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International Collaborative Study of the Endogenous Reference Gene *LAT*52 Used for Qualitative and Quantitative Analyses of Genetically Modified Tomato

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One tomato (*Lycopersicon esculentum*) gene, *LAT*52, has been proved to be a suitable endogenous reference gene for genetically modified (GM) tomato detection in a previous study. Herein are reported the results of a collaborative ring trial for international validation of the *LAT*52 gene as endogenous reference gene and its analytical systems; 14 GMO detection laboratories from 8 countries were invited, and results were finally received from 13. These data confirmed the species specificity by testing 10 plant genomic DNAs, less allelic variation and stable single copy number of the *LAT*52 gene, among 12 different tomato cultivars. Furthermore, the limit of detection of *LAT*52 qualitative PCR was proved to be 0.1%, which corresponded to 11 copies of haploid tomato genomic DNA, and the limit of quantification for the quantitative PCR system was about 10 copies of haploid tomato genomic DNA with acceptable PCR efficiency and linearity. Additionally, the bias between the test and true values of 8 blind samples ranged from 1.94 to 10.64%. All of these validated results indicated that the *LAT*52 gene is suitable for use as an endogenous reference gene for the identification and quantification of GM tomato and its derivates.

KEYWORDS: Collaborative trial; *LAT52*; endogenous reference gene; *Lycopersicon esculentum*; qualitative and quantitative PCR

INTRODUCTION

With the quick development of modern agricultural biotechnology, more than 100 kinds of genetically modified organisms (GMOs) have been developed and approved for commercialization worldwide, and the global plant area of GMOs reached 102 million hectares in 2006 (*1*). Due to consumers' concern on the safety of GMOs, regulations for GMO labeling are being set up in some countries; for instance, the European Union set the labeling threshold of GMOs as 0.9% (*2*), Korea, 3% (*3*), and Japan, 5% (*4*); China has set a zero threshold (*5*). To implement these regulations, much effort was taken to develop standard methods for GMO detection, and more than 20 different GMO detection standards have been published and used in practical GMO detection in Japan, Korea, and China, etc. To speed the standardization of GMO analysis methods at the global level, the International Organization for Standardization (ISO) has initiated some standards for GMO detection (*6–11*), which is helpful for the international harmonization of the detection approaches of GMO analysis, reducing national and international trade problems on GMO issues.

The international collaborative validation is the prerequisite step for pushing one method to be included as the ISO standard. Community Reference Laboratories (CRL) and others have organized some deliberate collaborative ring trials for CaMV35S promoter and NOS terminator quantitative detection methods (*12–15*) and event-specific quantitative detection methods of TC1507, MON863, GA21, and MON810 maize events (*16*). Recently, we finished one ring trial to validate an event-specific qualitative detection method of RT73 canola among 12 invited laboratories (*17*).

Crop endogenous reference gene is regarded as the gold standard for identifying one taxon, which is crucial for GMO detection. One ideal endogenous reference gene in GMO testing should display species specificity, stable low copy number in the genome, and low heterogeneity among different cultivars

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(*18*). To date, even though several endogenous reference genes have been reported for maize, soybean, tomato, rice, canola, and other plants, such as the *hmg*a, *Adh*1, *zein*, *ivr*1, *zssIIb* genes for maize (*19, 20*), the *lectin* gene for soybean (*21*), the *LAT*52 gene for tomato (*22*), the *SPS* gene for rice (*23*), the *HMG I/Y* gene for canola (*24*), and the *Sad1* gene for cotton (*25*), few endogenous reference genes have been validated by international collaborative ring trial. In this study, one collaborative ring trial for validating the qualitative and quantitative PCR methods of tomato endogenous reference gene *LAT*52 was first organized and reported. Also, this mode provides an example for other endogenous reference gene standardizations.

MATERIALS AND METHODS

Plant Materials. Twelve different tomato cultivars from different geographic and phylogenic origins were used and kindly provided by Prof. Weiming Zhu from Shanghai Academy of Agriculture Science (SAAS), such as Shengnong2, Jifan4, Zhongsu5, Yashu6, Jiafen1, Shenfeng2, Hongza9, R144 (introduced from Israel), Nongyou30, Dongnong704, Lichun, and Zaokui. Ten different plant species, such as eggplant (*Solanum melongena*), potato (*Solanum tuberosum*), capsicum (*Capsicum annuum*), maize (*Zea mays*), soybean (*Glycine max*), rapeseed (*Brassica rapa*), rice (*Oryza sativa*), petunia (*Petunia hybrida*), tobacoo (*Nicotiana tabacum*), and *Arabidopsis* (*Arabidopsis thaliana*), were saved in our laboratory.

Five tomato samples mixed as tomato powder with non-GM maize powder based on mass fractions were prepared, that is, 2, 0.5, 0.1, 0.05, and 0.01% (w/w), and each sample had one duplicate. These samples were mixed using the SPEX CertiPrep 6850 freezer/mill (SPEX CertiPrep Inc., Metuchen, NJ). The dried tomato cultivar (Jiafen1) seeds and non-GM maize seeds were first ground with the freezer/mill to obtain the pure dried powder, respectively. Then the 1.0000, 0.2500, 0.0500, 0.0250, or 0.0050 g of pure dried tomato powder and 49.0000, 49.7500, 49.9500, 49.9750, or 49.9950 g of pure dried non-GM maize powder were weighed with a Sartorius BS 224S balance (readability is 0.0001 g), respectively. The tomato powder and the corresponding non-GM maize powder were put into 50 mL grinding vials simultaneously (the total weight was 50.0000 g), and then the samples were mixed in the liquid nitrogen in the freezer/mill for 10 min. After removal from the freezer/mill, the vials were kept at room temperature for one or two days without the vial caps being opened. When the vials reached room temperature and the outside surfaces had no condensing water, these samples were put into small bottles.

Four tomato varieties, namely, R144, an Israeli variety introduced by Jiangsu province; Zhongsu5, a variety planted in northern China; Zaofeng, a variety planted in central China; and Lichun, a variety planted in southern China, were used for the construction of the standard curves and preparation of the blind DNA samples.

DNA Extraction. Plant genomic DNA samples were extracted by means of the CTAB method (ISO 21571) and purified by a silica column DNA purified kit (Ruifeng Agro-tech Co., Ltd., Shanghai, China). Briefly, 15 mL of 65 °C CTAB extraction buffer was added to 1 g ground samples with thorough shaking. Then, 100 μL of α-amylase and RNase A (10 mg/mL each) were added to the tube, followed by gentle shaking and 65 °C incubation for 30 min; 200 μL of proteinase K (200 mg/mL) was then added to the tube. After gentle shaking, the mixture was incubated at 65 °C for 30 min. After 10 min of centrifugation at 10000*g*, 1 volume of chloroform was added to the supernatant. The mixture was centrifuged for 15 min at 10000*g*, and the supernatant was precipitated using the CTAB precipitation buffer. The supernatant was discarded, and the pellet was dissolved in NaCl (1.2 μM) and extracted with chloroform (1 volume). After centrifugation (10 min at 12000*g*), the supernatant was treated with cold isopropanol (0.6 volume) and centrifuged for 15 min at 12000*g*. The supernatant was discarded, and the pellet was washed with 70% ethanol, vacuum-dried, and resuspended in 100 μL of Tris-EDTA (TE) buffer. Then, the DNA solutions were purified using a silica column according to the manufacturer's instructions.

The quality and quantity of the extracted DNA samples were evaluated by agarose gel electrophoresis analysis and ultraviolet spectrometric method according to ISO 21571. For quantitative standard curve construction, the final DNA solution was adjusted to a concentration of 50 ng/μL, and for the blind samples, to concentrations of 5 and 0.5 ng/μL, whereas other DNA samples were adjusted to a concentration of 20 ng/μL. Salmon sperm DNA (20 ng/μL) was used as the negative DNA target control, and the tomato (Jiafen1) genomic DNA was used as the positive DNA target control.

Qualitative PCR Reactions. In the *LAT*52 gene qualitative PCR assay, the previously reported primer with a 92 bp amplicon was used; the forward primer sequence was 5′ AGACCACGAGAACGATATTTGC 3′, and the reverse primer was 5′ TTCTTGCCTTTTCATATCCA-GACA 3′ (*22*). The qualitative PCR was carried out in a 30 μL reaction mixture containing 25 μL of Qualitative PCR Reaction Master Mix and 5 μL of the sample DNA. The Qualitative PCR Reaction Master Mix included 1× PCR buffer, 200 nM each of dNTPs, 400 nM each of the forward and reverse primers, and 1 unit of Hot Star Taq DNA polymerase (Shanghai RuiCheng Co., Ltd.). Thermal cycling conditions of the qualitative PCR were as follows: 94 °C for 15 min followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s, then the final extension of 72 °C for 7 min. This thermal cycling condition was confirmed to fit for various thermal cyclers, such as the PTC-100 Thermal Cycler (MJ Research), the ABI 2720 Thermal Cycler (Applied Biosystems), and the T1 Thermal Cycler (Biometra). The PCR amplified products were analyzed by 3% agarose gel electrophoresis in 0.5× TBE and stained with ethidium bromide.

Quantitative Real-Time PCR Reactions. In the *LAT*52 gene real-time PCR assay, the qualitative PCR primers and the TaqMan probe with the sequence of 5′HEX-CTCTTTGCAGTCCTCCCTTGGGCT-BHQ3′ were used. The quantitative PCR was carried out in a 25 μL reaction mixture containing 20 μL of Quantitative PCR Reaction Master Mix and 5 μL of the sample DNA. The Quantitative PCR Reaction Master Mix consisted of 1× Quantitative PCR buffer, 200 nM each of dNTPs, 400 nM each of the primers, 200 nM of the probe, and 1 unit of Hot Star Taq DNA polymerase (Shanghai RuiCheng Co., Ltd.). Thermal cycling conditions of the quantitative PCR were as follows: 95 °C for 15 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 45 s. Fluorescence data were collected during the elongation step of each cycle. This thermal cycling condition was confirmed to fit various fluorescence thermal cyclers, such as the Rotor Gene 3000A (Corbett Research) and the Prism ABI 7300 Sequence Detection System (Applied Biosystems). Each quantitative PCR plate required three performances, each time with three repeats.

Collaborative Trials. The collaborative trial was organized by the GMO Detection Laboratory of Shanghai Jiao Tong University (GMDL-SJTU). Fourteen GMO detection laboratories were invited to participate. Each participant received 12 tomato genomic DNAs coded U1−U12 (20 ng/μL), 10 other plant genomic DNAs coded W1−W10 (20 ng/μL), 10 DNA samples that were double-blind replicates of the five concentration levels of the mixed powder of maize and tomato coded S1−S10 (20 ng/μL), 4 purified tomato cultivar genomic DNA samples (R144, Zhongsu5, Zaofeng, and Lichun) coded A−D, respectively, and 9 blind DNA samples coded X1−X8. The eight blind DNA samples contained two concentration levels of four tomato cultivar genomic DNAs of 0.5 and 0.05 ng/μL. In addition, participants received one positive DNA target control consisting of the DNA solution of Jiafen1 tomato (20 ng/μL) and one negative DNA control consisting of the salmon sperm DNA solution (20 ng/μL). Furthermore, participants were provided with Qualitative PCR Reaction Master Mix (1 mL × 3), Quantitative PCR Reaction Master Mix (1 mL × 6), and the DNA dilute solution (1.2 mL × 2). DNA samples A−D providing 50 μL each at 50 ng/μL were used for the standard curve construction, and each of these four DNA samples was diluted to the concentration of 10, 1, 0.1, 0.01, or 0.002 ng/μL by the participants using the supplied DNA dilute solution. Five microliters of each sample DNA was used for the qualitative and quantitative PCR amplification under the PCR conditions described above. The DNA solutions and reagents were stored in one closed box filled with dry ice and express-shipped to each participating laboratory by DHL International GmbH (Shanghai, China). In the promissory time of 2 months, 13 laboratories sent back

Table 1. Results of the 2007 Collaborative Trial Validation of the Species Specificity of the *LAT*52 Gene

no. of labs	14
no. of labs submitting results	13
no. of samples per lab	10
no. of accepted results	130
no. of positive results	4
no. of negative results	126
false-positive rate	3.08%

the validated results report, and the following analyses were all based on the 13 received reports in this study; statistical difference among samples and participating laboratories, such as the *Q* test, was performed using SPSS 12.0 software.

All of the participating laboratories performed the real-time PCR reaction conditions provided described above except for one participant, and this laboratory used 20 μL of PCR reaction volumes for the quantitative PCR reaction. In this laboratory, the DNA samples A−D were diluted to the concentrations of 50, 5, 0.5, 0.05, and 0.01 ng/μL, and 19 μL of Quantitative PCR Reaction Master Mix and 1 μL of the diluted sample DNA were added to the quantitative PCR reactions. Also, the results from this laboratory were accepted and used for further analysis.

RESULTS AND DISCUSSION

Applicability of the *LAT*52 Gene Used as Tomato Endogenous Reference Gene. To further validate the applicability of *LAT*52 for tomato-specific endogenous reference gene at the international level, species specificity, consistent stable low copy number, and allelic variation among cultivars of the *LAT*52 gene were tested.

Species Specificity of *LAT*52. In our previous study, the low allelic variation of the specific sequence of tomato *LAT*52 gene (GenBank no. 19263) was analyzed in the Genbank Website by the BLASTN program, and the low allelic variation was further intralaboratory-tested employing 20 different plant species that were either evolutionarily related to tomato or frequently plant materials. These plant species included *S. vulgare* Pers., *T. repens*, *L. perenne*, barley (*H. vulgare*), cotton (*G. hirsutum*), maize (*Z. mays*), rice (*O. sativa*), canola (*B. napus*), wheat (*T. aestivum*), *A. thaliana*, sunflower (*H. annuus*), soybean (*G. max*), and more related plants in evolution from Solanaceae such as *L. chinense* Mill, *S. melongena* L., potato (*S. tuberosum*), sweet pepper (*C. annuum* L. var. *grossum*), pepper (*C. annuum*), *Lycopersicon peruvianum*, *S. muricacun*, and tobacco (*N. tabacum*). As expected, only tomato genomic DNA showed positive amplification, and no specific amplified products were observed with any of these tested species.

In this collaborative trail, the species specificity of the *LAT*52 gene for tomato was further validated using 10 different plant species coded W1−W10. We sent the prepared DNA samples extracted from these 10 plant materials and Qualitative PCR Reaction Master Mix to each participant. We requested them to repeat three times each qualitative PCR reaction and also return to us with results of positive and negative for each PCR plate. The results were acceptable when the positive control was amplified with the clear 92 bp fragment and no amplification in the negative and blank control was found.

In total, 13 laboratories returned the results of their analyses in this ring trial (**Table 1**). In the reported results, 4 false-positive results were reported in the test of a total 130 DNA samples of 10 other plant species in 13 laboratories. Thus, the deduced false-positive rate for this collaborative trial was about 3.08%, and the accuracy for the *LAT*52 gene was about 96.92%. Among the 4 false-positive results, 1 laboratory reported 2 false-positive results from potato and *Arabidopsis* DNA, respectively, 1 false result from 1 laboratory was from maize DNA, and 1 laboratory reported 1 false result from potato DNA. Furthermore, the positive result was only judged from the very faint gel electrophoresis band according to the returned results sheets of participants. Therefore, we believed that these 4 false-positive results were likely from DNA contamination during testing by these 3 laboratories. Anyway, these data demonstrated that the *LAT*52 gene was species-specific for the detection of tomato.

Table 2. Allelic Variation Testing Results of the *LAT*52 Gene in the 2007 Collaborative Trial

no. of labs	13
no. of labs submitting results	13
no. of samples per lab	12
no. of accepted results	156
no. of positive results	155
no. of negative results	1
false-negative rate	0.6%

Table 3. Square Regression Correlations (R^2) of Each Standard Curve from 13 Laboratories

	A		B		C		D	
lab	R^2	SD	R^2	SD	R^2	SD	R^2	SD
1	0.9997		0.9997		0.9994		0.9999	
2	0.9987		0.9948		0.9945		0.9992	
3	0.9994		0.9993		0.9998		0.9984	
5	0.9979		0.9961		0.9961		0.9965	
6	0.9991		0.9974		0.998		0.9989	
7	0.9969	0.0018	0.9943	0.0020	0.9985	0.0017	0.9995	0.0014
8	0.9994		0.9993		0.9998		0.9984	
9	0.9972		1.0000		0.9966		0.9981	
10	0.999		0.9999		0.9999		0.9996	
11	0.9968		0.9993		0.9984		0.9995	
12	0.9955		0.9988		0.9968		0.9951	
13	0.9939		0.9986		0.9992		0.9973	

Table 4. Difference of the Quantified Copy Numbers among Four Tomato Cultivar DNA Dilutions in This Trial

copy no.	mean A	mean B	mean C	mean D	SD	RSD (%)
55000	52772.6	58103.4	55494.5	57193.8	2343.3	4.19
5500	5206.3	5753.4	5619.5	5760.5	260.6	4.67
550	509.9	535.1	556.1	567.6	25.38	4.68
55	51.3	52.2	57.0	55.2	2.6	4.90
11	11.1	11.5	13.3	12.6	1.0	8.31

Less Allelic Variation among Different Tomato Cultivars. The allelic variation of the *LAT*52 gene in tomato was intralaboratory-tested using 16 DNA samples of tomato cultivars from different geographic and phylogenic origins, that is, Dongnong704, Shenfen2, Maohong1, Jifan4, Zaokuai, Xifen3, Lichun, Zaofen, Zhongsu5, Hongza9, R144, Nongyou30, Shuangfu, Shennong2, Jiafen1, and Yashu6, respectively. As expected, the 92 bp identical PCR products were obtained from all of the tested cultivars in conventional PCR.

In this collaborative trail, the allelic variation of the *LAT*52 gene for tomato was validated by 12 different tomato cultivars coded U1−U12. Referring to the total 156 DNA samples of the 12 tomato cultivars from 13 laboratories, 1 false-negative result was reported (**Table 2**), and 1 genomic DNA sample from a tomato cultivar named Lichun was detected as a negative result in 1 laboratory. The deduced false-negative rate observed in the collaborative trial was 0.6%, and the positive rate was 99.4%. Therefore, we believed that the *LAT*52 gene was a low allelic variation among different tomato cultivars.

Table 5. Difference of the Quantified Results of Tomato DNA Dilutions among 13 Laboratories in This Trial

	A			B			C			D		
copy no.	mean	SD	RSD (%)	mean	SD	RSD (%)	mean	SD	RSD (%)	mean	SD	RSD (%)
55000	52772.6	13409.2	25.41	58103.4	17116.7	29.46	55494.5	14772.8	26.62	57193.8	14999.3	26.23
5500	5206.3	1204.3	23.13	5753.4	1338.3	23.26	5619.5	1299.0	23.12	5760.5	1433.5	24.89
550	509.9	121.3	23.79	535.1	132.1	24.68	556.1	124.3	22.35	567.6	147.1	25.92
55	51.3	16.3	31.77	52.2	17.5	33.61	57.0	19.8	34.76	55.2	19.0	34.43
11	11.1	3.8	34.01	11.5	3.5	30.17	13.3	4.4	32.97	12.6	4.0	31.55

Table 6. Limit of Detection (LOD) of *LAT52* from the 2007 Qualitative PCR Assay

rel concn ($W_{tomato/maize}$)	2%	0.5%	0.1%	0.05%	0.01%
no. of labs	13	13	13	13	13
no. of labs submitting results	13	13	13	13	13
no. of samples per lab	2	2	2	2	2
no. of samples	26	26	26	26	26
positive results	25 (96.2%)	25 (96.2%)	26 (100%)	0 (0%)	2 (15.4%)

Stable Single Copy Number among Different Tomato Cultivars. The copy number of one gene in a genome could be assessed by means of Southern blot or real-time PCR; we previously proved that the copy number of *LAT52* was 1 single copy in 16 different tomato cultivars by means of Southern blotting (*22*). In this collaborative study, we prepared to assess the stable copy number among different cultivars using a real-time PCR method (*26, 27*). The participants were requested to construct four separate standard curves using series diluted genomic DNAs from four tomato cultivars (R144, Zhongsu5, Zaofeng, and Lichun with the defined labels of A, B, C, and D, respectively). The serially diluted genomic DNAs contained DNA concentrations at 10, 1, 0.1, 0.01, and 0.002 ng/μL. The tomato DNA amounts for each PCR reaction were 50, 5, 0.5, 0.05, and 0.01 ng (5 μL for each reaction), and the tomato haploid genomic DNA copy numbers were about 55000, 5500, 550, 55, and 11 in each PCR reaction according to the haploid tomato genomic DNA quantity (*28*).

The results of the constructed standards in this trial showed that the average PCR efficiencies of the four constructed standard curves from 9 repeats among 13 different laboratories ranged from 0.95 to 0.98. The average square regression correlations (R^2) of each standard curve ranged from 0.9939 to 1.0000 with a standard deviation (SD) of 0.017 (**Table 3**). Also, the copy numbers of the series diluted DNA samples from A to D were quantified on the basis of the constructed standard curves, and the differences of quantified results among the different cultivar samples and different laboratories were statistically analyzed.

The mean quantified copy numbers of the diluted DNA samples from A−D tomato samples in 13 laboratories are listed in **Table 4**, and the standard deviation (SD) and relative standard deviation (RSD) values were calculated according to the mean copy numbers. In the DNA dilutions of A−D samples with the copy number of tomato haploid genomic DNA from 55000 to 11, the SD values ranged from 2343.3 to 1.0 and the RSD values ranged from 8.31 to 4.19%. In addition, the quantified copy number of each tomato cultivar with the same dilution from 13 laboratories was statistically analyzed using the *Q* test, and the calculated *Q* test values showed that no significant difference was found among the quantified copy number of the four tomato cultivars with the same dilution (*Q* test, $P > 0.05$).

In **Table 5**, the RSD values of the quantified results of each diluted sample from A to D among 13 laboratories were analyzed; the RSDs ranged from 23.13 to 34.76%. The calculated RSDs indicated that the results from different participating laboratories were creditable. All of the RSD values calculated in **Tables 4** and **5** were below the acceptance threshold of RSD for one GMO detection method (*29*). All of the data from statistical analysis demonstrated that the single copy number of the *LAT52* gene in different tomato cultivars was stable.

Table 7. Efficiency and Square Regression Correlation of *LAT52* Quantitative PCR

lab	efficiency	SD	RSD (%)	R^2	SD	RSD (%)
1	0.83	0.021	2.56	0.9968	0.0025	0.25
2	0.90	0.030	3.30	0.9992	0.0006	0.06
3	0.83	0.033	3.97	0.9972	0.0043	0.43
4	0.92	0.016	1.75	0.9984	0.0008	0.08
5	0.97	0.096	9.92	0.9973	0.0023	0.23
6	1.06	0.040	3.80	0.9992	0.0006	0.06
7	0.97	0.032	3.27	0.9980	0.0015	0.15
8	0.92	0.008	0.88	0.9996	0.0004	0.04
9	1.17	0.078	5.94	0.9981	0.0033	0.33
10	0.94	0.026	2.79	0.9985	0.0012	0.12
11	1.04	0.099	9.50	0.9966	0.0017	0.17
12	1.05	0.047	4.50	0.9899	0.0057	0.58
13	0.94	0.047	4.94	0.9973	0.0024	0.24

Table 8. Repeatability and Reproducibility of the *LAT52* Gene Quantitative PCR

copy no.	SD^r	RSD^r (%)	SD^R	RSD^R (%)
55000	0.059	0.22	0.112	0.41
5500	0.042	0.14	0.100	0.33
550	0.110	0.33	0.089	0.26
55	0.141	0.38	0.121	0.33
11	0.143	0.36	0.160	0.40

Validation of the Qualitative PCR Method of *LAT52*. To assess whether the *LAT52* qualitative PCR method has sufficient sensitivity to meet the requirements of GMO detection, the limit of detection (LOD) of this method was further tested. In this ring trial, the LOD of the *LAT52* gene qualitative PCR was validated by the 10 DNA samples with serially diluted concentrations coded S1−S10. The 10 DNA samples were extracted from the mixed powder with maize and various contents of tomato seeds at 2.0, 0.5, 0.1, 0.05, and 0.01% and each tomato content level with two repeated samples with different codes. The returned results of 13 laboratories showed that all 13 laboratories detected the DNA samples from 0.1% tomato powder, and 2 in 13 laboratories could even detect the 0.01% mixed tomato samples, suggesting that the LOD of *LAT52* qualitative PCR system is as low as 0.1%, which corresponds to 11 copies haploid tomato genomic DNA (**Table 6**).

Validation of *LAT52* Quantitative PCR Method. To validate the developed quantitative PCR method of *LAT52*, four standard curves were constructed in each laboratory using tomato genomic DNA that was serially diluted to 50, 5, 0.5,

Table 9. Results of Blind DNA Samples Using Quantitative *LAT*52 Real-Time PCR

	0.5 ng				0.05 ng			
unknown samples	Zhongsu5	R144	Zaofeng	Lichun	Zhongsu5	R144	Zaofeng	Lichun
labs returning results	13	13	13	13	13	13	13	13
samples per lab	1	1	1	1	1	1	1	1
total data no.	117	117	117	117	117	117	117	117
data excluded	2	5	4	4	3	5	5	4
reason for exclusion	data not in the confidence area of 95%							
mean value	0.4468	0.4806	0.4875	0.4886	0.0464	0.0465	0.0515	0.0490
repeatability SD	0.0838	0.0769	0.0733	0.0913	0.008	0.011	0.007	0.006
repeatability RSD (%)	18.751	15.991	15.039	18.684	17.777	23.031	13.316	12.992
reproducibility SD	0.120	0.122	0.109	0.133	0.016	0.016	0.016	0.017
reproducibility RSD (%)	26.757	25.412	22.457	27.124	34.412	34.893	31.670	34.950
bias (absolute value)	0.053	0.019	0.012	0.011	0.004	0.004	0.001	0.001
bias (%)	10.64	3.88	2.50	2.29	7.28	7.06	2.99	1.94

0.05, and 0.01 ng (absolute amounts in each PCR reaction) from different tomato varieties, such as Zhongsu5, R144, Zaofeng, and Lichun.

For one ideal quantitative real-time PCR method, the efficiency and linearity of the quantitative standard curve, the LOD, repeatability, and reproducibility were very important parameters, and these parameters for *LAT*52 real-time PCR assay were all tested in this trial. Furthermore, eight practical blind tomato samples from four different cultivars with different quantities were quantified using the *LAT*52 real-time PCR assay.

Efficiency and Linearity of Quantitative Standard Curve. Each participant was requested to dilute the DNA standards, run the quantitative PCR, and submit the Ct values, and then the returned Ct values were plotted against the log of the absolute amount of the tomato genomic DNA (ng) to get the standard curves. PCR efficiency (E) was calculated using the equation $E = 10^{-1/\text{slope}} - 1$. **Table 7** shows the average values of efficiency and the linearity of four standard curves from the data of each laboratory.

The PCR reaction efficiencies ranged from 0.83 to 1.17, and the square regression correlation (R^2) values were at least 0.99 (**Table 7**). The PCR reaction efficiencies and R^2 values of the *LAT*52 real-time PCR assay from 13 laboratories were statistically analyzed as described under Stable Single Copy Number among Different Tomato Cultivars, and the high PCR efficiency and good linearity of the standard curves indicated the *LAT*52 gene was suitable for the quantitative analysis of the tomato samples.

LOD. For the *LAT*52 gene quantitative PCR assays, the dynamic range of the constructed standard curves wsd 0.01−50 ng in a 25 μL volume of PCR mixture. The high PCR efficiency and good linearity of the standard curves at this dynamic range were revealed in 13 laboratory results reports, and in each DNA dilution from 0.01 to 50 ng, the quantified results of each DNA dilution were within the 95% confidence interval, from which it was deduced that the LOD of the tomato *LAT*52 gene quantitative PCR was as low as 0.01 ng, that is, about 11 copies of haploid tomato genomic DNA, showing that the tomato *LAT*52 gene quantitative PCR has sufficient sensitivity for tomato sample quantification.

Repeatability and Reproducibility. Repeatability and reproducibility were both determined and calculated using the standard tomato genomic DNA dilutions. The standard deviation (SD^r) and relative standard deviation (RSD^r) of repeatability and standard deviation (SD^R) and relative standard deviation (RSD^R) of reproducibility were calculated from the data of three tests and each time with three replications. The RSD^r of the *LAT*52 gene quantitative PCR ranged from 0.14 to 0.36% and the RSD^R ranged from 0.26 to 0.41 (**Table 8**), demonstrating that this quantitative PCR assay is stable and reliable in tomato genomic DNA quantification.

Blind Sample Quantification. Eight blind tomato DNA samples at two concentration levels were then analyzed using the established *LAT*52 gene quantitative PCR, and the results are shown in **Table 9**.

After exclusion of the quantitative data (32 from a total of 936 with the rate of 3.4%) not in the confidence area of 95%, the average bias of the quantitative results among different laboratories ranged from 1.94 to 10.64%. In the blind sample quantification using *LAT*52 real-time PCR assay, the repeatability and reproducibility of the quantified results were calculated. The RSDs of repeatability ranged from 13.316 to 23.031%, and the RSDs of reproducibility ranged from 22.457 to 34.893%, indicating that the quantified results of blind samples were stable and reliable.

Conclusion. In summary, we report the validation results of *LAT*52 gene with tomato species specificity, low allelic variation, and single stable copy number from 13 participating laboratories, proving that the *LAT*52 gene is a suitable tomato endogenous reference gene in GMO analysis. Also, the participants confirmed that the developed *LAT*52 qualitative and quantitative real-time PCR assays have high efficiency, acceptable limits of detection, repeatability, and reproducibility, and creditable accuracy in blind sample quantification.

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