November 2005.

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

- ✓ Quantitative real-time PCR (Polymerase Chain Reaction). The methodology is an event-specific real-time quantitative TaqMan[®] PCR procedure for the determination of the relative content of events 281-24-236 and 3006-210-23 DNA to total cotton DNA. The procedure is a simplex system, in which cotton SAH7 (*Sinapis Arabidopsis Homolog 7*) endogenous assay (reference gene) and the target assays (281-24-236 and 3006-210-23) are performed in separate wells. The PCR assay has been optimised for use in real-time PCR instruments for plastic reaction vessels.

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).

2. List of Participants

The methods were tested in twelve ENGL laboratories to determine their performance. Each laboratory was requested to carefully follow the protocols provided. The participating laboratories are listed in Table 1 in alphabetical order.

Table 1. ENGL laboratories participating in the validation study of 281-24-236 and 3006-210-23 methods

Laboratory	Country
Agricultural Biotechnology Center	Hungary
Bundersinstitut fuer Risikobewertung (BfR)	Germany
CRA-W, Dépt. Qualité des productions agricoles	Belgium
Department of Plantgenetics and Breeding	Belgium
Ente Nazionale Sementi Elette	Italy
ICTP	Czech Republic
Institute of Public Health	Belgium
Istituto Zooprofilattico Sperimentale Lazio e Toscana	Italy
Laboratoire MDO – Unité PMDV	France
National Food Administration	Sweden
National Institute of Biology	Slovenia
Swiss Federal Research Station for Animal Production and Dairy Products	Switzerland

3. Materials

For the validation of the quantitative event-specific methods, a 281-24-236 x 3006-210-23 DNA stock solution, extracted from cotton variety PHY440W containing the events 281-24-236 and 3006-210-23 in homozygote form, and a DNA stock extracted from non-GM seeds of variety PSC355, with comparable genetic background, were provided by the applicant.

Samples containing mixtures of 0% and 100% 281-24-236 x 3006-210-23 cotton genomic DNA at different GMO concentrations were prepared by the JRC in a constant amount of total cotton DNA.

The participants received the following materials:

- ✓ Four calibration samples (200 µl of DNA solution each) labelled from S1 to S4 for 281-24-236 event.
- ✓ Four calibration samples (200 µl of DNA solution each) labelled from S1 to S4 for 3006-210-23 event.
- ✓ Twenty unknown DNA samples (88 µl of DNA solution each), labelled from U1 to U20 for 281-24-236 event.
- ✓ Twenty unknown DNA samples (88 µl of DNA solution each), labelled from U1 to U20 for 3006-210-23 event.
- ✓ Controls: 0%-GM DNA
- ✓ Reaction reagents as follows:
 - PCR buffer II 10X, two tubes 1408 µl each
 - Rox Reference Dye (50x), two tubes 197.12 µl each
 - Tween-20 (1%), two tubes 140.8 µl each
 - Glycerol (20%), two tubes 563.2 µl each
 - MgCl₂ (100mM), two tubes 845 µl each
 - dATP (10mM), two tubes 282 µl each
 - dCTP (10mM), two tubes 282 µl each
 - dGTP (10mM), two tubes 282 µl each
 - dUTP (20mM), two tubes 282 µl each
 - Ampli Taq Gold (5U/µl), two tubes 113 µl each
 - Distilled sterile water, two tubes 6 ml each
- ✓ Primers and probes as follows:
 - SAH7 system**
 - Sah7-uni-f1 (10 µM), two tubes 179.2 µl each
 - Sah7-uni-r1 (10 µM), two tubes 128 µl each
 - Sah7-uni-s1 (10 µM), two tubes 89.6 µl each
 - 281-24-236 cotton system**
 - 281-f1 (10 µM), one tube 247 µl
 - 281-r2 (10 µM), one tube 318 µl
 - 281-s1 (10 µM), one tube 125 µl

3006-210-23 cotton system

- 3006-f3 (10 µM), one tube 282 µl
- 3006-r2 (10 µM), one tube 282 µl
- 3006-s2 (10 µM), one tube 106 µl

Table 2 shows the GM contents of the unknown samples over the dynamic range.

Table 2. 281-24-236 and 3006-210-23 GM contents

281-24-236 GM % (GM copy number/cotton genome copy number *100)	3006-210-23 GM % (GM copy number/cotton genome copy number *100)
0.10	0.10
0.40	0.40
0.90	0.90
2.00	2.00
5.50	5.50

4. Experimental design

Twenty unknown samples (ten for each of two plates) for each event, representing five GM levels, were used in the validation study. On each PCR plate, samples were analyzed in parallel with both the 281-24-236 and SAH7 gene specific system and the 3006-210-23 and SAH7 gene specific system. Four plates in total were run, with two replicates for each GM level analysed on each run. The PCR analysis was triplicated for all samples. Participating laboratories carried out the determination of the GM% according to the instructions provided in the protocol and using the electronic tool provided (Excel spreadsheet).

5. Method

Description of the operational steps

For specific detection of event 281-24-236 genomic DNA, a 111-bp fragment of the recombination region of parts of the construct inserted into the plant genome is amplified using two specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with two fluorescent dyes: FAM as a reporter dye at its 5' end and TAMRA as a quencher dye at its 3' end.

For specific detection of event 3006-210-23 genomic DNA, a 90-bp fragment of the recombination region of parts of the construct inserted into the plant genome is amplified using two specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with two fluorescent dyes: FAM as a reporter dye at its 5' end and TAMRA as a quencher dye at its 3' end.

For relative quantification of events 281-24-236 and 3006-210-23 DNA, the cotton-specific reference system SAH7 (*Sinapis Arabidopsis* Homolog 7), a cotton endogenous gene, is used, with a pair of SAH7 gene-specific primers and an SAH7 gene-specific probe labelled with FAM and TAMRA. The SAH7 gene is present in both A-subgenome and D-subgenome, primers and probe of the cotton-specific reference PCR match perfectly to both subgenome gene copies. However, the sizes of the amplicons resulting from A- and D-subgenome differ slightly. For A-subgenome SAH7 system amplifies a 115-bp fragment while for D-subgenome, SAH7 amplifies a 123-bp fragment.

The standard curves are generated both for the SAH7, 281-24-236 and 3006-210-23 specific systems by plotting the Ct-values measured for the calibration points against the logarithm of the DNA copy numbers, and by fitting a linear regression line into these data.

Thereafter, the standard curves are used to estimate the copy numbers in the unknown sample DNA by interpolation from the standard curves.

For the determination of the amount of 281-24-236 and 3006-210-23 DNA in the unknown sample, the 281-24-236 or 3006-210-23 copy number is divided by the copy number of the cotton reference gene (SAH7) and multiplied by 100 to obtain the percentage value ($GM\% = 281-24-236/SAH7 * 100$ or $GM\% = 3006-210-23/SAH7 * 100$).

Calibration sample S1 was prepared by mixing the appropriate amount of 281-24-236 x 3006-210-23 DNA from the stock solution in control non-GM cotton DNA to obtain 10% 281-24-236 x 3006-210-23 in a total of 100 ng cotton DNA, corresponding to 4292 GM copies, per reaction. Samples S2-S4 were prepared by 1:6 serial dilutions of the S1 sample. The absolute copy numbers in the calibration curve samples are determined by dividing the sample DNA weight (nanograms) by the published average 1C value for cotton genome (2.33 pg) (Arumuganathan & Earle, 1991).

The copy number values, which were used in the quantification, are provided in Table 3.

Table 3. Copy number values of the standard curve samples.

Sample code	S1	S2	S3	S4
Total amount (ng) of DNA in reaction	100	16.7	2.8	0.5
Cotton genome copies	42918	7153	1192	199
281-24-236 GM copies	4291.8	715.3	119.2	19.9
3006-210-23 GM copies	4291.8	715.3	119.2	19.9

6. Deviations reported

Five laboratories reported no deviations

One laboratory inverted the positions of two samples for one system in one plate, but results were properly copied in the template results sheet.

One laboratory inverted the positions of two standards for one system in one plate, but results were properly copied in the template results sheet.

One laboratory inverted the positions of two controls for one system in one plate.

One laboratory inverted the positions of two standards with two controls for one system and both plates, but results were properly copied in the template results sheet.

One laboratory used optical caps instead of optical adhesion covers.

One laboratory made a "120 seconds, 50 C" cycle precede the cycling program as set in the validation protocol for one system.

One laboratory did not centrifuge the reaction plate but all samples were very carefully loaded into the bottom of the reaction tubes and no drop was present on the sides of the reaction tubes.

One laboratory in one plate deleted one replicate of Standard 4 because of deviated amplification pattern (nick in curve).

One laboratory carried out the validation with 20 µl instead of 25 µl per reaction well, re-calculating the volumes of the reaction mixes accordingly.

The dilution factor applied to the S1 sample, and carried forward to the S4 sample along the dilution series of the standard curve, was of 1:6 and not of 1:5 as declared in the validation protocol sent to the laboratories participating in the collaborative study. Corrections have been introduced by the CRL during data analysis, by modifying appropriately the copy number of the samples of the standard curves in the calculation sheets; no modifications were introduced by the CRL in the plate analysis settings (e.g. baseline, threshold) performed by each laboratory.

7. Summary of result

PCR efficiency and linearity

The values of the slopes [from which the PCR efficiency is calculated using the formula $((10^{(-1/\text{slope})}-1)*100)$ of the standard curves and of the R^2 (expressing the linearity of the regression) reported by participating laboratories for both PCR systems and runs for both events (reference gene and GM specific, plate A and B), are summarised in Table 4 and 5.

Table 4. Values of standard curve slope, PCR efficiency and linearity (R²) for the reference gene (SAH7) and the GM specific (281-24-236) systems. Data are reported as two-figure decimals.

Lab	Plate	SAH7			281-24-236		
		Slope	PCR Efficiency (%)	R ²	Slope	PCR Efficiency (%)	R ²
1	A	-3.35	98.83	1.00	-3.52	92.28	1.00
	B	-3.27	97.94	1.00	-3.35	98.66	1.00
2	A	-3.50	92.92	1.00	-3.39	97.39	1.00
	B	-3.68	87.11	0.99	-3.44	95.40	1.00
3	A	-3.38	97.67	0.99	-3.30	99.10	1.00
	B	-3.58	90.29	1.00	-3.41	96.64	1.00
4	A	-3.33	99.80	0.98	-3.35	98.71	1.00
	B	-3.48	93.78	0.99	-3.30	99.21	0.99
5	A	-3.00	84.34	0.98	-3.11	90.41	0.99
	B	-3.49	93.44	0.99	-3.08	88.63	0.98
6	A	-3.09	89.46	1.00	-3.40	96.98	1.00
	B	-3.17	93.20	0.99	-3.46	94.68	0.99
7	A	-3.30	99.28	0.99	-3.26	97.25	1.00
	B	-3.26	97.46	0.99	-3.25	96.70	1.00
8	A	-3.45	94.75	0.99	-3.30	99.08	0.99
	B	-3.27	97.91	0.99	-3.26	97.42	0.98
9	A	-3.51	92.86	0.99	-3.51	92.58	1.00
	B	-3.34	99.18	0.99	-3.40	96.88	1.00
10	A	-3.42	95.92	1.00	-3.39	97.17	1.00
	B	-3.64	88.20	1.00	-3.43	95.72	1.00
11	A	-3.01	85.21	0.99	-3.09	89.27	0.99
	B	-3.25	97.00	0.99	-3.13	91.15	0.99
12	A	-3.47	94.00	0.99	-3.53	92.14	1.00
	B	-3.42	96.17	0.99	-3.38	97.82	1.00
Mean		-3.36	94.03	0.99	-3.33	95.47	1.00

Table 5. Values of standard curve slope, PCR efficiency and linearity (R^2) for the reference gene (SAH7) and the GM specific (3006-210-23) systems. Data are reported as two-figure decimals.

Lab	Plate	SAH7			3006-210-23		
		Slope	PCR Efficiency (%)	R^2	Slope	PCR Efficiency (%)	R^2
1	A	-3.33	99.82	1.00	-3.35	98.71	1.00
	B	-3.71	86.06	1.00	-3.75	84.65	1.00
2	A	-3.39	97.06	0.99	-3.31	99.58	1.00
	B	-3.36	98.32	1.00	-3.40	96.69	1.00
3	A	-3.52	92.32	1.00	-3.51	92.86	1.00
	B	-3.31	99.30	0.99	-3.53	92.17	1.00
4	A	-3.42	96.04	0.99	-3.31	99.59	0.99
	B	-3.41	96.53	0.99	-3.35	98.80	1.00
5	A	-3.04	86.55	1.00	-2.86	76.31	0.98
	B	-3.23	95.81	1.00	-3.20	94.68	0.98
6	A	-3.23	96.22	0.96	-2.97	83.09	0.99
	B	-3.03	86.45	0.99	-3.30	99.05	0.99
7	A	-3.22	95.68	0.99	-3.10	89.72	1.00
	B	-2.99	84.26	0.99	-3.15	92.17	1.00
8	A	-3.35	98.83	1.00	-3.35	98.82	1.00
	B	-3.30	99.04	0.99	-3.31	99.66	0.99
9	A	-3.27	97.67	1.00	-3.40	96.89	1.00
	B	-3.37	97.90	0.99	-3.20	94.48	1.00
10	A	-3.30	99.06	1.00	-3.32	99.88	1.00
	B	-3.21	95.17	1.00	-3.44	95.38	1.00
11	A	-2.99	84.19	0.99	-2.99	84.10	1.00
	B	-2.89	78.39	0.98	-3.20	94.64	1.00
12	A	-3.31	99.46	0.99	-3.39	97.20	0.99
	B	-3.30	99.19	1.00	-3.30	99.01	1.00
Mean		-3.27	94.14	0.99	-3.29	94.09	1.00

Data reported in Table 4 and 5 confirm the good performance characteristics of the method tested.

The mean PCR efficiency for the event-specific and the reference-specific system are above 90% for both events while the linearity was 0.99 for the reference system and close to 1.00 for the two event-specific systems.

GMO quantitation

Table 6 shows the mean values of the four repetitions for each GM level for the 281-24-236 event as provided by all laboratories. Each mean value is the average of three PCR replicates.

Table 6. Replicates' mean value by laboratories and by all unknown samples in quantification of event 281-24-236.

Sample GMO content for the 281-24-236 event (GM% = GM copy number/cotton genome copy number *100)																				
LAB	0.1%				0.4%				0.9%				2.0%				5.5%			
	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4
1	0.10	0.10	0.08	0.07	0.40	0.45	0.34	0.31	1.05	1.07	0.77	0.87	2.37	2.90	2.34	2.07	6.15	7.73	5.43	5.40
2	0.12	0.11	0.14	0.13	0.42	0.42	0.51	0.49	0.97	0.98	0.92	1.00	2.11	2.02	2.11	2.00	5.26	5.40	5.04	5.34
3	0.14	0.11	0.12	0.10	0.48	0.42	0.53	0.45	1.13	1.03	0.87	1.01	2.26	2.94	1.99	1.99	5.61	6.57	5.32	5.46
4	0.09	0.10	0.09	0.10	0.39	0.46	0.45	0.45	0.89	0.92	1.09	1.00	2.28	2.57	2.26	2.74	6.53	6.95	6.80	6.06
5	0.09	0.09	0.16	0.17	0.37	0.28	0.46	0.34	0.75	0.76	1.16	1.35	2.18	1.85	2.02	2.02	4.41	3.80	5.75	6.85
6	0.11	0.09	0.19	0.14	0.59	0.33	0.53	0.55	1.06	1.08	0.98	2.13	2.65	2.76	2.13	3.14	10.29	5.23	10.43	8.64
7	0.13	0.10	0.07	0.12	0.51	0.53	0.38	0.30	1.50	1.27	0.82	0.93	2.70	2.36	1.88	2.07	6.63	8.00	5.34	6.07
8	0.17	0.16	0.12	0.13	0.53	0.57	0.44	0.49	1.05	1.24	0.97	1.09	2.13	2.29	1.75	2.26	5.76	6.14	4.99	4.69
9	0.13	0.14	0.10	0.09	0.55	0.47	0.42	0.34	1.13	0.97	1.10	0.87	2.18	2.33	2.13	1.84	5.80	6.37	4.82	4.67
10	0.09	0.12	0.10	0.11	0.42	0.43	0.44	0.45	0.99	1.15	0.99	0.88	2.29	2.05	2.20	2.28	6.31	5.57	5.30	5.05
11	0.06	0.06	0.07	0.10	0.26	0.32	0.32	0.40	0.66	0.82	0.90	1.00	1.98	2.78	2.06	1.92	6.02	6.19	5.72	5.44
12	0.07	0.11	0.07	0.08	0.43	0.39	0.31	0.29	0.68	0.71	0.63	0.63	2.18	2.54	1.53	1.46	6.28	4.48	4.32	4.03

In Figure 1 the relative deviation from the true value for each GM-level and per laboratory for event 281-24-236 is shown. The coloured bars represent the relative GM quantification obtained in the participating laboratories; the red bar represents the overall mean. In this computation, a few laboratories over- or underestimated the true value of the GM levels over the dynamic range. The mean overall bias of the GM quantitation, however, was well within the acceptance criteria for method performance at each GM-level, indicating the good correlation between estimated and true value.

Figure 1. Relative deviation (%) from the true value of 281-23-236 event for all laboratories (coloured bars) and the overall mean (red bar)

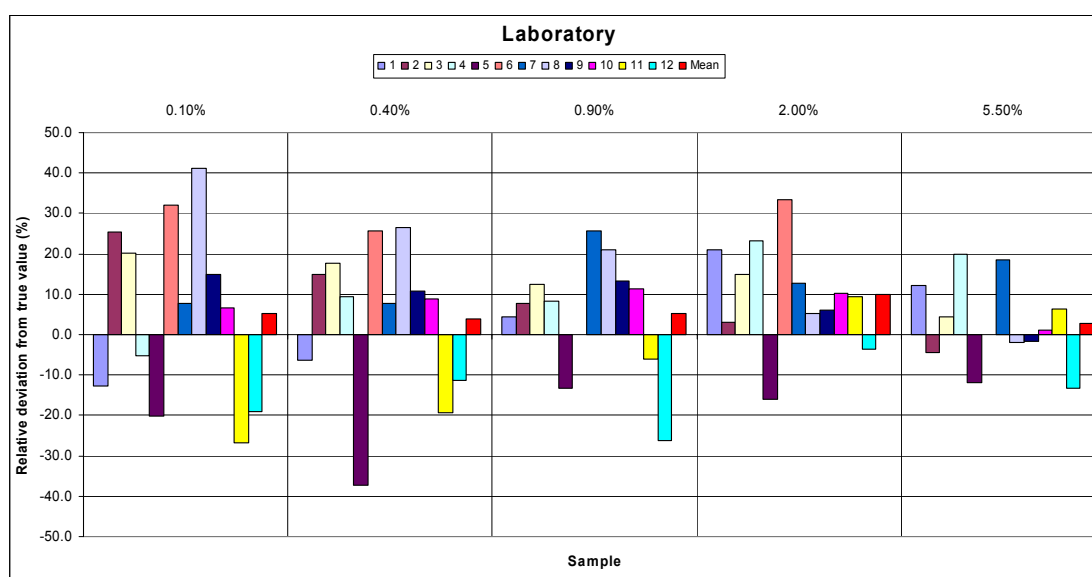


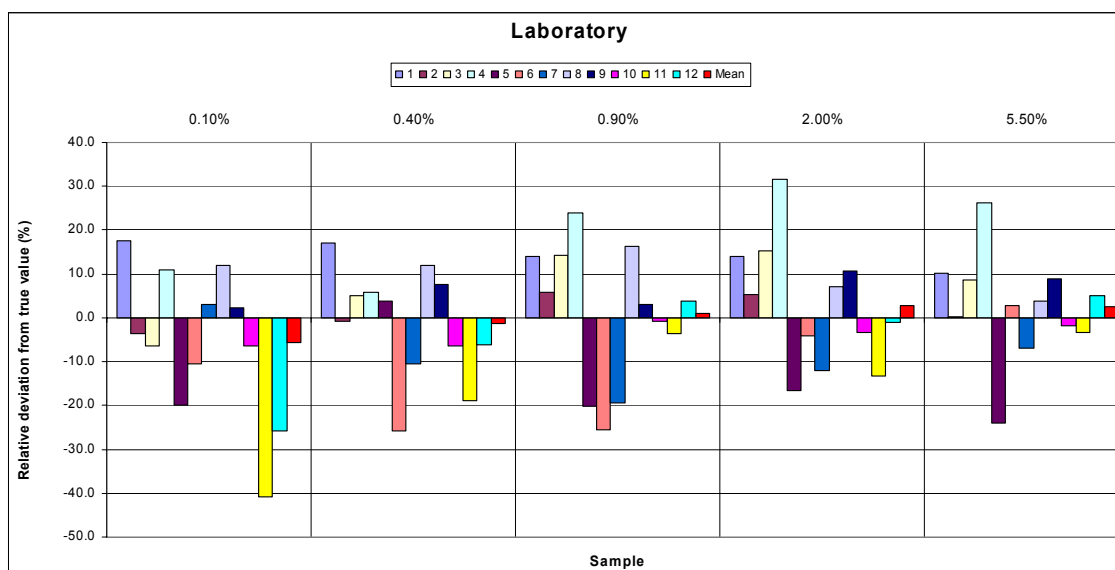
Table 7 shows the mean values of the four repetitions for each GM level for the 3006-210-23 event as provided by all laboratories. Each mean value is the average of three PCR replicates.

Table 7. Replicates' mean value by laboratories and by all unknown samples in quantification of event 3006-210-23.

Sample GMO content for the 3006-210-23 event (GM% = GM copy number/cotton genome copy number *100)																				
LAB	0.1%				0.4%				0.9%				2.0%				5.5%			
	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4
1	0.09	0.08	0.14	0.16	0.39	0.39	0.56	0.53	0.91	0.81	1.27	1.11	2.13	1.97	2.56	2.46	5.78	5.59	6.13	6.72
2	0.12	0.09	0.11	0.07	0.42	0.40	0.39	0.38	0.88	1.03	0.96	0.94	2.20	2.01	2.15	2.07	5.44	5.50	5.84	5.30
3	0.10	0.13	0.08	0.07	0.46	0.51	0.38	0.34	1.04	1.17	0.90	1.01	2.36	2.46	2.20	2.21	6.20	5.95	5.81	5.91
4	0.13	0.09	0.13	0.09	0.52	0.41	0.34	0.43	1.40	1.27	0.81	0.98	2.64	3.02	2.29	2.57	5.70	9.56	6.11	6.42
5	0.06	0.08	0.10	0.08	0.23	0.44	0.55	0.45	0.59	0.58	0.79	0.91	1.58	1.68	2.07	1.34	3.60	3.26	4.08	5.80
6	0.06	0.07	0.08	0.16	0.27	0.36	0.22	0.34	0.62	0.61	0.71	0.73	1.67	2.29	1.67	2.04	4.12	7.26	4.93	6.31
7	0.07	0.09	0.07	0.17	0.33	0.35	0.36	0.40	0.65	0.62	0.76	0.86	1.23	1.55	2.02	2.24	3.58	5.72	6.71	4.47
8	0.09	0.09	0.14	0.12	0.38	0.42	0.49	0.51	1.03	1.05	1.00	1.10	1.99	2.02	2.15	2.40	5.60	5.89	5.21	6.17
9	0.10	0.09	0.11	0.11	0.41	0.38	0.44	0.48	1.01	0.85	0.98	0.86	2.23	2.52	2.03	2.08	6.70	6.89	5.26	5.08
10	0.11	0.11	0.08	0.07	0.34	0.46	0.36	0.35	0.89	0.95	0.87	0.87	1.95	2.00	1.89	1.88	5.22	5.05	5.22	6.10
11	0.07	0.06	0.05	0.06	0.37	0.37	0.30	0.27	0.92	0.74	0.78	1.03	1.77	1.92	1.40	1.85	5.18	5.60	6.80	3.66
12	0.09	0.10	0.05	0.06	0.48	0.50	0.26	0.26	1.11	1.21	0.74	0.68	2.54	2.58	1.48	1.32	8.29	7.31	4.01	3.52

In Figure 2 the relative deviation from the true value for each GM level and per laboratory for event 3006-210-23 is shown. The coloured bars represent the relative GM quantification obtained in the participating laboratories; the red bar represents the overall mean. In this computation, the mean overall bias of the GM quantitation was well within the acceptance criteria for method performance at each GM-level, while very few laboratories under- or over-estimated the true value of more than 25% relative deviation across the GM-levels of the dynamic range.

Figure 2. Relative deviation (%) from the true value of 3006-210-23 for all laboratories (colored bars) and the overall mean (red bar)



8. Method performance requirements

Performance characteristics of the 281-24-236 method

The results of the collaborative trial are reported in table 8 for the 281-24-236 method. These 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 8 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.

Table 8. 281-24-236 validation data.

Unknown sample GM%	Expected value (GMO %)				
	0.10	0.40	0.90	2.00	5.50
Laboratories having returned results	12	12	12	12	12
Samples per laboratory	4	4	4	4	4
Number of outliers	0	0	1	0	1
Reason for exclusion	-	-	C	-	C
Mean value	0.105	0.416	0.948	2.199	5.646
Repeatability relative standard deviation (%)	22.35	16.37	16.50	14.66	14.64
Repeatability standard deviation	0.02	0.07	0.16	0.32	0.83
Reproducibility relative standard deviation (%)	28.63	23.14	20.27	17.27	16.58
Reproducibility standard deviation	0.03	0.10	0.19	0.38	0.94
Bias (absolute value)	0.005	0.016	0.048	0.199	0.146
Bias (%)	5.33	3.94	5.34	9.96	2.66

C. test = Cochran's test

The *relative reproducibility standard deviation* (RSD_R), 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 8, the method fully satisfies this requirement at all GM level tested. In fact, the highest value of RSD_R (%) is 28.63 at the 0.10% level, well within the acceptance criterion.

In the same table the *relative repeatability standard deviation* (RSD_r) values are also reported, as estimated from ring trial results for each GM-level. RSD_r should be below 25%, as indicated by ENGL. As it can be observed from the values reported in table 8, the method satisfies this requirement throughout the whole dynamic range tested.

In table 8 measures of method bias, which allows estimating trueness, are also shown for each GM level. Bias is estimated according to ISO 5725 data analysis protocol. According to ENGL method performance requirements, trueness should be $\pm 25\%$ throughout the whole dynamic range. In this case the method satisfies such requirement for all GM values tested, being the bias of the 281-24-236 method below 10% over the whole dynamic range.

Performance characteristics of the 3006-210-23 method

The results of the collaborative trial are reported in table 9 for the 3006-210-23 method. These 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 9 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.

Table 9. 3006-210-23 validation data.

Unknown sample GM%	Expected value (GMO %)				
	0.10	0.40	0.90	2.00	5.50
Laboratories having returned results	12	12	12	12	12
Samples per laboratory	4	4	4	4	4
Number of outliers	0	0	0	0	0
Reason for exclusion	-	-	-	-	-
Mean value	0.094	0.394	0.909	2.056	5.636
Repeatability relative standard deviation (%)	30.45	19.68	16.45	15.19	21.22
Repeatability standard deviation	0.03	0.08	0.15	0.31	1.20
Reproducibility relative standard deviation (%)	31.88	21.34	21.14	18.92	21.73
Reproducibility standard deviation	0.03	0.08	0.19	0.39	1.22
Bias (absolute value)	-0.006	-0.006	0.009	0.056	0.136
Bias (%)	-5.64	-1.40	0.95	2.79	2.48

C. test = Cochran's test

The *relative reproducibility standard deviation* (RSD_R), 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 9, the method fully satisfies this requirement at all GM-level tested. The highest value of RSD_R (%) is 31.88% at the 0.10% level, well within the acceptance criterion.

In the same table the *relative repeatability standard deviation* (RSD_r) values are also reported, as estimated from ring trial results for each GM-level. RSD_r should be below 25%, as indicated

by ENGL. As it can be observed from the values reported in table 9, the method satisfies this requirement throughout the dynamic range with a minor deviation at the 0.10% level.

In table 9 measures of method bias, which allows estimating trueness, are also shown for each GM-level. Bias is estimated according to ISO 5725 data analysis protocol. According to ENGL method performance requirements, trueness should be $\pm 25\%$ throughout the whole dynamic range. In this case the method satisfies such requirement for all GM values tested, being around 5% the maximum bias reported for the 3006-210-23 method at the 0.10% level.

9. Conclusions

The overall performance of the two methods has been evaluated with respect to the method acceptance criteria and method performance requirements recommended by the ENGL (available under <http://gmo-crl.jrc.it>). The method acceptance criteria were reported by the applicant and used to evaluate the method prior to the collaborative study.

The results obtained during the collaborative trial indicate that the methods can be considered as fit for enforcement purposes with respect to their intra- and inter-laboratory variability. The bias fully satisfies the performance requirements over the whole dynamic range for both methods.

In conclusion, the method is considered complying with the current labeling requirements in Europe.

10. References

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