Event-specific Method for the Quantification of Cotton Line MON 531 Using Real-time PCR v. 1.01

Validation Report and Validated Method

Marco Mazzara
Alessia Bogni
Nicoletta Foti
Guy Van den Eede

2013
The corrections made in the new document are:

### Page 7 §3

**MON 531 system**

- **MON 1445 primer forward** (10 µM): 240 µL
- **MON 1445 primer reverse** (10 µM): 240 µL
- **MON 1445 TaqMan® probe** (5 µM): 160 µL

Changed to

- **MON 531 primer forward** (10 µM): 240 µL
- **MON 531 primer reverse** (10 µM): 240 µL
- **MON 531 TaqMan® probe** (5 µM): 160 µL

**Note:**

Since 01/12/2009 the term "Community Reference Laboratory (CRL)" is changed into "European Union Reference Laboratory (EURL)."

Since 01/03/2009 the JRC-unit that hosts the EU-RL GMFF is named "Unit for Molecular Biology and Genomics" instead of "Biotechnology and GMO Unit."

JRC84173

EUR 26152 EN


ISSN 1831-9424

doi: 10.2788/18510


© European Union, 2013

Printed in Italy
Event-specific Method for the Quantification of Cotton Line MON 531 Using Real-time PCR

Validation Report
10 June 2008
Corrected version 1 - 28/08/2013 (see page 2)

Joint Research Centre
Institute for Health and Consumer Protection
Unit for Molecular Biology and Genomics

Executive Summary

The JRC as European Union Reference Laboratory for the GM Food and Feed (EURL 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 531 transformation event in cotton DNA (unique identifier MON-∅∅531-6). The collaborative trial was conducted according to internationally accepted guidelines (1, 2).

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 seeds containing the transformation event 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 seven European countries.

The results of the international collaborative trial mostly 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/.
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 apply 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 IHCP Institute provided by CERMET

Correction from the previous version:

Corrected version 1 - 28/08/2013

Page 1 and 2: new layout

Page 7 §3

MON 531 system

- MON 1445 primer forward (10 µM): 240 µL
- MON 1445 primer reverse (10 µM): 240 µL
- MON 1445 TaqMan® probe (5 µM): 160 µL

Changed to

MON 531 system

- MON 531 primer forward (10 µM): 240 µL
- MON 531 primer reverse (10 µM): 240 µL
- MON 531 TaqMan® probe (5 µM): 160 µL

Address of contact laboratory:
European Commission, Joint Research Centre (JRC)
Institute for Health and Consumer Protection (IHCP)
Unit for Molecular Biology and Genomics
European Union Reference Laboratory for GM Food and Feed
Via E. Fermi 2749, 21027 Ispra (VA) – Italy
Functional mailbox: EURL_GMFF@jrc.ec.europa.eu

EURL-GMFF: Validation Report MON 531 cotton
Report on Steps 1-3 of the Validation Process

Monsanto submitted the detection method and control samples for cotton event MON 531 (unique identifier MON-∅∅531-6) 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 European Union Reference Laboratory for GM Food and Feed (EURL 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 EURL 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, four 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 531 was positively concluded in April 2006.

Starting from February 2006, the EURL 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%-6.0% 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. The DNA extraction module of the method was tested by the EURL GMFF on samples of food and feed.

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

1. INTRODUCTION ........................................................................................................... 5
2. LIST OF PARTICIPATING LABORATORIES .............................................................. 6
3. MATERIALS .................................................................................................................. 7
4. EXPERIMENTAL DESIGN ......................................................................................... 8
5. METHOD ..................................................................................................................... 8
   DESCRIPTION OF THE OPERATIONAL STEPS FOLLOWED ........................................... 8
6. DEVIATIONS REPORTED .......................................................................................... 9
7. SUMMARY OF RESULTS ........................................................................................... 9
   PCR EFFICIENCY AND LINEARITY ............................................................................. 9
   GMO QUANTIFICATION .............................................................................................. 10
8. METHOD PERFORMANCE REQUIREMENTS ............................................................ 12
9. CONCLUSIONS .......................................................................................................... 13
10. QUALITY ASSURANCE ............................................................................................ 13
11. REFERENCES ............................................................................................................ 14
12. ANNEX 1: METHOD ACCEPTANCE CRITERIA AND METHOD PERFORMANCE
    REQUIREMENTS AS SET BY THE EUROPEAN NETWORK OF GMO
    LABORATORIES (ENGL) ............................................................................................ 15
1. Introduction

Monsanto submitted the detection method and control samples for cotton event MON 531 (unique identifier MON-∅∅531-6) under Article 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 European Union 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 531 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 in February-July 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 between July and August 2006.

A method for DNA extraction from cotton seeds, submitted by the applicant, was evaluated by the EURL 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/](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® PCR procedure for the determination of the relative content of event MON 531 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 531) are performed in separate wells.

The international collaborative trial was carried out in accordance with the following internationally accepted guidelines:

- 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 531

<table>
<thead>
<tr>
<th>Laboratory</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bundesinstitut fuer Risikobewertung (BfR)</td>
<td>Germany</td>
</tr>
<tr>
<td>Central Science Laboratory</td>
<td>UK</td>
</tr>
<tr>
<td>Chemisches und Veterinäruntersuchungsamt Freiburg</td>
<td>Germany</td>
</tr>
<tr>
<td>CRA-W, Dépt Qualité des productions agricoles</td>
<td>Belgium</td>
</tr>
<tr>
<td>Dr E.Wessling Chemical Laboratory</td>
<td>Hungary</td>
</tr>
<tr>
<td>Ente Nazionale Sementi Elette (central office in Milano)</td>
<td>Italy</td>
</tr>
<tr>
<td>Laboratorio Analisi Sementi</td>
<td>Italy</td>
</tr>
<tr>
<td>General Chemical State Laboratory, Food Division</td>
<td>Greece</td>
</tr>
<tr>
<td>Istituto Zooprofilattico Sperimentale Lazio e Toscana</td>
<td>Italy</td>
</tr>
<tr>
<td>Landesuntersuchungsanstalt für das Gesundheits- und Veterinärwesen Sachsen Amtliche Lebensmittelüberwachung</td>
<td>Germany</td>
</tr>
<tr>
<td>LAV Sachsen-Anhalt</td>
<td>Germany</td>
</tr>
<tr>
<td>LSGV Saarland (Landesamt für Soziales, Gesundheit und Verbraucherschutz)</td>
<td>Germany</td>
</tr>
<tr>
<td>Research Institute of Crop Production, Reference Laboratory of the Ministry of Agriculture</td>
<td>Czech Republic</td>
</tr>
</tbody>
</table>
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 531 event (Line DP448B, Lot number GLP-0403-14756-S) and;

ii) Seeds of conventional cotton (Line Stoneville, lot number GLP-0403-14786-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 531 cotton and non-GM cotton genomic DNA at different GMO concentrations were prepared by the EURL 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) for the preparation of the standard curve, labelled from S1 to S5.
✓ Twenty unknown DNA samples (80 µL of DNA solution each), labelled from U1 to U20.
✓ Reaction reagents:
  - Universal PCR Master Mix 2X, 3 vials: 5 mL each
  - Sterile distilled water, one vial: 12.2 mL

✓ Primers and probes (1 tube each) as follows:

  *acp 1 reference system*
  - acp 1 primer forward (10 µM): 240 µL
  - acp 1 primer reverse (10 µM): 240 µL
  - acp 1 TaqMan® probe (5 µM): 160 µL

  *MON 531 system*
  - MON 531 primer forward (10 µM): 240 µL
  - MON 531 primer reverse (10 µM): 240 µL
  - MON 531 TaqMan® 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). Two separated plates were run due to the difference in the annealing temperature between the two systems used. On each PCR plate, the samples were analysed either for the MON 531 specific system or 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. MON531 GM contents

<table>
<thead>
<tr>
<th>MON 531 GM% (GM copy number/cotton genome copy number x 100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
</tr>
<tr>
<td>2.5</td>
</tr>
<tr>
<td>0.9</td>
</tr>
<tr>
<td>0.5</td>
</tr>
<tr>
<td>0.1</td>
</tr>
</tbody>
</table>

5. Method

Description of the operational steps followed

For specific detection of MON 531 cotton genomic DNA, a 72-bp fragment of the integration region of the construct inserted into the plant genome (5’ insert-to-plant junction) is amplified using two specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with FAM dye at its 5’-end and TAMRA as quencher dye at 3’-end.

For the relative quantification of event MON 531 DNA, a cotton-specific reference system amplifies a 76-bp fragment of cotton reference 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 both for the acp1 and MON 531 specific systems by plotting the Ct values measured for the calibration points against the logarithm of the DNA copy numbers, and by fitting a linear regression line into these data. Thereafter, the standard curves are used to estimate the copy numbers in the unknown sample by interpolation from the standard curves.
For the relative quantification of event MON 531 DNA in the test sample, the MON 531 copy number is divided by the copy number of the cotton reference gene \((acp1)\) and multiplied by 100 to obtain the percentage value \((\text{GM}\% = \text{MON 531}/acp1 \times 100)\).

Calibration sample S1 was prepared by mixing the appropriate amount of MON 531 DNA in control non-GM DNA to obtain a 10% GM MON 531 in a total of 200 ng of cotton DNA. Sample S2 was prepared as a two-fold dilution from S1, sample S3 as a five-fold dilution from S2, sample S4 as a three-fold dilution from S3 and sample S5 as a four-fold dilution from S4.

The absolute copy numbers in the calibration curve samples are determined by dividing the sample DNA weight (in picograms) by the published average 1C value for cotton genome (2.33 pg)\(^{(3)}\). 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.

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

<table>
<thead>
<tr>
<th>Sample code</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
<th>S5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total amount (ng) of DNA in reaction</td>
<td>200</td>
<td>100</td>
<td>20</td>
<td>6.68</td>
<td>1.68</td>
</tr>
<tr>
<td>Cotton genome copies</td>
<td>85830</td>
<td>42910</td>
<td>8580</td>
<td>2860</td>
<td>710</td>
</tr>
<tr>
<td>531 cotton copies</td>
<td>8583</td>
<td>4291</td>
<td>858</td>
<td>286</td>
<td>71</td>
</tr>
</tbody>
</table>
Table 4. Values of the reference curve slope, PCR efficiency and linearity ($R^2$)

<table>
<thead>
<tr>
<th></th>
<th>MON 531</th>
<th></th>
<th></th>
<th></th>
<th>acp1</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LAB</td>
<td>Slope</td>
<td>PCR Efficiency (%)</td>
<td>Linearity ($R^2$)</td>
<td>Slope</td>
<td>PCR Efficiency (%)</td>
<td>Linearity ($R^2$)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-3.04</td>
<td>87</td>
<td>0.97</td>
<td>-3.13</td>
<td>91</td>
<td>0.99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>-2.64</td>
<td>60</td>
<td>0.98</td>
<td>-3.40</td>
<td>97</td>
<td>0.99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>-3.30</td>
<td>99</td>
<td>0.96</td>
<td>-3.37</td>
<td>98</td>
<td>0.99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>-3.04</td>
<td>87</td>
<td>0.99</td>
<td>-3.08</td>
<td>91</td>
<td>0.99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>-2.74</td>
<td>93</td>
<td>0.97</td>
<td>-3.05</td>
<td>87</td>
<td>0.99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>-3.17</td>
<td>68</td>
<td>0.97</td>
<td>-3.38</td>
<td>97</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>-2.90</td>
<td>79</td>
<td>0.99</td>
<td>-3.09</td>
<td>91</td>
<td>0.99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>-2.95</td>
<td>82</td>
<td>0.99</td>
<td>-3.01</td>
<td>91</td>
<td>0.99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>-2.79</td>
<td>72</td>
<td>0.98</td>
<td>-2.84</td>
<td>75</td>
<td>0.99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>-2.77</td>
<td>70</td>
<td>0.90</td>
<td>-3.01</td>
<td>85</td>
<td>0.98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>-2.82</td>
<td>74</td>
<td>0.97</td>
<td>-3.13</td>
<td>91</td>
<td>0.99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>-3.07</td>
<td>88</td>
<td>1.00</td>
<td>-3.12</td>
<td>91</td>
<td>0.98</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>-2.93</td>
<td>80</td>
<td>0.97</td>
<td>-3.13</td>
<td>90</td>
<td>0.99</td>
<td></td>
</tr>
</tbody>
</table>

The mean PCR efficiency for the event-specific MON 531 system was 80%, while that of the endogenous reference system acp1 was 90%.

The linearity of the method was on average 0.97 for the MON 531 system: this is slightly lower than the ENGL performance requirement ($\geq 0.98$), however it is worth noting that one laboratory contributes significantly to lowering the mean result (0.90). The mean $R^2$ of the acp1 reference system was 0.99.

**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 value determined by laboratories for all unknown samples.

<table>
<thead>
<tr>
<th>LAB</th>
<th>0.1</th>
<th>0.5</th>
<th>0.9</th>
<th>2.5</th>
<th>6.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.08</td>
<td>0.07</td>
<td>0.04</td>
<td>0.17</td>
<td>0.33</td>
</tr>
<tr>
<td>2</td>
<td>0.04</td>
<td>0.09</td>
<td>0.09</td>
<td>0.07</td>
<td>0.48</td>
</tr>
<tr>
<td>3</td>
<td>0.11</td>
<td>0.13</td>
<td>0.12</td>
<td>0.16</td>
<td>0.48</td>
</tr>
<tr>
<td>4</td>
<td>0.06</td>
<td>0.06</td>
<td>0.07</td>
<td>0.06</td>
<td>0.34</td>
</tr>
<tr>
<td>5</td>
<td>0.07</td>
<td>0.00</td>
<td>0.05</td>
<td>0.10</td>
<td>0.40</td>
</tr>
<tr>
<td>6</td>
<td>0.09</td>
<td>0.12</td>
<td>0.12</td>
<td>0.13</td>
<td>0.40</td>
</tr>
<tr>
<td>7</td>
<td>0.10</td>
<td>0.08</td>
<td>0.07</td>
<td>0.07</td>
<td>0.35</td>
</tr>
<tr>
<td>8</td>
<td>0.05</td>
<td>0.07</td>
<td>0.07</td>
<td>0.04</td>
<td>0.29</td>
</tr>
<tr>
<td>9</td>
<td>0.05</td>
<td>0.06</td>
<td>0.07</td>
<td>0.07</td>
<td>0.32</td>
</tr>
<tr>
<td>10</td>
<td>0.07</td>
<td>0.04</td>
<td>0.03</td>
<td>0.07</td>
<td>0.25</td>
</tr>
<tr>
<td>11</td>
<td>0.07</td>
<td>0.11</td>
<td>0.07</td>
<td>0.06</td>
<td>0.33</td>
</tr>
<tr>
<td>12</td>
<td>0.15</td>
<td>0.09</td>
<td>1.44</td>
<td>0.10</td>
<td>1.03</td>
</tr>
</tbody>
</table>

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; the light-yellow bar represents the overall mean for each GM level (%) tested.

Figure 1. Relative deviation (%) from the true value of MON 531 for all laboratories

Relative deviations from the true values are mainly negative at the lower GM levels (0.1%, 0.5% and 0.9%), meaning that the GM content of unknown samples tends to be underestimated at these GM levels.
However, the mean value of the relative deviation at all GM levels tested remains within the limit of the trueness acceptance level (25%), with a minor deviation for the 0.5% GM level (28%).

Overall this indicates a satisfactory trueness of the method through the GM% range tested.

8. Method performance requirements

Among the performance criteria established by ENGL and adopted by the EURL 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.

Table 6. MON531 summary of validation data.

<table>
<thead>
<tr>
<th>Unknown sample GM%</th>
<th>Expected value (GMO%)</th>
<th>0.1%</th>
<th>0.5%</th>
<th>0.9%</th>
<th>2.5%</th>
<th>6.0%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laboratories having returned results</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Samples per laboratory</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Number of outliers</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Reason for exclusion</td>
<td>1 C. test</td>
<td>1 C. test</td>
<td>2 C. test</td>
<td>1 C. test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean value</td>
<td>0.08</td>
<td>0.36</td>
<td>0.70</td>
<td>2.34</td>
<td>6.15</td>
<td></td>
</tr>
<tr>
<td>Relative repeatability standard deviation, RSDₐ (%)</td>
<td>34</td>
<td>22</td>
<td>21</td>
<td>15</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Repeatability standard deviation</td>
<td>0.03</td>
<td>0.08</td>
<td>0.15</td>
<td>0.36</td>
<td>1.30</td>
<td></td>
</tr>
<tr>
<td>Relative reproducibility standard deviation, RSDᵦ (%)</td>
<td>43</td>
<td>31</td>
<td>32</td>
<td>24</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Reproducibility standard deviation</td>
<td>0.03</td>
<td>0.11</td>
<td>0.22</td>
<td>0.57</td>
<td>1.73</td>
<td></td>
</tr>
<tr>
<td>Bias (absolute value)</td>
<td>-0.02</td>
<td>-0.14</td>
<td>-0.20</td>
<td>-0.16</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>Bias (%)</td>
<td>-22</td>
<td>-28</td>
<td>-22</td>
<td>-6.4</td>
<td>2.5</td>
<td></td>
</tr>
</tbody>
</table>

C. test = Cochran's test; G. test = Grubbs' test

The relative reproducibility standard deviation (RSDᵦ), 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 fully satisfies this requirement at all GM levels tested. In fact, the highest RSDᵦ (%) is of 43 at the 0.1% level, 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 EURL 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).

As it can be observed from the values reported in Table 6, the method satisfies this requirement for all GM levels with the exception of the 0.1% GM level for which the RSD_r is 34%.

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 ± 25% across the entire dynamic range. In this case the method satisfies this requirement at all GM levels tested, with a slight deviation at the 0.5% level with a bias of -28%.

9. Conclusions

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

The results obtained during the collaborative study indicate that the analytical module of the method submitted by the applicant complies with ENGL performance criteria, with the deviation of the method bias at the 0.5% GM level and of the relative repeatability standard deviation (RSD_r) at the 0.1% GM level. These divergences do not affect the estimation of the GM content at the higher GM levels, including the target level of 0.9%.

The method is therefore 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 EURL 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

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 = \([10^{-\left(-1/slope\right)}]} - 1\)

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

**R² Coefficient**

Definition: the R² coefficient is the correlation coefficient of a standard curve obtained by linear regression analysis.

Acceptance Criterion: the average value of R² should be ≥ 0.98.

**Repeatability Standard Deviation (RSDₚ)**

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 1/10th of the value of the target concentration with an RSDₚ ≤ 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 1/20th of the target concentration. Experimentally, quantitative methods should detect the presence of the analyte at least 95% of the time at the LOD, ensuring ≤ 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 ± 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 ($RSD_{\text{R}}$)

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 $RSD_{\text{R}} < 50 \%$ 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 ± 25% of the accepted reference value over the whole dynamic range.

Validated Method

10 June 2008

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

Method development:
Monsanto Company

Collaborative trial:
Community Reference Laboratory for GM Food and Feed (CRL-GMFF)
Biotechnology & GMOs Unit
Note:
Since 01/12/2009 the term "Community Reference Laboratory (CRL)" is changed into "European Union Reference Laboratory (EURL)".
Since 01/03/2009 the JRC-unit that hosts the EU-RL GMFF is named "Unit for Molecular Biology and Genomics" instead of "Biotechnology and GMO Unit".

Address of contact laboratory:
CRL-GMFF: protocol MON 531 cotton

European Commission, Joint Research Centre
Institute for Health and Consumer Protection (IHCP)
Biotechnology and GMOs Unit – Community Reference Laboratory for GM Food and Feed
Via Fermi 2749, 21027 Ispra (VA) - Italy

Content

1. GENERAL INFORMATION AND SUMMARY OF THE METHODOLOGY ....................................4
2. VALIDATION STATUS AND PERFORMANCE CHARACTERISTICS ....................................4
   2.1 GENERAL ...........................................................................................................................4
   2.2 COLLABORATIVE TRIAL ...................................................................................................5
   2.3 LIMIT OF DETECTION (LOD) ..........................................................................................5
   2.4 LIMIT OF QUANTIFICATION (LOQ) ..................................................................................5
   2.5 MOLECULAR SPECIFICITY .............................................................................................5
3. PROCEDURE ...................................................................................................................6
   3.1 GENERAL INSTRUCTIONS AND PRECAUTIONS ..........................................................6
   3.2 REAL-TIME PCR FOR QUANTITATIVE ANALYSIS OF COTTON EVENT MON 531 ..............7
      3.2.1 General ....................................................................................................................7
      3.2.2 Calibration ................................................................................................................7
      3.2.3 Real-time PCR set-up ..............................................................................................8
   3.3 DATA ANALYSIS ............................................................................................................9
   3.4 CALCULATION OF RESULTS .......................................................................................10
4. MATERIALS ................................................................................................................10
   4.1 EQUIPMENT ...................................................................................................................10
   4.2 REAGENTS ....................................................................................................................11
   4.3 PRIMERS AND PROBES ...............................................................................................11
5. REFERENCES ...............................................................................................................11
1. General information and summary of the methodology

This protocol describes a real-time quantitative TaqMan® PCR procedure for the determination of the relative content of event MON 531 DNA to total cotton DNA in a sample.

The PCR assay was optimised for the use in real-time PCR instruments for plastic reaction vessels.

Template DNA extracted by means of suitable methods should be tested for quality and quantity prior to the use in PCR assay. Tests for the presence of PCR inhibitors (e.g. monitor run of diluted series, use of DNA spikes) are recommended.

For the specific detection of event MON 531 genomic DNA, a 72-bp fragment of the integration region of the construct inserted into the plant genome (located at the 5' plant DNA region) is amplified using two specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with the fluorescent dye FAM as a reporter at its 5' end and TAMRA as a quencher dye at its 3' end.

For relative quantification of event MON 531 DNA, a cotton-specific reference system amplifies a 76-bp fragment of a cotton fibre-specific acy1 carrier protein gene (acy1), using two specific primers and one probe labelled with FAM and TAMRA as described above.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the “Ct” value. For quantification of the amount of event MON 531 DNA in a test sample, MON 531 and acy1 Ct values are determined for the sample. Standard curves are then used to estimate the relative amount of event MON 531 DNA to total cotton DNA.

2. Validation status and performance characteristics

2.1 General

The method was optimised for DNA extracted from cotton seeds or grains containing mixtures of genetically modified and conventional cotton.

The reproducibility and trueness of the method was tested through an international collaborative trial using samples at different GMO contents.
2.2 Collaborative trial

The method was validated in a collaborative study by the Joint Research Centre (JRC) of the European Commission. The study was undertaken with twelve laboratories in July-August 2006.

Each participant received twenty blind samples containing cotton MON 531 genomic DNA at five GM contents, ranging from 0.1% to 6.0%.

Each test sample was analysed by PCR in three repetitions. The study was designed as a blind quadruplicate collaborative trial; each laboratory received each level of GM cotton MON 531 in four unknown samples. Four replicates of each GM level were analysed on the same plate.

A detailed validation report can be found at http://gmo-crl.jrc.it/statusofdoss.htm

2.3 Limit of detection (LOD)

According to the method developer, the relative LOD of the method is at least 0.05% in 200 ng of total cotton DNA. The relative LOD was not assessed in the collaborative trial.

2.4 Limit of quantification (LOQ)

According to the method developer, the relative LOQ of the method is at least 0.1% in 200 ng of total cotton DNA. The lowest relative GM content included in the collaborative trial was 0.10%.

2.5 Molecular specificity

According to the method developer, the method exploits a unique DNA sequence in the region of recombination between the insert and the plant genome; the sequence is specific to cotton event MON 531 and thus imparts event-specificity to the method.

The specificity of the assay was experimentally tested by the applicant in real-time PCR against DNA extracted from plant materials containing the specific targets of MON 531, Roundup Ready® soybean 40-3-2, Roundup Ready® canola (RT200), Roundup Ready® canola (RT73), Roundup Ready® corn (GA21), Roundup Ready® corn (NK603), YieldGard® corn borer corn (MON 810), YieldGard® rootworm corn (MON 863), conventional corn, Bollgard® II cotton (MON 15985), conventional cotton, conventional Soybean, Roundup Ready® wheat (MON 71800), conventional wheat, Assoria rice, barley, basmati rice, lentil, quinoa, sunflower, oat, buckwheat, pinenuts, rye berries, millet, Teosinte, hard wheat.
According to the applicant, none of the plant materials tested, except the positive control cotton line MON 531, Assoria rice and cotton MON 15985 gave detectable amplifications; MON 15985 however contains event MON 531 and was expected to be positive in PCR.

Assoria rice reacted unexpectedly with the event-specific detection assay of MON 531, but this positive result is considered by the applicant to be an artefact. Bioinformatics analyses conducted by the CRL-GMFF confirmed the absence of relevant matches between the primers for MON 531 and the rice genome; this was also supported by additional tests conducted by the CRL-GMFF.

The specificity of the cotton reference assay \( acp1 \) was experimentally tested by the applicant against DNA extracted from plant materials containing Roundup Ready\textregistered soybean 40-3-2, Roundup Ready\textregistered canola (RT200), Roundup Ready\textregistered canola (RT73), conventional canola, Roundup Ready\textregistered corn (GA21), Roundup Ready\textregistered corn (NK603), YieldGard\textregistered corn borer corn (MON 810), YieldGard\textregistered rootworm corn (MON 863), conventional corn, Roundup Ready\textregistered cotton (MON 1445), Bollgard\textregistered cotton (MON 531), Bollgard II\textregistered cotton (MON 15985), conventional cotton, conventional soybean, Roundup Ready\textregistered wheat (MON 71800), conventional wheat, Assoria rice, barley, basmati rice, Teosinte, lentil, quinoa, sunflower, oat, buckwheat, pinenuts, rye berries, millet, peanut.

According to the applicant, none of the plant materials tested, except cotton MON 531, cotton MON 15985, cotton MON 1445, conventional cotton and Assoria rice yielded detectable amplifications. Assoria rice reacted unexpectedly with the \( acp1 \) assay, but this positive result is considered by the applicant to be an artefact. Bioinformatics analyses conducted by the CRL-GMFF confirmed the absence of relevant matches between the primers for \( acp1 \) and the rice genome; this was also supported by additional tests conducted by the CRL-GMFF.

### 3. Procedure

#### 3.1 General instructions and precautions

- The procedures require experience of working under sterile conditions.

- Laboratory organisation, e.g. “forward flow direction” during PCR-setup, should follow international guidelines, e.g. ISO 24276:2005.

- PCR-reagents should be stored and handled in a separate room where no nucleic acids (with exception of PCR primers or probes) or DNA degrading or modifying enzymes have been handled previously. All handling of PCR reagents and controls requires dedicated equipment, especially pipettes.
All the equipment used should be sterilised prior to use and any residue of DNA has to be removed. All material used (e.g. vials, containers, pipette tips, etc.) must be suitable for PCR and molecular biology applications. They must be DNase-free, DNA-free, sterile and unable to adsorb protein or DNA.

- Filter pipette tips protected against aerosol should be used.
- Powder-free gloves should be used and changed frequently.
- Laboratory benches and equipment should be cleaned periodically with 10% sodium hypochloride solution (bleach).
- Pipettes should be checked regularly for precision and calibrated, if necessary.
- All handling steps - unless specified otherwise - should be carried out at 0 - 4°C.
- In order to avoid repeated freeze/thaw cycles aliquots should be prepared.

3.2 Real-time PCR for quantitative analysis of cotton event MON 531

3.2.1 General

The PCR set-up for the taxon specific target sequence (acp2) and for the GMO (MON 531) target sequence should be carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.

The method is developed for a total volume of 50 µL per reaction mixture with the reagents as listed in Table 1 and Table 2.

3.2.2 Calibration

The calibration curves consist of five samples. The first point of the calibration curves is a 10% MON 531 in non-GM cotton DNA for a total of 200 ng of DNA (corresponding to approximately 85830 cotton genome copies with one genome assumed to correspond to 2.33 pg of haploid cotton genomic DNA) (1).

A calibration curve is produced by plotting the Ct-values against the logarithm of the target copy number for the calibration points. This can be done e.g. by means of spreadsheet software, e.g. Microsoft Excel, or directly by options available with the sequence detection system software. The
copy number measured for the unknown sample DNA is obtained by interpolation from the standard curves.

The ratio of GM copy number and reference gene copy number multiplied by 100 gives the % GM contents of the samples.

3.2.3 Real-time PCR set-up

1. Thaw, mix gently and centrifuge the required amount of components needed for the run. Keep thawed reagents at 1-4 °C on ice.

2. In two reaction tubes (one for the MON 531 system and one for the acp1 system) on ice, add the following components (Tables 1 and 2) in the order mentioned below (except DNA) to prepare the master mixes.

Table 1. Amplification reaction mixture in the final volume/concentration per reaction well for the MON 531 specific system.

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
<th>µL/reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqMan® Universal PCR Master Mix (2x)</td>
<td>1x</td>
<td>25</td>
</tr>
<tr>
<td>MON 531 primer forward (10 µM)</td>
<td>150 nM</td>
<td>0.75</td>
</tr>
<tr>
<td>MON 531 primer reverse (10 µM)</td>
<td>150 nM</td>
<td>0.75</td>
</tr>
<tr>
<td>MON 531 probe (5 µM)</td>
<td>50 nM</td>
<td>0.50</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>#</td>
<td>19</td>
</tr>
<tr>
<td>Template DNA (max 200 ng)</td>
<td>#</td>
<td>4.0</td>
</tr>
<tr>
<td>Total reaction volume:</td>
<td></td>
<td>50</td>
</tr>
</tbody>
</table>

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the cotton acp1 reference system.

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
<th>µL/reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqMan® Universal PCR Master Mix (2x)</td>
<td>1x</td>
<td>25</td>
</tr>
<tr>
<td>acp 1 primer forward (10 µM)</td>
<td>150 nM</td>
<td>0.75</td>
</tr>
<tr>
<td>acp 1 primer reverse (10 µM)</td>
<td>150 nM</td>
<td>0.75</td>
</tr>
<tr>
<td>acp 1 probe (5 µM)</td>
<td>50 nM</td>
<td>0.50</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>#</td>
<td>19</td>
</tr>
<tr>
<td>Template DNA (max 200 ng)</td>
<td>#</td>
<td>4.0</td>
</tr>
<tr>
<td>Total reaction volume:</td>
<td></td>
<td>50</td>
</tr>
</tbody>
</table>
3. Mix gently and centrifuge briefly.

4. Prepare two reaction tubes (one for the MON 531 and one for the acp I master mixes) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).

5. Add to each reaction tube the correct amount of master mix (e.g. 46 x 3 = 138 µL master mix for three PCR repetitions). Add to each tube the correct amount of DNA (e.g. 4 x 3 = 12 µL DNA for three PCR repetitions). Vortex each tubes for approx. 10 sec. This step is mandatory to reduce the variability among the repetitions of each sample to a minimum.

6. Spin down the tubes in a microcentrifuge. Aliquot 50 µL in each well. Seal the reaction plate with optical cover or optical caps. Centrifuge the plate at low speed (e.g. approximately 250 x g for 1 minute at 4 °C to room temperature) to spin down the reaction mixture.

7. Place the plate into the instrument.

8. Run the PCR with cycling conditions described in Tables 3 and 4:

Table 3. Cycling program for the MON 531 system

<table>
<thead>
<tr>
<th>Step</th>
<th>Stage</th>
<th>T°C</th>
<th>Time (sec)</th>
<th>Acquisition</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UNG</td>
<td>50 °C</td>
<td>120</td>
<td>No</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>Initial denaturation</td>
<td>95 °C</td>
<td>600</td>
<td>No</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Amplification</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Denaturation</td>
<td>95 °C</td>
<td>15</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Annealing &amp; Extension</td>
<td>55 °C</td>
<td>60</td>
<td>Yes</td>
<td>45</td>
</tr>
</tbody>
</table>

Table 4. Cycling program for the reference acpI system.

<table>
<thead>
<tr>
<th>Step</th>
<th>Stage</th>
<th>T°C</th>
<th>Time (sec)</th>
<th>Acquisition</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UNG</td>
<td>50 °C</td>
<td>120</td>
<td>No</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>Initial denaturation</td>
<td>95 °C</td>
<td>600</td>
<td>No</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Amplification</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Denaturation</td>
<td>95 °C</td>
<td>15</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Annealing &amp; Extension</td>
<td>60 °C</td>
<td>60</td>
<td>Yes</td>
<td>45</td>
</tr>
</tbody>
</table>

3.3 Data analysis

Subsequent to the real-time PCR, analyse the run following the procedure below:
a) **Set the threshold**: display the amplification curve in logarithmic mode. Locate the threshold line in the area where the amplification profiles are parallel (exponential phase of PCR) and where there is no “fork effect” between repetitions of the same sample. Press the “update” button to ensure changes affect Ct values. Switch to the linear view mode by clicking on the Y axis of the amplification plot, and check that the threshold previously set falls within the geometric phase of the curves.

b) **Set the baseline**: determine the cycle number at which the threshold line crosses the first amplification curve and set the baseline three cycles before that value (e.g. earliest Ct = 25, set the baseline crossing at Ct = 25 - 3 = 22).

c) Save the settings and export all the data to a text file for further calculations.

### 3.4 Calculation of results

After having defined a threshold value within the logarithmic phase of amplification as described above, the instrument’s software calculates the Ct-values for each reaction.

The standard curves are generated both for the *acp 1* and the MON 531 specific systems by plotting the Ct values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a linear regression line into these data.

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

For the determination of the amount of event MON 531 DNA in the unknown sample, the MON 531 copy number is divided by the copy number of the cotton reference gene (*acp 1*) and multiplied by 100 to obtain the percentage value (GM% = MON 531/*acp 1* x 100).

### 4. Materials

#### 4.1 Equipment

- Real-time PCR instrument for plastic reaction vessels
- Plastic reaction vessels suitable for real-time PCR instrument (enabling undisturbed fluorescence detection)
- Software for run analysis (mostly integrated in the software of the real-time PCR instrument)
- Microcentrifuge
- Micropipettes
4.2 Reagents

- TaqMan® Universal PCR Master Mix (2X). Applied Biosystems Part No 4304437

4.3 Primers and Probes

<table>
<thead>
<tr>
<th>Name</th>
<th>Oligonucleotide DNA Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MON 531 target sequence</td>
<td></td>
</tr>
<tr>
<td>531-F forward primer</td>
<td>5’ - TCC CAT TCG AGT TTC TCA CGT -3’</td>
</tr>
<tr>
<td>531-R reverse primer</td>
<td>5’ - AAC CAA TGC CAC CCC ACT GA -3’</td>
</tr>
<tr>
<td>531-P probe</td>
<td>FAM 5’ - TTG TCC CTC TTC TTC TC -3’ TAMRA</td>
</tr>
<tr>
<td>acp 1 forward primer</td>
<td>5’ - ATT GTG ATG GGA CTT GAG GAA GA -3’</td>
</tr>
<tr>
<td>acp 1 reverse primer</td>
<td>5’ - CTT GAA CAG TTG TGA TGG ATT GTG -3’</td>
</tr>
<tr>
<td>acp 1 probe</td>
<td>FAM 5’ - ATT GTC CTC TTC CAC CGT GAT TCC GAA -3’ TAMRA</td>
</tr>
</tbody>
</table>

5. References

How to obtain EU publications

Our priced publications are available from EU Bookshop (http://bookshop.europa.eu), where you can place an order with the sales agent of your choice.

The Publications Office has a worldwide network of sales agents.
You can obtain their contact details by sending a fax to (352) 29 29-42758.

European Commission
EUR 26152 EN – Joint Research Centre – Institute for Health and Consumer Protection


Author(s): Marco Mazzara, Alessia Bogni, Nicoletta Foti, Guy Van den Eede

Luxembourg: Publications Office of the European Union

2013– 34 pp. – 21.0 x 29.7 cm

EUR – Scientific and Technical Research series – ISSN 1831-9424

do: 10.2788/18510

Abstract

The JRC as European Union Reference Laboratory for the GM Food and Feed (EURL 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 531 transformation event in cotton DNA (unique identifier MON-00531-6). The collaborative trial was conducted according to internationally accepted guidelines (1, 2).

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 seeds containing the transformation event 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 seven European countries.

The results of the international collaborative trial mostly 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.
As the Commission’s in-house science service, the Joint Research Centre’s mission is to provide EU policies with independent, evidence-based scientific and technical support throughout the whole policy cycle.

Working in close cooperation with policy Directorates-General, the JRC addresses key societal challenges while stimulating innovation through developing new methods, tools and standards, and sharing its know-how with the Member States, the scientific community and international partners.

Key policy areas include: environment and climate change; energy and transport; agriculture and food security; health and consumer protection; information society and digital agenda; safety and security, including nuclear; all supported through a cross-cutting and multi-disciplinary approach.