VERIFICATION OF REAL-TIME PCR METHODS FOR QUALITATIVE AND QUANTITATIVE TESTING OF GENETICALLY MODIFIED ORGANISMS

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ABSTRACT

This article reports the experience of a university laboratory in accreditation of molecular biology methods for genetically modified (GM) organisms detection according to the International Organization for Standardization (ISO)/International Electrotechnical Commission 17025 standard. Verification studies were performed for one screening (35S promoter), one construct-specific (p35S/Chloroplast Transit Peptides-5-enolpyruvylshikimate-3-phosphate synthase [CTP-EPSPS]) and one event-specific (Bt11 maize) real-time polymerase chain reaction methods. Our procedures were performed as described in the ISO 21570 2005, except for the analysis done through a DNA Engine Opticon 2 MJ research instrument. The following method–performance characteristics were evaluated: amplification efficiency, linearity, dynamic range, limit of detection, limit of quantification, precision and trueness. Comparison of the performance results obtained for quantitative methods with the values of inter-laboratory ring trials, coordinated by the European Union Reference Laboratory for GM Food and Feed, confirmed that the laboratory is able to fulfill the published criteria.

PRACTICAL APPLICATIONS

This article illustrates the experience of a university laboratory with the International Organization for Standardization/International Electrotechnical Commission 17025 accreditation for genetically modified organisms testing. In-house verification has been carried out for one screening (35S promoter), one construct-specific (p35S/Chloroplast Transit Peptides-5-enolpyruvylshikimate-3-phosphate synthase [CTP-EPSPS]) and one event-specific (Bt11 maize) real-time polymerase chain reaction methods. We reported for the first time validation data obtained through a DNA Engine Opticon 2 MJ Research instrument. The described experimental procedure could represent a pattern for researchers interested in setting up verification of validated methods for genetically modified organism quantitative and qualitative testing.

INTRODUCTION

The use of genetically modified organisms (GMOs) and GMO-derived products in food and feed is subject to regulations that differ from one country to another. In the European Union (EU), the food labeling is mandatory when more than 0.9% of the food ingredients, considered individually, are of GM origin (EC regulation 1829/2003; Taverniers \textit{et al.} 2005; Elenis \textit{et al.} 2008). The implementation of labeling regulations is based on the reliability and precision of techniques for GMO detection. DNA is widely used as the target for GMO analysis because of its stability and high detectability in processed matrices and real-time polymerase chain reaction (PCR) technique is the method of choice used routinely in food control laboratories as it combines high sensitivity with the possibility to quantify...
the GMO content (Holst-Jensen and Berdal 2004; Chaouchi et al. 2007). The GMO detection strategy usually includes an initial screening for the mostly used genetic elements, including the 35S promoter of cauliflower mosaic virus and the nopaline synthase terminator of Agrobacterium tumefaciens. On the basis of the screening results, the construct- and/or event-specific elements are addressed to identify and quantify a specific GM event (Hohne et al. 2002; Gaudron et al. 2009; Del Gaudio et al. 2010). The amount of the transgenic target and the reference plant gene are determined by interpolation with a standard curve and the result is provided as the percentage of GMO in the sample tested (Von Gotz 2010).

The reference methods for the detection and quantification of transgenic crops in the EU are provided by applicants for authorization and validated by the EU Reference Laboratory for GM Food and Feed (EURL-GMFF), assisted by the European Network of GMO Laboratories (ENGL). In recent years, the International Organization for Standardization (ISO) 21569 2005 and 21570 2005, including some of the validated methods mentioned earlier, were made available for laboratories accredited according to the ISO/International Electrotechnical Commission (IEC) 17025 2005. Before a new method is introduced, the laboratory has to verify that it is able to achieve the performance characteristics of the method as published in the inter-laboratory validation report. As extensively reported in Zel et al. (2008), a series of parameters have to be tested to verify the agreement with the ENGL method–acceptance criteria (2008):

- amplification efficiency: the average value of the slope of the standard curve should be in the range of \((-3.1 \leq \text{slope} \leq -3.6)\), corresponding to an efficiency between 90 and 110%;
- \(R^2\) coefficient: the average value of the squared correlation coefficient \((R^2)\) of the standard curve obtained by linear regression analysis should be \(\geq 0.98\);
- dynamic range: the range of concentrations over which the method performs in a linear manner with an acceptable level of trueness and precision should include the 1/10 and at least 5 times the target concentration (0.09% and 4.5% for a 0.9% GMO concentration);
- limit of detection (LOD): the minimum level at which the analyte can reliably be detected should be less than 1/20th of the target concentration (LOD < 0.045% for a 0.9% GMO concentration);
- limit of quantification (LOQ): the minimum level at which the analyte can reliably be quantified should be less than 1/10th of the target concentration (LOQ < 0.09% for a 0.9% GMO concentration);
- precision: the relative repeatability standard deviation should be below 25% over the entire dynamic range of the method;
- trueness: the bias between mean measured value and accepted reference value should be within \(\pm 25\%\) of the accepted reference value across the entire dynamic range of the method.

This article illustrates the experience of a university laboratory with the ISO/IEC 17025 accreditation for GMO testing. In-house verification has been carried out for one screening (35S promoter), one construct-specific (p35S/Chloroplast Transit Peptides-5-enolpyruvylshikimate-3-phosphate synthase [CTP-EPSPS]) and one event-specific (Bt11 maize) real-time PCR method.

In summary, the described experimental procedure could be a representative pattern for accredited laboratories interested in implementation of validated methods for GMO quantitative and qualitative testing.

**MATERIALS AND METHODS**

**Samples**

Powdered certified reference materials (CRM) containing 0, 0.1, 1, 5% Bt11 maize (ERM-BF412) and GTS 40-3-2 soy (ERM-BF410), respectively, prepared by the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium) were obtained commercially (Sigma-Aldrich, Seelze, Germany). Test materials from the GeMMA proficiency tests were from Food Analysis Performance Assessment Scheme (FAPAS) (Central Science Laboratory, Sand Hutton, UK).

**DNA Extraction**

The cetyltrimethylammonium bromide DNA extraction protocol was used in accordance with the UNI EN ISO 21571 2005. All extractions were made in duplicate and the DNA concentrations were measured through the Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE).

**TaqMan Probes and Primers**

The sequence of primers and TaqMan probes were used in this study as suggested in the UNI EN ISO 21570 2005 (Table 1). All primers and probes were supplied by Eurofins MWG Operon (Ebersberg, Germany).

**Real-Time PCR**

The real-time PCR reactions were performed on a DNA Engine Opticon 2 MJ research (Biorad, Hercules, CA). Five micro liters of DNA were amplified in a total volume of 25 μL containing 1X TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and the thermal
TABLE 1. LIST OF PRIMERS AND TaqMAN PROBES

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer and probe name</th>
<th>Sequence</th>
<th>Conc. (nM)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>adh1</td>
<td>ADHFF3</td>
<td>5′- GTCGTTTCCATCTTCCCTCC - 3′</td>
<td>300</td>
<td>134</td>
</tr>
<tr>
<td></td>
<td>ADHRR4</td>
<td>5′- CCACTCCAGGACACCTAGTC - 3′</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ADH1-MDO</td>
<td>5′- FAM - AATCCAGGGCTATTTCTGCCTTCA - TAMRA - 3′</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GM1-F</td>
<td>5′- CGACGCTCGGCCTTCCCTTC - 3′</td>
<td>600</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>GM1-R</td>
<td>5′- GAAGGCAAGGCCCAT TGCAAGCC - 3′</td>
<td>600</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe GM1</td>
<td>5′- FAM - CTTACCCTTCTAGGCCCTGACAC - TAMRA - 3′</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>p35S</td>
<td>35S-F</td>
<td>5′- GCCTCTGCGACAGTGGT - 3′</td>
<td>300</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>35S-R</td>
<td>5′- AAGACGTGTTGGAAGCTTCTTC - 3′</td>
<td>900</td>
<td></td>
</tr>
<tr>
<td></td>
<td>35S-TMP</td>
<td>5′- FAM - CAAAGATGGACCCCCACCCAC - TAMRA - 3′</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>GTS 40-3-2 construct</td>
<td>RR1-F</td>
<td>5′- CATTGGAGAGGACAGGTGA - 3′</td>
<td>740</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>RR1-R</td>
<td>5′- GAGCCATGTTGTAATTGTGCCC - 3′</td>
<td>600</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>Probe RR1</td>
<td>5′- FAM - CAAGCTGACTCTAGCAGATCTTTC - TAMRA - 3′</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>Bt11 event</td>
<td>Bt113JFor</td>
<td>5′- GCCGAACCCCCATTTTCAA - 3′</td>
<td>750</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>Bt113JRev</td>
<td>5′- TCCAAGAATCCCTATGAA - 3′</td>
<td>750</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bt113JFT</td>
<td>5′- FAM - AAATACATTTGAATATGATCCGCTCA - TAMRA - 3′</td>
<td>250</td>
<td></td>
</tr>
</tbody>
</table>

From left to right target, name, sequence, concentration used and amplicon size are shown.

RESULTS AND DISCUSSION

In this study, we performed a verification procedure of a real-time PCR method for qualitative detection of 35S promoter and two quantitative methods targeting, respectively, the construct (p35S/ CTP-EPSPS) specific for GTS 40-3-2 soy and the maize event Bt11-specific sequence. The procedure involves the amplification of soy-specific lectin (le1) and maize-specific alcohol dehydrogenase (adh1) reference genes. Previous validation studies concerning EURL-GMFF developed methods carried out with Applied Biosystems
(Hubner et al. 2001; Ronning et al. 2003), Bio-Rad i-Cycler (Scholtens et al. 2010) or capillary Light Cycler real-time PCR instrument (Sieradzki and Kwiatek 2009). This article reports for the first time validation data obtained through a DNA Engine Opticon 2 MJ Research instrument.

The primers and probes were the same as published in the standard UNI EN ISO 21570 2005. Primer and probe concentrations were unchanged with respect to the reference protocol and the TaqMan Universal PCR Master Mix was used for all the assays in order to minimize the optimization procedures. Before real-time amplification, the quality and amount of DNA were assessed spectrophotometrically (data not shown). Because the methods were previously validated by EURL-GMFF, we did not evaluate some parameters as applicability, practicability and specificity (Zel et al. 2008).

The first step of verification consisted in verifying that all the real-time PCR assays showed an acceptable efficiency and linearity, in agreement with the requirements of the ENGL (2008). The values in Table 2 are the mean of nine (le1 and GTS 40-3-2) and 10 (adh1 and Bt11) repeated PCR runs, while for the p35 screening method, three PCR reactions were performed. The average slope of the regression line should be within –3.1 and –3.6; all the slopes met the ENGL criteria (2008), reaching the ideal value of –3.32 for le1 and Bt11 assays. Consequently, the average reaction efficiencies ranged between 96.8 and 100%. Only the slope for p35S was –3.70, corresponding to an efficiency of 86.6%. The linearity of reaction was very high, as $R^2$ coefficient was over 0.992 for all the tested assays. The dynamic range of all methods was limited by the amount of target DNA copies isolated from 5% CRM at the higher value and by the LOQ at low-range value (Hubner et al. 2001; Ronning et al. 2003; Sieradzki and Kwiatek 2009; Scholtens et al. 2010). As shown in Table 2, this range expanded to four orders of magnitude for reference gene assays (adh1 and le1) and to three orders of magnitude for GM assays (GTS 40-3-2 soy and Bt11 maize). The p35S method worked in a linear manner with an acceptable precision in a limited range of concentrations.

The LOD and LOQ are defined as the lowest content that can be detected and can be measured with reasonable statistical certainty, at least 95% probability, respectively. Our results met the acceptance criteria (maximum 25 copies for LOD and 50 copies for LOQ) for soy and maize quantitative assays required from ENGL (2008, 2011). For GTS 40-3-2 quantification method the corresponding relative LOD was 0.015% and relative LOQ was 0.046%. Previous validation by EURL-GMFF reported a 0.06% as relative LOQ (ISO 21570 2005). Similarly, Hubner et al. (2001) reported a 0.01% as relative LOD and a 0.06% as relative LOQ, while Sieradzki and Kwiatek (2009) found that LOD was 0.025% and LOQ was 0.075%, although using alternative construct-specific primers and TaqMan probes. As regards Bt11 quantification method, the relative LOD was 0.015% and the relative LOQ was 0.07%. This means that we reached a sensitivity higher than EURL-GMFF, which made the validation of Bt11 event-specific assay coupled with adh1 reference gene reporting a 0.1% as relative LOD and LOQ (ISO 21570 2005). Other authors determined relative LOQ at 0.05% (Ronning et al. 2003) and 0.06% (Sieradzki and Kwiatek 2009) with maize invertase being selected as reference gene. Instead for p35S screening LOD and LOQ were, respectively, 114 and 198 copies, corresponding to high-relative LOD (0.14%) and LOQ (0.24%). Major efforts will be needed to improve the sensitivity of this assay.

The precision of the tested quantitative methods was expressed in terms of RSDr value, which is the ratio of the standard deviation of the mean divided by the mean value of measurements. We calculated the precision by repeated measurements at three distinct levels of GMO, i.e., 0.1, 1 and 5%. RSDr value should be below 25% over the entire dynamic range. As can be seen in Table 3, our results complied with ENGL criteria (2008, 2011) except for the RSDr value relative to 0.1% Bt11 maize (35.7%). Similar result was obtained by EURL-GMFF whose RSDr at the 0.1% Bt11 level was 33.5% (ISO 21570 2005). For GTS 40-3-2 assay, we reached better values of precision than those reported by EURL-GMFF in ISO 21570 2005 (from 10 to 33%), as our RSDr ranged between 8.52 and 22.2%. Therefore these results were very close to the precision values reported by Hubner et al. (2001) and Sieradzki and Kwiatek (2009) for their method.

Expanded uncertainty was below 25% of the mean measured value as required by ENGL criteria (2008, 2011) except for 0.1% GTS 40-3-2 (66%) and 0.1% Bt11 (35%).

**Table 2. Slope, Efficiency, Squared Coefficient of Correlation ($R^2$), Dynamic Range, LOD and LOQ for Tested Assays**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Slope</th>
<th>Efficiency (%)</th>
<th>$R^2$</th>
<th>Dynamic range (copies)</th>
<th>LOD (copies)</th>
<th>LOQ (copies)</th>
</tr>
</thead>
<tbody>
<tr>
<td>adh1</td>
<td>–3.40</td>
<td>96.8</td>
<td>0.996</td>
<td>94,676–49</td>
<td>4</td>
<td>49</td>
</tr>
<tr>
<td>le1</td>
<td>–3.37</td>
<td>98.0</td>
<td>0.997</td>
<td>88,495–43</td>
<td>10</td>
<td>43</td>
</tr>
<tr>
<td>p35S</td>
<td>–3.70</td>
<td>86.6</td>
<td>0.992</td>
<td>4,425–198</td>
<td>114</td>
<td>198</td>
</tr>
<tr>
<td>GTS 40-3-2 construct</td>
<td>–3.32</td>
<td>100.0</td>
<td>0.992</td>
<td>4,425–39</td>
<td>12</td>
<td>39</td>
</tr>
<tr>
<td>Bt11 event</td>
<td>–3.32</td>
<td>100.0</td>
<td>0.994</td>
<td>4,700–26</td>
<td>5</td>
<td>26</td>
</tr>
</tbody>
</table>

LOD, limit of detection; LOQ, limit of quantification.

This result could be explained with the contribution of CRM uncertainty, as reported on the IRMM certificate, which has a considerable weight on the final value of expanded uncertainty at this level of GMO content.

Finally, we determined the trueness by measuring the bias at 0.1, 1 and 5% GMO level. The expanded uncertainty was larger than the difference between measured and certified mean value. This means that the measured mean value was not significantly different from the certified value and then the tested methods had no bias. According to the ENGL method performance requirements, trueness should be within ±25% across the entire dynamic range. In this case, both the tested methods satisfied these requirements, because the highest values of relative bias were 13% at 1% level GM soy and 16.6% at 0.12% Bt11 maize.

It is a requirement for accreditation to ISO/IEC 17025 that the laboratory takes part to a proficiency testing scheme. Our laboratory participated to the GeMMA proficiency test coordinated by FAPAS, obtaining satisfactory performance results. In fact, $z$ score value of 1.0 for GTS 40-3-2 soy (GeM SU33 round) and a $z$ score of 0.4 for BT11 maize (GeM SU09 round) have been obtained, included in the accepted range ($|z| \leq 2$).

**CONCLUSIONS**

A verification study has been carried out for one screening (35S promoter), one construct-specific (p35S/ CTP-EPSPS) and one event-specific (Bt11 maize) real-time PCR methods. We optimized these PCR assays, originally developed for an Applied Biosystems instrument, on a DNA Engine Opticon 2 MJ Research thermocycler. The method performance has been evaluated with respect to the ENGL criteria and our results were compared with those reported for inter-laboratory ring-trial carried out by the EURL-GMFF and to literature available studies. While the screening method requires an additional optimization work, satisfying performance characteristics allowed the applicability of tested quantitative methods for GMO analysis in our accredited ISO/IEC 17025 laboratory. Together with the use of validated methods, proficiency testing is an essential element of laboratory quality assurance.

**ACKNOWLEDGMENTS**

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