Report on the Verification of the Performance of MON 531 and MON 1445 Event-specific Methods on the Cotton Event MON 531 x MON 1445 Using Real-Time PCR

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Joint Research Centre
Institute for Health and Consumer Protection
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Executive Summary

The JRC as Community Reference Laboratory for GM Food and Feed (CRL-GMFF), established by Regulation (EC) No 1829/2003, has carried out an in-house verification study to assess the performance of two quantitative event-specific methods on the cotton event MON 531 x MON 1445 (unique identifier MON-ØØ531-6 x MON-Ø1445-2) which combines the MON 531 and MON 1445 transformation events. The two methods have been previously validated individually on single-trait events, to detect and quantify each event in cotton samples; a validation report for each method is available at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm. This study was conducted according to internationally accepted guidelines [1, 2].

In accordance to Regulation (EC) No 1829/2003 of 22 September 2003 on genetically modified food and feed and to Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003, Monsanto Europe S.A. provided the detection methods and the control samples: whole seeds of MON 531 x MON 1445 cotton (ST5599BR) and whole conventional cotton seeds of ST474. The JRC prepared the in-house verification samples (calibration samples and blind samples at different GM percentages).

The results of the in-house verification study were evaluated with reference to ENGL method performance requirements (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm) and to the validation results on the individual parental events (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

The results of this CRL-GMFF in-house verification studies are made publicly available at http://gmo-crl.jrc.ec.europa.eu/.

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Report on Steps 1-3 of the Validation Process

Monsanto Europe S.A. submitted the detection methods and control samples of the cotton event MON 531 x MON 1445 (unique identifier MON-ØØ531-6 x MON-Ø1445-2) under Article 5 and 17 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The Community Reference Laboratory for GM Food and Feed (CRL-GMFF), following reception of the documentation and material, including control samples, (step 1 of the validation process) carried out the scientific assessment of documentation and data (step 2) in accordance with Commission Regulation (EC) No 641/2004 "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" and according to its operational procedures ("Description of the CRL-GMFF Validation Process", http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm).

The scientific assessment focused on the method performance characteristics assessed against the method acceptance criteria set out by the European Network of GMO Laboratories and listed in the "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (http://gmo-crl.jrc.ec.europa.eu/doc/Method%20requirements.pdf) (see Annex 1 for a summary of method acceptance criteria and method performance requirements). During step 2, five scientific assessments were performed and three requests of complementary information were addressed to the applicant. Upon reception of the complementary information, the scientific assessment of the detection method for the MON 531 x MON 1445 cotton was concluded in May 2006.

The event-specific detection methods for the two cotton lines hosting the single events MON 531 and MON 1445 were validated by the CRL-GMFF following the conclusion of the respective international collaborative studies and the publication of the validation reports (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm). Hence, the detection methods applied on the cotton event MON 531 x MON 1445 did not undergo a full validation process. The CRL-GMFF performed an in-house verification of the detection methods to verify that they exhibit a comparable performance on samples of event MON 531 x MON 1445 combining the two traits (as provided in accordance to Annex 1.2.C.2 of Commission Regulation (EC) No 641/2004).

In October 2006, the CRL-GMFF concluded the experimental verification of the method characteristics (step 3, experimental testing of the samples and methods) by quantifying, with each specific method, five blind GM-levels within the range 0.1 %-6 %, on a DNA/DNA ratio. The experiments were performed under repeatability conditions and demonstrated that the PCR efficiency, linearity, trueness and repeatability of the quantification were within the limits established by the ENGL.

A Technical Report summarising the results of tests carried out by the CRL-GMFF (step 3) is available on request.

Content

1. Introduction

The applicant Monsanto Europe S.A. submitted the detection methods for MON 531 and MON 1445 and the control samples of the cotton event MON 531 x MON 1445 (unique identifier MON-ØØ531-6 x MON-Ø1445-2) under Article 5 and 17 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The Joint Research Centre (JRC, Molecular Biology and Genomics Unit of the Institute of Health and Consumer Protection) as Community Reference Laboratory for GM Food and Feed, established by Regulation (EC) 1829/2003, carried out an in-house verification of the two event-specific methods for the detection and quantification of MON 531 and MON 1445 in the MON 531 x MON 1445 cotton event combining the two traits. The single methods had been previously validated by international collaborative studies on the single-trait cotton events (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

Upon reception of methods, samples and related data (step 1), the CRL-GMFF carried out the assessment of the documentation (step 2) and the in-house evaluation of the methods (step 3) according to the requirements of Regulation (EC) 641/2004 and following CRL-GMFF operational procedures. The CRL-GMFF method verification was concluded in October 2006.

A method for DNA extraction from cotton seeds, submitted by the applicant, was evaluated by the CRL-GMFF in order to confirm its performance characteristics. The protocols for DNA extraction are available at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm.

The operational procedure of the in-house verification included the following module:

- ✓ Quantitative real-time PCR (Polymerase Chain Reaction). The methodology consists of two event-specific real-time quantitative TaqMan® PCR procedures for the determination of the relative content of events MON 531 and MON 1445 DNA to total cotton DNA in the MON 531 x MON 1445 cotton event. The procedures are simplex systems, in which the events MON 531 and MON 1445 were quantified in reference to the cotton *acp1* (acyl carrier protein) endogenous gene.

The study was carried out in accordance to the following internationally accepted guidelines:

- ✓ The IUPAC "Protocol for the design, conduct and interpretation of method performance studies" (1).
- ✓ ISO 5725:1994 (2).

2. Materials

For the verification of the quantitative event-specific methods, control samples consisting of:

- genomic DNA extracted from homogenised seeds of MON 531 x MON 1445 cotton (ST5599BR),
- genomic DNA extracted from homogenised seeds of conventional cotton ST474,

were provided by the applicant, in accordance to the provisions of Regulation (EC) No 1829/2003, Art 2.11 ["control sample defined as the GMO or its genetic material (positive sample) and the parental organism or its genetic material that has been used for the purpose of the genetic modification (negative sample)"].

Samples containing mixtures of 100 % MON 531 x MON 1445 and conventional cotton genomic DNA at different GMO concentrations were prepared by the CRL-GMFF in a constant amount of total cotton DNA, using the control samples provided.

The protocols (reagents, concentrations, primer/probe sequences) followed in the in-house verification are those already published as validated methods for the individual MON 531 and MON 1445 events and available at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm.

Table 1 shows the five GM levels of blind samples used in the verification of the MON 531 and MON 1445 methods.

Table 1. MON 531 and MON 1445 GM contents in cotton event MON 531 x MON 1445

MON 531 GM% (GM DNA / Non-GM DNA x 100)	**MON 1445 GM%** (GM DNA / Non-GM DNA x 100)
0.10	0.10
0.50	0.50
0.90	0.90
2.50	2.50
6.00	6.00

3. Experimental design

Eight runs for each event-specific method were carried out. In each run, samples were analysed in parallel with both the GM-specific system and the reference system (*acp1*). Five GM levels per run were examined and two replicates for each GM level were analysed. PCR analysis was performed in triplicate for all samples. In total, for each method (MON 531 and MON 1445), the quantification of the five GM levels was performed as an average of sixteen replicates per GM level. An Excel spreadsheet was used for determination of GM%.

4. Method

Description of the operational steps

For specific detection of events MON 531 and MON 1445 in cotton event MON 531 x MON 1445, two specific fragments of the integration regions of the constructs inserted into the plant genome, of 72 bp and 87 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 two fluorescent dyes: 6-FAM is used for MON 531 and MON 1445 as reporter dye at its 5′ end and TAMRA as a quencher dye at its 3′ end.

For relative quantification of events MON 531 and MON 1445 DNA, a cotton-specific reference system which amplifies a 76 bp fragment of the cotton endogenous gene *acp1* (acyl carrier protein), using two *acp1* gene-specific primers and an *acp1* gene-specific probe labelled with FAM and TAMRA, was used.

Standard curves are generated for each GM specific system (MON 531 or MON 1445), by plotting Ct values of the calibration standards against the logarithm of the DNA copy numbers MON 531 or MON 1445 DNA, and fitting a linear regression into these data. Thereafter, the Ct values of the blind samples are measured and, by means of the regression formula, the relative amount of event MON 531 or MON 1445 DNA is estimated respectively.

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

5. Deviations reported

No deviations were introduced.

6. Summary of results

PCR efficiency and linearity

The values of the slopes of the standard curves, from which the PCR efficiency is calculated using the formula [10^(-1/slope)-1] x 100, and of the R^2 (expressing the linearity of the regression) reported for all PCR systems in the eight runs, are presented in Table 2 and 3 for MON 531 and MON 1445 methods, respectively.

Table 2. Values of standard curve slope, PCR efficiency and linearity (R^2) for the MON 531 method on event MON 531 x MON 1445.

Run	MON 531			*acp1*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.22	104	0.99	-3.46	94	1.00
2	-3.22	104	0.99	-3.46	94	1.00
3	-3.12	109	1.00	-3.38	98	1.00
4	-3.12	109	1.00	-3.38	98	1.00
5	-3.21	105	1.00	-3.42	96	1.00
6	-3.21	105	1.00	-3.42	96	1.00
7	-3.25	103	1.00	-3.41	97	1.00
8	-3.25	103	1.00	-3.41	97	1.00
Mean	-3.20	105	1.00	-3.42	96	1.00

Table 3. Values of standard curve slope, PCR efficiency and linearity (R^2) for the MON 1445 method on event MON 531 x MON 1445.

Run	MON 1445			*acp1*		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.28	102	0.98	-3.38	98	0.99
2	-3.32	100	0.98	-3.30	101	0.99
3	-3.19	106	0.99	-3.28	102	0.99
4	-3.06	112	0.99	-3.20	105	0.99
5	-3.29	101	0.99	-3.19	106	0.99
6	-3.33	100	0.99	-3.23	104	0.99
7	-3.21	105	0.99	-3.24	103	0.99
8	-3.14	108	0.98	-3.19	106	0.99
Mean	-3.23	104	0.99	-3.25	103	0.99

The mean PCR efficiencies of the GM specific systems are 105 % and 104 % respectively for the MON 531 and MON 1445 specific systems. The linearity of the methods is equal to or

above 0.98 for both systems. Overall, data reported in Table 2 and 3 confirm the appropriate performance characteristics of the two methods tested on MON 531 x MON 1445 cotton samples in terms of PCR efficiency and linearity.

7. Method performance requirements

The results of the in-house verification study for the MON 531 and MON 1445 detection methods applied to event MON 531 x MON 1445 cotton DNA are reported in Tables 4 and 5, respectively. Results were evaluated with respect to the method acceptance criteria, as established by ENGL and adopted by the CRL-GMFF (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm, see also Annex 1). In addition, Tables 4 and 5 report estimates of the trueness and repeatability standard deviation for each GM level and for both methods.

Table 4. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the MON 531 method on event MON 531 x MON 1445 cotton DNA.

MON 531					
Blind sample GM%	**Expected value (GMO%)**				
	0.1	**0.5**	**0.9**	**2.5**	**6**
Mean	0.12	0.57	0.89	2.3	5.44
SD	0.02	0.05	0.05	0.21	0.65
RSD_r (%)	14	8.3	5.2	9.3	12
Bias (%)	23	15	-1.2	-8.1	-9.4

Table 5. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation of the MON 1445 method on event MON 531 x MON 1445 cotton DNA

MON 1445					
Blind sample GM%	**Expected value (GMO%)**				
	0.1	**0.5**	**0.9**	**2.5**	**6**
Mean	0.08	0.42	0.9	2.47	6.29
SD	0.01	0.05	0.08	0.29	0.60
RSD_r (%)	9.6	13	8.6	12	9.5
Bias (%)	-16	-17	0.5	-1.1	4.8

The *trueness* of the method is estimated using the measures of the method bias for each GM level. According to the ENGL acceptance criteria and method performance requirements, the trueness of the method, measured as bias from the accepted value, should be within ± 25% across the entire dynamic range. As shown in Tables 4 and 5, both methods satisfy the above requirement throughout their respective dynamic ranges.

Tables 4 and 5 further document the relative repeatability standard deviation (RSD_r) as estimated for each GM level. In order to accept methods for collaborative trial evaluation, the

CRL-GMFF requires that RSD_r values are below 25%, as indicated by ENGL (Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" [http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm]). As it can be observed from the values reported in Tables 4 and 5, the two methods satisfy this requirement throughout their respective dynamic ranges.

8. Comparison of method performance between event MON 531 x MON 1445 and the single trait events

A synoptic comparison of the two method performances on the cotton event MON 531 x MON 1445 and the single trait events is shown in Tables 6 and 7. The performance of the methods on the single lines was previously assessed through international collaborative trials.

Table 6. Trueness (bias %) and relative repeatability standard deviation (RSD_r %) of the MON 531 detection method on event MON 531 x MON 1445 and on event MON 531.

Trueness and repeatability of MON 531 quantification on MON 531 x MON 1445			**Trueness and repeatability of MON 531 quantification on single event MON 531***		
GM %	**Bias (%)**	**RSD_r (%)**	**GM %**	**Bias (%)**	**RSD_r (%)**
0.1	23	14	**0.1**	-22	34
0.5	15	8.3	**0.5**	-28	22
0.9	-1.2	5.2	**0.9**	-22	21
2.5	-8.1	9.3	**2.5**	-6.4	15
6	-9.4	12	**6**	2.5	21

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

Table 7. Trueness (bias %) and relative repeatability standard deviation (RSD_r %) of the MON 1445 detection method on event MON 531 x MON 1445 and on event MON 1445.

Trueness and repeatability of MON 1445 quantification on MON 531 x MON 1445			**Trueness and repeatability of MON 1445 quantification on single event MON 1445***		
GM %	**Bias (%)**	**RSD_r (%)**	**GM %**	**Bias (%)**	**RSD_r (%)**
0.1	-16	9.6	0.1	41	14
0.5	-17	13	0.5	25	18
0.9	0.5	8.6	0.9	4.7	14
2.5	-1.1	12	2.5	11	11
6	4.8	9.5	6	5.2	17

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

For trueness, the MON 531 even-specific method (Table 6), when applied to event MON 531 x MON 1445 and compared to the single line, shows a higher bias (%) at 6 % and a lower bias at GM levels 0.5 and 0.9 %. The MON 1445 event-specific method (Table 7), when applied to

event MON 531 x MON 1445, shows a lower bias (%) at all levels of GM. The trueness is within the acceptance value set by ENGL (± 25%) for the dynamic range between 0.5 and 6.0% GM. However, the method for MON 1445 when applied to the single line event shows a bias of 41% at the 0.1 % GM level.

With the exception of the 2.5 % GM level of the MON 1445 method both the MON 531 and MON 1445 (Table 6 and 7) event-specific methods show lower values for relative repeatability standard deviation (RSD_r %) when applied to the hybrid compared to the single events. With the exception of the 0.1 % GM level of the MON 531 event-specific method, the results are in all cases below the ENGL acceptance level established at maximum 25%.

Therefore, the in-house method verification has demonstrated that the MON 531 and MON 1445 detection methods developed to detect and quantify the single events can be equally applied for the quantification of the respective events combined in event MON 531 x MON 1445.

9. Conclusions

The overall method performance of the two event-specific methods for the quantitative detection of events MON 531 and MON 1445 combined in cotton event MON 531 x MON 1445 have been evaluated with respect to the method acceptance criteria and the method performance requirements recommended by the ENGL (as detailed under http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm), and to the validation results obtained for the single trait events (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

The results obtained during the present verification study indicate that the analytical modules of the methods submitted by the applicant comply with ENGL performance criteria. The methods are therefore applicable to the control samples provided (see section 3 "Materials"), in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004.

10. Quality assurance

The CRL-GMFF carries out all operations according to ISO 9001:2000 (certificate number: CH-32231) and ISO 17025:2005 (certificate number: DAC-PL-0459-06-00) [DNA extraction, qualitative and quantitative PCR in the area of Biology (DNA extraction and PCR method validation for the detection and identification of GMOs in food and feed materials)].

11. References

1. Horwitz W., 1995. Protocol for the design, conduct and interpretation of method performance studies, *Pure and Appl. Chem*, 67: 331-343.

2. International Standard (ISO) 5725:1994. Accuracy (trueness and precision) of measurement methods and results. International Organization for Standardization.

12. Annex 1: method acceptance criteria and method performance requirements as set by the European Network of GMO Laboratories (ENGL)

Method Acceptance Criteria should be fulfilled at the moment of submission of a method (Phase 1: acceptance for the collaborative study).

Method Performance Requirements should be fulfilled in a collaborative study in order to consider the method as fit for its purpose (Phase 2: evaluation of the collaborative study results).

Method Acceptance Criteria

Applicability

Definition: The description of analytes, matrices, and concentrations to which a method can be applied.

Acceptance Criterion: The applicability statement should provide information on the scope of the method and include data for the indices listed below for the product/s for which the application is submitted. The description should also include warnings to known interferences by other analytes, or inapplicability to certain matrices and situations.

Practicability

Definition: The ease of operations, the feasibility and efficiency of implementation, the associated unitary costs (e.g. Euro/sample) of the method.

Acceptance Criterion: The practicability statement should provide indication on the required equipment for the application of the method with regards to the analysis *per se* and the sample preparation. An indication of costs, timing, practical difficulties and any other factor that could be of importance for the operators should be indicated.

Specificity

Definition: Property of a method to respond exclusively to the characteristic or analyte of interest.

Acceptance Criterion: The method should be event-specific and be functional only with the GMO or GM based product for which it was developed. This should be demonstrated by empirical results from testing the method with non-target transgenic events and non-transgenic material. This testing should include closely related events and cases where the limit of the detection is tested.

Dynamic Range

Definition: The range of concentrations over which the method performs in a linear manner with an acceptable level of accuracy and precision.

Acceptance Criterion: The dynamic range of the method should include the 1/10 and at least 5 times the target concentration. Target concentration is intended as the threshold relevant for legislative requirements. The acceptable level of accuracy and precision are described below. The range of the standard curve(s) should allow testing of blind samples throughout the entire dynamic range, including the lower (10%) and upper (500%) end.

Accuracy

Definition: The closeness of agreement between a test result and the accepted reference value.

Acceptance Criterion: The accuracy should be within ± 25% of the accepted reference value over the whole dynamic range.

Amplification Efficiency

Definition: The rate of amplification that leads to a theoretical slope of –3.32 with an efficiency of 100% in each cycle. The efficiency of the reaction can be calculated by the following equation: Efficiency = $[10^{(-1/slope)}] - 1$

Acceptance Criterion: The average value of the slope of the standard curve should be in the range of (- 3.1 ≥ slope ≥ - 3.6)

R^2 Coefficient

Definition: The R^2 coefficient is the correlation coefficient of a standard curve obtained by linear regression analysis.

Acceptance Criterion: The average value of R^2 should be ≥ 0.98.

Repeatability Standard Deviation (RSD_r)

Definition: The standard deviation of test results obtained under repeatability conditions. Repeatability conditions are conditions where test results are obtained with the same method, on identical test items, in the same laboratory, by the same operator, using the same equipment within short intervals of time.

Acceptance Criterion: The relative repeatability standard deviation should be below 25% over the whole dynamic range of the method.

Note: Estimates of repeatability submitted by the applicant should be obtained on a sufficient number of test results, at least 15, as indicated in ISO 5725-3 (1994).

Limit of Quantitation (LOQ)

Definition: The limit of quantitation is the lowest amount or concentration of analyte in a sample that can be reliably quantified with an acceptable level of precision and accuracy.

Acceptance Criterion: LOQ should be less than $1/10^{th}$ of the value of the target concentration with an RSD_r ≤ 25%. Target concentration should be intended as the threshold relevant for legislative requirements. The acceptable level of accuracy and precision are described below.

Limit of Detection (LOD)

Definition: The limit of detection is the lowest amount or concentration of analyte in a sample, which can be reliably detected, but not necessarily quantified, as demonstrated by single laboratory validation.

Acceptance Criterion: LOD should be less than $1/20^{th}$ of the target concentration. Experimentally, quantitative methods should detect the presence of the analyte at least 95% of the time at the LOD, ensuring $\leq$ 5% false negative results. Target concentration should be intended as the threshold relevant for legislative requirements.

Robustness

Definition: The robustness of a method is a measure of its capacity to remain unaffected by small, but deliberate deviations from the experimental conditions described in the procedure.

Acceptance Criterion: The response of an assay with respect to these small variations should not deviate more than $\pm$ 30%. Examples of factors that a robustness test could address are: use of different instrument type, operator, brand of reagents, concentration of reagents, and temperature of reaction.

Method Performance Requirements

Dynamic Range

Definition: In the collaborative trial the dynamic range is the range of concentrations over which the reproducibility and the trueness of the method are evaluated with respect to the requirements specified below.

Acceptance Criterion: The dynamic range of the method should include the 1/10 and at least five times the target concentration. Target concentration should be intended as the threshold relevant for legislative requirements.

Reproducibility Standard Deviation (RSD_R)

Definition: The standard deviation of test results obtained under reproducibility conditions. Reproducibility conditions are conditions where test results are obtained with the same method, on identical test items, in different laboratories, with different operators, using different equipment. Reproducibility standard deviation describes the inter-laboratory variation.

Acceptance Criterion: The relative reproducibility standard deviation should be below 35% at the target concentration and over the entire dynamic range. An RSD_R < 50% is acceptable for concentrations below 0.2%.

Trueness

Definition: The closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. The measure of trueness is usually expressed in terms of bias.

Acceptance Criterion: The trueness should be within $\pm$ 25% of the accepted reference value over the whole dynamic range.