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Applicability of Plasmid Calibrant pTC1507 in Quantification of TC1507 Maize: An Interlaboratory Study

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ABSTRACT: To enforce the labeling regulations of genetically modified organisms (GMOs), the application of DNA plasmids as calibrants is becoming essential for the practical quantification of GMOs. This study reports the construction of plasmid pTC1507 for a quantification assay of genetically modified (GM) maize TC1507 and the collaborative ring trial in international validation of its applicability as a plasmid calibrant. pTC1507 includes one event-specific sequence of TC1507 maize and one unique sequence of maize endogenous gene *zSSIIb*. A total of eight GMO detection laboratories worldwide were invited to join the validation process, and test results were returned from all eight participants. Statistical analysis of the returned results showed that real-time PCR assays using pTC1507 as calibrant in both GM event-specific and endogenous gene quantifications had high PCR efficiency (ranging from 0.80 to 1.15) and good linearity (ranging from 0.9921 to 0.9998). In a quantification assay of five blind samples, the bias between the test values and true values ranged from 2.6 to 24.9%. All results indicated that the developed pTC1507 plasmid is applicable for the quantitative analysis of TC1507 maize and can be used as a suitable substitute for dried powder certified reference materials (CRMs).

KEYWORDS: *genetically modified maize, collaborative ring trial, plasmid calibrant, pTC1507*

INTRODUCTION

With the rapid development and application of modern agricultural biotechnologies, more than 150 genetically modified organisms (GMOs) have been developed and commercialized worldwide on 148 million hectares of land, including 46.8 million hectares of genetically modified (GM) maize by the end of 2010.[1] To protect consumers, labeling regulations for GMO products have been issued and executed in more than 50 countries, and much attention has been paid to the development of flexible, accurate, and cheap analysis methods and certified reference materials (CRMs).

For GMO identification and quantification, DNA-based PCR analysis is still the most widely used approach today, even when many new techniques based on the analysis of proteins or metabolites have been developed.[2−4] Real-time PCR has been the key technique in determining GM amounts for the execution of GMO labeling regulations. Four different PCR strategies based on different target DNA sequences have been developed for GMO detection. Among them, event-specific PCR, which targets the unique junction sequence between the host genomic DNA and the inserted DNA, has been widely used for GMO identification and quantification due to its high specificity.[5] To date, several event-specific PCR assays have been developed and applied in practical quantification of GM soybean and maize.[6−8]

As the core for the GMO detection and quantity traceability system, CRMs are very important in GMO quantification. Today, the Institute for Reference Materials and Measurements (IRMM) and the American Oil Chemists' Society (AOCS) are the two major developers of CRMs for GMO detection. Many CRMs for different GM events have been developed and commercialized by IRMM and AOCS, including more than 30 dried powder CRMs, 10 genomic DNA CRMs, and 3 plasmid CRMs.[9−13] However, there are still many GMO events that are lacking corresponding CRMs. There are two types of CRMs for GMO analysis: dried powder CRMs and plasmid calibrants. The dried powder CRM was developed by mixing GM and non-GM seed powders in fixed ratios of mass/mass. The plasmid calibrants are recombinant plasmids containing event-specific sequence and endogenous reference gene sequence with a ratio of 1:1 (copy/copy). The dried powder CRMs were used in earlier days for GM amounts and quantity traceability analysis due to their similar properties with blind samples and easy traceability to the International System of Units (SI) of gram. However, there are some limitations with the dried powder CRMs for GMO analysis, such as limited quantification range, inconvenient preparation procedures, difficulty to get homogeneous candidate samples, and high cost. To solve these problems, plasmid calibrants have been used as CRMs since 1991 and have gained popularity. Plasmids as calibrants in GMO quantification have been demonstrated to be a good alternative to genomic DNAs extracted from conventional CRMs.[14,15] Burns et al. compared plasmid DNA with genomic

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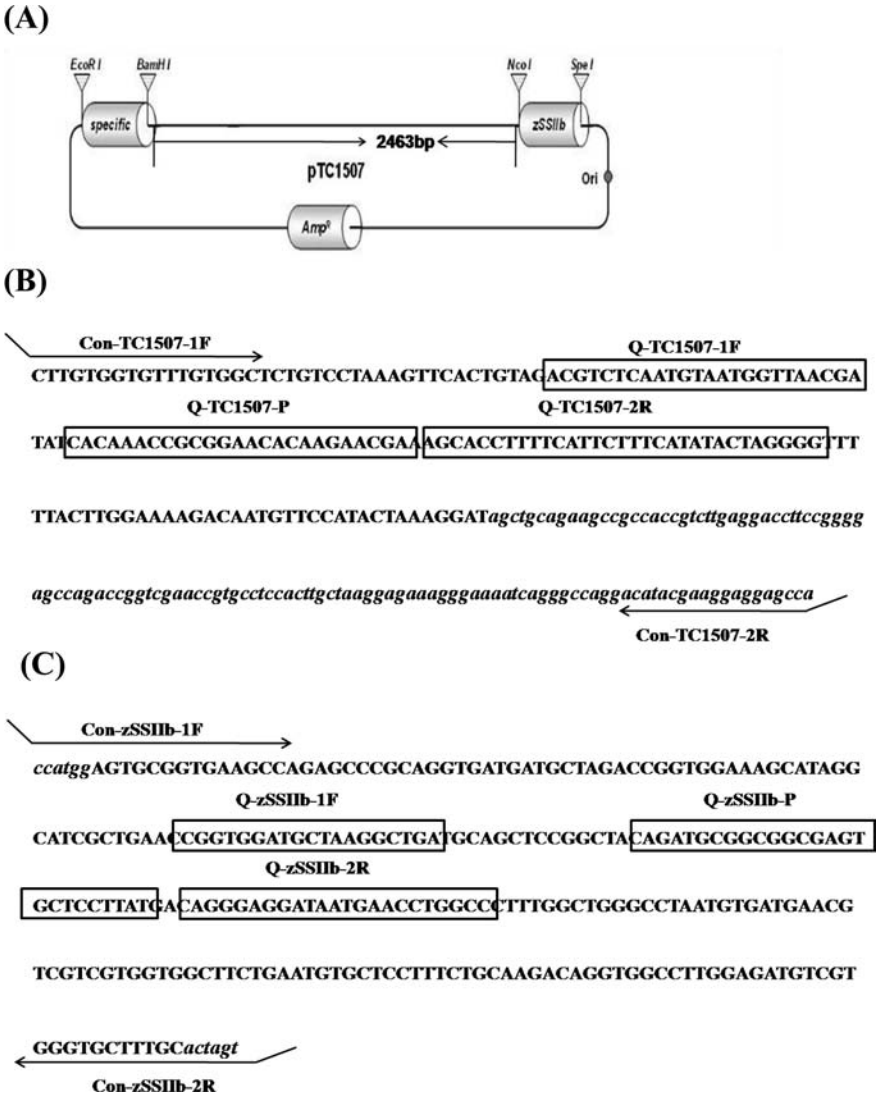

Figure 1. Structure and integration sequences of pTC1507. (A) Schematic diagram of pTC1507 (5950 bp), Amp^R, ampicillin-resistant gene; an event-specific sequence of the 3′ flanking region of TC1507 maize; partial sequence of maize endogenous reference gene; *Eco*RI, *Bam*HI, *Nco*I, and *Spe*I indicate the corresponding restriction endonuclease sites. (B) Nucleotide sequences of the 3′ flanking sequence of TC1507 maize. (C) Partial sequence of maize endogenous reference gene. The black arrows indicate the primer pairs used for the construction of pTC1507. The rectangular boxes indicate the location and direction of primer pairs and TaqMan probes used in real-time PCR assays of TC1507 event and endogenous reference gene.

DNA as calibrants for quantification of GM ingredients, and their results showed that the standard curve made with plasmid calibrant gave a better determination of the true GM percentage in blind samples.[16] Furthermore, plasmid calibrant has several advantages over dried powder CRMs, such as easier production, lower cost, and higher stability. Although plasmid calibrants have some contamination problems during experimental procedures, the problems can be easily solved if certain precaution measures are taken. Because of their convenience and low cost, plasmid calibrants have been considered a good substitute for CRMs using raw plant materials. To date, more than 20 plasmid calibrants have been successfully constructed and used for the detection and quantification of several GM maize, soybean, and cotton events.[17−19] Two plasmid calibrants have been validated for the detection of Roundup Ready soybean event GTS-40-3-2 by interlaboratory ring trials.[20,21] Three other plasmid calibrants, ERM-AD413, ERM-AD415, and ERM-AD427, have also been certified and commercialized worldwide by IRMM.[11−13]

International collaborative validation is the prerequisite step for proposing a CRM for a GMO. In this study, we developed a plasmid calibrant, pTC1507, for GM maize event TC1507 and organized an interlaboratory ring trial to validate the applicability and feasibility of pTC1507 as a plasmid calibrant for the quantification assay of TC1507 maize.

MATERIALS AND METHODS

Materials and DNA Extraction. Seeds of TC1507 maize were provided by Monsanto Co. Wild-type maize was purchased from local markets in Shanghai, China. Maize genomic DNAs were extracted and purified from ground powders using a Plant DNA Mini-Prep Kit (Ruifeng Agro-tech Co. Ltd., Shanghai, China) according to the manufacturer's instructions. Plasmid DNAs were extracted and purified using a Plasmid Mini Extraction Kit (Axygen Scientific Inc.).

DNA concentrations were determined by measuring UV absorption at 260 nm using an ND-1000 spectrophotometer (NanoDrop

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Table 1. Primers and Probes for Real-Time PCR Analysis

target	primer name	sequence (5′−3′)	amplicon size (bp)
TC1507	Con-TC1507-1F	CCCAAGCTTCTTGTGGTGTTTGTGGCTCT	306
	Con-TC1507-2R	AAAGATATCTGGCTCCTCCTTCGTATGT	
	Q-TC1507-1F	GACGTCTCAATGTAATGGTTAACGA	83
	Q-TC1507-2R	CCTAGTATATGAAAGAATGAAAAGGTGCTT	
	Q-TC1507-P	FAM-TCACAAACCGCGGAACACAAGAACG-TAMRA	
zSSIIb	Q-zSSIIb-1F	CGGTGGATGCTAAGGCTGATG	88
	Q-zSSIIb-2R	AAAGGGCCAGGTTCATTATCCTC	
	Q-zSSIIb-P	HEX-TAAGGAGCACTCGCCGCCGCATCTG-TAMRA	

Technologies Inc., Rockland, DE). The purity of the extracted DNA was evaluated on the basis of the ratio of absorbance at 260 and 280 nm (accepted ratios are between 1.7 and 2.0). The copy number of plasmid DNA was calculated on the basis of the DNA quantity and the size of genomic DNA. For conversion factor (Cf) evaluation, three different concentrations of genomic DNAs from 100% positive TC1507 maize (2.0, 1.0, and 0.5 ng/μL) were used. A total of five blind DNA samples containing different GM contents (0.05, 0.1, 0.5, 1.0, and 5.0%) were prepared by mixing the purified TC1507 maize DNAs with non-GM maize DNAs, and each blind DNA sample was supplemented with the concentration of 10 ng/μL. Salmon sperm DNA (10 ng/μL) was used as negative DNA control.

Oligonucleotides Primers and Probes. Primers and TaqMan probes were designed using Oligo software (version 6.31, Molecular Biology Insights). To construct plasmid pTC1507, primer pair Con-TC1507-1F/2R was used. Primer pair Q-TC1507-1F/2R and probe Q-TC1507-P were used for 3′ event-specific quantification of TC1507 maize. For detection of maize endogenous reference gene *zSSIIb*, primer pair Q-zSSIIb-1F/2R and probe Q-zSSIIb-P were used. The 5′ end of the probe Q-zSSIIb-P was labeled with 5-hexachlorofluorescein (HEX) and the 5′ end of the probe Q-TC1507-P for event-specific detection of TC1507 maize was labeled with 6-carboxyfluorescein (FAM) as the fluorescent reporters. The primers and probes are shown in Figure 1. All of the oligonucleotide primers and TaqMan probes used in this study were synthesized by TaKaRa Co. Ltd. (Dalian, China) and are listed in Table 1.

Construction of pTC1507. Plasmid pTC1507 was constructed from plasmid pMaize. Plasmid pMaize was constructed to facilitate the construction of a series of plasmid CRMs suitable for different GM maize events in previous studies.[17] In pMaize, the partial sequence of maize endogenous reference gene *zSSIIb* and a 2463 bp DNA fragment were inserted into cloning vector pBSK in tandem. The 3′ event-specific sequence (306 bp in length) of GM maize TC1507 was inserted into pMaize by *Eco*RI and *Bam*HI restriction endonuclease digestion (Figure 1). Thus, the 2463 bp fragment between the event-specific sequence and endogenous gene *zSSIIb* could be seen as a physical barrier to avoid unspecific amplifications during duplex analysis.[19]

For the construction of plasmid pTC1507, PCR reactions were performed in a 50 μL volume containing 1× PCR buffer, 0.2 mM dNTP, 2.5 mM $MgSO_4$, 0.3 μM of each primer, 1.5 units of KOD-Plus DNA Polymerase (Toyobo Co., Japan), and 2 μL of 20 ng TC1507 maize genomic DNA as PCR templates. All PCR amplifications were carried out using a PTC-100 Peltier Thermal Cycler (MJ Research, Waltham, MA) with the following program: predenaturation at 95 °C for 8 min, 35 cycles of 30 s at 95 °C, 30 s at 58 °C, and 50 s at 72 °C. Final extension was at 72 °C for 8 min.

Real-Time PCR. All real-time PCR reactions were performed in a 25 μL volume consisting of 1× PCR buffer, 0.4 mM dNTPs, 1.5 Uunits of *Taq* DNA polymerase, 6 mM $MgCl_2$, optimized concentrations of oligonucleotides/probe (0.4 μM of each primer and 0.16 μM probe), and 5 μL of template DNA. The PCR program was as follows: 95 °C for 10 min, 50 cycles of 95 °C for 15 s, and 60 °C for 60 s. Fluorescent signals were monitored during each PCR cycle at the annealing and extension step (60 °C). Participants of the interlaboratory validation carried out PCR amplifications using various thermal cyclers such as Rotor Gene 3000A (Corbett Research), Prism ABI 7300, and 7500 sequence detection system (Applied Biosystems). Each reaction was repeated three times, and each time with three parallels.

Collaborative Ring Trial. The collaborative trial was organized by the GMO Detection Laboratory, Shanghai Jiao Tong University (GMDL-SJTU), in accordance with internationally accepted guidelines.[20,22] A total of eight GMO detection laboratories were invited to participate in this trial. Each participant received one tube of plasmid calibrant pTC1507 (10^7 copies/μL, 40 μL per tube), three tubes of genomic DNA from TC1507 maize seeds (coded C1−C3 at concentrations of 2, 1, and 0.5 ng/μL, 120 μL per tube), five blind DNA samples (coded X1−X5 at 10 ng/μL, 120 μL per tube), and one tube of negative DNA control consisting of salmon sperm DNA (10 ng/μL, 140 μL per tube). Furthermore, all participants were provided with ready-to-use real-time PCR reaction master mix (1 mL × 4) and DNA dilute buffer (10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1.2 mL × 2). All DNA samples and reagents were shipped on dry ice (DHL International GmbH, Shanghai, China). One hard copy of ring trial introduction, protocols, and data report forms was also provided.

In this collaborative ring trial, three groups of quantitative analyses were performed. First, the pTC1507 DNA samples were used for the construction of standard curves using template DNA quantities as abscissae and relative Ct values as ordinates. Participants were requested to dilute the provided pTC1507 plasmid DNA to concentrations of 10^6, 10^5, 10^4, 10^3, 10^2, and 10 copies/μL using the supplied DNA dilution buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Salmon sperm DNA was used as negative template control (NTC).

Second, the conversion factor (Cf) of pTC1507 was tested using the C1−C3 DNA samples (0.5, 1.0, and 2.0 ng/μL). The copy numbers of TC1507 maize genomic DNA were calculated using the calibration curves constructed in the first step. The Cf values were then calculated using the following formula:

$$\text{Cf} = (\text{copy number of TC1507sequence})/(\text{copy number of endogenous gene zSSIIb sequence}) \quad (1)$$

Finally, five blind DNA samples with different GM contents were quantified using the constructed standard curves and tested Cf values of pTC1507. The GM amounts (percent) were calculated by using the following formula:

$$\text{GM amount (\%)} = (\text{copy number of TC1507 sequence} \times 100)/(\text{copy number of endogenous zSSIIb sequence} \times \text{Cf}) \quad (2)$$

Statistical Analysis. After all participants sent back their validation reports, several critical parameters were analyzed statistically, including PCR efficiency, linearity of standard curve, Cf value, and three ISO validation measures (the average quantified bias of blind samples (*B* %), relative standard deviation of repeatability (RSD^R), and relative

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Table 2. Efficiency and Linearity (R^2) of Each Standard Curve from Eight Laboratories

	TC1507						*zSSIIb*					
lab code	efficiency	SD	RSD (%)	R^2	SD	RSD (%)	efficiency	SD	RSD (%)	R^2	SD	RSD (%)
1	0.87	0.11	11.72	0.9966	0.0015	0.15	0.87	0.10	10.55	0.9953	0.0031	0.31
2	0.80			0.9994			0.80			0.9989		
3	0.85			0.9993			0.85			0.9993		
4	1.15			0.9981			1.10			0.9965		
5	0.99			0.9961			1.03			0.9959		
6	0.98			0.9973			1.01			0.9965		
7	1.08			0.9959			1.03			0.9921		
8	0.99			0.9998			0.99			0.9996		

standard deviation of reproducibility (RSD^r). Two-factor ANOVA analysis of two experimental factors (laboratory and GM amounts) was also applied to analyze the variance of the test results of blind samples in all eight laboratories.

RESULTS AND DISCUSSION

Construction of pTC1507. In this study, a dual-target plasmid suitable for quantitative analysis of TC1507 maize was constructed that includes a 3′ event-specific sequence of TC1507 maize, a nonspecific 2463 bp DNA fragment, and a partial sequence of maize endogenous reference gene *zSSIIb*. The length of the constructed pTC1507 was 5950 bp (Figure 1). Purified pTC1507 plasmid DNA was diluted to 10^7 copies/μL, which equaled 6.22 ng/μL on the basis of the size of the plasmid and the mean DNA quantity (1 pg = 965 Mb).[23]

After the construction of pTC1507, real-time PCR assays of both TC1507 event-specific sequence and endogenous *zSSIIb* gene using pTC1507 as a calibrant were evaluated through in-house validation. For determination of the limit of detection (LOD) and limit of quantification (LOQ) of these two real-time PCR assays, five diluted pTC1507 DNA solutions with lower concentrations of 100, 50, 25, 10, and 5 copies were prepared and amplified. Each reaction was performed with three replicates and repeated by three operators. The test results showed that all of the reactions with 5 copies of pTC1507 DNA could be detected in all three parallels and repeats in both TC1507 event-specific and *zSSIIb* assays. The LOD of these two assays were 5 initial copies of pTC1507. However, slightly high bias values (>25%) were observed in the reactions with 5 copies of pTC1507 DNA as template. Therefore, we concluded that the LOQs of these two assays were 10 initial copies of pTC1507. These results suggested that two event-specific quantitative systems employing pTC1507 as calibrant have acceptable and high sensitivity. Furthermore, the test results of standard curves showed that the real-time PCR assays employing plasmid pTC1507 had high PCR efficiency, good linearity, good repeatability, good reproducibility, and acceptable dynamic range of quantification (data not shown).

Interlaboratory Validation of pTC1507 as a Plasmid Calibrant in TC1507 Maize Quantification. Before the deadline of 3 months after receiving the validation package, all eight participants sent back the validated result reports with the data of Ct values, PCR efficiency, linear correlation (R^2), and the formula of standard curves. All of these data without elimination were used for further statistical analysis on the basis of Cochran's test.

Efficiency and Linearity. In the TC1507 assay, the returned data showed that the PCR efficiency among eight different laboratories ranged from 0.80 to 1.15 with a standard deviation (SD) of 0.11 and a relative standard deviation (RSD) of 11.72% (Table 2). The average R^2 ranged from 0.9959 to 0.9998 with a SD of 0.0015 and a RSD of 0.15% (Table 2). For the endogenous reference gene *zSSIIb* assay, the returned data showed that the PCR efficiency ranged from 0.80 to 1.10 with a SD of 0.10 and a RSD of 10.55%. The average R^2 ranged from 0.9921 to 0.9996 with a SD of 0.0031 and a RSD of 0.31%. All returned data indicated that pTC1507 had high PCR efficiency, acceptable linear correlation, and acceptable bias that were within the limits proposed by the European Network of GMO Laboratories (ENGL) guidelines.[24] Thus, pTC1507 is an acceptable plasmid calibrant for quantitative measurements of TC1507 maize.

Repeatability and Reproducibility. Repeatability and reproducibility of both event-specific and endogenous gene real-time PCR assays using pTC1507 as calibrants were determined. The SD^r and RSD^r were calculated from three tests; each test had three parallel PCR reactions. For the TC1507 event-specific assay, real-time PCR SD^r ranged from 0.068 to 0.338, SD^R ranged from 0.106 to 0.256, RSD^r ranged from 0.25 to 0.98%, and RSD^R ranged from 0.35 to 0.94%. For the zSSIIb endogenous gene assay, SD^r ranged from 0.070 to 0.564, SD^R ranged from 0.014 to 0.191, RSD^r ranged from 0.28 to 1.54%, and RSD^R ranged from 0.08 to 0.75%. Both SD and RSD values were in the acceptable range according to relative criteria,[24] demonstrating that the real-time PCR assay was stable and reliable in maize genomic DNA quantification.

Measurement of Conversion Factors. To minimize the amplification rate difference between plasmid DNA and plant genomic DNA, Cf was used for GM amount (percent) calculation of blind samples. Cf was calculated as the ratio of the copy number of event-specific targets to the copy number of the endogenous gene.[21] The differences of quantification results among all eight participating laboratories were statistically analyzed. The mean Cf values from the eight laboratories ranged from 0.38 to1.37 with SD ranging from 0.04 to 0.262 and RSD rangingfrom 7.39 to 23.09% (Table 3). The calculated Cf values from participating laboratories were around 0.5 except for those for two laboratories that had values of 0.38 and 1.37. The slightly large discrepancy in those two laboratories might be generated from different PCR efficiencies and fluorescent threshold settings. Although the Cf values were different among the eight laboratories, the final quantification results of blind samples were all close to the true values, indicating that the Cf values from plasmid calibrants were not always stable and should be analyzed further in blind sample analysis.

Blind Sample Quantification. On the basis of the quantification results returned from the eight participating laboratories, the mean value of the GM contents of the five blind samples were 5.569, 1.275, 0.513, 0.123, and 0.058%

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Table 3. Conversion Factor (Cf) Values from Eight Laboratories in the Interlaboratory Study

lab code	Cf value C1	C2	C3	mean Cf	SD[a]	RSD[b]
1	1.34	1.65	1.13	1.37	0.262	19.05
2	0.58	0.57	0.50	0.55	0.044	7.93
3	0.81	0.67	0.74	0.74	0.07	9.46
4	0.46	0.39	0.30	0.38	0.08	20.92
5	0.62	0.74	0.75	0.70	0.07	10.29
6	0.39	0.55	0.49	0.48	0.08	16.96
7	0.85	0.53	0.70	0.69	0.16	23.09
8	0.57	0.57	0.50	0.55	0.04	7.39

[a]Standard deviation. [b]Relative standard deviation. Values were calculated by dividing the standard deviation by mean value and are given in %.

(Table 4). The deviations of the quantification results of the eight laboratories for each GM level tested are shown in Figure 2. The RSD of the quantification results of all five GM contents were within the acceptable range of the ENGL except that two laboratories overestimated the sample of 0.05% GM level with the bias over 40%.[24,25]

The repeatability and reproducibility of quantification results among eight laboratories were also evaluated and are listed in Table 5. These values were evaluated with respect to the method acceptance criteria and method performance requirements, which were established by ENGL and adopted by CRL (RSD^r <25% and RSD^R <35%). The average bias of the quantification results among eight laboratories ranged from 2.60 to 24.9%. In the quantification assay of blind samples using pTC1507 as calibrant, the RSD^r ranged from 8.08 to 17.24%, and RSD^R ranged from 13.11 to 34.48%. The resulting RSD^R of low GM amount blind samples were slightly higher, whereas the RSD^r and RSD^R of high GM amounts are all acceptable. All results indicated that the quantification results of blind samples were stable and reliable using the measures designed by us.

Two-Factor ANOVA Analysis. From the quantification results discussed above, some deviations exist, so further validation of the results is necessary. Therefore, the conventional parametric approach was applied in the fully noncrossed two-factor ANOVA. Table 6 shows the breakdown of the total variance into contributing components to perform statistical inferences for two experimental factors (laboratory and GM amounts). The results showed that there are no significant effects on the quantification results under different laboratory conditions and with different GM amounts using pTC1507 DNA as calibrant.

In conclusion, the results obtained from the interlaboratory validation demonstrated that real-time PCR assays employing pTC1507 as plasmid calibrant had high PCR efficiency, good repeatability and reproducibility, and credible accuracy in blind sample quantification, indicating that pTC1507 is suitable for use as a substitute CRM for dried powder CRMs in the quantification of TC1507 maize or its derivates.

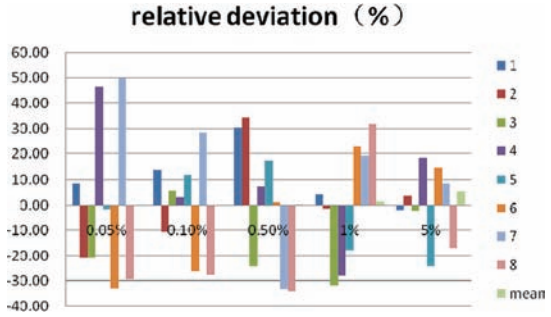

Figure 2. Relative deviation (percent) of the quantification results of five blind samples in the interlaboratory study.

Table 5. Statistical Analysis of the Quantification Results of Blind Sample Detection from Eight Laboratories

unknown samples GM%	blind sample X1	X2	X3	X4	X5
labs returning results	8	8	8	8	8
sample per lab	1	1	1	1	1
total data number	9	9	9	9	9
data excluded	0	0	0	0	0
reason for exclusion					
mean value (%)	0.058	0.123	0.513	1.275	5.569
true value (%)	0.05	0.10	0.50	1.00	5.00
repeatability SD	0.01	0.02	0.07	0.17	0.45
repeatability RSD (%)	17.24	16.26	13.65	13.33	8.08
reproducibility SD	0.02	0.02	0.12	0.25	0.73
reproducibility RSD (%)	34.48	16.26	23.39	19.61	13.11
bias (absolute value)	0.008	0.023	0.013	0.275	0.569
bias (%)	16.00	23.00	2.60	24.9	11.38

Table 6. Evaluation of the Variation from Laboratory and GM Amounts in Interlaboratory Study Using Two-Factor ANOVA Analysis

source of variation	sum of squares	degrees of freedom	mean squares	F	$p(F)$
total	1.39	39			
lab (L)	0.33	7	0.05	1.41	3.36
GM amount (A)	0.12	4	0.03	0.87	4.07
error	0.94	28	0.03		

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Table 4. Quantification Results of Blind Samples from Eight Laboratories

true value (%)	lab 1	2	3	4	5	6	7	8	mean (%)
0.05	0.063	0.046	0.046	0.085	0.057	0.039	0.087	0.041	0.058
0.1	0.14	0.11	0.13	0.127	0.138	0.091	0.158	0.089	0.123
0.5	0.67	0.69	0.39	0.55	0.603	0.519	0.343	0.339	0.513
1.0	1.33	1.26	0.87	0.92	1.05	1.57	1.52	1.678	1.275
5.0	5.46	5.77	5.44	6.61	4.23	6.39	6.04	4.61	5.569

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Author Contributions

[‖]These two authors contributed equally to this work.

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