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Report on the Verification of the Performance of 281-24-236, 3006-210-23 and MON 88913 Event-specific PCR-based Methods Applied to DNA Extracted from GM Stack 281-24-236 x 3006-210-23 x MON 88913 Cotton

European Union Reference Laboratory for Genetically Modified Food and Feed

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Joint Research Centre

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29 February 2016

European Union Reference Laboratory for GM Food and Feed

Executive Summary

An application was submitted by Dow AgroSciences Ltd to request the authorisation of the genetically modified stack (GM stack) 281-24-236 x 3006-210-23 x MON 88913 cotton (providing protection against cotton bollworm, tobacco budworm, beet armyworm, fall armyworm, soybean loopers, cabbage loopers and pink bollworm and being glyphosate herbicide tolerant) and all sub-combinations of the individual events as present in the segregating progeny, for food and feed uses, and import and processing, in accordance with articles 5 and 17 of Regulation (EC) N° 1829/2003 on GM Food and GM Feed. The unique identifier assigned to GM stack 281-24-236 x 3006-210-23 x MON 88913 cotton is DAS-24236-5 x DAS-21023-5 x MON-88913-8.

The GM stack 281-24-236 x 3006-210-23 x MON 88913 cotton has been obtained by conventional crossing between the genetically modified cotton events: DAS-24236-5 x DAS-21023-5 (WideStrike™) and MON 88913, without any new genetic modification.

The EURL GMFF has previously validated individually, and declared fit for purpose, the detection methods for the single events 281-24-236, 3006-210-23 and MON 88913 (see http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx). In line with the approach defined by the ENGL (http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf), the EURL GMFF has carried out only an *in-house* verification of the performance of each validated method when applied to genomic DNA extracted from GM stack 281-24-236 x 3006-210-23 x MON 88913 cotton. Noteworthy the 281-24-236 and 3006-210-23 event-specific methods could be applied to the GM stack DNA with acceptable performance after adjusting the pH value of PCR buffer II (pH 8.0 instead of pH 8.3).

This report is published at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx.

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Quality assurance

The EURL GMFF is ISO 17025:2005 accredited [certificate number: ACCREDIA 1172 (Flexible Scope for DNA extraction and qualitative/quantitative PCR) - Accredited tests are available at http://www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7].

The original version of the document containing evidence of internal checks and authorisation for publication is archived within the EURL GMFF quality system.

The EURL GMFF is also ISO 17043:2010 accredited (proficiency test provider) and applies the corresponding procedures and processes for the management of ring trials during the method validation.

The EURL GMFF conducts its activities under the certification ISO 9001:2008 of the Institute for Health and Consumer Protection (IHCP) provided by SGS.

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1. Introduction

The EU legislative system [1, 2] for genetically modified food and feed provides that any GMO for food and feed use shall undergo the authorisation process before it can be placed on the market. This holds true also for a GMO containing more than one single GM event obtained by conventional crossing, co-transformation or re-transformation (genetically modified stack).

Consequently, the application for authorisation of a GM stack shall be accompanied, among others, by an event-specific method for detection, identification and quantification for each GM event composing the stack, and by samples of the stack and food and feed derived from it. The EURL GMFF shall validate the event specific methods of detection proposed by the applicant with regard to their performance when applied to DNA extracted from the stack, and shall report to the European Food Safety Authority, who will include the EURL GMFF report in the overall opinion concerning the risk assessment and potential authorisation of the assessed stack. In line with the approach defined by the ENGL (http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf) the EURL GMFF carries out an *in-house* verification of the performance of each event-specific methods if this method has previously been validated by the EURL GMFF for the parental single-line event and these events have been stacked by conventional crossing. These criteria are met for the GM stack 281-24-236 x 3006-210-23 x MON 88913 cotton.

Upon reception of methods, samples and related data (step 1), the EURL GMFF carried out the assessment of the documentation (step 2) and the *in-house* verification of the methods (step 3) according to the requirements of Regulation (EC) No 641/2004 (Annex I).

The results of the *in-house* verification study were evaluated with reference to ENGL method performance requirements and to the validation results on the individual events.

2. Step 1 (dossier reception and acceptance)

Dow AgroSciences Ltd submitted the detection methods, data demonstrating the adequate performance of the methods for quantitative detection of events 281-24-236 and 3006-210-23 on the triple stacked cotton material, and the corresponding control samples (DNA extracted from GM stack cotton 281-24-236 x 3006-210-23 x MON 88913 and from non GM cotton and ground seeds from GM stack cotton 281-24-236 x 3006-210-23 x MON 88913 and non GM cotton). In particular, detection methods and data for the previously validated GM event MON 88913 were submitted by Monsanto, in support to the application of the stack event.

The dossier was found to be complete and thus was moved to step 2.

3. Step 2 (dossier scientific assessment)

The data provided by the applicant were assessed against the method acceptance criteria set out by the ENGL[3] and with regard to their documentation and reliability.

Table 1 shows values of trueness (expressed as bias %) and precision (expressed as RSDr %) calculated by the applicant for the three methods on the stack DNA. Means are the average of sixteen replicates for 281-24-236 and 3006-210-23 and fifteen for MON 88913; they were obtained through one run performed with ABI PRISM® 7500 Sequence Detection System. Percentages are expressed as GM DNA / total DNA x 100.

Table 1. Trueness (expressed as bias %) and precision (expressed as relative repeatability standard deviation, RSDr %) for the 281-24-236, 3006-210-23 and MON 88913 methods applied to GM stack 281-24-236 x 3006-210-23 x MON 88913 cotton.

281-24-236*				
Unknown sample GM%	**Expected value (GMO %)**			
	0.085	**0.90**	**2.0**	**5.0**
Mean	0.10	0.90	1.8	5.5
RSD_r (%)	23	18	15	16
Bias (%)	23	-0.53	-9.5	10
3006-210-23*				
Unknown sample GM%	**Expected value (GMO %)**			
	0.085	**0.90**	**2.0**	**5.0**
Mean	0.10	0.90	1.65	4.9
RSD_r (%)	21	16	15	18
Bias (%)	14	-0.58	-17	-1.7
MON 88913				
Unknown sample GM%	**Expected value (GMO %)**			
	0.085	**0.90**	**2.0**	**5.0**
Mean	0.09	0.89	2.20	4.38
RSD_r (%)	20	14	16	10
Bias (%)	5.7	-1.6	9.9	-12

* These data were obtained by the applicant using commercial PCR buffer II at pH 8.3

The EURL GMFF verified the data and concluded that they were reliable and seemed to confirm that the methods meet the ENGL performance criteria [3].

Three requests for complementary information regarding the genetic background of the control samples and the food and feed samples were addressed to the applicant. The EURL GMFF verified the data and the complementary information received and accepted the received clarifications as satisfactory.

The dossier was therefore moved to step 3.

4. Step 3 (EURL GMFF experimental testing)

In step 3 the EURL GMFF implemented the three methods in its own laboratory and performed a verification of their performance when applied to DNA extracted from GM stack 281-24-236 x 3006-210-23 x MON 88913 cotton.

During step 3, two of the three methods (281-24-236 and 3006-210-23) showed underperformance in repeated tests (high relative standard deviation RSDr% for both GM events).

Hence, the EURL GMFF requested the applicant to provide complementary information, reagents and additional control samples for the verification of 281-24-236 and 3006-210-23 methods on the stacked material (September 2012). In April 2013 the EURL GMFF received from the applicant a report named "*Identification of a suitable Reference Dye Batch-Experiments performed in response to the EURL request concerning the EFSA-GMO-NL-2009-68 application*". Preliminary experiments performed identified the ABI PCR buffer II as the reason for underperformance of the methods. A more detailed study was therefore asked to the applicant (May 2013) which was received, together with the reagents and control samples, in September 2013 ["*Identification of PCR buffer as key component for unsatisfying performance of the event-specific detection methods for the 281-24-236 and 3006-210-23 transformation events in the cotton line 281-24-236 x 3006-210-23 x MON 88913*" *(Experiments performed in response to the EURL GMFF request concerning the EFSA-GMO-NL-2009-68 application)*. In that report, the experiments performed by the applicant identified the batch-to-batch variability of ABI PCR buffer II as the cause of the methods performance variations. Moreover, the applicant also demonstrated that *in-house* preparations of PCR buffer of identical composition as ABI PCR buffer II 10x but with a pH of 8.0 instead of 8.3 improved the performance of the methods. Therefore, the EURL GMFF verified the performance of the methods for detection of events 281-24-236 and 3006-210-23 on DNA extracted from the GM stack cotton 281-24-236 x 3006-210-23 x MON 88913 with *in-house* preparations of PCR buffer II at pH 8.0 instead of pH 8.3 (see Annex for the composition of the buffer). Further to this modification (detailed in § 4.5 'Deviations from the validated methods') the methods were shown to meet the ENGL performance criteria when applied to the cotton GM stack 281-24-236 x 3006-210-23 x MON 88913 (§ 4.6 'Results').

4.1 Materials

The following control samples were provided by the applicant:

- GM cotton seeds of the GM stack 281-24-236 x 3006-210-23 x MON 88913 (07CA-HM204)
- Ground conventional cotton seeds (04-CA-BK351) (used to extract DNA for the verification of the method of MON88913)

- DNA extracted from GM stack 281-24-236 x 3006-210-23 x MON 88913

- DNA extracted from non-GM cotton (used for the verification of the methods for detection of 281-24-236 and 3006-210-23)

The EURL GMFF prepared test samples of different GMO concentrations by mixing genomic DNA extracted from GM stack 281-24-236 x 3006-210-23 x MON 88913 cotton and genomic DNA extracted from non GM cotton in a constant amount of total cotton DNA. The same concentrations as in the validation of the GM stack methods 281-24-236 x 3006-210-23 were achieved. Table 2 shows the five GM concentrations used in the verification of the 281-24-236, 3006-210-23 and MON 88913 methods when applying them to genomic DNA extracted from the GM stack 281-24-236 x 3006-210-23 x MON 88913 cotton. These are the same concentrations used in the validation of these methods for the parental GM stack 281-24-236 x 3006-210-23.

Table 2. Percentage of 281-24-236, 3006-210-23 and MON 88913 in 281-24-236 x 3006-210-23 x MON 88913 in the verification samples.

281-24-236 GM% (GM DNA / Non-GM DNA x 100)	**3006-210-23 GM%** (GM DNA / Non-GM DNA x 100)	**MON 88913 GM%** (GM DNA / Non-GM DNA x 100)
5.50	5.50	8.00
2.00	2.00	3.00
0.90	0.90	0.90
0.40	0.40	0.30
0.10	0.10	0.09

The protocols (reagents, concentrations, primer/probe sequences) described by the applicant were implemented in the EURL GMFF laboratory. The *in-house* verification followed the protocols already published as validated methods for 281-24-236 x 3006-210-23 and MON 88913 events (available at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx) with the deviations reported in paragraph 4.5.

4.2 DNA extraction

A method for DNA extraction from cotton seeds was previously evaluated by the EURL GMFF with regard to its performance characteristics and was considered valid i.e. fit the purpose of providing cotton DNA of appropriate quality and amount to be used in subsequent PCR experiments. The protocol for the DNA extraction method is available at http://gmo-crl.jrc.ec.europa.eu/summaries/281-3006%20Cotton_DNAExtr.pdf.
Consequently, the EURL GMFF did not verify the DNA extraction method proposed by the applicant.

4.3 Experimental design

Eight PCR runs for each method were carried out. In each run, samples were analysed in parallel: for 281-24-236 and 3006-210-23 GM-specific systems the relevant reference system *SAH7* (*Sinapis Arabidopsis Homolog 7*) was used, while for MON 88913, the *acp*1 (endogene

encoding a fiber-specific acyl carrier protein) reference system was used. Five GM levels were examined per run for each GM level in duplicate. PCR analysis was performed in triplicate for all samples. In total, for each method (281-24-236, 3006-210-23 and MON 88913), the quantification of the five GM levels was performed as an average of sixteen replicates per GM level (8 runs x 2 replicated levels per run). An Excel spreadsheet was used for determination of GM%.

4.4 PCR methods

During the verification study, the EURL GMFF carried out parallel tests on DNA extracted from GM stack 281-24-236 x 3006-210-23 x MON 88913 cotton using the detection methods previously validated for the respective GM events 281-24-236, 3006-210-23 and MON 88913.

For detection of GM cotton events 281-24-236, 3006-210-23 and MON 88913, DNA fragments of 111-bp, 90-bp and, 94-bp respectively are amplified using specific primers. PCR products are measured during each cycle (real-time) by means of target-specific oligonucleotide probes labelled with fluorescent dyes: FAM (6-carboxyfluorescein) as reporter dye at their 5′-end and TAMRA (6-carboxytetramethylrhodamine) as a quencher dye at their 3′-end for events 281-24-236 and 3006-210-23; for MON 88913, the fluorescent dye used was FAM as well, but the non-fluorescent quencher MBGNFQ was used instead of TAMRA.

In the case of quantification of GM cotton events 281-24-236 and 3006-210-23, the taxon-specific reference system amplifies two fragments of *Sinapis Arabidopsis Homolog* 7 (*SAH7)*: a 115-bp fragment in the A sub-genome and a 123-bp fragment in the D subgenome. Two *SAH7* gene-specific primers and a *SAH7* gene-specific probe labelled with FAM and TAMRA are used. As far as MON 88913 event is concerned, its quantification is achieved by using the cotton reference system *acp*1 whose amplification generates a 76-bp fragment. Two *acp*1 gene specific primers and an *acp*1 specific probe labelled with FAM and MBGNFQ are used.

For the quantification of the cotton GM events, standard curves are generated both for the 281-24-236, 3006-210-23, MON 88913 and for the *SAH7* and *acp*1 specific systems by plotting Cq values of the calibration standards against the logarithm of the DNA copy numbers and by fitting a linear regression into these data. Thereafter, the normalised Cq values of the unknown samples are measured and, by means of the regression formula, the relative amount of each event is estimated.

For detailed information on the preparation of the respective standard curve calibration samples please refer to the protocols of the validated methods at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx.

4.5 Deviations from the validated methods

No deviations were introduced in the verification of the MON88913 method. For the 281-24-236 and 3006-210-23 event specific methods, an *in-house* prepared buffer with the same

composition as the commercially available ABI PCR buffer II 10x was used, with the pH adjusted to 8.0 instead of pH 8.3 as originally validated. This modification was deemed necessary by the applicant after a study done on different lots of ABI PCR buffer II 10x and demonstrating a variable performance of the methods of detection of respectively cotton events 281-24-236 and 3006-210-23 in function of the buffer lot. An acceptable method performance could be re-established by either adjusting the pH of commercial buffers or by preparing *in-house* the PCR buffer II at pH 8.0 (see Annex for the composition of the buffer). The EURL GMFF made use of a PCR buffer II 10x pH 8.0 prepared by the applicant in the verification of the method for detection of event 281-24-236 and of a EURL GMFF-prepared PCR buffer II 10x pH 8.0 to verify the method for detection of event 3006-210-23 in the triple stack cotton material.

Further to updated bioinformatics analyses indicating that the *acp1* target is present in two copies per cotton haploid genome, the method for quantification of MON88913, originally validated as ratio of GM DNA copy numbers to target taxon-specific DNA copy numbers, in the current report has been reported as mass fractions of GM DNA in the cotton stacked event 281-24-236 x 3006-210-23 x MON 88913 (§ 4.1).

4.6 Results

Tables 3, 4 and 5 present the values of the slopes of the different standard curves generated by the EURL GMFF when using DNA extracted from the GM stack, from which the PCR efficiency is calculated using the formula $[10^{(-1/slope)} - 1] \times 100$, and of the R^2 (expressing the linearity of the regression) reported for all PCR systems in the eight runs, for GM cotton events 281-24-236, 3006-210-23 and MON 88913.

Table 3. Values of standard curve slope, PCR efficiency and linearity (R^2) for the 281-24-236 method on GM stack 281-24-236 x 3006-210-23 x MON 88913 cotton.*

Run	281-24-236			*SAH7*		
	Slope	**PCR Efficiency (%)**	**Linearity (R^2)**	**Slope**	**PCR Efficiency (%)**	**Linearity (R^2)**
1	-3.41	97	1.00	-3.45	95	1.00
2	-3.39	97	1.00	-3.38	97	1.00
3	-3.41	96	1.00	-3.37	98	1.00
4	-3.34	99	1.00	-3.40	97	1.00
5	-3.41	97	1.00	-3.29	102	1.00
6	-3.35	99	1.00	-3.20	105	1.00
7	-3.46	95	1.00	-3.41	96	1.00
8	-3.41	96	1.00	-3.32	100	1.00
Mean	-3.40	97	1.00	-3.35	99	1.00

* Results obtained with PCR buffer II, prepared by the applicant and adjusted to pH 8.0.

Table 4. Values of standard curve slope, PCR efficiency and linearity (R^2) for the 3006-210-23 method on GM stack 281-24-236 x 3006-210-23 x MON 88913 cotton.**

Run	3006-210-23			*SAH7*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.30	101	0.99	-3.34	99	1.00
2	-3.34	99	1.00	-3.14	108	0.98
3	-3.33	100	1.00	-3.31	100	0.99
4	-3.38	98	1.00	-3.25	103	0.99
5	-3.31	101	1.00	-3.14	108	0.99
6	-3.32	100	1.00	-3.19	106	0.99
7	-3.37	98	1.00	-3.24	103	0.99
8	-3.18	106	1.00	-3.45	95	0.99
Mean	-3.32	100	1.00	-3.26	103	0.99

** Results obtained with PCR buffer II, prepared at the EURL GMFF and adjusted to pH 8.0.

Table 5. Values of standard curve slope, PCR efficiency and linearity (R^2) for the MON 88913 method on GM stack 281-24-236 x 3006-210-23 x MON 88913 cotton.

Run	MON 88913			*acp1*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.22	105	1.00	-3.41	96	1.00
2	-3.26	103	1.00	-3.33	100	1.00
3	-3.24	103	1.00	-3.37	98	1.00
4	-3.28	102	1.00	-3.34	99	1.00
5	-3.30	101	1.00	-3.34	99	1.00
6	-3.33	100	1.00	-3.35	99	1.00
7	-3.26	103	1.00	-3.43	96	1.00
8	-3.34	99	1.00	-3.37	98	1.00
Mean	-3.28	102	1.00	-3.37	98	1.00

The mean PCR efficiencies of the GM and species-specific systems were 97% for the 281-24-236, 100% for the 3006-210-23 and 102% for the MON 88913 system, respectively. The *SAH7* species-specific system showed PCR efficiency of 99% and 103%, respectively and the *acp*1 system 98%. The linearity of the methods (R^2) was 1.00 for all systems except *SAH7* which in one case was 0.99. The data presented in Tables 3, 4 and 5 confirm the appropriate performance characteristics of the methods when tested on GM stack cotton in terms of PCR efficiency and linearity.

The EURL GMFF also assessed the values of trueness (expressed as bias %) and precision (expressed as relative repeatability standard deviation, RSDr %) of the three methods applied to samples of DNA extracted from GM stack cotton, see tables 6, 7 and 8.

Table 6. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the 281-24-236 method applied to genomic DNA extracted from GM stack 281-24-236 x 3006-210-23 x MON 88913 cotton.*

281-24-236					
Unknown sample GM%	**Expected value (GMO%)**				
	0.10	**0.40**	**0.90**	**2.0**	**5.5**
Mean	0.12	0.47	1.08	2.22	5.94
SD	0.02	0.03	0.12	0.23	0.67
RSD_r (%)	16	5.9	11	10	11
Bias (%)	25	16	20	11	8

* Results obtained with PCR buffer II, prepared by the applicant and adjusted to pH 8.0.

Table 7. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the 3006-210-23 method applied to genomic DNA extracted from GM stack 281-24-236 x 3006-210-23 x MON 88913 cotton.**

3006-210-23					
Unknown sample GM%	**Expected value (GMO%)**				
	0.10	**0.40**	**0.90**	**2.0**	**5.5**
Mean	0.12	0.39	1.03	2.05	5.55
SD	0.02	0.07	0.23	0.35	1.14
RSD_r (%)	18	18	22	17	21
Bias (%)	15	-2.5	15	2.6	0.84

** Results obtained with PCR buffer II, prepared at the EURL GMFF and adjusted to pH 8.0.

Table 8. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the MON 88913 method applied to genomic DNA extracted from GM stack 281-24-236 x 3006-210-23 x MON 88913 cotton.

MON 88913					
Unknown sample GM%	**Expected value (GMO%)**				
	0.09	**0.30**	**0.90**	**3.0**	**8.0**
Mean	0.09	0.33	1.05	3.69	9.75
SD	0.01	0.02	0.05	0.17	0.35
RSD_r (%)	11	7	4.8	4.5	3.6
Bias (%)	-4.3	11	17	23	22

The trueness of the method is estimated using the measurements of the method bias for each GM level. According to the ENGL acceptance criteria and method performance requirements, the trueness of the method should be within ± 25% across the entire dynamic range. As

shown in Tables 6, 7 and 8, the values range from 8% to 25% for 281-24-236, from 0.84% to 15% for 3006-210-23 and from -4.3% to 23% for MON 88913. Therefore, the three methods satisfy the above mentioned requirement throughout their respective dynamic ranges, also when applied to DNA extracted from GM stack 281-24-236 x 3006-210-23 x MON 88913 cotton.

Tables 6, 7 and 8 also show the relative repeatability standard deviation (RSD_r) as estimated for each GM level of each event. According to the ENGL acceptance criteria and method performance requirements, the EURL GMFF requires RSD_r values to be below 25%. As the values range between 5.9% and 16% for 281-24-236, between 17% and 22% for 3006-210-23, and between 3.6% and 11% for MON 88913, the three methods satisfy this requirement throughout their respective dynamic ranges when applied to DNA extracted from GM stack 281-24-236 x 3006-210-23 x MON 88913 cotton.

5. Comparison of method performance on 281-24-236 x 3006-210-23 x MON 88913 and on the single events

An indicative comparison of the performance (bias, RSDr %) of the three methods applied to GM stack 281-24-236 x 3006-210-23 x MON 88913 cotton and on the 281-24-236 x 3006-210-23 and MON 88913 GM events is shown in Tables 9, 10 and 11. The performance of the methods was previously validated through international collaborative trials (http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx).

Note: the comparison of data generated in different testing conditions and different times is intended to be only of qualitative nature; differences in the figures reported are not necessarily statistically significant.

Table 9. Qualitative comparison of the performance of the 281-24-236 detection method applied to genomic DNA extracted from GM stack 281-24-236 x 3006-210-23 x MON 88913 cotton and to genomic DNA extracted from GM stack 281-24-236 x 3006-210-23.

Trueness and repeatability of 281-24-236 quantification on 281-24-236 x 3006-210-23 x MON 88913			**Trueness and repeatability of 281-24-236 quantification on event 281-24-236 x 3006-210-23***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.10	25	16	**0.10**	5.3	22
0.40	16	5.9	**0.40**	3.9	16
0.90	20	11	**0.90**	5.3	17
2.0	11	11	**2.0**	10	15
5.5	8.1	11	**5.5**	2.7	15

*method validated in inter-laboratory study (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm)

Table 10. Qualitative comparison of the performance of the 3006-210-23 detection method applied to genomic DNA extracted from GM stack 281-24-236 x 3006-210-23 x MON 88913 cotton and to genomic DNA extracted from GM stack 281-24-236 x 3006-210-23.

Trueness and repeatability of 3006-210-23 quantification on 281-24-236 x 3006-210-23 x MON 88913			**Trueness and repeatability of 3006-210-23 quantification on event 281-24-236 x 3006-210-23 ***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.10	15	18	**0.10**	-5.6	30
0.40	-2.5	18	**0.40**	-1.4	20
0.90	15	22	**0.90**	0.95	16
2.0	2.6	17	**2.0**	2.8	15
5.5	0.84	21	**5.5**	2.5	21

*method validated in inter-laboratory study (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm)

Table 11. Qualitative comparison of the performance of the MON 88913 detection method applied to genomic DNA extracted from GM stack 281-24-236 x 3006-210-23 x MON 88913 cotton and to genomic DNA extracted from the single line event MON 88913.

Trueness and repeatability of MON 88913 quantification on 281-24-236 x 3006-210-23 x MON 88913			**Trueness and repeatability of MON 88913 quantification on single event MON88913***		
GM%	**Bias (%)**	**RSD_r (%)**	**GM%**	**Bias (%)**	**RSD_r (%)**
0.09	-4.3	11	**0.09**	-16	13
0.30	11	7.0	**0.30**	0.5	10
0.90	17	4.8	**0.90**	-27	13
3.0	23	4.5	**3.0**	-12	11
8.0	22	3.6	**8.0**	-7.2	12

*method validated in inter-laboratory study (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm)

6. Conclusions

The performance of the three event-specific methods for the detection and quantification of cotton events 281-24-236, 3006-210-23 and MON 88913, when applied to genomic DNA extracted from GM stack 281-24-236 x 3006-210-23 x MON 88913 cotton, meets the ENGL performance requirements, as assessed on the control samples provided by the applicant. The method verification has demonstrated that the PCR efficiency, linearity, trueness and repeatability of the methods were within the limits established by the ENGL. However, the pH of the PCR buffer II should be 8.0 (Annex). The careful preparation of the PCR buffer II appears very important as slight changes may affect method performance. In addition, when converting copy numbers into mass fraction, the fact that the *acp1* target is present in two copies per cotton haploid genome must be taken into account.

In conclusion, the verification study confirmed that the three methods are capable to detect, identify and quantify each of the GM events when applied to genomic DNA of suitable quality, extracted from GM stack 281-24-236 x 3006-210-23 x MON 88913 cotton.

Therefore these methods, developed and validated to detect and quantify the single events, can be equally applied for the detection and quantification of the respective events combined in GM stack 281-24-236 x 3006-210-23 x MON 88913 cotton.

7. References

1. Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed (Text with EEA relevance). OJ L 268, 18.10.2003, p. 1–23.

2. Commission Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003 of the European Parliament and of the Council as regards the application for the authorisation of new genetically modified food

and feed, the notification of existing products and adventitious or technically unavoidable presence of genetically modified material which has benefited from a favourable risk evaluation (Text with EEA relevance). OJ L 102, 7.4.2004, p. 14–25.

3. European Network of GMO Laboratories: Definition of minimum performance requirements for analytical methods of GMO testing. 13 October 2008. http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf

Annex

Composition of the 10x PCR buffer II, pH 8

500 mM KCl, 100 mM Tris-HCl.

Preparation for 12 ml
Mix at room temperature:
1.2 ml Tris-HCl 1M, pH8.0
2.0 ml KCl 3M
8.8 ml nuclease-free water

Reagents

Tris-HCl solution 1M, pH8	(Sigma, T-3038)
KCl 3M	(Fluka, 60135)
Nuclease-free Water	(Promega, P1193)

Abbreviations

Tris	Tris(hydroxymethyl)aminomethane hydrochloride
KCl	potassium chloride

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Abstract

An application was submitted by Dow AgroSciences Ltd to request the authorisation of genetically modified stack (GM stack) 281-24-236 x 3006-210-23 x MON 88913 cotton (providing protection against cotton bollworm, tobacco budworm, beet armyworm, fall armyworm, soybean loopers, cabbage loopers and pink bollworm and being glyphosate herbicide tolerant) and all sub-combinations of the individual events as present in the segregating progeny, for food and feed uses, and import and processing, in accordance with articles 5 and 17 of Regulation (EC) N° 1829/2003 GM Food and GM Feed. The unique identifier assigned to GM stack 281-24-236 x 3006-210-23 x MON 88913 cotton is DAS-24236-5 x DAS-21023-5 x MON-88913-8.

The GM stack 281-24-236 x 3006-210-23 x MON 88913 cotton has been obtained by conventional crossing between the genetically modified cotton events: DAS-24236-5 x DAS-21023-5 (WideStrike™) and MON 88913, without any new genetic modification.

The EURL GMFF has previously validated individually, and declared fit for purpose, the detection methods for the single events 281-24-236, 3006-210-23 and MON 88913 (see http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx). In line with the approach defined by the ENGL (http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf) the EURL GMFF has carried out only an in-house verification of the performance of each validated method when applied to genomic DNA extracted from GM stack 281-24-236 x 3006-210-23 x MON 88913 cotton. Noteworthy the 281-24-236 and 3006-210-23 event-specific methods could be applied to the GM stack DNA with acceptable performance after adjusting the pH value of PCR buffer II (pH 8.0 instead of pH 8.3).

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