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METHODS

Inter-laboratory validation by event-specific qPCR methods for the detection of genetically modified insect and herbicide-tolerant maize DBN9501

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Abstract

DBN9501 is a new maize transgenic event characterised by being resistance to insects and herbicides. To meet genetically modified (GM)-labeling requirements and monitor the unintended release of genetically modified organisms (GMOs), developing a creditable and applicable method for identifying and quantifying GM events is essential. Herein we developed an event-specific method and validated it through inter-laboratory ring trials using blind samples. The limit of detection (LOD) and limit of quantification (LOQ) of copy number ratio were confirmed at 0.05% and 0.1%, respectively. The quantitative bias ranged from -3.52 to 10.38%, and the relative standard deviation (RSD) of the method was < 25%. Furthermore, the expanded uncertainty for the blind samples S1 – S5 was 0.22%, 0.10%, 0.05%, 0.03%, and 0.02%. These results demonstrated that the event-specific quantitative PCR method could identify and quantify GM DBN9501 for further routine lab analysis.

Keywords Genetically modified maize · DBN9501 · GMO · Event-specific method · Inter-laboratory ring trial · RT-PCR

1 Introduction

Transgene technology has bestowed the most benefits on enhancing crop productivity, and no direct safety hazard has been reported from any GMOs (Teferra 2021). However, controversial concerns over GMOs and their products regarding consumer safety and environmental sustainability remain unchanged after a worldwide rapid and vast adoption for the last 2 decades (Teferra 2021). Governments have established the strictest testing measures to fulfill effective regulatory compliance requirements (Davison and Ammann 2017; Hartung and Schaub 2018). For instance, the European Union, Korea, and Japan have issued labeling regulations to monitor GMO events with specific threshold values of 0.9%, 3%, and 5%, respectively (Fraiture et al. 2015; Zhang and Guo 2011). In addition, many detection methods have been developed for identifying event traits, assessing risk, post-release monitoring of new GM events, and addressing consumer concerns and legal disputes. Most of the methods are based on the techniques of nucleic acid, including PCR (Li et al. 2011), real-time PCR (Takabatake

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et al. 2016; Yang et al. 2007), micro-droplet PCR implemented capillary gel electrophoresis (MPIC) (Guo et al. 2011), loop-mediated isothermal amplification (LAMP) (Singh et al. 2019), microarray (Turkec et al. 2016), and CRISPR-associated method (Huang et al. 2020). Because of the high degree of accuracy, sensitivity, and specificity, real-time (RT) PCR is the gold standard for identifying and quantifying GMOs in crops and food products (Wei et al. 2016), such as the significant commercialized GM events of maize (Long et al. 2021), canola (Akiyama et al. 2010), soybean (Charles et al. 2013), cotton (Savini et al. 2009), and rice (Mazzara et al. 2013).

An inter-laboratory collaborative study has already developed and validated RT-PCR-based event-specific quantitative methods (Scholtens et al. 2017). For instance, 11 laboratories from 3 countries participated in the linseed event CDC Triffid FP967 (Grohmann et al. 2011), 11 laboratories from China collaborated in the ring trial of Huanong No.1 GM papaya (Wei et al. 2016), so as the soybean (Grohmann et al. 2017; Kodama et al. 2011), rice (Grohmann et al. 2015), and tomato (Yang et al. 2008). Many RT-PCR methods of different GM events have been validated through the collaborative inter-laboratory ring trial and used as standards of the international standard organization (ISO 21569 2005; ISO 21,570 2005; ISO 24276 2006). Moreover, inter-laboratory ring trials are recommended for routine GMO analysis (ENGL 2015; ENGL 2017) to confirm that the detection methods fit for purpose and are transferable to multiple laboratories.

Maize, one of the most commercialized biotechnological crops, was popularized through compound traits, especially herbicide and insect resistance. The lepidopteran insects, such as *Agrotis Ypsilon*, cotton bollworm, and Spodoptera litura, seriously destroyed maize and other crops. Meanwhile, the herbicide glufosinate is a widely used resistant trait of GM crops worldwide (Lean 2011; Shwe et al. 2020). DBN9501 is a new maize event resistant to lepidopteran insects and herbicide glufosinate. It is likely to appear soon on the market, since it has been approved for use in food and feed in China, like DBN9936 (Li et al. 2022) and DBN9858 (Kang et al. 2016). However, no event-specific method has been developed for DBN9501 and its derivatives. This research aimed to provide a method and technical support for routing analysis and implementation. In this study, we established the event-specific method for detecting DBN9501 and successfully validated the applicability and suitability of the method.

2 Materials and methods

2.1 Plant material

DBN9501, recipient maize variety, and all GM materials, including the GM event mixed samples were provided by the Development Center of Science and Technology, Ministry of Agriculture and Rural Affairs of the People's Republic of China, were preserved in the laboratory. The 6 mixed samples were:

- 14 GM maize events without DBN9501 (Bt11, Bt176, MON810, MON863, GA21, NK603, T25, TC1507, MON89034, 59122, MIR604, MON88017, 3272, and MON87460);
- 5 GM rice events (Kefeng6, Kefeng8, KMD, M12, and TT51);
- 7 GM soybean events (356043, 305423, CV127, MON89788, A5547-127, A2704-12, and GTS40-3-2);
- 5 GM cotton events (MON1445, MON531, MON15985, LLCOTTON25, and MON88913);
- 8 GM rape events (MS1, MS8, RF1, RF2, RF3, T45, Oxy235, and Topas19/2);
- Non-GM maize mixed samples. Each event contained 1% (w/w) content levels in these GM mixed samples. The amount of DNA from the mixed samples was 50 ng per PCR reaction.

2.2 DNA extraction and purification

Total genomic DNA of all the samples were extracted and purified from leaves by the CTAB extraction according to ISO 21570 (2005). The quality of DNA was estimated by agarose gel electrophoresis and the ultraviolet spectrometric method using a NanoDrop 2000 UV/vis spectrophotometer (NanoDrop, Wilmington, DE, USA), according to ISO 21571 (2005). Subsequently, DNA concentrations were measured using the Qubit™ dsDNA BR Assay Kits in a Qubit® 2.0 Fluorometer (Invitrogen, Thermofisher, MA, USA). Finally, the concentration of DNA samples was adjusted to 50 ng/μL for further experiment analysis.

2.3 Blind sample preparation

Blind samples were directly prepared first by adjusting the DNA concentration to 50 ng/μL with a 0.1×TE solution. Second, DNA of the non-GM transformant and GM were mixed with the ratio of 9:1 to obtain a sample with expected mass fractions of 10% (w/w). Finally, a serial dilution was performed with non-GM rice DNA to obtain respective copies number ratio of 5%, 2%, 1%, 0.5%, and 0.1% DBN9501.

Each sample was adjusted to 100 µL with the concentration of 50 ng/µL for further application.

2.4 Primer and probes

The sequence information of the exogenous vector insert fragment in DBN9501 was provided by Beijing DaBeiNong Biotechnology Co., Ltd. Primer and probes of the event-specific PCR were designed according to the 3' flanking sequences. The 5' and 3' ends of the probe were labeled with 6-carboxyfluorescein (FAM) and Black Hole Quencher 1 (BHQ1), respectively. The probe of the maize reference gene *zSSIIb* (Yang et al. 2005) was labeled with 5'-VIC and 3'-BHQ. All primer and probes used for this research were synthesized by Thermo Fisher Scientific (Table 1).

2.5 Real-time PCR

RT- PCR reactions took place in a fluorometric thermal cycler (ABI7500 USA), in a volume of 25 µL, including 1×iTaq universal probes supermax mix, 400 nM for each primer and probe, and 50 ng genomic DNA, unless otherwise specified. Each reaction followed the program of a deactivation step at 95°C for 5 min, followed by 40 cycles of 15 s at 95°C, 1 min at 60°C, and fluorescence measurements after annealing and extension. Unless otherwise specified, each qPCR reaction was performed in triplicates and 3 parallels each. The negative and blank control used the recipient maize variety DNA and ddH_2O as a template, respectively. Results were analyzed using the SDS Version 1.3.1 (Applied Biosystems, Foster City, CA, USA).

2.6 Digital PCR

Digital PCR was carried out according to the manufacturer's instructions (QuantStudio™ 3D digital PCR, Thermo Fisher). Briefly, the reaction was performed in 15 µL containing 1×3D digital PCR Master Mix v2, 20 ng DNA, 560 nM forward and reverse primers and 280 nM probe. Then, the mixture was loaded onto the chip automatically and sealed by Immersion Fluid. Next, the chip was amplified by QuantStudio™ 3D digital PCR with the amplification program: 96°C, pre-denaturation for 10 min; denaturation for 30 s at 98°C and annealing for 2 min at 60°C (40 cycles in total). Finally, the chip data was read by QuantStudio™ 3D digital PCR and analyzed using Analysis Suite™ Cloud Software (QuantStudio™ 3D digital PCR, Thermo Fisher). No template reactions were prepared for quality control. All data were reported as the mean value of triplicates for each sample.

Table 1 Primer and probe sequences

Target	Primer/probe	Sequence(5'-3')	Amplicon size (bp)
zSSIIb	*zSSIIb*-3 F	CGGTGGATGCTAAGGCTGATG	88
	zSSIIb-4R	AAAGGGCCAGGTTCATTATCCTC	
	zSSIIb-P[a]	TAAGGAGCACTCGCCGCCGCATCTG	
DBN9501	DBN9501-qF	aacgtgactcccttaattctcc	96
	DBN9501-qR	ccggactactataccatttatagttaca	
	DBN9501-qP[b]	ACTGAAGGCGGGAAACGACAATCT	

[a]labelled with 5'-VIC and 3'-Black Hole Quencher 1 (BHQ1)

[b]labelled with 5'-6-carboxy-fluorescein (FAM) and 3'-BHQ1

2.7 Inter-laboratory ring trials

The ring trial included 8 GMO detection laboratories (affiliated with the Ministry of Agriculture and Rural Affairs, China). Each laboratory received 12 gDNA samples, including a positive and a negative control labeled DBN9501 and non-GM, 5 blind samples labeled S1, S2, S3, S4, and S5, and 5 samples labeled G1, G2, G3, G4, and G5, respectively, for the standard curve. The blind sample S1 – S5 solutions with respective copy number ratios of 5%, 2%, 1%, 0.5%, and 0.1% were sequentially prepared by mixing the DNA solution of DBN9501 and non-GM maize using a gravimetric method (Li et al. 2022). G1 – G5 represented the genomic DNA of DBN9501 diluted by EASY Dilution (for RT-PCR) (Takara) with different copy numbers of endogenous gene *zSSIIb* (44453, 7409, 1235, 206, and 34 copies) and DBN9501 (22226, 3704, 617, 103, and 17 copies), respectively, in each reaction as calibrator to construct the standard curves. The samples (G1 – G5, S1 – S5) were simultaneously amplified on each PCR plate for the DBN9501-specific and *zSSIIb*-specific assay. Each sample included 3 parallels, each in triplicates.

2.8 Data analysis

The participants were asked to record the cycle threshold (Ct) of the RT-PCR, and to send back their records within a specified time, in total 180 Ct values (2 genes×(5 calibrators + 5 samples)×3 parallels×3 repeats = 180). Data were analyzed using Microsoft Excel and SPSS 22.0 software to determine the characteristics of the DBN9501-specific method. The PCR efficiency, linearity of regression, accuracy, repeatability, and reproducibility were calculated according to ISO 5725-2 (1994) and ISO 5725-3 (1994). Measurement uncertainty, LOD, and LOQ of the DBN9501 were estimated based on the performance data provided

by the 8 participants, according to the guidance document (Trapmann et al. 2020).

3 Results and discussion

3.1 Development of an event-specific detection method for DBN9501

We designed event-specific primer and probes for RT-PCR using the 3' junction sequence of DBN9501. Their specificity was proved *in silico* through online databases (https://blast.ncbi.nlm.nih.gov/; http://www.phytome.org/). After optimization of the specificity, annealing temperature, and reaction concentration by PCR, primer DBN9501-qF/R and the TaqMan probe DBN9501-qP were selected, yielding a product of 96 bp, which confirmed the requirements of ISO, CRL-GMFF, and the labeling policies of most countries (ISO 21569 2005; ENGL 2015) (Table 1; Fig. S1). DBN9501-qF/P and DBN9501-qR were located in the inserted DNA sequence and the maize genome, respectively (Fig. 1). Their specificity was confirmed and met the requirements of ENGL (ENGL 2015). The primer and probe of the endogenous reference gene *zSSIIb* (Table 1) was validated by Yang et al. (2005). To test the specificity of the primer and probe, we used mixed samples instead of individual GM events and non-GM samples. Mixed samples can decrease the number of tested samples and costs without compromising specificity (Wei et al. 2016). As a result, only DBN9501 showed amplification curves and Ct-values, but not the other 6 mixed samples using DBN9501-qF/R primer, while the amplicons of *zSSIIb* was detected in all maize samples (Fig. S1). Moreover, participants from 8 laboratories confirmed this result using the mixed samples, DBN9501 (1% and 0.05%), positive and negative control by PCR instruments with 6 brands, and each sample was performed with 6 replicates. As a result, each repeat of any one sample showed consistent results, expressed as either positive or negative (Table S1). It also indicated the robustness of different real-time PCR cyclers since no specific difficulties or unusual observations were reported or identified evaluating the results. Given the widely used RT-PCR as a method for GMO detection (Gang et al. 2010; Yang et al. 2013) and its specificity (ENGL 2015), this RT-PCR method was confirmed to be specific to DBN9501 and no cross-reactivity with other GM events and non-GM maize cultivars.

Additionally, we tested the copy number of the DBN9501 in triplicates on a digital PCR platform. The haploid genome size of maize was estimated 2,319 Mbp, corresponding to a weight of 2.5 pg (Arumuganathan et al. 1991). Therefore, the copy number of the haploid maize genome was calculated by the weight of the maize DNA divided by 2.5 pg. One ng DNA of maize contains about 400 copies of endogenous genes. We analyzed the copy number of the endogenous gene and DBN9501 specific fragment after we obtained the data from the digital PCR. Compared with the endogenous gene, the percentage of copy number of DBN9501 was 50%, which indicated that the DBN9501 transformant was a heterozygote (Fig. S2). To testify the LOD value, the ring trials from 8 participants showed that the LOD of real-time PCR could reach 0.05%, and no false-positive and false-negative result was found in the returned reports (Table S1). Since digital PCR is superior to real-time PCR in quantification (Teruaki et al. 2021), this result was more reliable. These results showed that the event-specific real-time PCR assay had high specificity and sensitivity to DBN9501, and we developed an event-specific detection method for DBN9501.

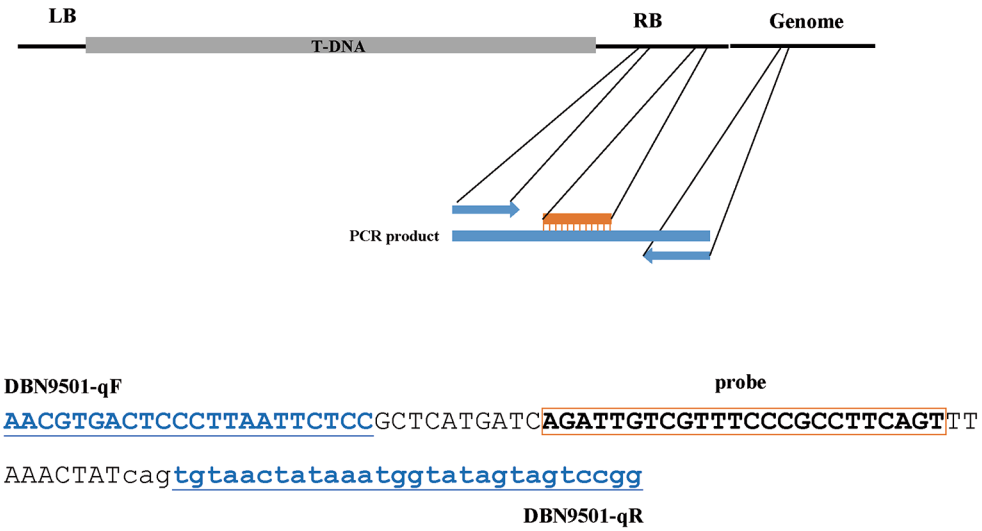

Fig. 1 Primer and probe design for event-specific PCR detection methods for DBN9501. The PCR product of GM-DBN9501 included 65 bp T-DNA insert sequence (capital letter) and maize genome DNA sequence 31 bp (lower case letter). The upstream and downstream sequences with blue underline were DBN9501-qF and DBN9501-qR, respectively. The probe was located on the T-DNA insert sequence and labeled with an orange frame

3.2 PCR efficiency and linearity of qPCR

The participating laboratories provided the Ct values of the serial reference solutions (G1 − G5) without missing data (Table S2). We performed Cochran's and Grubbs' tests to check for outliers of estimated values according to ISO 5725-2 (1994), and no outliers were found. Thus, we generated separate standard curves of the DBN9501 and *zSSIIb* assays by plotting the returned Ct values against the logarithm of the copy number ranging from 44452.8 to 34.3. As a result, the mean slope of the standard curves of *zSSIIb* and DBN9501 was − 3.39 and − 3.38, respectively, between − 3.13 to -3.54. The mean regression coefficients (R^2) were both 1.00 for the *zSSIIb* and DBN9501 (above the minimum acceptable value of 0.98). The PCR efficiency (E) of *zSSIIb* and DBN9501 was calculated based on the formula $E=(10^{-1/slope}-1)\times100\%$, and the mean efficiencies were all not less than 97% (Table 2). The mean values of these parameters were all within the allowed range of the requirements for GMO analytical methods issued by the MPR document (ENGL 2015). Therefore, these results revealed that this detection method had good linearity and high PCR efficiency between Ct-values and copy numbers.

3.3 Limits of detection and quantification

The LOD and LOQ are critical parameters that should be < 1/20 and 1/10 of the target concentration, respectively, according to Announcement No. 2259-5-2015 in China (MARA 2015). Moreover, the LOD should be < 25 copies with a confidence level of 95%, ensuring ≤ 5% false negative results (ENGL 2015). Considering the strictest labeling regulation of the European Union (0.9% threshold), we proposed 1% as a reference value, since China has no threshold yet. To satisfied the requirement, we respectively performed the RT- PCR reactions with 60 parallels (1 repeat) with the concentration of DBN9501 at 0.1% and 0.05%. In both, 0.1% and 0.05% levels of DBN9501, the typical amplification curves could be detected in each parallel (Fig. S3). Simultaneously, the inter-laboratory ring trials obtained the same results with 6 parallel reactions (Table S1). Therefore, the LOD and LOQ for DBN9501 can reach 0.05% and 0.1% levels, respectively. The copy number of LOD of 0.05% equaled 50 ng×0.05%×400 = 10 copies, which also complied with the request of fewer than 25 copies.

To verify whether 0.1% level suit for the LOQ, we performed the RT-PCR using 15 transformants of DBN9501 with a 0.1% concentration (Table S3). After calculating the copy ratios of DBN9501 and *zSSIIb*, we confirmed the

Table 2 The values of the slopes and the regression coefficients of DBN9501 and *zSSIIb*

Laboratory #	*zSSIIb*			DBN9501		
	slope	Efficiency (%)	R^2	slope	Efficiency (%)	R^2
1	-3.36	99	1.00	-3.13	109	1.00
	-3.28	102	1.00	-3.14	108	1.00
	-3.37	98	1.00	-3.18	107	1.00
2	-3.54	92	0.99	-3.45	95	0.98
	-3.44	95	0.98	-3.48	91	0.99
	-3.30	101	0.99	-3.29	101	0.98
3	-3.47	94	1.00	-3.51	93	1.00
	-3.42	96	1.00	-3.38	98	0.99
	-3.45	95	1.00	-3.53	92	1.00
4	-3.40	97	1.00	-3.29	102	1.00
	-3.35	99	1.00	-3.34	99	1.00
	-3.38	98	1.00	-3.37	98	1.00
5	-3.43	96	1.00	-3.47	94	1.00
	-3.46	94	1.00	-3.54	91	1.00
	-3.54	91	1.00	-3.54	92	1.00
6	-3.46	95	1.00	-3.40	97	1.00
	-3.44	95	1.00	-3.46	94	1.00
	-3.37	98	0.99	-3.43	96	0.99
7	-3.37	98	1.00	-3.36	98	1.00
	-3.37	98	1.00	-3.38	98	1.00
	-3.31	101	1.00	-3.41	97	0.99
8	-3.34	99	1.00	-3.37	98	1.00
	-3.34	99	1.00	-3.31	100	1.00
	-3.27	102	1.00	-3.28	102	1.00
Mean	-3.39	97	1.00	-3.38	98	1.00

average percentage was 0.1%. Moreover, the mean bias and relative standard deviation (RSD) were 8.3% and 9.6%, respectively, compared with the original dilution concentration. Since the values of RSD were all < 25%, it suggested that the LOQ could reach 0.1%. Furthermore, the participants performed the RT-PCR with 0.1% DBN9501 in 3 parallels to verify the value of LOQ. The values of relative repeatability standard deviation (RSD_r) in laboratories was < 25% and ranged from 0.00 to 8.33%. Meanwhile, the relative reproducibility standard deviation (RSD_R) among laboratories was < 35% (7.83%, Table S4). For the stability of LOQ, the 0.1% level contained about 100 ng×0.1%×400 = 40 copies of DBN9501. Therefore, the LOQ value of this standard method was 0.1%. Since the value of LOD or LOQ is not lower than 10 copies, so we did not need to estimate the probability of a positive PCR response for the targeted DNA sequence, known as the probability of the detection (POD) (Grohmann et al. 2015). Thus, we concluded that the LOD and LOQ of the event-specific RT-PCR was approximately 0.05% and 0.1% of the haploid genome.

3.4 Qualification of blind samples

Based on the handed-in Ct-values of the blind samples S1–S5 (Table S2), standard curves were constructed and calculated for the copy numbers of the DBN9501 genome (Fig. 2). For blind samples, the GM contents were determined by the formula GM% = GM copy number/maize genome copy number×100. The results in triplicates of each participating laboratory are listed in Table 3. The quantified GM DBN9501 content in S1–S5 samples (5%, 2%, 1%, 0.5%, and 0.1%) were 4.82%, 1.98%, 0.99%, 0.51%, and 0.11%, respectively (Table 4). The quantitative bias for the 5 samples ranged from −3.52 to 10.38% (Table 4), which is within the dynamic range according to the ENGL (2015) acceptance criterion (±25%). These results show

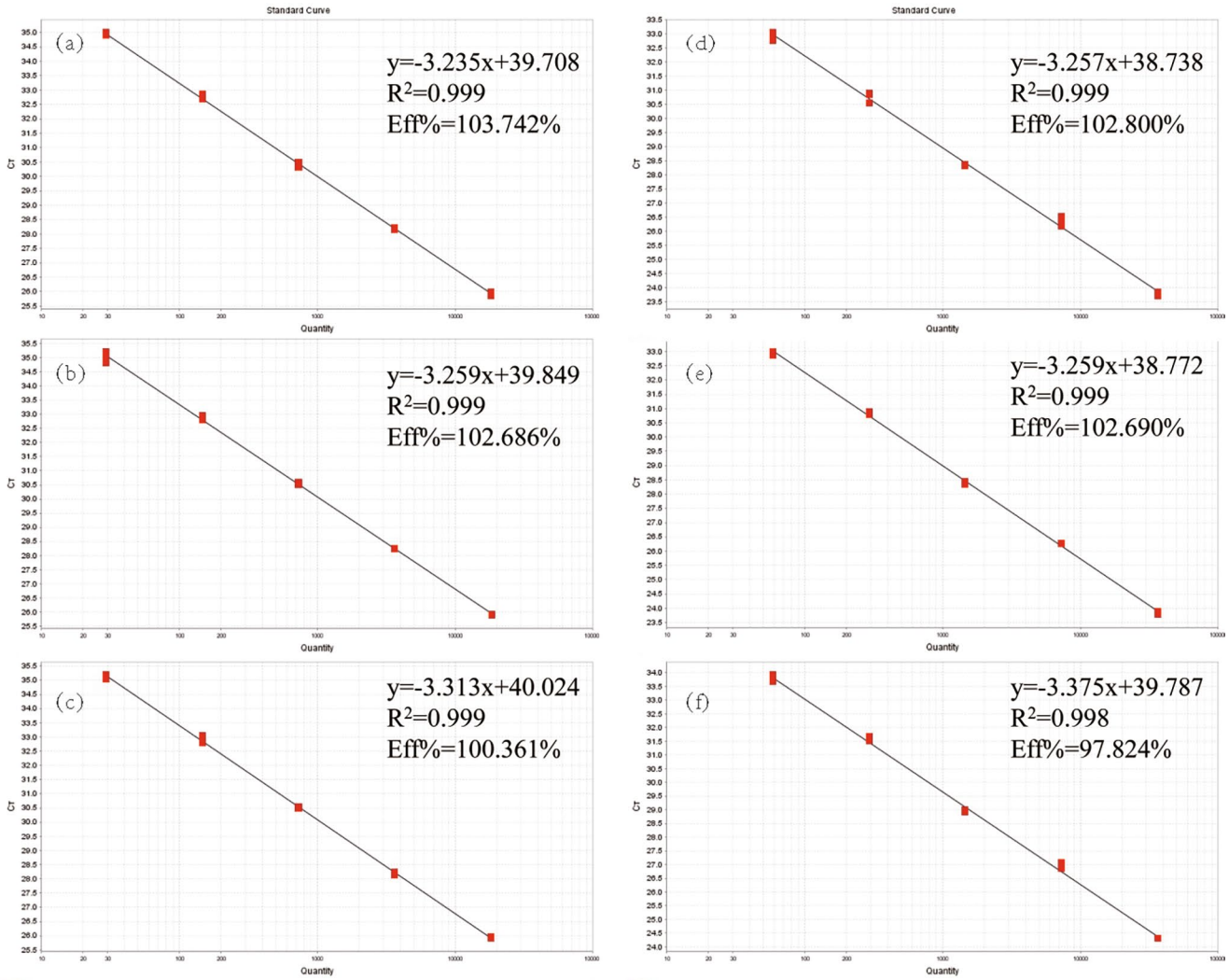

Fig. 2 Standard curves of DBN9501 and *zSSIIb* for the event-specific qPCR using gradient-diluted DBN9501 genomic DNA as the template. The standard curves of DBN9501 (**a-c**) and *zSSIIb* (**d-f**) in triplicates

Table 3 GM DBN9501 content in blind samples from 8 laboratories

Sample GMO content (GM% =GM copy number/maize genome copy number×100)						
Laboratory #	Replicates	S1	S2	S3	S4	S5
1	Rep1	4.77	1.87	0.92	0.49	0.10
	Rep2	4.97	1.96	0.95	0.48	0.09
	Rep3	5.11	1.97	1.07	0.51	0.10
2	Rep1	5.13	2.16	1.14	0.56	0.12
	Rep2	4.95	2.21	1.03	0.57	0.12
	Rep3	4.18	1.82	0.87	0.39	0.10
3	Rep1	5.51	2.00	1.15	0.55	0.12
	Rep2	4.48	1.96	1.14	0.48	0.12
	Rep3	5.36	2.08	1.15	0.56	0.11
4	Rep1	4.47	1.81	0.91	0.45	0.12
	Rep2	4.31	1.82	0.89	0.47	0.11
	Rep3	4.69	2.00	1.02	0.49	0.13
5	Rep1	4.45	1.85	0.95	0.48	0.12
	Rep2	4.71	2.09	0.82	0.62	0.12
	Rep3	4.69	1.86	0.90	0.52	0.12
6	Rep1	5.01	2.01	0.96	0.50	0.11
	Rep2	5.04	1.91	1.04	0.51	0.09
	Rep3	4.84	1.98	0.98	0.54	0.11
7	Rep1	4.94	1.96	0.99	0.51	0.10
	Rep2	5.01	2.03	1.00	0.50	0.10
	Rep3	4.99	1.99	1.04	0.55	0.10
8	Rep1	5.08	1.99	1.00	0.52	0.11
	Rep2	4.57	2.12	0.98	0.48	0.12
	Rep3	4.52	1.97	0.93	0.45	0.11

Table 4 Summary of validation results from collaborative trials results

Blind samples	Expected value				
	5%	2%	1%	0.5%	0.1%
Laboratories that returned results	8	8	8	8	8
Samples per laboratory	5	5	5	5	5
Number of outlies	0	0	0	0	0
Mean value	4.82%	1.98%	0.99%	0.51%	0.11%
Relative repeatability standard deviation, RSD_r	4.27%	3.72%	4.83%	6.66%	4.54%
Relative reproducibility standard deviation, RSD_R	4.11%	2.86%	6.98%	4.40%	7.83%
Bias	-3.52%	-1.21%	-0.71%	1.50%	10.38%

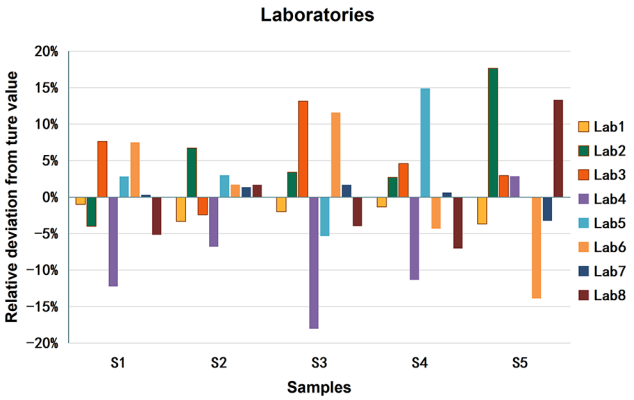

Fig. 3 The relative deviation of the quantification results of blind samples from 8 laboratories

that the established RT-PCR assay is suitable for quantifying DBN9501.

3.5 Accuracy

Based on the slope of the standard curve derived from the DBN9501 genomic DNA dilution series (G1 − G5), this event-specific quantitative system had almost 100% efficiency (Fig. 2). The relative deviation of the true value for blind samples was mainly positive for most participants, ranging from -18.02 to 17.65% (Fig. 3). The bias ranged from − -3.52 to 10.38%, which is < 25%. Intra- and inter-laboratory variation were separately characterized by RSD_r and RSD_R for each sample according to ISO5725-2 (Table S5 and Table 4). The RSD_r values for sample S1, S2, S3, S4, and S5 were 4.27%, 3.72%, 4.83%, 6.66%, and 4.54%, respectively. All RSD_r values were < 25%. The RSD_R values were < 35% (2.86–7.83%) (Table 4). These results illustrated that the real-time PCR method was creditable in practical sample quantification.

3.6 Measurement uncertainty of the tested results

To estimate the absolute standard uncertainty (u_0) and relative standard uncertainty (RSU), the reproducibility standard deviation (s_R) values were plotted against the mean quantities of the tested blind samples (c) and calculated for linear regression (Fig. 4). The u_0 and RSU equals the linear regression intercept ($u_0 = 0.0106$) and the linear regression slope (RSU = 0.0228), respectively. The standard uncertainty (u) associated with a measurement result c is calculated by the formula $u=(u_0^2+(c\times RSU)^2)^{1/2}==(0.0106^2+(c\times 0.0228)^2)^{1/2}$ (Trapmann et al. 2020). The measurement uncertainty is usually calculated from a standard using a coverage factor of 2 expanded uncertainties (U = 2×u). It is equivalent to a confidence level of 95%. For the blind samples S1 – S5, the c-values were 4.82%, 1.98%, 0.99%, 0.51%, and 0.11%. The U-values of the expanded uncertainty were 0.22%, 0.10%, 0.05%, 0.03%, and 0.02% for the tested samples. Therefore, the measurement concentrations were (4.82 ± 0.22%) for S1, (1.98 ± 0.10%) for S2, (0.99 ± 0.05%) for S3, (0.51 ± 0.03%) for S4, and (0.11 ± 0.02%) for S5. These analytical results indicated that different laboratories could use the established DBN9501 event-specific RT-PCR system to produce acceptable, reproducible, and comparable results for a given analysis.

4 Conclusion

This inter-laboratory validation demonstrated that an event-specific RT-PCR method of DBN9501 transgenic maize had high specificity and sensitivity, including excellent PCR efficiency, LOD and LOQ, as well as PCR performance in blind sample quantification. The results of this study provide a creditable application to detect, identify, and quantify genetic modified DBN9501.

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Author contributions J.W. wrote most of the manuscript and helped perform the experiment; B.X., Y.Y., Y.J.D. performed most of the experiment; R.Y., X.L.Z. performed statistical analyses; L.Y.L. helped write the manuscript; H.T.W. and H.T.G. provided the study design and materials and refined the manuscript; J.G.L. and R.Y.Z. conceived of the study, secured funding, and coordinated collaborators. All authors reviewed and approved the manuscript.

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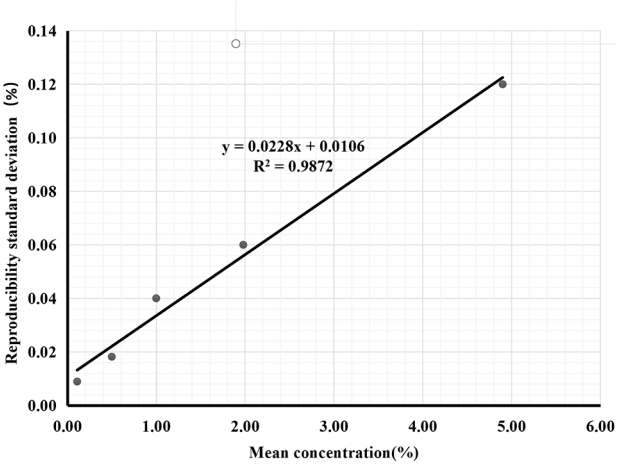

Fig. 4 Linear regression is produced by plotting mean measurement concentration against reproducibility standard deviation

Data availability The data underlying this article are available in the article and its online supplementary material.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Not applicable.

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