



# Event-specific Method for the Quantification of Cotton Line MON 15985 Using Real-time PCR

## Validation Report

19 June 2008

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

### Executive Summary

The JRC as Community Reference Laboratory for GM Food and Feed (CRL-GMFF), established by Regulation (EC) No 1829/2003, in collaboration with the European Network of GMO Laboratories (ENGL), has carried out a collaborative study to assess the performance of a quantitative event-specific method to detect and quantify the MON 15985 transformation event in cotton DNA (unique identifier MON-15985-7). The collaborative trial was conducted according to internationally accepted guidelines <sup>(1, 2)</sup>.

In accordance with Regulation (EC) No 1829/2003 of 22 September 2003 on genetically modified food and feed and with Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003, Monsanto provided the detection method and the samples (cotton containing the transformation event MON 15985 and conventional cotton seeds). The JRC prepared the validation samples (calibration samples and blind samples at unknown GM percentage [DNA/DNA]). The collaborative trial involved twelve laboratories from nine European countries.

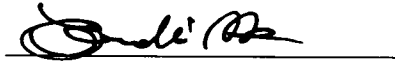
The results of the international collaborative trial met the ENGL performance requirements. The method is therefore considered applicable to the control samples provided, in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004.

The results of the collaborative study are made publicly available at <http://gmo-crl.jrc.it/>.

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## Report on Steps 1-3 of the Validation Process

Monsanto submitted the detection method and control samples for cotton event MON 15985 (unique identifier MON-15985-7) under Article 8 and 20 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.it/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.it/guidancedocs.htm>) (see Annex 1 for a summary of method acceptance criteria and method performance requirements). During step 2, five scientific assessments were performed and requests of complementary information addressed to the applicant. Upon reception of complementary information, the scientific evaluation of the detection method for event MON 15985 was positively concluded in May 2006.

Between February and April 2006, the CRL-GMFF verified experimentally the method characteristics (step 3, experimental testing of samples and methods) by quantifying five blind GM-levels within the range 0.1%-6% on a copy number basis. The experiments were performed in repeatability conditions and demonstrated that the PCR efficiency, linearity, accuracy and precision of the quantifications 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.

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## 1. Introduction

Monsanto submitted the detection method and control samples for cotton event MON 15985 (unique identifier MON-15985-7) under Article 8 and 20 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, Biotechnology and GMOs Unit of the Institute for Health and Consumer Protection) as Community Reference Laboratory for GM Food and Feed (see Regulation EC No 1829/2003) organised the international collaborative study for the event-specific method for the detection and quantification of MON 15985 cotton. The study involved twelve laboratories, all members of the European Network of GMO Laboratories (ENGL).

Upon reception of method, samples and related data (step 1), the JRC carried out the assessment of the documentation (step 2) and the in-house evaluation of the method (step 3) according to the requirements of Regulation (EC) No 641/2004 and following its operational procedures.

The internal in-house experimental evaluation of the method was carried out between February and April 2006.

Following the evaluation of the data and the results of the in-house laboratory tests, the international collaborative study was organised (step 4) and took place in June-July 2006.

A method for DNA extraction from cotton seeds, submitted by the applicant, was evaluated by the CRL-GMFF; laboratory testing of the method was carried out in order to confirm its performance characteristics. The protocol for DNA extraction and a report on method testing is available at <http://gmo-crl.jrc.it/>.

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

- ✓ Quantitative real-time PCR (Polymerase Chain Reaction). The methodology is an event-specific real-time quantitative TaqMan<sup>®</sup> PCR procedure for the determination of the relative content of event MON 15985 DNA to total cotton DNA. The procedure is a simplex system, in which a cotton *acp1* (*Acyl carrier protein*) endogenous assay (reference gene) and the target assay (MON 15985) are performed in separate wells.

The international collaborative study 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 participating laboratories

As part of the international collaborative study the method was tested in twelve ENGL laboratories to determine its performance. Clear guidance was given to the laboratories with regards to the standard operational procedures to follow for the execution of the protocol. The participating laboratories are listed in alphabetical order in Table 1.

Table 1. Laboratories participating in the validation of the detection method for cotton line MON 15985.

| Laboratory  | Country         |
|---|-----------------|
| Bavarian Health and Food Safety Authority   | Germany         |
| Crop Research Institute - Reference Laboratory for GMO Detection and DNA                        | Czech Rep.      |
| Institute of Chemical Technology Prague   | Czech Rep.      |
| LGC Limited   | UK              |
| National Centre for Food, Spanish Food Safety Agency  | Spain           |
| National Institute of Engineer (INETI)  | Portugal        |
| RIKILT Institute of Food Safety   | The Netherlands |
| Scientific Institute of Public Health (IPH)   | Belgium         |
| Service Commun des Laboratoires du MINEFI - Laboratoire de Strasbourg                           | France          |
| The Food and Consumer Product Safety Authority  | The Netherlands |
| Veterinary Public Health Institute for Lazio and Toscana Regions; National Reference Laboratory | Italy           |
| Walloon Agricultural Research Centre (CRA-W) - Department Quality of Agricultural Products      | Belgium         |

### 3. Materials

For the validation of the quantitative event-specific method, genomic DNA was extracted from samples consisting of:

- i) seeds of cotton harbouring the MON 15985 event (Line Bollgard II, lot number GLP-0304-13873-S) and;
- ii) seeds of conventional cotton (Line DP5415, lot number GLP-0403-14754-S)

Samples 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 15985 cotton and non-GM cotton genomic DNA at different GMO concentrations were prepared by the CRL-GMFF, using the control samples provided, in a constant amount of total cotton DNA.

Participants received the following materials:

- ✓ Five calibration samples (160 µL of DNA solution each) labelled from S1 to S5.
- ✓ Twenty 'unknown' DNA samples (80 µL of DNA solution each), labelled from U1 to U20.
- ✓ Amplification reagent control was used on each PCR plate.
- ✓ Reaction reagents as follows:
  - Universal PCR Master Mix, three bottles: 5 mL each
  - Distilled sterile water, one tube: 12.2 mL
- ✓ Primers and probes (1 tube each) as follows:
  - acp1* reference system
    - *acp1* primer forward (10 µM): 240 µL
    - *acp1* primer reverse (10 µM): 240 µL
    - *acp1* Taqman<sup>®</sup> probe (5 µM): 160 µL
  - MON 15985* system
    - MON 15985 primer forward (10 µM): 240 µL
    - MON 15985 primer reverse (10 µM): 240 µL
    - MON 15985 Taqman<sup>®</sup> probe (5 µM): 160 µL

### 4. Experimental design

Twenty unknown samples (labelled from U1 to U20), representing five GM levels, were used in the validation study (Table 2). On each PCR plate, the samples were analysed for the MON 15985 specific system and for the *acp1* specific system. In total, two plates were run per participating laboratory and four replicates for each GM level were analysed. PCR analysis was performed in triplicate 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).

Table 2. MON 15985 GM contents

| MON 15985 GM%<br>(GM copy number/cotton genome copy number x 100) |
|---|
| 0.1   |
| 0.4   |
| 0.9   |
| 2.5   |
| 6.0   |

## 5. Method

### *Description of operational steps followed*

For the specific detection of event MON 15985 DNA, an 82-bp fragment of the integration region of the construct inserted into the plant genome (3' insert-to-plant junction) is amplified using two specific primers. PCR products are measured at each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with FAM dye and TAMRA as quencher dye.

For the relative quantification of event MON 15985 DNA, a cotton-specific reference system 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.

Standard curves are generated for both the MON 15985 and the *acp1* specific systems by plotting the Ct values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a 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 relative quantification of event MON 15985 DNA in a test sample, the MON 15985 copy number is divided by the copy number of the cotton reference gene (*acp1*) and multiplied by 100 to obtain the percentage value (GM% = MON15985 / *acp1* x 100).

Calibration sample S1 was prepared by mixing the appropriate amount of MON 15985 DNA in control non-GM cotton DNA to obtain a 10% GM MON 15985 in a total of 200 ng cotton DNA. Samples S2 was prepared by two-fold dilution from the S1 sample; sample S3 was prepared by five-fold dilution from S2 sample; sample S4 was prepared by three-fold dilution of sample S3 and sample S5 was prepared by four-fold dilution from the S4 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)<sup>(3)</sup>. The copy number values used in the quantification, the GM contents of the calibration



samples and total DNA quantity used in PCR are provided in Table 3 (% GM calculated considering the 1C value for cotton genome as 2.33 pg) <sup>(3)</sup>.

Table 3. % GM values of the standard curve samples.

| Sample code                     | S1    | S2    | S3   | S4   | S5   |
|---------------------------------|-------|-------|------|------|------|
| Total amount of DNA in reaction | 200   | 100   | 20   | 6.68 | 1.68 |
| Cotton genome copies            | 85830 | 42910 | 8580 | 2860 | 710  |
| MON 15985 cotton copies         | 8583  | 4291  | 858  | 286  | 71   |

## 6. Deviations reported

Six laboratories reported no deviations from the protocol.

One laboratory received thawed samples and reagents due to improper storage and late delivery of the samples by the courier. This occurrence was considered as a major violation of the validation protocol and as such the data from this laboratory were not included in the study.

One laboratory swapped the U10 and C0 samples in one plate with no consequences since sample label was correctly attributed in subsequent analysis.

One laboratory performed two sets of runs (total of four runs instead of two requested) with two different operators. One set of runs was randomly selected for data analysis.

Four laboratories considered the S5 calibration sample as outlier.

Overall, in the vast majority of the data reported by laboratories, the S5 calibration sample did display an unexpected behaviour (higher than expected Ct figures) with clear deviation from the linearity represented by the regression line fitting the calibration points from S1 to S4. As a consequence, the standard curves including the S5 calibration samples resulted in extremely low reaction efficiencies with severely biased estimation of GM-content in the 'unknown' samples. In particular, the outcome of the ring-trial indicated that an apparent marked decrease of total DNA content occurred in the S5 sample. Presence of DNase activity cannot be excluded as a carry-over from the DNA purification procedures [that might affect to a greater extent samples at low concentration], despite careful execution of the submitted DNA extraction protocol. Tests previously performed during the step 3 of the CRL-GMFF validation process (experimental testing of samples and method) had shown a good performance of the method with standard curve parameters meeting the ENGL requirements and no aberrant behaviour on the same calibration sample S5. Therefore, the CRL-GMFF took the decision to eliminate the S5 calibration sample from the standard curves of both the GM and the cotton-specific reference systems from the data of all participating laboratories and to perform

accordingly the data analysis whose outcome is described in the present validation report. Therefore, the estimation of accuracy (trueness and precision) of the sample at lowest GM concentration (0.1%) has been performed by extrapolation rather than interpolation due to Ct values falling outside those of the accepted standard curves (S1-S4).

## 7. Summary of results

### *PCR efficiency and linearity*

The values of the slopes [from which the PCR efficiency is calculated using the formula  $((10^{(-1/\text{slope})}) - 1) \times 100$ ] of the reference curve and of the  $R^2$  (expressing the linearity of the regression) reported by participating laboratories for the MON 15985 system and the *acp1* reference system are summarised in Table 4.

Table 4. Values of reference curve slope, PCR efficiency and linearity ( $R^2$ )

| Lab | Plate | MON 15985    |                    |             | <i>acp1</i>  |                    |             |
|-----|-------|--------------|--------------------|-------------|--------------|--------------------|-------------|
|     |       | Slope        | PCR Efficiency (%) | $R^2$       | Slope        | PCR Efficiency (%) | $R^2$       |
| 1   | A     | -3.02        | 114                | 1.00        | -3.20        | 105                | 0.99        |
|     | B     | -3.02        | 114                | 1.00        | -3.21        | 105                | 0.99        |
| 2   | A     | -3.00        | 115                | 0.99        | -3.21        | 105                | 0.99        |
|     | B     | -3.03        | 114                | 0.99        | -3.24        | 104                | 0.99        |
| 3   | A     | -3.28        | 102                | 1.00        | -3.48        | 94                 | 1.00        |
|     | B     | -3.03        | 114                | 0.99        | -3.22        | 104                | 0.99        |
| 4   | A     | -4.09        | 76                 | 0.96        | -4.19        | 73                 | 0.98        |
|     | B     | -3.22        | 104                | 0.97        | -3.31        | 101                | 0.99        |
| 5   | A     | -3.53        | 92                 | 1.00        | -3.40        | 97                 | 0.98        |
|     | B     | -4.05        | 77                 | 0.99        | -3.32        | 100                | 1.00        |
| 6   | A     | -3.17        | 107                | 1.00        | -3.22        | 105                | 0.99        |
|     | B     | -3.15        | 108                | 0.99        | -3.21        | 105                | 0.99        |
| 7   | A     | -2.31        | 171                | 0.95        | -2.77        | 130                | 0.94        |
|     | B     | -2.43        | 158                | 0.97        | -2.61        | 141                | 0.87        |
| 8   | A     | -3.17        | 107                | 0.99        | -3.37        | 98                 | 1.00        |
|     | B     | -3.14        | 108                | 0.99        | -3.41        | 96                 | 1.00        |
| 9   | A     | -3.31        | 100                | 1.00        | -3.29        | 101                | 0.99        |
|     | B     | -3.20        | 105                | 1.00        | -3.23        | 104                | 0.99        |
| 10  | A     | -3.48        | 94                 | 1.00        | -3.45        | 95                 | 1.00        |
|     | B     | -3.27        | 102                | 1.00        | -3.43        | 96                 | 1.00        |
| 11  | A     | -            | -                  | -           | -            | -                  | -           |
|     | B     | -            | -                  | -           | -            | -                  | -           |
| 12  | A     | -3.01        | 115                | 0.99        | -3.36        | 98                 | 1.00        |
|     | B     | -3.08        | 111                | 0.99        | -3.29        | 101                | 1.00        |
|     | Mean  | <b>-3.20</b> | <b>110</b>         | <b>0.99</b> | <b>-3.30</b> | <b>103</b>         | <b>0.98</b> |

The mean PCR efficiency was 110% for the MON 15985 and 103% for the *acp1* system, with both values within the ENGL acceptance criteria. The linearity of the method was 0.99 for the MON 15985 system and 0.98 for the cotton-specific reference system, therefore within the acceptance limits. Data reported confirm the appropriate performance characteristics of the method tested in terms of efficiency and linearity.

### ***GMO quantification***

Table 5 shows the mean values of the four replicates for each GM level as provided by all laboratories. Each mean value is the average of three PCR repetitions.

Table 5. GM% mean values determined by laboratories for unknown samples.

| LAB | GMO content<br>(GM% = GM copy number/cotton genome copy number x 100) |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
|-----|---|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
|     | 0.1   |       |       |       | 0.4   |       |       |       | 0.9   |       |       |       | 2.5   |       |       |       | 6.0   |       |       |       |
|     | 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   | 0.07  | 0.08  | 0.08  | 0.06  | 0.36  | 0.33  | 0.33  | 0.31  | 0.65  | 0.83  | 0.84  | 0.86  | 2.34  | 2.04  | 2.46  | 2.85  | 6.72  | 5.72  | 5.84  | 6.48  |
| 2   | 0.06  | 0.06  | 0.07  | 0.07  | 0.27  | 0.25  | 0.31  | 0.32  | 0.63  | 0.72  | 0.63  | 0.75  | 2.01  | 2.16  | 2.03  | 2.29  | 5.50  | 5.17  | 5.16  | 5.25  |
| 3   | 0.13  | 0.09  | 0.06  | 0.07  | 0.38  | 0.52  | 0.31  | 0.35  | 1.34  | 0.95  | 0.73  | 0.67  | 2.52  | 2.61  | 1.99  | 2.3   | 7.40  | 9.23  | 6.64  | 4.74  |
| 4   | 0.22  | 0.25  | 0.11  | 0.08  | 0.73  | 0.64  | 0.34  | 0.31  | 1.36  | 1.44  | 0.64  | 0.92  | 3.38  | 2.95  | 2.04  | 2.21  | 7.20  | 6.07  | 5.47  | 4.18  |
| 5   | 0.07  | 0.14  | 0.49  | 0.67  | 0.50  | 0.50  | 1.73  | 1.39  | 1.28  | 1.48  | 2.10  | 1.65  | 2.17  | 2.52  | 2.24  | 4.93  | 5.8   | 1.06  | 10.3  | 8.84  |
| 6   | 0.07  | 0.07  | 0.07  | 0.08  | 0.27  | 0.31  | 0.31  | 0.32  | 0.80  | 0.74  | 0.65  | 0.66  | 2.21  | 2.39  | 2.18  | 2.25  | 5.43  | 4.68  | 5.87  | 5.15  |
| 7   | 0.02  | 0.03  | 0.01  | 0.01  | 0.12  | 0.13  | 0.11  | 0.09  | 0.42  | 0.5   | 0.43  | 1.00  | 0.37  | 3.67  | 2.00  | 1.62  | 7.31  | 4.14  | 0.71  | 6.28  |
| 8   | 0.08  | 0.06  | 0.06  | 0.05  | 0.32  | 0.28  | 0.29  | 0.28  | 0.8   | 0.73  | 0.73  | 0.73  | 2.68  | 1.90  | 2.38  | 1.85  | 5.43  | 5.97  | 6.64  | 6.49  |
| 9   | 0.12  | 0.14  | 0.10  | 0.10  | 0.50  | 0.56  | 0.34  | 0.44  | 0.88  | 0.96  | 1.05  | 1.07  | 3.16  | 3.35  | 2.85  | 2.80  | 6.40  | 6.75  | 6.09  | 6.53  |
| 10  | 0.13  | 0.12  | 0.11  | 0.10  | 0.35  | 0.41  | 0.39  | 0.4   | 0.91  | 0.89  | 0.84  | 0.96  | 2.76  | 2.96  | 2.41  | 2.55  | 7.53  | 5.17  | 6.28  | 5.73  |
| 11  | -   | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     |
| 12  | 0.09  | 0.12  | 0.11  | 0.11  | 0.34  | 0.36  | 0.38  | 0.43  | 0.91  | 1.07  | 0.83  | 0.9   | 2.87  | 3.09  | 2.31  | 2.78  | 6.83  | 5.80  | 5.80  | 5.66  |

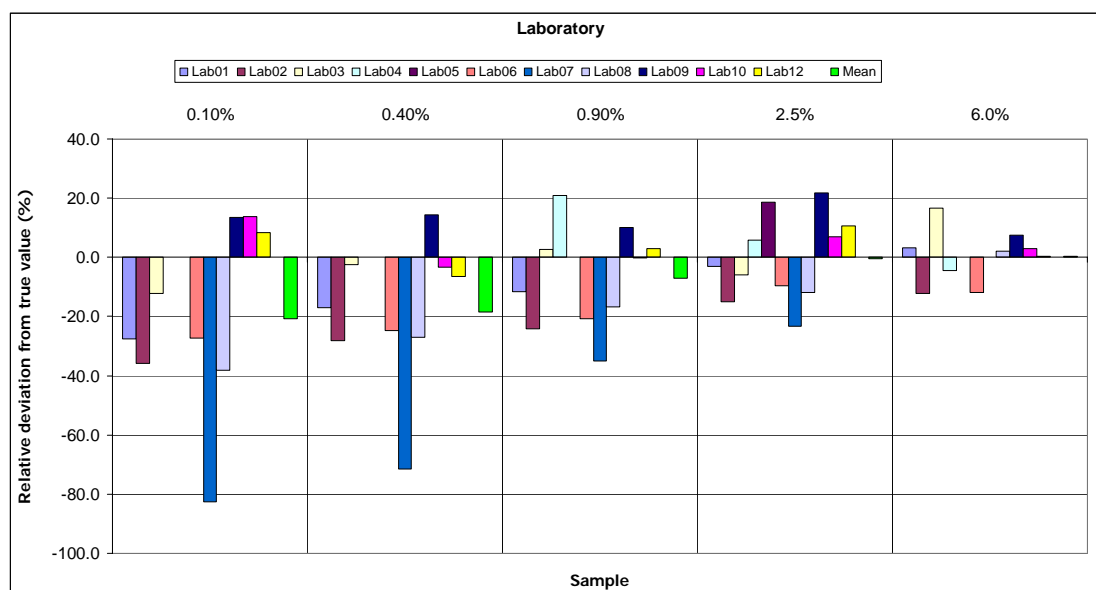
In Figure 1 the relative deviation from the true value for each GM level tested is shown for each laboratory. The coloured bars represent the relative GM quantification obtained by the participating laboratories as well as the mean value (represented by the green bar).

As observed in Figure 1, relative deviations from the true values are mainly negative for GM levels of 0.1% and 0.4%, meaning that the GM content tends to be underestimated at these GM levels. One laboratory showed very large relative deviations from the true values at 0.1% and 0.4% GM levels.

The bias generated by all laboratories at the 0.9% and higher GM levels is randomly distributed and of values below 20%.

Overall, the average relative deviation is within the acceptance criterion at all GM levels tested, indicating a satisfactory trueness of the method.

Figure 1. Relative deviation (%) from the true value of MON 15985 for all laboratories



## 8. Method performance requirements

Among the performance criteria established by ENGL and adopted by the CRL-GMFF (<http://gmo-crl.jrc.it/guidancedocs.htm>, see also Annex 1), repeatability and reproducibility are assessed through an international collaborative trial, carried out with the support of ENGL laboratories (see Table 1).

Table 6 illustrates the estimation of repeatability and reproducibility at various GM levels, according to the range of GM percentages tested during the collaborative trial.

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 6, the method satisfies this requirement at all GM levels tested. In fact, the highest values of  $RSD_R$  (%) are 42% at the 0.1% level and 33% at 0.4%, thus within the acceptance criterion.

Table 6 further documents the relative repeatability standard deviation ( $RSD_r$ ), as estimated for each GM level. In order to accept methods for collaborative study evaluation, the CRL requires that  $RSD_r$  values be below 25%, as indicated by ENGL (Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (<http://gmo-crl.jrc.it/guidancedocs.htm>)).

Table 6. MON 15985: summary of validation results.

| 'unknown' sample GM%  | Expected value (GMO %) |       |       |       |      |
|---|------------------------|-------|-------|-------|------|
|   | 0.1*                   | 0.4   | 0.9   | 2.5   | 6.0  |
| Laboratories having returned valid results                        | 11                     | 11    | 11    | 11    | 11   |
| Samples per laboratory  | 4                      | 4     | 4     | 4     | 4    |
| Number of outliers  | 2                      | 2     | 1     | -     | 2    |
| Reason for exclusion  | 2C                     | 2C    | 1G    | -     | 2C   |
| Mean value  | 0.08                   | 0.33  | 0.84  | 2.49  | 6.03 |
| Relative repeatability standard deviation, RSD <sub>r</sub> (%)   | 19                     | 16    | 22    | 26    | 15   |
| Repeatability standard deviation                                  | 0.01                   | 0.05  | 0.19  | 0.65  | 0.90 |
| Relative reproducibility standard deviation, RSD <sub>R</sub> (%) | 42                     | 33    | 27    | 27    | 16   |
| Reproducibility standard deviation                                | 0.03                   | 0.11  | 0.23  | 0.67  | 0.95 |
| Bias (absolute value)   | -0.02                  | -0.07 | -0.06 | -0.01 | 0.03 |
| Bias (%)  | -21                    | -18   | -7.2  | -0.5  | 0.5  |

C = Cochran's test; G = Grubbs' test; identification and removal of outliers through Cochran and Grubbs tests, according to ISO 5725-2.

Bias is estimated according to ISO 5725 data analysis protocol.

\* Extrapolation from S1-S4 standard curves.

As it can be observed from the values reported in Table 6, the method satisfies this requirement, with the minor deviation of a relative repeatability standard deviation slightly of 26% at the 2.5% GM level.

The trueness of the method is estimated using the measures of the method bias for each GM level. According to ENGL method performance requirements, trueness should be  $\pm 25\%$  across the entire dynamic range. In this case the method fully satisfies this requirement across the entire dynamic range tested; in fact, the highest deviation from true value (bias %) is -21% at the 0.1% level, thus within the acceptance criterion.

## 9. Conclusions

The overall method performance has been evaluated with respect to the method acceptance criteria and method performance requirements recommended by the ENGL (as detailed under <http://gmo-crl.jrc.it/guidancedocs.htm>). The method acceptance criteria were reported by the applicant and used to evaluate the method prior to the international collaborative study (see Annex 1 for a summary of method acceptance criteria and method performance requirements).

The results obtained during the collaborative study, indicate that the analytical module of the method submitted by the applicant complies with ENGL performance criteria.

Therefore, the method is considered applicable to the control samples provided (see paragraph 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-32232) 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, Genève, Switzerland.
3. Arumuganathan K., Earle E. D., 1991. Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter* 9: 208-218.

## 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  $\pm 25\%$  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:  $\text{Efficiency} = [10^{(1/\text{slope})} - 1]$

Acceptance Criterion: the average value of the slope of the standard curve should be in the range of  $(-3.1 \geq \text{slope} \geq -3.6)$

### ***R<sup>2</sup> Coefficient***

Definition: the R<sup>2</sup> coefficient is the correlation coefficient of a standard curve obtained by linear regression analysis.

Acceptance Criterion: the average value of R<sup>2</sup> should be  $\geq 0.98$ .

### ***Repeatability Standard Deviation (RSD<sub>r</sub>)***

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<sup>th</sup> of the value of the target concentration with an  $\text{RSD}_r \leq 25\%$ . 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  $1/20^{\text{th}}$  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.