Rapid Quantification Methods for Genetically Modified Maize Contents Using Genomic DNAs Pretreated by Sonication and Restriction Endonuclease Digestion for a Capillary-Type Real-Time PCR System with a Plasmid Reference Standard

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For rough quantitative analysis of genetically modified maize contents, rapid methods for measurement of the copy numbers of the cauliflower mosaic virus 35S promoter region (P35S) and MON810 construct-specific gene (MON810) using a combination of a capillary-type real-time PCR system with a plasmid DNA were established. To reduce the characteristic differences between the plasmid DNA and genomic DNA, we showed that pretreatment of the extracted genomic DNA by a combination of sonication and restriction endonuclease digestion before measurement is effective. The accuracy and reproducibility of this method for MON810 content (%) at a level of 5.0% MON810 mixed samples were within a range from 4.26 to 5.11% in the P35S copy number quantification. These methods should prove to be a useful tool to roughly quantify GM maize content.

Key words: genetically modified maize; capillary-type real-time PCR system

The cultivation areas of genetically modified (GM) crops, especially Yield Guard® Maize (MON810) and Roundup Ready® Soybean (RRS), have increased in the United States over the past several years.1) In some countries, controversial issues still exist regarding the acceptance of GM crops, and concerns about their safety persist in public opinion. In many countries, the labeling of grains and foodstuffs is mandatory if the genetically modified organism (GMO) content exceeds a certain level of approved GM varieties. The European Union (EU), Japan, Korea, and Taiwan have set threshold values for foods at 0.9%, 5%, 3%, and 5% respectively, of GMO material in a non-GM background as the basis for labeling.2–4) The enforcement of these threshold values has created a demand for the development of reliable GMO analysis methods with rapid and inexpensive features.5–21) Hence many real-time PCR systems based on fluorescent detection, such as TaqMan® chemistry, have been developed to identify and quantify GM maize, GM soybeans, and GM varieties of other agricultural commodities.22–33)

Real-time PCR systems using TaqMan® chemistry are based on the use of a fluorescent TaqMan® probe that monitors the formation of the PCR product during each cycle of the reaction. In addition, most commonly, GMO quantification by real-time PCR methods is calculated from the ratio of the target transgenic specific DNA sequence copy number vs. the DNA sequence copy number of the respective target plant species (taxon-specific sequence). Determination of the copy number by real-time PCR methods involves the establishment of calibration curves based on the analysis of a set of calibrators, such as genomic DNA (gDNA) or plasmid DNA (pDNA). In Japan, pDNA reference material containing the target sequences used for PCR detection have been used and are being increasingly promoted as the standards of choice for GMO analysis, because pDNA is semi-infinitely available in the same quality.

We have reported a real-time PCR method for the construct-specific quantification of GM soybeans and maize using reference pDNAs as the standard molecule and an ABI PRISM 7700 system as the real-time PCR
equipment.26) The official Japanese method is applied for the purpose of monitoring the mixing level of GM in non-GM materials, which can be controlled by the identity preserved handling system. In GM maize quantification in the official Japanese method, a screening system for rough quantification of GM maize contents using a combination of quantification systems for copy number quantification of the cauliflower mosaic virus 35S promoter region (P35S) and GA21 event construct-specific region has been adopted. This system is the most practical method as a first step when maize grains imported from the United States and Canada arrive in Japan because there are many authorized GM maize lines.

For such quantification of GM crop contents using real-time PCR equipment, the establishment of appropriate quantification methods corresponding to real-time PCR equipment or GM crops is necessary because equipment properties or crop properties are significantly different among the respective varieties. When the quantification method of the ABI PRISM 7700 system was applied to the LightCycler® real-time PCR system, it was reported that the value of the GM maize contents measured by the LightCycler® real-time PCR system appeared to be significantly different from the expected theoretical value on a weight-to-weight basis.33) In addition, a recent study suggested that DNA extraction methods affect the quantified value of the GM maize contents using real-time PCR equipment because the integrity and purity of the extracted genomic DNA differs depending on the DNA extraction method.34)

We have also developed a system for Roundup Ready® Soybean (RRS) quantification using a combination of the LightCycler® real-time PCR system and pDNA as the reference standard. In addition, comparable studies between pDNA and gDNA have been performed for GM soybeans,35) but there has been little information on quantification of GM maize contents using a combination of capillary-type real-time PCR system and pDNA as the reference standard.

In the present study, we established a rapid screening quantification method to measure roughly GM maize contents by measurement of the relative copy numbers of the P35S and MON810 maize construct-specific sequences (MON810) using a combination of reference plasmid DNA and capillary-type real-time PCR equipment. We further evaluated the established method, including determination of the C_{T}, to calculate roughly the GM maize contents as the MON810 content (%). In addition, we found that treatment of gDNA with a combination of sonication and restriction endonuclease digestion is required for the equivalence of gDNA and pDNA as reference standards in the established methods using the LightCycler® real-time PCR system.

Materials and Methods

Materials. The non-GM maize sample was purchased from Quality Technology International (Elgin, IL). MON810 genuine seeds were kindly provided by Monsanto (St. Louis, MO). Dried maize powder of European reference material ERM®, 1% and 5% MON810 (ERM1 and ERM5), were commercially purchased (Fluka, Buchs, Switzerland). As a standard material for the calibration curve, the Maize Detection Plasmid Set-ColE1/TE (Nippon Gene, Toyama, Japan) was used.

Preparation of test samples. To prepare the GM mixed test samples, maize grains (GM seeds and non-GM seeds) were separately milled into a fine powder using grinders (Retsch, Haan, Germany), and then mixed with 1% and 5% GM maize on a weight-to-weight basis, according to a previously reported procedure.26) Extraction and purification of gDNA. DNA extraction and purification were carried out using an anion exchange resin-type kit (Genomic-tip 20/G; Qiagen, Hilden, Germany) according to a previously reported procedure.34) The extracted DNA was dissolved in 200 µl of sterile distilled water.

Sonication and restriction enzyme treatment of extracted gDNA. Seventeen µl of the extracted DNA solution (including 2 µg of DNA) was transferred to fresh microtubes. The microtubes were sonicated at 100% output power for 5 min using a Sharp UT-10SS (Sharp, Osaka, Japan). Two µl of 10 × high buffer and 1 µl EcoRI (15 U) were added to the sonicated extracted gDNA and incubated at 37 °C for 1 h. The solution was then mixed with a 0.1 volume of 3 M sodium acetate at pH 4.8 and 2.5 volumes of ethanol. The mixture was centrifuged at 8,000 × g for 20 min at 4 °C. The supernatant was discarded and the pellets were washed with 70% ethanol. Finally, the DNA was dissolved in 200 µl of TE buffer (10 mM Tris–Cl, pH 8.0, 1 mM EDTA). The DNA concentration in the solutions was determined by measuring the UV absorption at 260 nm using a GeneQuant pro spectrophotometer (Amersham Biosciences, Piscataway, NJ). The purity of the extracted DNA was evaluated using a ratio of 260/280 nm. The ratio was in the range of 1.7 and 2.0 for most of the test samples. The pretreated gDNA with sonication followed by EcoRI digestion was diluted with an appropriate volume of sterile distilled water to a final concentration of 10 ng/µl and stored at −20 °C until used. These DNA samples were used for subsequent PCR analysis.

Real-time quantitative PCR of the endogenous Starch Synthase IIb gene (SSIib), P35S, and the MON810 construct-specific gene (MON810). Fluorescence resonance energy transfer (FRET) hybridization probes were used for quantification in the LightCycler® real-time PCR system (Roche Diagnostics, Mannheim, Germany). In the Japanese official standard real-time PCR methods, the SSIib 3 system (SSIib 3-5’ and SSIib 3-3’ with
SSIIb-Taq) is used as primers and probe for quantification of the taxon specific gene encoding SSIIb,19) and the P35S-1 system (P35S 1-5’ and P35S 1-3’ with P35S-Taq) is used for quantification of P35S as the screening quantification method of GM maize. The target sequence of the P35S-1 system to detect the 35S promoter region is derived from the cauliflower mosaic virus. This 35S sequence has been found widely in the recombinant DNA of almost all GM commercialized events to date except for the GA21 event.20) The MON810 system (MON810 2-5’ and MON810 2-3’ with MON810-Taq) is used as primers and probe for quantification of the construct-specific gene encoding MON810.20) All the sets of primer pairs and probes (the GM Maize Detection SSIIb-3 Oligonucleotide Set, the GM Maize Detection P35S Oligonucleotide Set, and the GM Maize Detection MON810 Oligonucleotide Set) were purchased from Fasmac (Kanagawa, Japan). PCR was performed in glass capillary tubes (Roche). Unless otherwise specified, the total reaction volume of 20 μl contained 50 ng of the DNA template, 0.25 μM of each primer, 0.2 μM of the probe, and a LightCycler® 480 Probes Master (Roche), which contained FastStart Taq DNA polymerase, hybridization probes for detection by the LightCycler® real-time PCR system, reaction buffer, MgCl2, and dNTPs. The PCR step-cycle condition was as follows: pre-incubation at 95 °C for 10 min, 50 cycles consisting of denaturation at 95 °C for 15 s (20 °C/s), and annealing at 59 °C for 30 s (1 °C/s). Standard curves were calibrated using five concentrations of the control plasmids, viz., 40, 250, 3,000, 40,000, and 500,000 copies per reaction. A no-template control was also prepared as the negative control. Each control sample was run in one capillary for each target, and unless otherwise specified, each maize sample was run in duplicate for each target. The data were analyzed using LightCycler® real-time PCR system Data Analysis software version 3.5.5 and the “Fit Points” algorithm. The cycle at which the amplification curve crosses the threshold was defined as Ct (cycle of threshold), and the standard curve was constructed from the mean Ct values of the triplicate determination.

Measurement of PCR efficiency. PCR efficiency was calculated using the slope of the standard curve according to Formula 1, as follows.31)

Formula 1:

\[ \text{PCR efficiency (E)} = 10^{(-1/\text{slope})} \]

Measurement of conversion factor and calculation of GM maize content. The copy number of each sample was obtained as the mean value of triplicates compared to the optimal standard curve.26,27) The ratio of the copy number of SSIIb and the specific gene (P35S or MON810) in the MON810 genuine seed was calculated using Formula 2, listed below, and was defined as the conversion factor (Cf). The GM maize contents (%) were calculated using Formula 3, listed below, and the defined Cf.

Formula 2:

\[ \text{Cf} = \frac{\text{(copies of SSIIb in the DNA extracted from GM seeds)}/(\text{copies of P35S or MON810 in the DNA extracted from GM seeds})}{\text{copies of SSIIb in the DNA extracted from an unknown sample} \times 100}/ \]

Formula 3:

\[ \text{GM maize content (%)} = \frac{\text{(copies of P35S or MON810 in the DNA extracted from an unknown sample})}{\text{copies of SSIIb in the DNA extracted from an unknown sample} \times \text{Cf}} \]

Statistical analysis. All values are expressed as means ± standard error of the mean. Statistical comparisons were performed by Student’s t-test. In all cases, probability (P) values below 0.05 were considered significant.

Results and Discussion

Examination of preparation of genomic DNA for determination using the LightCycler® real-time PCR system

We have reported a real-time PCR quantification method for the construct-specific quantification of GM soybeans and maize using reference pDNA as a standard molecule and the ABI PRISM 7700 system as the real-time PCR equipment.20) We have also developed a rapid quantification system for Roundup Ready® Soybean (RRS) using a combination of a LightCycler® real-time PCR system and pDNA as the reference standard, and comparable studies between pDNA and gDNA have been performed for GM soybeans only.34) However, it also has been reported that the value of the MON810 maize content measured using a LightCycler® real-time PCR system was significantly high in a laboratory-performance study.33) In the present study, the reported RRS quantification conditions were first applied to GM corn quantification, and comparable studies between pDNA and gDNA for quantitative analysis of GM maize were performed. As shown in Table 1, it is clear that the slopes for gDNA and pDNA are significantly different for all the SSIIb, P35S, and MON810. The average PCR efficiencies (E) for pDNA and gDNA were 2.020 and 1.777 in SSIIb, 1.970 and 1.716 in P35S, and 2.000 and 1.802 in MON810, respectively. These results suggest that pDNA and gDNA might behave in different ways in the detection system. We considered that the difference might be due to the tertiary structure of the extracted gDNA, because a recent study suggests that the integrity of gDNA can have a significant influence on the results obtained by real-time PCR.34) To solve this problem, we attempted to reduce the effect of the tertiary structure of the extracted gDNA using boiling treatment, sonication treatment, and restriction endonuclease digestion. As shown in Fig. 1, the boiling treatment significantly degraded the extracted gDNA. We could not determine the SSIIb copy number in real-time PCR using extracted
The enzyme was chosen to degrade the tertiary structure of the extracted gDNA without digesting the sequences for amplification of SSIIb, P35S, and MON810. Restriction endonuclease (EcoRI) digestion moderately degraded the extracted gDNA, but it was thought that only restriction endonuclease (EcoRI) digestion was still inadequate. Because the GM maize contents measured using pretreated gDNA with only the restriction endonuclease still significantly higher that the expected value (data not shown), although the extracted gDNA appeared to be rather degraded. As Fig. 1 shows, the combination treatment with sonication and restriction endonuclease (EcoRI) digestion further degraded the extracted gDNA compared to either the sonication treatment alone or the restriction endonuclease (EcoRI) digestion alone. Therefore, to degrade the tertiary structure of the extracted gDNA appropriately, we found that a combination of sonication treatment and restriction endonuclease digestion of EcoRI digestion should be effective before measurement of SSIIb, P35S, and MON810 copy numbers using the LightCycler® real-time PCR.

Analysis of PCR efficiencies for pDNA and pretreated gDNA with sonication followed by EcoRI digestion using the real-time quantitative system

To assess the validity of the reference pDNA and pretreated gDNA with sonication followed by EcoRI digestion for quantitative analysis of GM maize, we compared the PCR efficiencies of pDNA and pretreated gDNA (Formula 1). The standard curves of pDNA were calibrated by the pDNA series (five concentrations of the control plasmids, 40, 250, 3,000, 40,000, and 500,000 copies per reaction). The standard curves of pretreated gDNA with sonication followed by EcoRI digestion were calibrated with the pretreated gDNA series (five

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<th>Mean of data set</th>
<th>Variance of data set</th>
<th>n</th>
<th>T Statistical data</th>
<th>P (T &lt; t) two-sided</th>
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E, PCR efficiency was calculated using the slope of the standard curve according to Formula 1 (Formula 1: PCR efficiency (E) = 10^-1/slope).

Fig. 1. DNA Degradation of MON810 Genome by Various Pretreatments.

Extracted DNA from MON810 maize was separated on an 0.4% agarose gel after treatment by lane 1, boiling; lane 2, sonication; lane 3, EcoRI digestion; lane 4, sonication and EcoRI digestion, and lane 5, no treatment (control).
concentrations of diluted DNA extracted from 100% MON810, 0.005, 0.05, 0.5, 5, and 50 ng per reaction). As shown in Fig. 2, the average calculated R^2 values of the standard curves using pDNA for SSIIb, P35S, and MON810 copy number quantification were 0.9998, 0.9995, and 0.9996 respectively. The average calculated R^2 values of the standard curves using gDNA for SSIIb, P35S, and MON810 copy number quantification were 0.9992, 0.9997, and 0.9985 respectively. Since approximately 50,000 copies of SSIIb are contained in 50 ng of maize DNA, 40 copies of P35S and MON810 in 50 ng of maize DNA correspond to a GM maize content of approximately 0.1%. These results suggest that the detection limit is 0.1% for GM maize quantification by measurement of both the P35S and MON810 copy numbers. Table 2 shows a statistical comparative analysis of the calibration curves set up with pDNA and pretreated gDNA series with sonication followed by EcoRI digestion using this system. As shown in Table 2, it is clear that the slopes of pDNA and pretreated gDNA with sonication followed by EcoRI digestion are not significantly different for the SSIIb, P35S, and MON810 copy number quantification. The average PCR efficiencies for pDNA and pretreated gDNA with sonication followed by EcoRI digestion were 1.934 and 1.954 in SSIIb copy number quantification, 1.944 and 1.910 in P35S copy number quantification, and 1.967 and 1.998 in MON810 copy number quantification, respectively. These results suggest that pDNA and pretreated gDNA with sonication followed by EcoRI digestion behave in a similar way using this system. We concluded that these studies are important, because comparable behavior between the pDNA and pretreated gDNA with sonication followed by EcoRI digestion in the PCR is reflected in the characteristics of the standard curves obtained.

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y = -3.4978x + 40.989 \\
R^2 = 0.9998
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y = -3.5766x + 31.847 \\
R^2 = 0.9997
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y = -3.3584x + 31.419 \\
R^2 = 0.9985
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y = -3.4034x + 39.727 \\
R^2 = 0.9996
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y = -3.4645x + 39.986 \\
R^2 = 0.9995
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y = -3.4381x + 31.694 \\
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SSIIb

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<th>Variance of data set</th>
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<th>P (T &lt; t) two-sided</th>
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<td>3.75 × 10⁻⁴</td>
<td>5</td>
<td>1.078</td>
<td>0.342</td>
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<tr>
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<td></td>
<td>gDNA</td>
<td>−3.573</td>
<td>4.75 × 10⁻²</td>
<td>5</td>
<td>−6.264</td>
<td>(0.003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(−3.251)</td>
<td>(6.58 × 10⁻³)</td>
<td>(5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MON810</td>
<td>E</td>
<td>pDNA</td>
<td>1.944</td>
<td>5.22 × 10⁻⁵</td>
<td>5</td>
<td>0.967</td>
<td>0.388</td>
</tr>
<tr>
<td></td>
<td></td>
<td>gDNA</td>
<td>1.910</td>
<td>5.75 × 10⁻³</td>
<td>5</td>
<td>−5.853</td>
<td>(0.004)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(2.031)</td>
<td>(1.25 × 10⁻²)</td>
<td>(5)</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>slope</td>
<td>pDNA</td>
<td>−3.403</td>
<td>1.97 × 10⁻³</td>
<td>5</td>
<td>−0.275</td>
<td>0.797</td>
</tr>
<tr>
<td></td>
<td></td>
<td>gDNA</td>
<td>−3.358</td>
<td>1.16 × 10⁻¹</td>
<td>5</td>
<td>−1.388</td>
<td>(0.237)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(−3.344)</td>
<td>(5.42 × 10⁻³)</td>
<td>(5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>pDNA</td>
<td>1.967</td>
<td>2.98 × 10⁻⁴</td>
<td>5</td>
<td>−0.529</td>
<td>0.624</td>
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<tr>
<td></td>
<td></td>
<td>gDNA</td>
<td>1.998</td>
<td>1.41 × 10⁻²</td>
<td>5</td>
<td>−1.415</td>
<td>(0.230)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(1.991)</td>
<td>(8.95 × 10⁻⁴)</td>
<td>(5)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The results obtained using gDNA pretreated only by sonication are shown in parentheses.

*E.* PCR efficiency was calculated using the slope of the standard curve according to Formula 1 (Formula 1: PCR efficiency (E) = 10⁰⁻¹⁰^slope^).

According to Formula 3, described in “Materials and Methods,” we had to determine the conversion factor (C_f) as described in Formula 2. Measurements of C_f in the DNA extracted from 100% MON810 were performed by different researchers from three laboratories using the LightCycler® real-time PCR system equipment in each laboratory, and were run in triplicate for SSIIb, P35S, or MON810 copy number quantification. The C_f values determined in the three laboratories were 0.43, 0.51, and 0.44 for GM maize quantification by measurement of the SSIIb and P35S copy numbers, and 0.54, 0.49, and 0.48 for GM maize quantification by measurement of the SSIIb and MON810 copy numbers. The average values obtained from the three laboratories were defined as C_f for calculation of the GM maize content (%) in the maize samples. The averaged C_f values for GM maize content quantification were 0.46 for GM maize quantification by measurement of the SSIIb and P35S copy numbers, and 0.50 for GM maize quantification by measurement of the SSIIb and MON810 copy numbers. The relative standard deviation (RSD) values of C_f were 8.75% and 8.20% respectively.

**Accuracy, repeatability, and reproducibility for the quantification of P35S and MON810 in maize samples**

The accuracy, repeatability, and reproducibility of GM maize contents by measurement of the P35S and MON810 copy numbers using the established method were assessed. DNA extracted from the 1% and 5% MON810 mixed samples was repeatedly amplified five times per d. Each sample was run in duplicate for SSIIb, P35S, and MON810 copy number quantification. The MON810 content (%) of each sample was calculated using Formula 3, as described in “Materials and Methods.” Table 3 shows the results of this reproducibility study of the calculated MON810 content (%) for the same day and three different days using the method established. As can be seen in Table 3, the calculated MON810 (1%) content measured by the P35S copy number quantification ranged from 0.92 to 1.05% for the three different days. The RSD values ranged from 3.57 to 10.70% for one day, and the RSD value was 8.71% for the three different days. The calculated MON810 (5%) content measured by P35S copy number quantification ranged from 0.96 to 1.14% for the three different days. The RSD values ranged from 4.67 to 5.11% for the three different days. The calculated MON810 (5%) content measured by MON810 copy number quantification ranged from 7.02 to 10.70% for one day, and the RSD value was 9.09% for the three different days. The calculated MON810 (5%) content measured by MON810 copy number quantification ranged from 4.79 to 5.12% for the three different days. These results indicate that we detected the MON810 content in
the samples in both screenings, and that a specific target should be detectable with a good accuracy and precision by the method established in this study.

Quantitative results of MON810 content in the maize samples

To attempt further to apply this established method to other samples, GM maize content (%) was measured using the established method and calculated for ERM1 (1%) and ERM5 (5%) as certified reference samples. As shown in Table 4, the calculated GM maize contents of ERM1 and ERM5 measured by P35S copy number quantification were 1.38 and 5.56% respectively. The RSD values of ERM1 and ERM5 were 6.66 and 4.25% respectively. The calculated GM maize contents of ERM1 and ERM5 measured by MON810 copy number quantification were 1.31 and 5.29% respectively. The RSD values of ERM1 and ERM5 were 5.21 and 6.32% respectively. These results indicate that all the calculated MON810 contents for these samples were very close to the expected theoretical values, and were reasonable. For examination of the extracted gDNA without pretreatment, the calculated GM maize contents of the 1.0 and 5.0% MON810 mixed samples measured by P35S quantification were 1.66 and 8.18%, and their contents measured by MON810 copy number quantification were 1.51 and 9.89% respectively (Table 4). This study indicates that the previously described method using pDNA and the ABI PRISM 7700 equipment can be adapted to a capillary-type real-time PCR system for GM maize and still perform acceptably if the gDNA is pretreated with a combination of sonication and restriction endonuclease digestion before copy number quantification of SSIIb, P35S, and MON810 using LightCycler® real-time PCR. Furthermore, we demonstrated for the first time that pDNA and pretreated gDNA with sonication followed by EcoRI digestion can similarly perform as reference molecules. With respect to the similarity between pDNA and pretreated gDNA with sonication followed by EcoRI digestion, we believe that these data have not been demonstrated before for GM maize quantification, and that they can address the ongoing criticism of amplification differences when using pDNA as a reference.

In the official Japanese method, to monitor the unintentional mixing level of GM maize in non-GM materials controlled by an identity-preserved handling system, a screening quantification system using a combination of quantifications for the P35S and GA21 event construct-specific regions has been adopted, and this system is the most practical method as the first step for maize grains imported from the United States and Canada. Hence we are considering future studies to develop a quantitative method for GA21 maize using a combination of the LightCycler® real-time PCR system and pDNA as the reference standard.

In conclusion, we established rapid quantification methods of GM maize contents in maize grains by measurement of the P35S copy number and MON810 copy number using a combination of the LightCycler® real-time PCR system and pDNA as the reference standard. In addition, we found that pDNA and gDNA pretreated with a combination of sonication and restric-

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Table 3. Accuracy, Repeatability, and Reproducibility of Quantitative Results of 1% and 5% MON810 Mixing Sample

<table>
<thead>
<tr>
<th>Target</th>
<th>Same day (n = 5)</th>
<th>Three days (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Calculated (%)</td>
<td>RSD (%)</td>
</tr>
<tr>
<td>MON810</td>
<td>1% Mixing Sample</td>
<td></td>
</tr>
<tr>
<td>P35S</td>
<td>0.98</td>
<td>3.57</td>
</tr>
<tr>
<td></td>
<td>1.05</td>
<td>10.70</td>
</tr>
<tr>
<td></td>
<td>0.92</td>
<td>3.71</td>
</tr>
<tr>
<td></td>
<td>0.98</td>
<td>8.71</td>
</tr>
<tr>
<td></td>
<td>5% Mixing Sample</td>
<td></td>
</tr>
<tr>
<td>P35S</td>
<td>4.26</td>
<td>2.94</td>
</tr>
<tr>
<td></td>
<td>4.83</td>
<td>6.24</td>
</tr>
<tr>
<td></td>
<td>5.11</td>
<td>5.52</td>
</tr>
<tr>
<td></td>
<td>4.73</td>
<td>9.09</td>
</tr>
</tbody>
</table>

Table 4. Quantitative Results of the Maize Samples Including MON810 Using the System Established

<table>
<thead>
<tr>
<th>Target</th>
<th>Sample</th>
<th>n</th>
<th>GMO Amount (%)</th>
<th>Actual</th>
<th>Calculated</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MON810</td>
<td></td>
<td></td>
<td>P35S</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MON0</td>
<td>3</td>
<td>0</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ERM1</td>
<td>15</td>
<td>1</td>
<td>1.38</td>
<td>6.66</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ERM5</td>
<td>15</td>
<td>5</td>
<td>5.56</td>
<td>4.25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MON1*</td>
<td>6</td>
<td>1</td>
<td>1.66</td>
<td>9.78</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MON5*</td>
<td>6</td>
<td>5</td>
<td>8.18</td>
<td>9.20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MON0</td>
<td>3</td>
<td>0</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ERM1</td>
<td>15</td>
<td>1</td>
<td>1.31</td>
<td>5.21</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ERM5</td>
<td>15</td>
<td>5</td>
<td>5.29</td>
<td>6.32</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MON1*</td>
<td>6</td>
<td>1</td>
<td>1.51</td>
<td>10.33</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MON5*</td>
<td>6</td>
<td>5</td>
<td>9.89</td>
<td>14.52</td>
<td></td>
</tr>
</tbody>
</table>

*Quantitative PCR was performed with extracted gDNA without pretreatment by sonication and EcoRI digestion.

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Quantitative results of MON810 content in the maize samples

To attempt further to apply this established method to other samples, GM maize content (%) was measured using the established method and calculated for ERM1 (1%) and ERM5 (5%) as certified reference samples. As shown in Table 4, the calculated GM maize contents of ERM1 and ERM5 measured by P35S copy number quantification were 1.38 and 5.56% respectively. The RSD values of ERM1 and ERM5 were 6.66 and 4.25% respectively. The calculated GM maize contents of ERM1 and ERM5 measured by MON810 copy number quantification were 1.31 and 5.29% respectively. The RSD values of ERM1 and ERM5 were 5.21 and 6.32% respectively. These results indicate that all the calculated MON810 contents for these samples were very close to the expected theoretical values, and were
tion enzyme digestion are similar to the established PCR system, and are thus commutable. These methods can monitor a labeling system rapidly and have sufficient levels of accuracy and precision.

Acknowledgment

We are very grateful to the Monsanto Company, St. Louis, MO, for providing reference MON810 seed materials. We thank Dr. Doris Dixon and Mr. Hiroo Wakimori for useful suggestions, and Dr. Satoshi Futo, Ms. Ai Miyazaki, and Ms. Chiseko Wakui for teaching us the technique of using the LightCycler® real-time PCR system. This study was supported by Health and Labor Sciences Research Grants from the Research on Food Safety from the Ministry of Health, Labor, and Welfare of Japan.

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