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Practicable Group Testing Method to Evaluate Weight/Weight GMO Content in Maize Grains

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Supporting Information

ABSTRACT: Because of the increasing use of maize hybrids with genetically modified (GM) stacked events, the established and commonly used bulk sample methods for PCR quantification of GM maize in non-GM maize are prone to overestimate the GM organism (GMO) content, compared to the actual weight/weight percentage of GM maize in the grain sample. As an alternative method, we designed and assessed a group testing strategy in which the GMO content is statistically evaluated based on qualitative analyses of multiple small pools, consisting of 20 maize kernels each. This approach enables the GMO content evaluation on a weight/weight basis, irrespective of the presence of stacked-event kernels. To enhance the method's user-friendliness in routine application, we devised an easy-to-use PCR-based qualitative analytical method comprising a sample preparation step in which 20 maize kernels are ground in a lysis buffer and a subsequent PCR assay in which the lysate is directly used as a DNA template. This method was validated in a multilaboratory collaborative trial.

KEYWORDS: GMO detection, detection, group testing, subsampling

INTRODUCTION

Industrial use of genetically modified organisms (GMOs) has been advancing, and many genetically modified (GM) crops have been put on the market in the past 15 years.[1] In maize, which is one of the four major GM crops, along with soybeans, cotton, and canola, stacked-event seeds, generated by crossing two or more single GM events, have been widely used.[1] Numerous safety assessments of GM crops and their derived foods and feeds have been conducted by authorities in countries around the world, and commercially available GM crops are considered to be as safe as their conventional (non-GM) counterparts. In many countries, however, the use of GM crops is controversial among general consumers, and the demand for conventional crops is deeply rooted. To expand consumers' choices, many countries have introduced legislation requiring labels to be applied to agricultural products that happen to contain approved GMOs at more than a certain threshold level. For example, the thresholds are set as 0.9%, 3%, and 5% in the European Union (EU), Korea, and Japan, respectively.[2] For products that do not carry GM labels, compliance with these regulations is checked at various points of the supply chain, often starting with the crops.

The regulations in some countries refer to the GM material in terms of weight/weight percentages, although the most commonly used technique for GMO quantification in grain is quantitative real-time polymerase chain reaction (PCR) analysis of bulk sample homogenates, and the analysis typically measures GMO contents based on the ratio of GM DNA to plant-species DNA. Because the GM stacked events contain the GM DNA corresponding to two or more single events, the GMO content of non-GMO maize samples with a small number of stacked-event kernels measured by real-time PCR leads to an overestimation as compared to the actual weight/weight GMO content.[3] In light of the increasing use of GM stacked events, it has become virtually impossible to accurately measure low-level GMO content on a weight/weight basis with current methodology. Recently, a single-kernel-based analytical system was developed and implemented in Japan as an official method, as one possible solution for the potential overestimation due to stacked events.[3−5] In this method, individual maize kernels are analyzed to determine their GM or non-GM status, and the weight/weight GMO content is evaluated based on the assumption that the ratio of GM kernels relative to the total number of kernels is equal to the weight/weight ratio.

Since Dorfman's report on blood testing for syphilis in 1943, the group testing strategy has been exploited in epidemiology,

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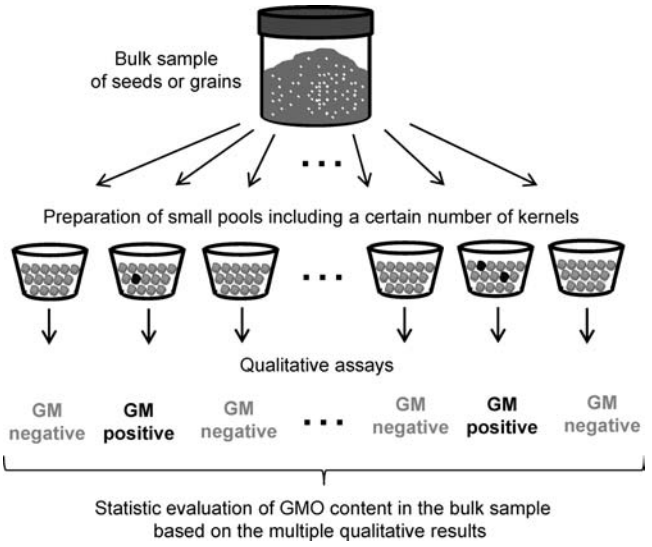

Figure 1. Group testing applied to GMO analysis.

genetics, blood-bank screening, drug discovery, biology, and plant pathology.[6,7] In this strategy, groups of units that make up an analytical sample are prepared. Then, qualitative analyses of multiple groups are individually performed, and the contents of the analyte are evaluated statistically.[8] When applied to GMO analysis of seeds or grains, each group contains a defined number of kernels from a larger bulk sample, and the GMO content is statistically evaluated based on qualitative results for multiple groups (Figure 1). Irrespective of the presence of stacked-event kernels, such a strategy enables the evaluation of GMO content on a weight/weight basis. Additionally, this strategy would be more efficient than a single-kernel-based strategy. In fact, the theoretical application of group testing strategy to GMO analysis has been investigated.[9−11] Meanwhile, to the best of our knowledge, a practical and accurate testing method to perform group testing for maize grains has not yet been reported. Accordingly, we present the development and validation of an efficient, easy-to-use PCR-based testing method for GMO detection in small pools of maize kernels.

MATERIALS AND METHODS

Cereal Materials. The representative GM maize events used were Bt11, Event176, GA21, MON810, MON863, NK603, T25, TC1507, DAS59122, MON88017, and MIR604. F1-generation seeds of Bt11 and Event176 and ground F1-generation seeds of GA21 and MIR604 were kindly provided by Syngenta Seeds (Basel, Switzerland). F1-generation seeds of MON810, MON863, NK603, and MON88017 were kindly provided by Monsanto (St. Louis, MO, USA), and F1-generation seeds of TC1507 and DAS59122 were kindly provided by Pioneer Hi-Bred International (Johnston, IA, USA). F1-generation seeds of T25 were imported directly from the United States. Five conventional maize seeds were used as non-GM maize: DK537 and RX740 maize from Monsanto; QC9651 maize from Quality Technology International (Huntley, IL, USA); and Strike5512 and LG2265 maize, obtained in Japan. Dry conventional soybean seeds directly imported from the United States were used as non-GM soy. Seeds of the conventional rice variety Kinuhikari (*Oryza sativa*), the conventional wheat variety Haruyutaka (*Triticum aestivum*), and the conventional barley variety Harrington (*Hordeum vulgare*) were obtained in Japan.

Preparation of Genomic DNAs. For the preparation of purified DNA extracts, all dry seeds were ground with a P-14 speed rotor mill (Fritsch, Idar-Oberstein, Germany). For maize, soy, wheat, barley, and rice, DNA extraction was performed using a DNeasy Plant Maxi Kit (Qiagen, Hilden, Germany) as described previously.[13] The DNA concentration of solutions was determined by measuring ultraviolet (UV) absorbance with a spectrophotometer (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, DE, USA). DNA concentration was calculated with 1 optical density unit at 260 nm equal to 50 ng/μL. All extracted DNAs were diluted to 20 ng/μL with sterile distilled water. Genomic DNAs were analyzed using a real-time PCR array system as previously reported,[14] and the purity of the samples was confirmed.

Preparation of Plasmid DNAs. To establish the method for group testing, we developed two duplex real-time PCR assays: a GM maize screening assay and an experimental control assay. The GM maize screening assay was designed to detect the 35S promoter region (P35S) and NOS terminator region (TNOS) widely introduced into commercially available GM maize events. The experimental control assay was intended to detect both the starch synthase IIb gene derived from *Zea mays* (SSIIb) as the endogenous reference DNA and an artificial sequence on the pART plasmid as an internal positive control (IPC). The pUC19 plasmids harboring each of the target sequences, namely, P35S, TNOS, SSIIb, and IPC, were prepared after cloning in *Escherichia coli* DH5α and are denoted pP35S, pTNOS, pSSIIb, and pART, respectively. The target sequences were confirmed to be single and correct by nucleotide sequence analyses. The sequence information is included in the Supporting Information. The plasmids were purified by cesium chloride/ethidium bromide equilibrium centrifugation[15] and then diluted to the given concentration with 5 ng/μL ColE1 plasmid solution in Tris/ethylenediaminetetraacetic acid (EDTA) buffer (Nippon Gene, Tokyo, Japan).

PCR Assays. The two duplex real-time PCR assays, GM maize screening and experimental control assays, were developed as described above. The reaction mixture for the GM maize screening assay consisted of 12.5 pmol of P35S 1-5′ (5′-ATTGATGTGATATCTCCACTGACGT-3′), P35S 1-3′ (5′-CCTCTCCAAATGAAATGAACTTCCT-3′), TNOS 2-5′ (5′-GTC-TTGCGATGATTATCATATAATTTCTG-3′), and TNOS 2-3′ (5′-CG-CTATATTTTGTTTTCTATCGCGT-3′) primers; 2.5 pmol of P35S-Taq (5′-CCCACTATCCTTCGCAAGACCCTTCCT-3′) and TNOS-Taq (5′-AGATGGGTTTTTATGATTAGAGTCCCGCAA-3′) probes; 2.5 μL of DNA template; 0.5 μL of ROX Reference Dye (Life Technologies); 0.625 units of BIOTAQ HS DNA polymerase (Shimadzu, Kyoto, Japan); and 12.5 μL of 2 × Ampdirect Plus buffer (Shimadzu) in a total volume of 25 μL. The reaction mixture for the experimental control assay consisted of 12.5 pmol of IPC 1-5′ (5′-CCGAGCTTACAAGGCAGGTT-3′), IPC 1-3′ (5′-TGGCTCGTACACCAGCATACTAG-3′), SSIIb 1-5′ (5′-CTCCCAATCCTTTGACATCTGC-3′), and SSIIb 1-3′ (5′-TCGAT-TTCTCTCTTGGTGACAGG-3′) primers; 2.5 pmol of IPC 1-Taq (5′-TA-GCTTCAAGCATCTGGCTGTCGGC-3′) and SSIIb-Taq (5′-AGCAA-AGTCAGAGCGCTGCAATGCA-3′) probes; 40 theoretical copies of the pART plasmid; 2.5 μL of DNA template; 0.5 μL of ROX Reference Dye, 0.625 units of BIOTAQ HS DNA polymerase; and 12.5 μL of 2 × Ampdirect Plus in total volume of 25 μL. The oligonucleotide DNAs for PCR primers and TaqMan probes were synthesized by Fasmac (Atsugi, Japan) and Biosearch Technologies (Novato, CA, USA), respectively. P35S-Taq, TNOS-Taq, and IPC 1-Taq were labeled with 6-carboxyfluorescein (FAM) and black hole quencher 1 (BHQ1) dyes at the 5′ and 3′ terminals, respectively. For SSIIb-Taq, hexachlorofluorescein (HEX) was used in place of FAM dye. Thermal cycling of reaction mixtures was carried out with a 7900HT real-time PCR instrument (Life Technologies, Carlsbad, CA, USA) unless otherwise specified. The thermal cycling condition was set as 10 min at 95 °C and 45 cycles of 15 s at 95 °C and 1 min at 65 °C under 9600 emulation mode. Data analysis was performed using Sequence Detection Software, version 2.3. The manual Ct mode (threshold, 0.256 for FAM and 0.064 for HEX) and manual baseline mode (start of baseline, 3; end of baseline, 15)

were set at the "Delta Rn vs. Cycle" view of the "Amplification Plot" feature. DNA amplifications with threshold-cycle (Ct) values below 40 were determined to be positive. Concerning PCR assays performed with a 7500 real-time PCR instrument (Life Technologies), all experiments were carried out as described above, except that the volume of ROX reference dye was set as 0.05 μL and Sequence Detection Software, version 1.4, was used for data analysis.

Design of Testing Protocol. We designed the following testing protocol:

Step 1. Groups comprising 20 maize kernels each are prepared using a grain counter plate on which only 20 holes are available (For 100 Soybeans; Fuji Kinzoku, Tokyo, Japan), and they are put into glass vessels with a capacity of 75 mL for use with a Milser 800-DG household food processor (Iwatani, Tokyo, Japan). The number of groups in one experiment is variable depending on the analyst's purpose.

Step 2. Twenty milliliters of a lysis buffer is added to each glass vessel. One liter of the lysis buffer contains of 20 mL of 1 mol/L Tris-HCl buffer solution (Nacalai Tesque, Kyoto, Japan), 10 mL of 0.5 mol/L EDTA solution (Nacalai Tesque), 80 mL of 5 mol/L sodium chloride (NaCl) solution (Nacalai Tesque), and 30 mL of 10% sodium dodecyl sulfate (SDS) solution (Nacalai Tesque) in distilled water. Each group is ground for 20 s with the household food processor. After 10 min of incubation at room temperature, the lysate in each glass vessel is vigorously shaken by hand. After 10 min of static standing to allow solid–liquid separation, 50 μL of the supernatant is moved to a plastic tube. Each portion of supernatant is diluted 2-fold with sterile distilled water. The diluted solution is centrifuged at more than 1000*g* on a personal benchtop centrifuge for 1 min and then used for the following PCR assay.

Step 3. PCR mixtures are prepared with the supernatant for both GM maize screening and experimental control assays, and thermal cycling is performed as described above.

Step 4. The data from real-time PCR are analyzed with Sequence Detection Software as described in the PCR Assays section. If SSIIb or IPC detection is determined to be negative by the experimental control assay, the group in question is rejected. If both SSIIb and IPC are positive, the group is determined to be either GM-positive or GM-negative based on the result of the GM maize screening assay.

Evaluation of the Testing Protocol. To evaluate the linearity of the PCR assays, we analyzed the respective plasmid DNAs with theoretical numbers of copies of 250000, 20000, 1500, 125, and 20 (n = 3). Then, we calculated the regression lines from the averages of triplicate PCR results. To evaluate the specificity of the PCR assays, we analyzed the genomic DNAs derived from various kinds of GM events and plant materials using both assays (n = 6). Ct values of P35S/TNOS detection by the GM maize screening assay and those of SSIIb and IPC detection by the experimental control assay were measured. To evaluate the sensitivity of the PCR assays, we analyzed plasmid DNAs with low theoretical numbers of copies (40, 20, 10, and 0; n = 21 for each dilution level) and counted the number of positive results. For the comparative analysis of PCR inhibition between P35S, TNOS, and IPC detections, we selected SDS, NaCl, EDTA, and crude maize extracts as PCR inhibitors. Under the coexistence of these possible inhibitors at various concentrations, positive and negative detections were examined both in the GM maize screening assay with 40 copies of the pP35S or pTNOS plasmid and in the experimental control assay with 40 copies of the pART plasmid. For the preparation of the crude maize extract, 1 g of the ground DK537 maize sample was incubated for 1 h with vigorous shaking at room temperature in 3 mL of the lysis buffer. After centrifugation at 15000*g* for 1 min, the supernatant was used as the crude maize extract.

We prepared simulated groups, each of which consisted of 19 kernels of non-GM maize and one MON810 kernel. As non-GM materials, DK537, RX740, QC9651, LG2265, and Strike5512 maize were individually used. The simulated groups were analyzed in accordance with the testing protocol (n = 6 for each material).

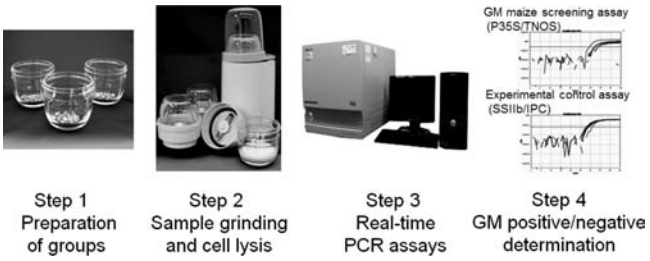

Figure 2. Overview of the testing protocol.

Collaborative Trial for Method Validation. For the collaborative trial, DK537 maize and F1-generation seeds of MON810 maize were used as non-GM and GM maize materials, respectively. All MON810 kernels were cut in half with a knife to inhibit germination, and simultaneously, approximately 2-mg fragments were scraped off individual half-cut GM kernels. To check for an adventitious presence of non-GM kernels in the GM seed lot, we suspended these fragments in 50 μL of the lysis buffer with sterile toothpicks and then analyzed them according to the testing protocol beginning with the 10-min incubation in step 2. We confirmed GM-positive detection for each MON810 kernel. The AOAC guideline specifies 10 laboratories reporting 2 analyte levels per matrix, 6 test samples per level, and 6 negative controls per matrix as the minimum criteria for the validation of qualitative methods.[16] These criteria also satisfy the requirements outlined in McClure's report.[17] Accordingly, we prepared groups consisting of 2 GM kernels and 18 non-GM kernels, groups consisting of 1 GM kernel and 19 non-GM kernels, and groups consisting of 20 non-GM kernels. These groups were named A, B, and C groups, respectively. As a set of blind samples for a laboratory, 6 A groups, 6 B groups, and 6 C groups were sorted at random and numbered from 1 to 18. A set of blind samples was provided to each of 12 laboratories. In each laboratory, the blind samples were analyzed in one experiment according to the testing protocol.

RESULTS AND DISCUSSION

Design of the Testing Protocol. To perform group testing for maize grains in a practical manner, it is essential to develop an easy-to-use, high-throughput, and cost-effective sample pretreatment and GM maize screening assay for groups of kernels. A series of immunoassays for individual GM traits might be a good candidate, but a protein-based methodology limits the range of detectable GM events. We designed a testing method comprising a sample pretreatment step in which a group of maize kernels is ground in a lysis buffer with a household food processor and a subsequent PCR assay step in which the lysed sample is directly analyzed as a DNA template. We experimentally adjusted testing conditions, and the testing protocol was fixed as described in the Materials and Methods section. An overview of the testing protocol is shown in Figure 2. In this testing protocol, we fixed the number of kernels in a group to 20. This was because our preliminary investigation indicated that a group consisting of 20 kernels was easy to handle and gave stable PCR assay results. For sample preparation, we recommend using a grain counter plate to make small pools efficiently without intentional bias. For the sample pretreatment step, we used a household food processor that permitted sequential grinding of many samples just by changing glass vessels. The use of a household food processor promises a high-throughput treatment with a

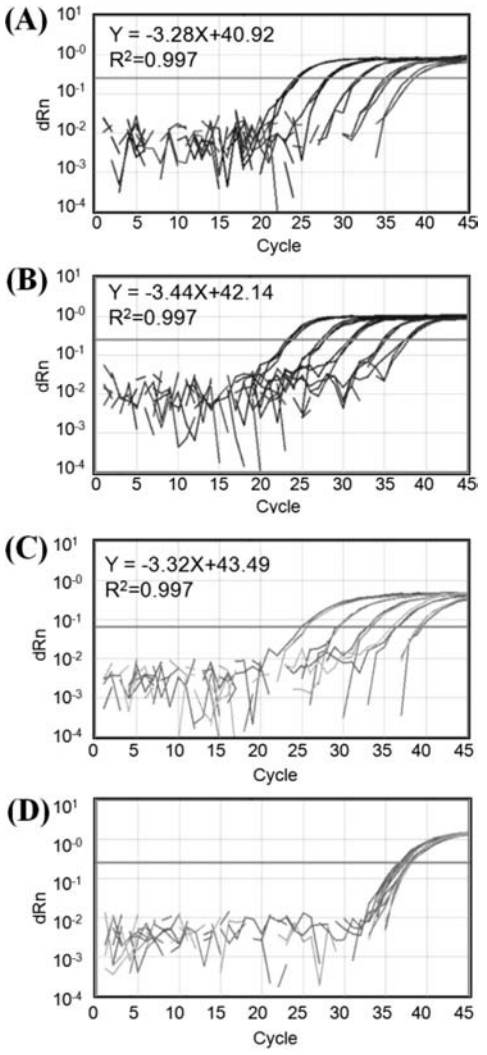

Figure 3. DNA amplification lines and parameters of their regression lines. Dilution series of plasmid DNAs were analyzed in triplicate. (A) pP35S series in the GM maize screening assay, (B) pTNOS series in the GM maize screening assay, (C) pSSIIb series in the experimental control assay, and (D) IPC detection results in the experimental control assay with the pSSIIb series. Regression lines were calculated from the mean values of triplicate analyses, and their parameters are shown in plots A−C.

minimum investment for grinding instruments. In addition, sample grinding in a lysis buffer does not require handling of dry flour, which simplifies the method and reduces the chance of contamination. For the PCR assay step, we designed two qualitative duplex real-time PCR assays, namely, GM maize screening and experimental control assays, using Ampdirect technology as a PCR reagent, which reduces the influence of PCR inhibitors. For the GM maize screening assay, the P35S and TNOS regions were selected as targets, because commercially available GM events have at least one, if not both, of these regions as part of their recombinant DNAs. TaqMan probes both of P35S and TNOS were labeled with FAM as a reporter dye, because P35S and TNOS detections were not necessarily distinguished. Meanwhile, we developed an experimental control assay to individually detect both SSIIb and 40 copies of pART, as the endogenous reference DNA and as an IPC, respectively. TaqMan probes for SSIIb and IPC detections were labeled with HEX and FAM, allowing us to distinguish between SSIIb and IPC. The experimental control assay was designed to confirm that the reaction mixture contained sufficient DNA extraction without PCR inhibition. To avoid detecting very tiny amounts of contamination, in terms of analytical robustness, we decided that DNA amplifications with Ct values of up to 40 were positive.

Evaluation of PCR Assays. We evaluated analytical performances of the PCR assay step. To do so, we prepared plasmid DNAs, each of which had a single target sequence for P35S, TNOS, SSIIb, and IPC detection. We confirmed the amplification linearity by using dilution series of plasmids except for pART (Figure 3). Detection results for P35S, TNOS, and SSIIb showed high coefficient values (>0.990). IPC detections were successfully obtained, irrespective of predominant SSIIb amplification in the same reaction mixture. Then, we confirmed the detection specificity with genomic DNAs from commercially distributed GM maize events and non-GM crops (Figure 4). P35S and/or TNOS regions were detected for all GM maize events, and these Ct values roughly corresponded to the numbers of copies of the P35S and/or TNOS regions in each event. Meanwhile, for non-GM maize, soy, wheat, barley, and rice, nonspecific detection was not observed, as expected. Although the specificity evaluation was carried out using only the single-GM-event samples, the results suggested that the GM stacked events derived from the single events would be detected in the developed assays. By analyzing the plasmid dilution series, we confirmed that the detection sensitivity of our method was high enough to detect 40 copies of target DNAs (Table 1). Then, we compared PCR inhibitions between P35S, TNOS, and IPC detections. We selected SDS, NaCl, EDTA, and crude maize extracts as PCR inhibitors that exist in PCR mixtures. Under the coexistence of these inhibitors at various concentrations, positive and negative detections were counted both in the GM maize screening assay with the pP35S or pTNOS plasmid and in the experimental control assay with the pART plasmid (Table 2). The results indicated that the IPC detection was sensitive to PCR inhibitors as well as P35S and TNOS detections when at least 40 copies of the P35S or TNOS regions were included in a reaction mixture. We also evaluated the PCR assays on the 7500 real-time PCR instrument, and the results are available in the Supporting Information. There were no large differences in results between the 7900HT and 7500 real-time PCR instruments.

Analysis of Simulated Samples According to the Testing Protocol. We performed analyses with the simulated groups of a maize sample that included one GM kernel among 20 kernels (Figure 5). As the GM maize kernel, we used F1-generation seeds of the MON810 event, which has the lowest number of copies of the target of the GM maize screening assay. No false negative result was observed in the GM maize screening assay, suggesting that the testing protocol had the capacity to detect at least one GM kernel in a group. Homogeneity of Ct-value variances of SSIIb detection between different non-GM materials was confirmed by Bartlett's test ($\alpha = 0.05$), and one-way analysis of variance (ANOVA) ($\alpha = 0.05$) of the data showed no significant differences ($p = 0.08$). This indicated that stable DNA extraction was achieved, irrespective of maize materials. In these results, the numbers of copies of SSIIb from 20 kernels were calculated as being between 5200 and 21000 copies (mean value of all results, 15000 copies) based on the calibration curve of the pSSIIb plasmid dilution series. This suggested that, if there was one kernel of GM maize in a group, at lease 5200/20 copies of P35S and/or TNOS regions would be expected to be present in a

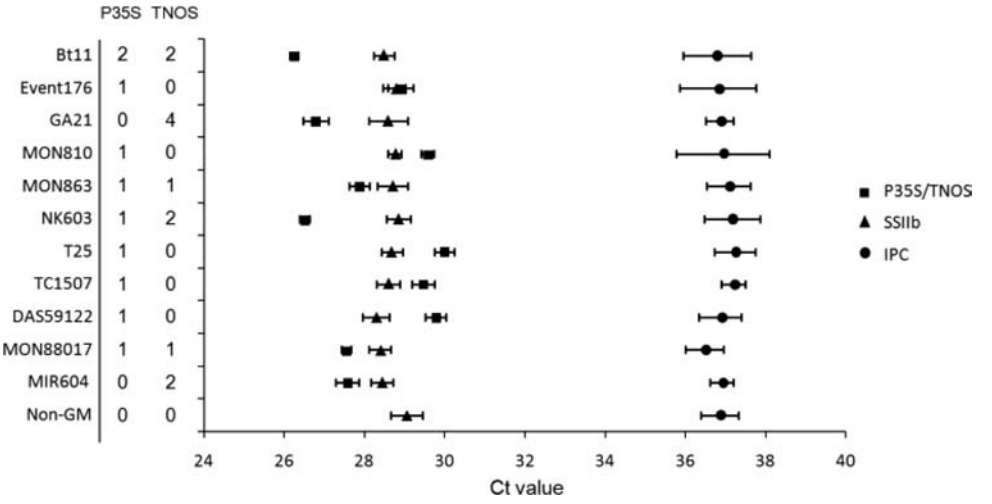

Figure 4. Specificity evaluation of PCR assays. DNA samples from GM events and non-GM maize were subjected to PCR assays ($n = 6$). The means of Ct values ± standard deviations are shown in the graphs. The numbers of P35S and TNOS regions in each GM-event haploid genome are summarized beside the names of the GM events.

Table 1. Sensitivity Evaluation of PCR Assays

detection	theoretical number of plasmid copies	number of positives	positive rate (%)
P35S	40	21/21	100
	20	21/21	100
	10	16/21	76
	0	0/21	0
TNOS	40	21/21	100
	20	21/21	100
	10	20/21	95
	0	0/21	0
SSIIb	40	21/21	100
	20	18/21	86
	10	12/21	57
	0	0/21	0

Table 2. Comparative Analyses of PCR Inhibition between IPC, P35S, and TNOS Detections

inhibitor	final concentration	IPC detection	P35S detection	TNOS detection
SDS	0.01%	3/3	3/3	3/3
	0.05%	3/3	3/3	3/3
	0.5%	0/3	0/3	0/3
NaCl	2 mM	3/3	3/3	3/3
	10 mM	3/3	3/3	3/3
	100 mM	0/3	0/3	0/3
EDTA	0.1 mM	3/3	3/3	3/3
	0.2 mM	3/3	3/3	3/3
	1 mM	0/3	0/3	0/3
crude maize extract	1/1000	3/3	3/3	3/3
	1/500	2/3	3/3	3/3
	1/10	0/3	0/3	0/3

reaction mixture, and thus the IPC detection designed to have 40 copies of plasmid DNA as a template would be capable of checking PCR inhibition in the GM maize screening assay.

Evaluation of Robustness in the Sample Pretreatment Step. We evaluated the robustness of the sample pretreatment step with groups of non-GM kernels by slightly changing pretreatment conditions (n = 6 per condition). The modified conditions were the grinding time (10 s, 15 s, 20 s, or 25 s), the lysis time (5 min, 10 min, or 20 min), and the lysis temperature (15, 20, or 25 °C). The Ct values of SSIIb and IPC detections in the experimental control assay were evaluated (Figure 6). The homogeneity of Ct-value variances between conditions was confirmed by Bartlett's test ($\alpha = 0.05$), and then Ct values were analyzed by one-way ANOVA ($\alpha = 0.05$). Ct values under various lysis times and temperatures did not significantly differ. Meanwhile, Ct values of SSIIb detection under the various grinding times showed significant differences, suggesting that the amount of extracted DNA was influenced by the grinding time. We concluded that the sample pretreatment step was sufficiently robust in terms of the lysis time and temperature, but that the grinding time should be strictly controlled.

Collaborative Trial for Method Validation. A collaborative trial was carried out according to the procedure described in a previous report and guideline.[16,17] The results for the individual groups are available in the Supporting Information. All of the results showed the expected positive/negative determinations corresponding to the presence/absence of GM kernel(s) in each group, except for a result that was rejected because of unsuccessful IPC detection. The results indicated that the method accurately detected the presence of GM and the absence of cross-contamination between groups. After removal of the result rejected because of the unsuccessful IPC detection, the false-negative rates were calculated separately for the A and B groups, and the false-positive rate was calculated for the C groups (Table 3). Both false-negative rates were 0%, which fulfilled the criterion for the limit of detection for qualitative GMO detection methods as described in the ISO standard regarding GMO analysis.[18] In addition, the Ct values of the detections were found to be stable even under inter-laboratory evaluation (Figure 7). Thus, the testing method was validated to have sufficient performance for the reliable detection of one GM maize kernel in a group.

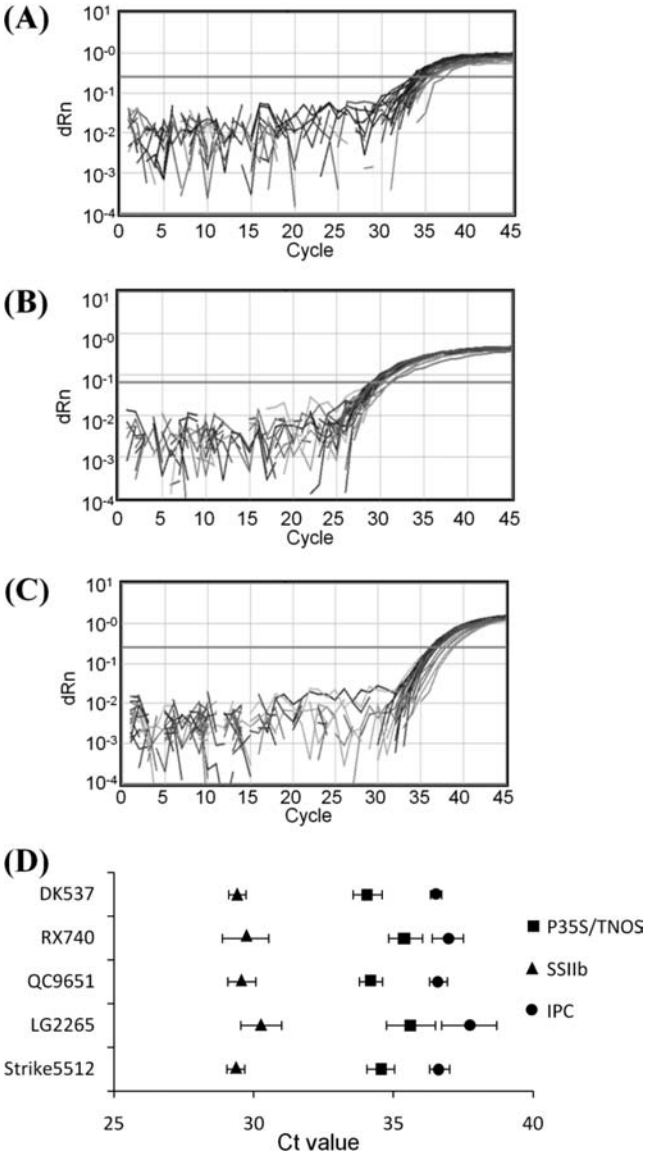

Figure 5. Testing results for the simulated samples including a MON810 kernel mixed in with five kinds of non-GM maize materials. (A) Amplification lines in the GM maize screening assay, (B) amplification lines of SSIIb detection in the experimental control assay, (C) amplification lines of IPC detection in the experimental control assay, and (D) summary of Ct value data for each non-GM background (means ± standard deviations, $n = 6$).

Practical Use of the Group Testing Based on the Developed Method. In summary, we have described an easy-to-use analytical method for group testing. This method was efficient enough to analyze 18 groups within 3 h at a low cost. Although our method harnesses two targets, namely, P35S and TNOS, to cover the commercially distributed GM maize events so far, this might become insufficient as new GMO events become available. There have been some reports describing highly multiplexing real-time PCRs for qualitative GMO detection.[19−22] The availability of the PCRs described in these studies suggests that it will be possible to update our method to test for new GMOs as they enter the market.

To practice group testing, it is indispensable to first determine the appropriate testing conditions, that is, the number of kernels per group (group size), the number of groups, and the maximum number of GM-positive groups for acceptance. Statistical calculation programs previously reported, such as Seedcalc, facilitate the determination of the optimal testing conditions depending on the analyst's purpose.[9,12] In our method, the group size was fixed at 20; however, the other parameters could be freely chosen. We confirmed that, even when the group size was fixed at 20, the testing conditions suitable for various threshold levels of GMO content such as 0.9%, 3%, and 5% could be selected by using the already existing calculation programs.

As an official method in Japan, the single-kernel-based method has already been used to determine whether the GMO content in a bulk maize sample exceeds 5%. The testing procedure requires analysis of 90 kernels for the first screening. If there are 3 or more GM kernels in the first 90 kernels tested, another set of 90 kernels must be tested. If the total number of GM kernels in the two tests (180 kernels) is 9 or less, then the GMO content of the bulk

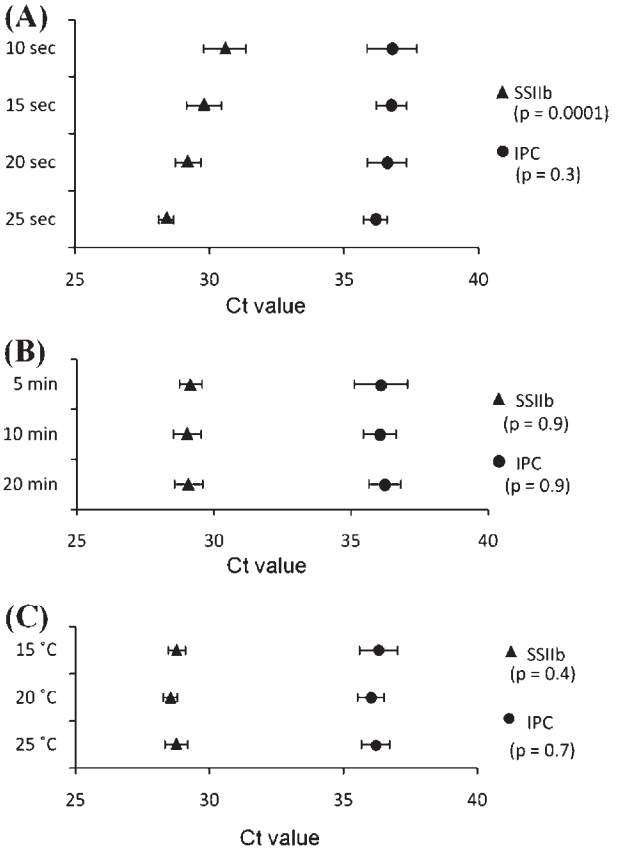

Figure 6. Robustness evaluation for the pretreatment step by simulated sample analyses. Effects by (A) grinding time, (B) lysis time, and (C) lysis temperature. Data are shown as means of Ct values ± standard deviations ($n = 6$). p values given by one-way ANOVA ($\alpha = 0.05$) are shown under the graph legends.

Table 3. Summary of Results in the Interlaboratory Study

group	number of rejections	false-positive results	false-positive rate (%)	false-negative results	false-negative rate (%)
A	1/72	–	–	0/71	0
B	0/72	–	–	0/72	0
C	0/72	0/72	0	–	–

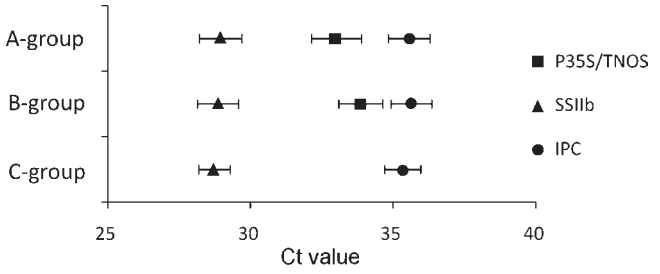

Figure 7. Summary of Ct value data in the interlaboratory study. The definitions of the groups A−C are given in the Materials and Methods section. Data are shown as the means ± standard deviations (n = 71 for the A group and n = 72 for the B and C groups).

sample is below 5% and is acceptable.[23] Based on the operating characteristic curve calculated by the Seedcalc program, we can design group testing that has approximately the same accuracy of judgment as the single-kernel-based method. The designed sampling plan is as follows: A group contains 20 maize kernels, and 10 groups are analyzed for the first screening. If there are 7 or more GM-positive groups in the first screening, another set of 10 groups will be tested. If the total number of GM-positive groups in the two tests (20 groups) combined is 12 or less, the GMO content of the bulk sample is determined to be below 5%. A comparison of operating characteristic curves between the single-kernel-based method and our group testing is provided in the Supporting Information. The slope of an operating characteristic curve represents the uncertainty of judgment that is caused by the sampling, and an analyst should take it into consideration. The introduction of group testing using our method will significantly decrease time and cost for inspection.

Furthermore, calculation programs permit the estimation of a GMO content value with confidence intervals from the testing results. For example, when 8 groups are determined as GM-positive in the testing of 20 groups containing 20 kernels per group, the GMO content will be estimated as 2.52% and its two-sided confidence interval will be between 1.06% and 4.97% at the 95% confidence level. In this manner, one can obtain quantitative information on the GMO content of the bulk sample based on the qualitative testing results and the established statistics.

We believe that group testing is a useful measure for weight/weight GMO content evaluation in maize grains, irrespective of increasing GM stacked events. Certainly, the strategy limits a sample to only seeds or grains and is not applicable to processed foods. However, group testing would contribute to the assured segregation of GM and non-GM maize through the production and transportation systems.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Nucleotide sequence information on PCR amplicons, results of analytical performance evaluation on a 7500 real-time PCR instrument, results of interlaboratory studies, and comparative analysis of the single-kernel-based method and the designed group testing. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

ANOVA, analysis of variance; BHQ1, black hole quencher 1; EDTA, ethylenediaminetetraacetic acid; EU, European Union; GM, genetically modified; GMO, genetically modified organism; HEX, hexachlorofluorescein; IPC, internal positive control; PCR, polymerase chain reaction; NaCl, sodium chloride; SDS, sodium dodecyl sulfate; TNOS, NOS terminator region; SSIIb, starch synthase IIb gene of *Zea mays*; P35S, 35S promoter region; Ct, threshold cycle; UV, ultraviolet; FAM, 6-carboxyfluorescein.

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