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Article

Development and Validation of a New Robust Detection Method for Low-Content DNA Using ΔΔCq-Based Real-Time PCR with Optimized Standard Plasmids as a Control Sample

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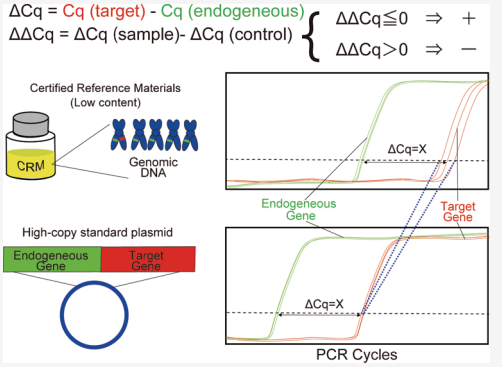
ABSTRACT: Real-time polymerase chain reaction (PCR) is the gold standard for DNA detection in many fields, including food analysis. However, robust detection using a real-time PCR for low-content DNA samples remains challenging. In this study, we developed a robust real-time PCR method for low-content DNA using genetically modified (GM) maize at concentrations near the limit of detection (LOD) as a model. We evaluated the LOD of real-time PCR targeting two common GM maize sequences (P35S and TNOS) using GM maize event MON863 containing a copy of P35S and TNOS. The interlaboratory study revealed that the LOD differed among laboratories partly because DNA input amounts were variable depending on measurements of DNA concentrations. To minimize this variability for low-content DNA samples, we developed ΔΔCq-based real-time PCR. In this study, ΔCq and ΔΔCq are as follows: ΔCq = Cq (P35S or TNOS) − Cq (SSIIb; maize endogenous gene), ΔΔCq = ΔCq (analytical sample) − ΔCq (control sample at concentrations near the LOD). The presence of GM maize was determined based on ΔΔCq values. In addition, we used optimized standard plasmids containing SSIIb, P35S, and TNOS with ΔCq equal to the MON863 genomic DNA (gDNA) at concentrations near the LOD as a control sample. A validation study indicated that at least 0.2% MON863 gDNA could be robustly detected. Using several GM maize certified reference materials, we have demonstrated that this method was practical for detecting low-content GM crops and thus for validating GM food labeling. With appropriate standards, this method would be applicable in many fields, not just food.



INTRODUCTION

Many technologies are used for qualitative nucleic acid detection, such as the conventional polymerase chain reaction (PCR), real-time PCR, loop-mediated isothermal amplification, and recombinase polymerase amplification.[1] Notably, real-time PCR has become a well-established method for the detection of genetically modified (GM) organisms, food allergens, microbial agents, viruses and clinical diagnostics because of its high specificity and sensitivity.[2] Robust qualitative real-time PCR is crucial to obtain the reproducibility among different laboratories that is required for regulatory management. However, robust detection of low nucleic acid concentrations remains difficult.[3−5]

Qualitative real-time PCR is the most commonly used detection tools for GM crops. A large number of GM crops for animal feed and human consumption have been developed and authorized throughout the world.[6] To allow consumer choice, many countries mandate the labeling of foods as "GM" if they contain products of GM crops. However, the regulatory threshold level of GM content for labeling differs among countries (e.g., 0.9% in the European Union and Russia and 5% in the United States).[7,8] In Japan, non-GM crops have been segregated and imported from foreign countries using an identity-preserved handling system that requires documentary certification at each step of the production, distribution, and processing. Using this handling system, Japanese regulations regard ≤5% GM content.[9] From 2023, this regulatory threshold will be reduced from 5% to "undetectable".[10] However, conventional GM crop screening methods are only designed to meet the 5% threshold.[11,12] Moreover, there is no detection method that can robustly guarantee "undetectable" GM content. Therefore, new methods with robustness are needed to detect and regulate GM crop content in foods.

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Among GM crops, maize has the largest number of authorized events.[6] As of 2022, 209 GM maize events, including stacked events, have been authorized for foods in Japan, in addition to 48 cotton events, 29 soybean events, 23 canola events, and 12 potato events.[13] To monitor this abundance of authorized GM maize events, a practical screening strategy is crucial. In Japan, real-time PCR targeting the cauliflower mosaic virus 35S promoter (P35S) and *Agrobacterium tumefaciens* nopaline synthase terminator (TNOS) for kernels and processed foods has been officially implemented.[11,12] This strategy, targeting P35S and TNOS, is also prevalent in many other countries[14−18] and currently detects approximately 97% of the GM maize events authorized in Japan. However, the robustness of P35S- and TNOS-based methods remains unclear.

The limit of detection (LOD) may vary among laboratories partially because different real-time PCR and related instruments are used in each laboratory. Thus, to develop a robust detection method, it is important to evaluate the LOD among laboratories and then identify the causes of variations. Moreover, to avoid laboratory bias, it is desirable for all laboratories to use a method based on the same criteria to detect low-content DNA.

In this study, to develop a robust detection method for low-content DNA using real-time PCR, we used GM maize as a model. The LOD of the real-time PCR targeting P35S and TNOS was evaluated. However, the quantitative measurements of DNA concentrations varied among laboratories, resulting in differences in the LOD. Based on this result, a comparative threshold cycle (ΔΔCq)-based real-time PCR was developed and validated.

EXPERIMENTAL SECTION

Samples. GM maize certified reference material (CRM), MON863 maize powder (ERM-BF416b; 0.1%, ERM-BF416c; 1%, ERM-BF416d; 10% GMO), was purchased from Sigma-Aldrich (St. Louis, MO). DNA extracted from the CRM was used as reference material (RM). Non-GM maize was obtained from Snow Brand Seed Co. (Hokkaido, Japan). GM maize standard plasmid containing the starch synthase IIb (SSIIb), P35S, and TNOS (GM Maize ΔΔCq Standard Plasmid Set) sequences was synthesized by NIPPON GENE (Tokyo, Japan). For evaluations using CRM, GM maize powders 59122 (ERM-BF424c), MIR604 (ERM-BF423b; 0.1%, ERM-BF423c; 1% GMO), MON810 (ERM-BF413a; blank, ERM-BF413ek; 0.5% GMO), 1507 (ERM-BF418c), and 3272 (ERM-BF420b) were purchased from Sigma-Aldrich.

DNA Extraction. Non-GM maize kernels (50 g) were ground using a food processor. Non-GM or GM maize genomic DNA (gDNA) was extracted and purified from 1 g of corn powder using an anion-exchange resin Genomic-tip 100/G (QIAGEN, Hilden, Germany). The powder was completely resuspended with 7 mL of buffer G2, 100 μL of protease K (QIAGEN), and 5 μL of RNase A (100 mg/mL) (NIPPON GENE) at 50 °C for 60 min. The supernatant was loaded onto Genomic-tip equilibrated with 4 mL buffer QBT. The Genomic-tip was washed twice with 7.5 mL buffer QC. Maize gDNA was eluted with 3 mL pre-warmed (50 °C) buffer QF and purified by isopropanol precipitation. The obtained DNA pellet was rinsed with 70% ethanol, dried, and resuspended with ultrapure water. The quantity and quality of DNA were evaluated using a NanoDrop1000 UV spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Each sample was diluted to a concentration of 10 ng/μL in ultrapure water. MON863 standard samples were prepared by diluting GM maize gDNA with non-GM maize gDNA to concentrations of 0.2, 0.1, 0.05, 0.02, 0.01, 0.005, and 0.001%. MON863 standard samples are hereafter defined as MON863-RM.

Real-Time PCR. Singleplex real-time PCR assays were used to detect SSIIb and GM constructs (P35S and TNOS). Real-time PCR primers and probes for SSIIb, P35S, and TNOS (Table S1) were described in a previous study.[11] Oligonucleotide TaqMan probes SSIIb-Taq, P35S-Taq, and TNOS-Taq were labeled with 6-carboxyfluorescein (FAM) at the 5′ terminus and 6-carboxytetramethylrhodamine (TAMRA) at the 3′ terminus. All primers and fluorescent probes were synthesized and purified as NGS-grade reagents by Eurofins Genomics (Louisville, KY). Five microliters of a solution containing 50 ng of DNA was mixed with 12.5 μL of FastStart Universal Probe Master (ROX) (Roche Diagnostics, Basel, Switzerland); then, final concentrations of 0.8 μM for each primer and 0.1 μM probe were added to yield a final volume of 25 μL. The PCR protocol comprised 95 °C incubation for 10 min, followed by 45 cycles of 30 s at 95 °C and 1 min at 59 °C. An Applied Biosystems PRISM 7900 Sequence Detection System (7900HT; Thermo Fisher Scientific) and a LightCycler 480 Instrument II (LC480; Roche Diagnostics) were used. Data from the 7900HT instrument were analyzed using SDS 2.4 sequence detection software (Thermo Fisher Scientific). The baseline was set between cycles 3 and 15. The normalized reporter signal (ΔRn) threshold cycle (Cq) value was set to 0.2 during exponential amplification. Data from the LC480 instrument were analyzed using in-built software (Roche Diagnostics) and the second derivative maximum method.

Interlaboratory Study. The first study was organized at the National Institute of Health Sciences (NIHS) to evaluate the LOD among laboratories. Based on the guidelines for the single-laboratory validation of qualitative real-time PCR,[19] the LOD_{12} in each laboratory was defined as the lowest dilution at which 12 replicates were all GM-positive. Twenty-six laboratories, including four in the prestudy and 22 in the main study, participated in the first interlaboratory study. Maize gDNA extracts containing approximately 100 ng/μL non-GM maize and 10% MON863 together with PCR master mix, ultrapure water, primers, and probes were distributed to each lab and stored at −20 °C until use. The following drop-based spectrophotometers were used (Tables S2 and S4): NanoDrop1000 (ND-1000; Thermo Fisher Scientific), NanoDrop2000 (ND-2000; Thermo Fisher Scientific), NanoDrop One (ND-One; Thermo Fisher Scientific), and NanoPhotometer Pearl (Implen, Munich, Germany). Cuvette-based spectrophotometers included GeneQuant Pro (GE Healthcare, Chicago, IL), UV-1700 (Shimazu, Kyoto, Japan), UV-1600PC (Shimazu), UV-1800 (Shimazu), UV-2450 (Shimazu), BioSpec-mini (Shimazu), GeneSpecIII (Hitachi, Tokyo, Japan), U-3900/3900H (Hitachi), SpectraMax M2e (Molecular Devices, San Jose, CA), and Multiskan GO (Thermo Fisher Scientific). PCR instruments used in the study (Table S2 and S4) included LC480, LightCycler96 (LC96) (Roche Diagnostics), 7900HT, Applied Biosystems 7000 (ABI7000) (Thermo Fisher Scientific), Applied Biosystems 7500 (ABI7500) (Thermo Fisher Scientific), Applied Biosystems QuantStudio 12K Flex (QS12K) (Thermo Fisher Scientific), and Applied Biosystems StepOnePlus (Thermo Fisher Scientific). Participants measured the gDNA concentration of

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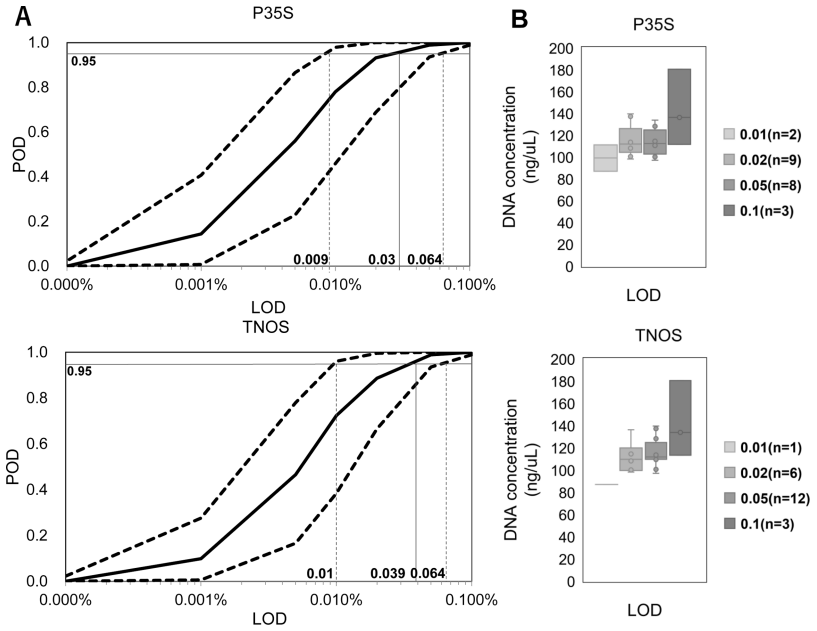

Figure 1. Evaluation of the limit of detection (LOD) in the first interlaboratory study. (A) Prediction interval for the LOD. The solid bold line indicates the mean probability of detection (POD), and bold dotted lines indicate the lower (5%) and upper (95%) limits of its prediction interval. The horizontal line indicates POD = 0.95. The solid vertical line indicates the mean number of copies of the target sequence having POD = 0.95 ($LOD_{95\%}$), and the vertical dotted ines indicate the lower (5%) and upper (95%) limits of the $LOD_{95\%}$ prediction interval. (B) Correlation between LOD and DNA concentration obtained in each laboratory. The lowest dilution level at which 12 replicates were all GM-positive (LOD_{12}) was evaluated at MON863-RM concentrations of 0.01, 0.02, 0.05, and 0.1%.

each sample, and a 10 ng/μL MON863 GM maize gDNA standard solution was prepared in ultrapure water and serially diluted with non-GM maize gDNA solution. For each dilution level, 12 replicate PCR measurements of P35S and TNOS and two replicate measurements of SSIIb were performed. Participants submitted the results to a spreadsheet, and the data were analyzed at the NIHS. The interlaboratory prediction interval of $LOD_{95\%}$ (the mean number of copies of the target sequence having a probability of detection of 0.95) was statistically calculated using the inverse β distribution, following an established protocol (Table 1).[20]

The second interlaboratory study was organized to validate the method developed at the NIHS. The number of laboratories was determined using a previously established method,[21] and 15 participated in this study. Maize gDNA of non-GM and MON863 (0.05, 0.2, and 0.5%), standard plasmids for SSIIb (1,000,000 copies/5 μL) and GM constructs P35S and TNOS (500 copies/5 μL), PCR master mix, ultrapure water, primers, and probes were distributed to each lab and stored at −20 °C until use. Participants performed real-time PCR using the following instruments (Table 2): QuantStudio 5 (QS5; Thermo Fisher Scientific), QS12K, 7900HT, ABI7500, LC480, and LC96. Six replicates of MON863 sample and three replicates of standard plasmid were measured. Participants submitted the results to a spreadsheet, and the data were analyzed at the NIHS using Cochran's Q test ($\alpha = 0.05$) to exclude outlier laboratories, as described elsewhere.[21] After the outlier (no. 13 laboratory in the second interlaboratory study) was excluded, the concordance, accordance, and concordance odds ratio (COR) were calculated from the data of 14 laboratories as previously described,[22] and cumulative distributions of ΔΔCq values were analyzed. A summary of the interlaboratory study data is shown in Table S12.

The ΔΔCq values of P35S and TNOS were calculated independently. The ΔCq and ΔΔCq values were calculated using the following formulas

$$\Delta Cq(\text{P35S or TNOS}) = Cq(\text{P35S or TNOS}) - Cq(\text{SSIIb})$$

$$\Delta\Delta Cq(\text{P35S}) = \Delta Cq(\text{P35S[analytical sample]}) - \Delta Cq(\text{P35S[control sample]})$$

$$\Delta\Delta Cq(\text{TNOS}) = \Delta Cq(\text{TNOS[analytical sample]}) - \Delta Cq(\text{TNOS[control sample]})$$

where the Cq values obtained from control sample wells were averaged and used for the calculation. Samples with $\Delta\Delta Cq \leq 0$ were GM-positive, while those with $\Delta\Delta Cq > 0$ were GM-negative.

Statistics. Differences between pairs of Cq samples were evaluated using Student's t-test (equal sample distributions) or Welch's t-test (unequal sample distributions).

RESULTS AND DISCUSSION

Evaluation of the Lowest Detectable GM Concentration among Laboratories. To develop a GM maize detection method suitable for the new Japanese labeling system, we evaluated the LOD of real-time PCR targeting SSIIb,[23] P35S,[24] and TNOS.[11] Generally, LOD is defined as the lowest concentration of an analyte detected consistently with good repeatability. For molecule testing including GM crops, LOD is most commonly expressed as copy number according to some guidelines or articles.[19,25−27] However, the regulatory threshold for GM crops in each country is generally expressed as a mass ratio.[7] Since the number of copies in DNA samples could theoretically be calculated from the mixing level, the gDNA quantity, and the genome size of the sample, we

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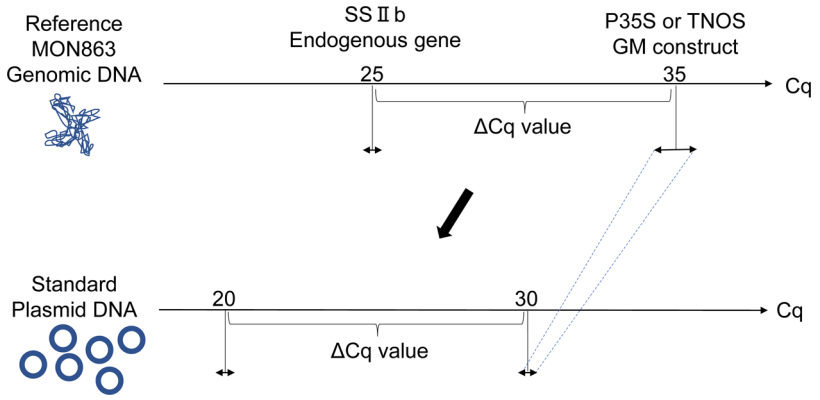

Figure 2. Benefit of using a large number of plasmid DNA for measuring threshold cycle (Cq) values relative to an endogenous control (ΔCq). To reduce measurement errors, a large number of standard plasmid DNA were substituted for reference MON863 gDNA. Two-way arrows indicate the magnitudes of the errors in Cq values.

decided to use and evaluate the mixing level of GM as the LOD in this study. For the GM model sample, GM maize MON863, which has a copy of P35S- and TNOS-containing transgene construct per gDNA, was used. For the negative control sample containing non-GM maize gDNA, real-time PCR was performed 21 times using P35S or TNOS primers and probes, but no specific amplification was detected (Figure S1). Prior to the interlaboratory study, we examined the practical concentration range of RM among four laboratories. Approximately 100 ng/μL gDNA of 10% MON863-RM and non-GM maize was distributed to each laboratory, and the steps from sample dilution to real-time PCR were performed to evaluate errors originating from differences in handling and instrumentation. Each laboratory diluted MON863-RM with non-GM gDNA samples and measured the DNA concentration using its own spectrophotometer (Tables S2 and S3). The DNA concentration measured using cuvette-based spectrophotometers was higher than that measured using drop-based spectrophotometers. As a result, the LOD_{12} values were 0.02−0.05 and 0.05−0.1% MON863-RM in laboratories using drop-based and cuvette-based spectrophotometers, respectively. No false-positive result was obtained in any laboratory (Table S2). In all 48 tests, P35S and TNOS sequences were detected for both 0.1 and 0.2% MON863-RM (Table S3).

On the basis of the above result, we determined the LOD of MON863-RM among 22 laboratories. As shown in Tables S4 and S5, these data were obtained using 16 different spectrophotometers and 7 different PCR instruments. For each detection method, 264 data for each GM dilution level and 132 data for non-GM samples were submitted from 22 laboratories. No specific amplification was detected for any non-GM gDNA samples, indicating that the false-positive rate was zero for both methods (Table S4).

The $LOD_{95\%}$ values were 0.030 and 0.039% MON863-RM using P35S and TNOS detection, respectively (Figure 1A). Based on the size of maize gDNA, mixing levels of 0.030 and 0.039% were expected to contain approximately 3 and 4 copies, respectively, of maize genome haploid per 50 ng of DNA sample,[28] which satisfies established guidelines.[19,25,26] Likewise, prediction intervals of the $LOD_{95\%}$ values were 0.009−0.064 and 0.010−0.064% MON863-RM using P35S and TNOS detection, respectively.

Notably, the value of LOD_{12} varied among laboratories because of differences in DNA input amounts depending on the measured DNA concentration (Figure 1B), which possibly resulted from the use of different types of spectrophotometers (Table S4). The relative standard deviation (SD) of DNA concentrations was 0.17−0.18, and the corresponding values were 0.04−0.05 and 0.22 for the drop-based and cuvette-based spectrophotometers, respectively (Table S6). However, an official Japanese method does not prescribe the use of a specific spectrophotometer. Thus, it is necessary to develop a robust GM detection method that is not affected by variations in DNA input amounts.

The variability of Cq values among laboratories was also problematic. The average SD of Cq values obtained from 0.1% MON863-RM using real-time PCR was 0.82 (max, 40.03; min, 36.23) for P35S and 0.65 (max, 38.19; min, 35.01) for TNOS (Table S4). Therefore, if a threshold level is expressed as a Cq value, the reported GM content and thus the "non-GM" determination of the same product may vary greatly among laboratories. These results suggest that a threshold expressed as a Cq value is not practical for robust detection of GM crops.

In-House Validation of the ΔΔCq-Based Real-Time PCR for Qualitative Assays. Since the LOD differed among laboratories because of variations in DNA input amounts, this error needed to be standardized. The ΔΔCq-based real-time PCR is convenient for comparing samples without a calibration curve[29] and can determine relative quantities of target DNA and internal control DNA by comparing ΔCq values.[11] Thus, we used the ΔΔCq-based real-time PCR to standardize errors due to differences in DNA input amounts. We also demonstrated that ΔCq had a lower variance than Cq for DNA concentrations in the 50−200 ng/μL range (Figure S2). In general, the ΔΔCq-based real-time PCR is only valid if the amplification efficiencies of the target taxon-specific assay and the GM-specific assay are similar.[30] Previously, we showed that the PCR methods used in this study have similar amplification efficiencies.[11] Generally, reliable detection of a control sample is crucial for the ΔΔCq-based real-time PCR. Our data indicated that 0.1% MON863-RM could be detected among all laboratories (Table S3 and S5); therefore, this concentration was used as a control sample. Next, the positive rate of the ΔΔCq-based real-time PCR was evaluated and found to be >95% for 0.2% MON863-RM using both P35S- and TNOS-based methods (Table S7). This indicated that the ΔΔCq-based real-time PCR could confirm the presence of at least 0.2% MON863-RM.

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Because the SD of Cq values near the LOD was high, a large number of plasmid DNA containing SSIIb, P35S, and TNOS were examined as a control sample, instead of MON863-RM (Figure 2). Our data indicated that plasmid DNA with more than 313 copies yielded a low SD using both P35S- and TNOS-based methods with the LC480 instrument (Figure S3 and Table S8). Thus, we decided to use 500 copies of GM maize standard plasmid DNA as a control sample. In this manner, 1,000,000 copies of SSIIb plasmid were found to yield a ΔCq value comparable with 0.1% MON863-RM using the LC480 instrument (*P* values: P35S, 0.73; TNOS; 0.83) (Figure S4 and Table S9). Therefore, to minimize the variance of the control sample, we used a large number of plasmids, namely, 1,000,000 copies of SSIIb and 500 copies each of P35S and TNOS.

Next, we evaluated positive rates using the ΔΔCq-based real-time PCR with these plasmids. Our data showed that at least 0.2% MON863-RM was required for a GM-positive result in all tests using the LC480 (Table 1). Like MON863-RM, equivalent results were obtained using the powder CRM (Table S10). Because 0.2% MON863-RM (50 ng) was estimated to be equivalent to 20 haploid genomes, the ΔΔCq-based real-time PCR satisfied the LOD criteria described in the guidelines.[19,25,26] Moreover, all 0.05% MON863-RM tested negative for GM using these plasmids as a control sample (Table 1), but some tested positive for using 0.1% MON863-RM as a control sample (Table S7), indicating that samples with lower concentrations would be tested more rigorously.

Interlaboratory Validation of the ΔΔCq-Based Real-Time PCR for Qualitative Assays. To validate the performance of the developed ΔΔCq-based real-time PCR in other locations, 15 laboratories assayed blind samples of DNA solutions containing four different concentrations of MON863-RM. Laboratory 13 was excluded using Cochran's *Q* test ($\alpha = 0.05$) because the *Q* value for the TNOS method was 32.3 > 23.7.[21] After exclusion, the *Q* values were 10.4 < 22.4 (P35S) and 17.4 < 22.4 (TNOS).[21] The designed test fulfilled McClure's equation,[21] namely, Lm^2 (L is the number of laboratories and m is the number of test samples per laboratory) = $14 \times 6^2 = 504 > 362$. No positive results were obtained from any non-GM sample in any laboratory, indicating that the false-positive rate was zero (Table 2). The 0.2% MON863-RM samples yielded GM-positive rates of 96 and 100% using the P35S- and TNOS-based methods, respectively (Table 2). The cumulative distribution of ΔΔCq values also indicated a positive rate of more than 96% for the 0.2% MON863-RM samples (Figure 3). The COR value at 0.2% MON863-RM was 0.97−1.00, indicating that the variability of GM-positive results was low. These findings suggest that our method can robustly determine a low-GM content.

Table 1. In-House Validation of ΔΔCq-Based Real-Time PCR Using LC480 with a Large Number of Plasmid DNA as a Control Sample

method	target	positive/total	positive rate (%)	mean ΔΔCq
P35S	1.0% MON863-RM	12/12	100	−2.98
	0.5% MON863-RM	12/12	100	−2.01
	0.2% MON863-RM	12/12	100	−0.79
	0.1% MON863-RM	4/12	33	0.27
	0.05% MON863-RM	0/12	0	2.11
	0.02% MON863-RM	0/12	0	3.38
TNOS	1.0% MON863-RM	12/12	100	−3.50
	0.5% MON863-RM	12/12	100	−2.52
	0.2% MON863-RM	12/12	100	−0.98
	0.1% MON863-RM	7/12	58	−0.03
	0.05% MON863-RM	0/12	0	1.27
	0.02% MON863-RM	0/12	0	2.84

There are two problems with using a CRM as a control to confirm that maize is GM-free. First, there are concerns about individual lots of CRMs having a sufficient supply for GM crop testing around the world because of the difficulty in CRM manufacture. Second, the Cq of 0.1% RM was too variable (SD = 0.22−0.95), despite being a control sample for the ΔΔCq-real-time PCR (Table S4). We think that plasmid DNA is a more suitable control sample than gDNA extracted from powdered CRM. Although the matrix matching error between gDNA and plasmid DNA remains an open issue,[31,32] a qualitative assay for allergen detection using standard plasmid DNA was recently developed to minimize the differences between real-time PCR runs and instruments.[33] In our developed method, plasmid DNA was also used as a control sample, yielding robust ΔCq values. However, plasmid DNA equal to 0.1% MON863 (50 ng gDNA) is estimated to contain approximately ten copies, and preparing and controlling the quality of a small number of plasmid DNA (<20 copies) are difficult. In addition, Cq values obtained using a small number of DNA are highly variable, resulting in low-precision analyses. In contrast, the ΔΔCq-based real-time PCR is expected to yield robust results, given that a range of ΔCq values for 0.1% MON863-RM is obtained even using a large number of plasmid DNA. Our data suggest that the ΔΔCq-based real-time PCR using a large number of plasmid DNA as a control sample yields a performance equivalent to the method using MON863-RM, despite the Cq values of a large number of plasmid DNA having lower SDs than those of MON863-RM (Table S9) in 13 (P35S) and 14 (TNOS) out of 14 laboratories (Table S11). Therefore, using a large number of plasmid DNA as a control sample for the ΔΔCq-based real-time PCR would overcome the two difficulties mentioned above.

Tests of GM Maize CRMs. GM maize CRMs were used to assess the practicability of our method. The data from two replicate measurements of extracted gDNA are shown in Table 3. The ΔΔCq values of MON810-blank gDNA samples for P35S were found to be positive numbers. Consistent with the results using MON863-RM (Table 1), the ΔΔCq values of 1% 59122, 0.1% and 1% MIR604, 0.5% MON810, 1% 1507, and 1% 3272 were negative, indicating that these samples contained GM crops. The CRM containing two or more copies of the same GM target in haploid gDNA such as MIR604 (Table 3) could be detected in even 0.1% powdered samples. These results suggest that our method can determine the presence of low-content GM in actual crop samples.

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Table 2. Interlaboratory Validation of ΔΔCq-Based Real-Time PCR

		P35S test				TNOS test			
lab. no.	PCR instrument	0%	0.05%	0.20%	0.50%	0%	0.05%	0.20%	0.50%
1	QuantStudio 5	0/6	0/6	6/6	6/6	0/6	1/6	6/6	6/6
2	QuantStudio 12K	0/6	0/6	6/6	6/6	0/6	0/6	6/6	6/6
3	LC480	0/6	0/6	5/6	6/6	0/6	1/6	6/6	6/6
4	LC480	0/6	0/6	5/6	6/6	0/6	0/6	6/6	6/6
5	LC96	0/6	0/6	6/6	6/6	0/6	0/6	6/6	6/6
6	LC480	0/6	0/6	6/6	6/6	0/6	0/6	6/6	6/6
7	7900HT	0/6	0/6	6/6	6/6	0/6	1/6	6/6	6/6
8	ABI7500	0/6	0/6	6/6	6/6	0/6	3/6	6/6	6/6
9	QuantStudio 5	0/6	0/6	6/6	6/6	0/6	1/6	6/6	6/6
10	QuantStudio 12K	0/6	0/6	6/6	6/6	0/6	0/6	6/6	6/6
11	QuantStudio 5	0/6	1/6	6/6	6/6	0/6	2/6	6/6	6/6
12	ABI7500	0/6	0/6	6/6	6/6	0/6	0/6	6/6	6/6
14	QuantStudio 5	0/6	0/6	6/6	6/6	0/6	2/6	6/6	6/6
15	ABI7500	0/6	0/6	5/6	6/6	0/6	0/6	6/6	6/6
total		0/84	1/84	81/84	84/84	0/84	11/84	84/84	84/84
positive rate		0.00	0.01	0.96	1.00	0.00	0.13	1.00	1.00
accordance (%)		100	97.6	92.9	100	100	76.0	100	100
concordance (%)		100	97.6	93.0	100	100	73.3	100	100
COR		1.00	1.00	0.97	1.00	1.00	1.10	1.00	1.00

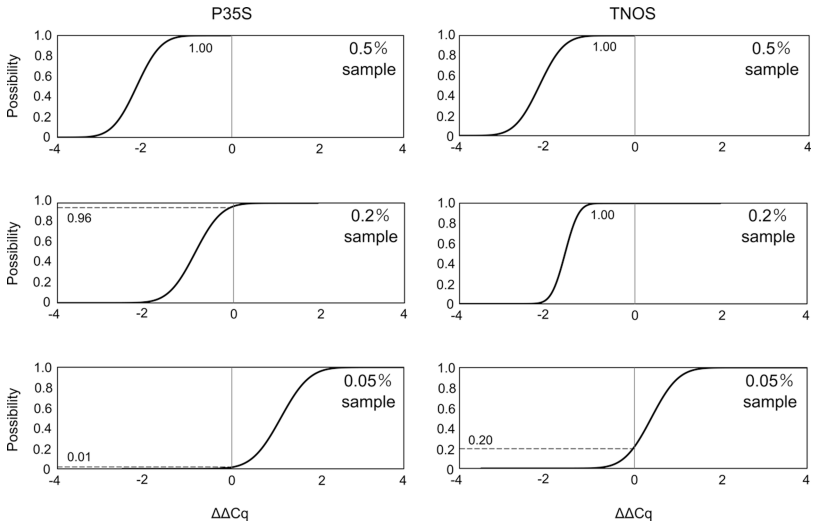

Figure 3. Probability distribution of the difference between ΔCq values of analytical samples and control samples (ΔΔCq) in the interlaboratory study. Horizontal dotted lines indicate the value of the cumulative distribution function at ΔΔCq = 0.

Because GM maize is not commercially cultivated in Japan, domestic maize should be non-GM. However, the screening method targets sequences from viruses or soil bacteria and can therefore result in false positives if these exogenous DNA molecules have adhered to the surface. Therefore, it is important to estimate the risk of false-positive results. Although MON810-blank CRM was confirmed to be GM-negative in our investigation (Table 3), indicating that the false-positive rate is low, targeting elements originating from natural organisms requires scrutiny. Positive results using P35S- or TNOS-based methods do not necessarily imply the presence of GM crop-derived DNA.[34,35] For crops with soils, kernels should be carefully washed in advance. For genuine non-GM samples (e.g., maize produced in Japan), false positives remain a possibility. Thus, positive results near ΔΔCq = 0 need to be interpreted with caution.

The final determination that a sample contains GM crops is based on single- or double-positive results using P35S- and TNOS-based methods. Authorized GM maize that includes a copy of either the P35S or TNOS sequence, such as MON810 or MIR162, is available in Japan (Figure S5). Assuming that kernels containing 0.1% MON810 and 0.1% MIR162 are included in a lot, the developed method may determine that the lot is GM-negative even if it contains 0.2% GM content (Figure S5B). Thus, a sample deemed to be at the 0.2% GM-positive threshold might have up to 0.4% GM content if the sample contains two single events, one including only a copy of the P35S sequence and the other including only a copy of the TNOS sequence (Figure S5C). In addition, many stacked events in which multiple GM constructs have been developed have been distributed in Japan (Figure S5D).[6,36,37] In such cases, our method could monitor the GM content of samples more strictly than ever before. Like GM maize, a new detection method for detecting GM soybean has been developed in Japan.[38]

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Table 3. Tests of GM Maize CRM

CRM code	event name	conc. (%)	extract	copy number in the genome		ΔΔCq value			
				P35S	TNOS	P35S method		TNOS method	
ERM-	MON810-blank	≤0.02	1	1		4.37	1.99	ND	ND
BF413a			2	1		2.63	ND	ND	ND
ERM-	MON810	0.5	1	1		−1.86	−2.22	ND	ND
BF413ek			2	1		−1.24	−0.81	ND	ND
ERM-	59122	1	1	1		−2.61	−2.62	ND	4.46
BF424c			2	1		−1.62	−1.38	ND	ND
ERM-	1507	1	1	1		−3.52	−2.48	ND	ND
BF418c			2	1		−4.25	−4.48	2.29	ND
ERM-	3272	1	1		1	ND	3.97	−2.37	−2.58
BF420b			2		1	ND	ND	−2.78	−3.28
ERM-	MIR604	0.1	1		2	ND	ND	−0.53	−1.13
BF423b			2		2	ND	ND	−1.78	−1.13
ERM-	MIR604	1	1		2	ND	ND	−4.12	−3.75
BF423c			2		2	ND	ND	−3.82	−3.70

In this study, a ΔΔCq-based real-time PCR method for robust detection of low-content DNA using a large number of standard plasmids as a control sample was developed and applied to GM maize detection. Our data indicated that the method reduced the variability due to DNA input amounts and a small number of plasmids and is therefore useful for detecting low-content GM maize. On the basis of our results, we determined a practical threshold for regulatory purposes. If the practical criteria are defined, our concept could readily be applied to other fields, such as clinical diagnostics. Robust detection based on validated criteria among laboratories is crucial for regulatory testing.

CONCLUSIONS

To solve the problem concerning the robustness of low-content DNA detection methods, we developed a new ΔΔCq-based real-time PCR using low-content GM maize as a model. In particular, the LOD among laboratories, taking into account DNA preparation and real-time PCR, was evaluated, and practical criteria for defining a regulatory threshold were determined. The ΔΔCq-based real-time PCR using a large number of plasmid DNA as a control sample was developed, and in-house and interlaboratory studies validated universal testing criteria. This new approach would offer a sustainable supply of RM to laboratories to ensure robust detection. Thus, our method would meet the performance requirements of the new labeling system in Japan and enable monitoring of GM-labeled maize more strictly than ever before. Moreover, the screening method could be used worldwide, enabling the reduction of relatively high regulatory thresholds (e.g., 5% in the United States) to more stringent levels (e.g., 0.9% in the European Union and Russia) by adjusting the plasmid range between an internal control and a target sequence.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.2c03680.

Further details of real-time PCR methods, primer sequences, DNA concentration quantification, interlaboratory studies, and in-house validation (PDF)

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Author Contributions

K.S., K.N., K.K., N.S., and K.K. conceived and conceptualized the study. K.S. and T.E. acquired and analyzed data. K.N., K.K.,

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R.T., N.S., and K.K. supervised the study. J.N., S.Y., M.K., and J.M. provided critical advice. The manuscript was drafted by K.S. and N.S. and revised through the contribution of all authors. All authors approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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