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Digital PCR-Based characterization of a Zhonghuang 6106 soybean genomic DNA reference material for its food and feed detection

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The genomic DNA reference material of genetically modified soybean Zhonghuang6106, developed in this study, was prepared by extracting genomic DNA from the leaves of both homozygous transgenic soybean Zhonghuang 6106 and non-transgenic soybean Zhonghuang10, followed by proportional mixing of the genomic DNA. Eight qualified laboratories independently validated the certified reference material (CRM) using the digital polymerase chain reaction (dPCR) method. The certified value for the transgene-specific sequence copy number concentration was determined to be (1.04 ± 0.16)×10³ copies·μL^{-1}, while the copy number ratio of Zhonghuang 6106 to Lectin was 0.047 ± 0.006.Homogeneity and stability assessments revealed that this batch of CRMs exhibited excellent homogeneity and could be stably stored for up to 10 days at 37 °C. Additionally, it remained stable for up to 6 months at -20 °C. This reference material can be used for qualitative and quantitative detection of genetically modified soybean Zhonghuang 6106 and its related products, as well as for the evaluation of specific detection methods and laboratory quality control.

Keywords Certified reference material, Zhonghuang 6106 soybean, Homogeneity, Digital PCR

Genetically modified (GM) soybeans are the predominant GM crop, accounting for 48.9% of the global GM crop planting area and 73.7% of the global soybean planting area[1]. Given this prevalence, GM soybeans are likely to be used as raw materials in food processing. However, the safety of GM crops has long been a focal point of public concern, prompting many countries to implement quantitative labeling of GM ingredients in food[2,3]. To accurately determine the content of GM crops in food, standard reference materials for GM detection have been developed[4,5]. By integrating corresponding molecular biotechnologies and rigorous statistical methods, these reference materials have become a crucial foundation for GMO safety supervision, qualitative and quantitative testing of GMO products, and the research and standardization of detection methods[6]. They effectively enhance the scientific validity, reliability, and traceability of GMO detection results, thereby ensuring high-quality analytical data.

Genomic DNA reference materials are one of the three primary types of standard materials used for testing genetically modified (GM) products[7–10]. They are typically extracted from tissues of GM and non-GM products, such as seeds and leaves. This type of reference material offers several advantages, including ease of large-scale preparation, excellent uniformity, precise quantification, and convenient application. As a result, they have been widely adopted in the detection of genetically modified organisms (GMOs). However, compared to plasmid reference materials and matrix reference materials, genomic DNA reference materials tend to be less stable and require more stringent conditions for storage and transportation[11].Digital PCR (dPCR) technology has emerged as a powerful tool for the absolute quantification of nucleic acids. By directly measuring the copy number of DNA molecules, dPCR eliminates the need for a standard curve, thereby enhancing accuracy and reliability[12–16]. This feature makes it particularly suitable for quantifying the characteristic values of DNA standard substances, including genomic DNA reference materials.

Transgenic soybean line Zhonghuang 6106 is a novel herbicide-tolerant soybean variety developed by the Institute of Crop Sciences, Chinese Academy of Agricultural Sciences. It incorporates the *GAT* and *G2-EPSPS* genes, which confer herbicide tolerance. Currently, China has established corresponding qualitative testing standards for this variety[17]. However, there have been no reports on the development of nucleic acid reference

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materials for this specific transgenic soybean. Given this gap, the present study focuses on the development of a genomic DNA reference material for the herbicide-tolerant transgenic soybean Zhonghuang6106. The aim is to provide a scientific and effective basis for food-safety supervision and the detection of GMO components, thereby enhancing the accuracy and reliability of GMO testing and regulatory oversight.

Results

The results of gDNA assessment

The qualified gDNA was diluted to a concentration of 25 ng·μL^{-1} using a 0.1× TE solution. Subsequently, the gDNA from Zhonghuang 6106 soybean was mixed with that from its non-transgenic recipient (Zhonghuang 10) at a ratio of 1:20. Three aliquots were taken from the top, middle, and bottom of the mixture tube, respectively, yielding nine samples in total. The copy number concentration and copy number ratio of each DNA solution were then measured by dPCR to assess mixing uniformity. Results (Table S1) showed no significant differences in either metric among samples taken from different positions.

Sanger sequencing was performed on the dPCR amplicons, and the resulting sequences were aligned—using DNAMAN v9.0 software—against the signature sequence of Zhonghuang 6106 and the corresponding specific regions of other genetically modified crops (Fig. S1). The results showed that the specific sequence of Zhonghuang 6106 displayed partial homology with the promoter regions of GM soybean MON89788,GM maize 3272, GM canola MON88302 and GM rice T1c-19. Importantly, the diagnostic primers for Zhonghuang 6106 were designed outside this region, ensuring the PCR assay's specificity. To confirm specificity, real-time PCR specificity assays were performed on 100% samples of the above high-homology transformants; none produced detectable amplification (Fig. S2). Moreover, the amplified sequence of Zhonghuang 6106 did not align with any sequences from other GM crops. To further assess the amplification specificity of the Zhonghuang 6106 specific primers and probe in the dPCR system, the following templates were tested: gDNA from Zhonghuang 6106 soybean, GM soybean, GM rice, GM corn, GM cotton, GM canola, non-GM soybean (NTC), and ddH_2O (CK). Only the Zhonghuang 6106 gDNA produced positive droplets, whereas all other samples were negative (Fig. S3). This confirms that the dPCR system exhibits excellent specificity for Zhonghuang6106.

The results of homogeneity assessment

Forty-five randomly selected gDNA samples of Zhonghuang 6106 were analyzed using dPCR, and the measured copy values are presented in Table S2. The copy number ratio of Zhonghuang 6106 to Lectin was calculated based on the specific sequence and the copy number of the internal standard gene. The measured data were analyzed by one-way ANOVA (*F*-test), and the results showed that $F < F_{0.05(14,30)}$ (Table 1; Fig. 1A-B). This indicates no significant difference in between-tube homogeneity of the gDNA solution after packaging. Owing to $s_1^2 > s_2^2$, the uncertainty introduced by the homogeneity of copy number ratio was estimated as: $u_{bb}=s_{bb} = \sqrt{(s_1{}^2 - s_2{}^2)/n} = \sqrt{\frac{6.60\times10^{-7}-5.56\times10^{-7}}{3}}=0.00018$. The corresponding relative uncertainty: $u_{rel(bb)}=u_{bb}/\bar{x}$ $=0.00018/0.05=0.0036$. For the copy number concentration of Zhonghuang6106, the homogeneity uncertainty was: $u_{bb}=s_{bb} = \sqrt{(s_1{}^2 - s_2{}^2)/n} = \sqrt{\frac{3.74\times10^{3}-2.97\times10^{3}}{3}}=16.00$. The relative uncertainty: $u_{rel(bb)}=u_{bb}/\bar{x}$ $=16.00/1.01\times10^{3}=0.016$. Therefore, the gDNA CRM of Zhonghuang 6106 exhibits excellent homogeneity.

The results of stability assessment

The results of short-term stability assessment

Through digital PCR analysis, it was found that after 10 days of storage at −20 °C, 4 °C, 25 °C, and 37 °C, the copy number ratio of Zhonghuang 6106 to Lectin remained within the acceptable range, indicating good short-term stability (Table S3, Fig. 2A-H). The slope was not significant, as determined by the condition $|\beta 1| < t_{0.95,n-2} \cdot s(\beta 1)$. Based on the standard deviation with the temperature conditions that can

Parameters	Copy numbers/Unit	Ratio/Unit
Mean	1010	0.05
Q_1	52339.91	9.24×10^{-6}
V_1	14	14
$S_1{}^2$	3738.57	0.66×10^{-6}
Q_2	89,120	0.17×10^{-4}
V_2	30	30
$S_2{}^2$	2971	0.56×10^{-6}
F	1.26	1.19
$F_{0.05(14,30)}$	2.04	2.04
Result	$F < F_{0.05(14,30)}$	
Conclusion	Homozygote	

Table 1. Results of homogeneity analysis.

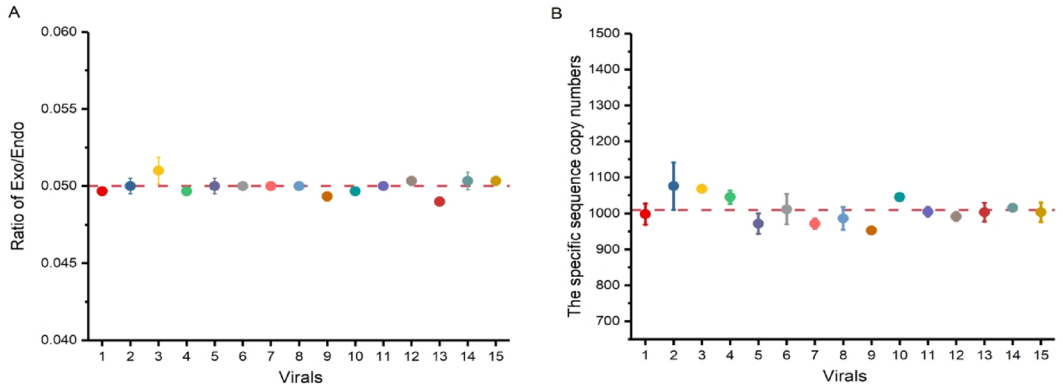

Fig. 1. Schematic diagram of homogeneity test results. Each data point represents the measured value from individual vials, with error bars indicating the standard error. The dashed red line denotes the certified value.

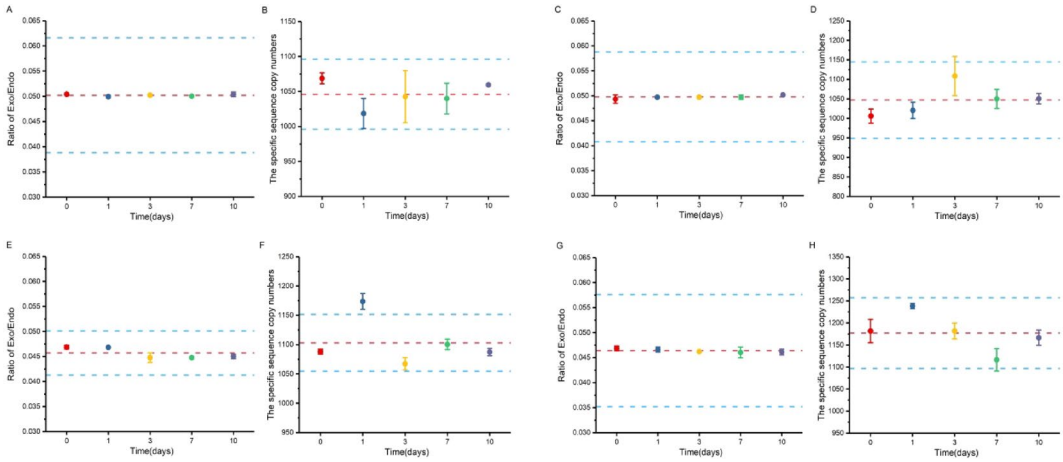

Fig. 2. Short-term stability of the candidate CRM at − 20 °C (**A** and **B**), at 4 °C (**C** and **D**), 25 °C (**E** and **F**), and 37 °C (**G** and **H**). Dashed lines indicate the certified value (red) and range of the standard uncertainty (blue).

be guaranteed during actual transportation, the short-term stability uncertainty was calculated at 25 °C. The results are as follows: Copy number ratio: $u_{s(ss)} = s(\beta_1) \cdot X = 0.001 \times 10 = 0.001$. The relative uncertainty is: $u_{rel(sts)} = u_{s(sts)} / \bar{x} = 0.001/0.0457 = 0.022$. Copy number concentration of Zhonghuang6106: $u_{s(ss)} = s(\beta_1) \cdot X = 5.35 \times 10 = 53.5$. The relative uncertainty was: $u_{rel(sts)} = u_{s(sts)} / \bar{x} = 53.5/1.103 \times 10^3 = 0.049$. In conclusion, the gDNA CRM of Zhonghuang 6106 can be stably stored and transported for 10 days at temperatures up to 37 °C without significant changes in the characteristic values.

The results of long-term stability assessment

Data analysis showed that after storage at −20℃ for 0, 1, 2, 4 and 6 months, the copy number ratio of Zhonghuang 6106 to Lectin remained stable, with $|\beta 1| < t_{0.95,n-2} \cdot s(\beta 1)$; the slope was not significant, confirming good long-term stability (Table S4, Fig. 3A-B). Uncertainty calculations under these conditions are as follows: Copy number ratio: $u_{s(lts)} = s(\beta_1) \cdot X = 3.71 \times 10^{-5} \times 6 = 2.26 \times 10^{-4}$, the relative uncertainty was: $urel(ls) = u_{s(ls)} / \bar{x} = 2.26 \times 10^{-4}/0.05 = 0.0045$. Copy number concentration of Zhonghuang6106: $u_{s(lts)} = s(\beta_1) \cdot X = 2.27 \times 6 = 13.62$. The relative uncertainty was: $urel(ls) = u_{s(ls)} / \bar{x} = 13.62/1.07 \times 10^3 = 0.013$. Therefore, the gDNA CRM of Zhonghuang 6106 is stable for at least 6 months when stored at − 20 °C.

The results of collaborative validation of laboratories

Raw data from the eight-laboratory joint calibration were collected and compiled; complete dPCR datasets are presented in the supplementary materials. Specifically, the exogenous-to-endogenous gene ratio is reported in Table S5 and Fig. 4A, while the corresponding specific sequence copy numbers for each laboratory are listed in Table S6 and depicted in Fig. 4B.

After conducting the Dixon and Grubbs tests, all data points obtained from the eight laboratories were found to be within the normal range and were retained for downstream analyses. To further assess the distribution of the data, a normality test was performed using the D'Agostino-Pearson test. The test statistic was calculated

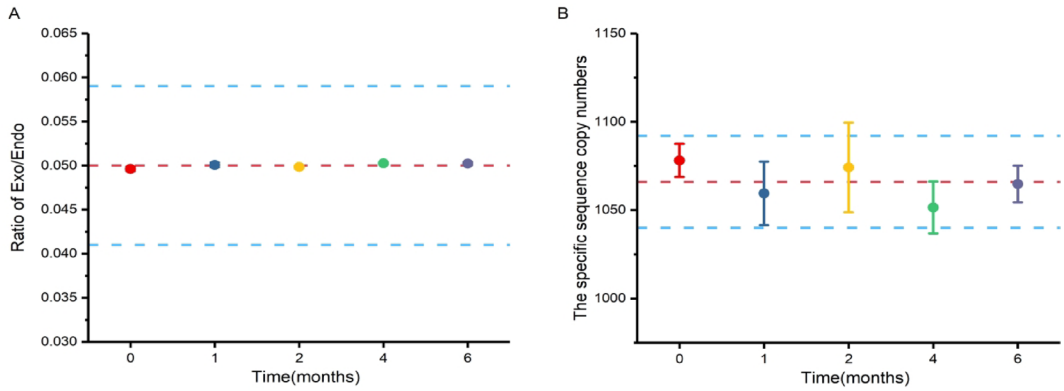

Fig. 3. Long-term stability of the candidate CRM at − 20 °C. Dashed lines indicate the certified value (red) and range of the standard uncertainty (blue).

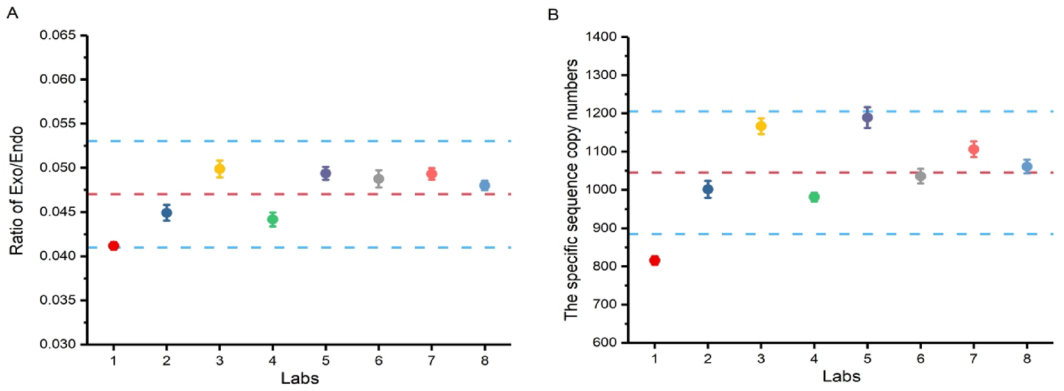

Fig. 4. Absolutely quantified Zhonghuang 6106 content of CRMs by eight laboratories. The dashed lines indicate the certified value (red) and range of the standard uncertainty (blue).

as follows: $Y=\sqrt{n}\left[\frac{\sum\left[\left(\frac{n+1}{2}-K\right)\left(X_{n+1-K}-X_k\right)\right]}{n^2\sqrt{m_2}}-0.28209479\right]/0.02998598=-0.216$. This value falls within the critical interval of the D'Agostino-Pearson test, indicating that the measurement data are normally distributed. Therefore, the data collected from the laboratories can be considered to follow a normal distribution.

Given that each group of experimental data was obtained using the same method (digital PCR), it is assumed to have equal precision. Under this assumption, a Cochran test was used to verify the absence of any suspicious data sets among the eight groups of data, which are presumed to have the same accuracy. The Cochran test statistic was calculated as follows: $C=\frac{{s_{max}}^2}{\sum_{i=1}^{m}{s_i}^2}=\frac{0.0034^2}{0.0027^2+0.0023^2+0.0028^2+0.0024^2+0.0019^2+0.0034^2+0.0017^2+0.0014^2}$ =0.2442. The critical value for the Cochran test at a 0.05 significance level with 8 groups and 16 degrees of freedom is C(0.05,8,16)=0.2462. Since C < C(0.05,8,16), no outlying data sets were identified, confirming that all 8 data sets are of equal accuracy.

The total uncertainty of the CRMs' value is composed of three parts. The first part is the combined relative uncertainty $u_{rel(char)}$ arising from both Class A and Class B uncertainties associated with the collaborative valuation process of the CRMs. The second part is the standard uncertainty $u_{rel(bb)}$ caused by material homogeneity. The third part is the standard uncertainty $u_{rel(sts)}$ and $u_{rel(lts)}$ caused by substance instability during the shelf life.

The relative uncertainty introduced during the collaborative valuation process of the CRMs is synthesized as follows: $u_{rel(char)}=\sqrt{u_{rel(A)}^2+u_{rel(B)}^2}$ (Table 2).

The synthetic standard uncertainty of the copy number concentration of the transformer-specific sequence is calculated as follows: $u_{rel(CRM)}=\sqrt{u_{rel(char)}^2+u_{rel(bb)}^2+u_{rel(sts)}^2+u_{rel(lts)}^2}=\sqrt{0.059^2+0.016^2+0.049^2+0.013^2}=0.079$. The relative uncertainty was: $u=C\cdot u_{rel(CRM)}=1.04\times10^3\times0.079=0.082\times10^3$. Its extanded uncertainty was: $U=ku=2\times0.082\times10^3=0.16\times10^3$ (k=2, confidence level 95%). Therefore, the value and uncertainty of the copy number concentration of the transformer-specific sequence are: (1.04±0.16) $\times10^3$ copies • μL^{-1}.

Targets	Mean	$u_{rel(A)}$	$u_{rel(B)}$	$u_{rel(char)}$
Copy numbers of Zhonghuang6106	1.04×10^3	0.040	0.043	0.059
Exogenous/endogenous copy number ratio	0.047	0.026	0.043	0.050

Table 2. Uncertainties brought by the process of CRMs cooperative value.

The synthetic standard uncertainty of the copy number ratio of Zhonghuang 6106 to Lectin is calculated as follows: $u_{rel(CRM)} = \sqrt{0.050^2 + 0.0036^2 + 0.022^2 + 0.0045^2} = 0.055$. The absolute standard uncertainty was: $u = 0.047 \times 0.060 = 0.003$, Its extanded uncertainty was: $U = 2 \times 0.003 = 0.006$ ($k = 2$, confidence level 95%). Therefore, the value and uncertainty of the copy number ratio of Zhonghuang 6106 to Lectin are: 0.047±0.006.

Discussion

Currently, international genomic DNA reference materials are primarily produced by the American Oil Chemists' Society (AOCS) and are supplied as purified products[18]. For instance, the genomic DNA reference material for non-GM soybean is designated AOCS 0707-A2, with an assigned transgenic content of < 0.10 ng·μg^{-1}. The genomic DNA reference material for GM soybean event A2704-12 is numbered AOCS 0707-B3, with an assigned transgenic content of > 999.9 ng·μg^{-1}. The transgene content assigned to AOCS genomic DNA reference materials is determined primarily by the limit of detection, which reflects the sensitivity of the detection method[19,20].

When used for the quantitative detection of GM products, genomic DNA reference materials typically provide two key quantitative parameters: the transgenic content and the DNA copy number concentration value. Consequently, the genomic DNA reference material described in this study is characterized by these two values.

The European Union's Joint Research Centre (JRC) has developed a comprehensive suite of standard materials for GMO testing, encompassing more than twenty events with different content levels. For instance, the Bt-176 maize genomic DNA standard material includes seven transgenic test standards with content levels of 0%, 0.1%, 0.5%, 1%, 2%, 5%, and 100%. Similarly, the Bt-11 maize genomic DNA reference material comprises five transgenic detection standards with content levels of 0%, 0.1%, 1%, 10%, and 100%[21]. In most catalogues of standard materials from the JRC and AOCS, the assigned values are established mainly by qPCR. Compared to qPCR, dPCR offers greater sensitivity[22–24] and higher tolerance to inhibitors in samples[25,26]. This is particularly true for food samples that have undergone processing methods such as baking, frying, and microwaving, where dPCR is less affected and delivers more robust and accurate results than qPCR[27]. Hence, dPCR has greater application potential.

Currently, the detection methods for Zhonghuang 6106 rely primarily on conventional PCR and qPCR. This study is the first to apply droplet dPCR to Zhonghuang 6106, bridging the methodological gap for this transgenic line. Moreover, the development of its certified reference material represents a global first. Although commercial cultivation of Zhonghuang 6106 has not yet begun, the results will advance the commercialization of biotech breeding, align China's GM soybeans with international standards, and promote harmonized detection protocols worldwide.

While this study centres on the 5% (w/w) reference material, developing a series of gravimetrically certified standards covering a wider concentration range is essential to broaden the regulatory scope and improve the accuracy and precision of monitoring. Work on additional concentrations is already under way. Our next goal is to establish a complete family of Zhonghuang 6106 genomic DNA reference materials, creating a robust analytical framework for detecting this GM soybean in food and feed matrices. The methodological framework developed here can be directly transferred to any GM crop with a fully characterised genome, eliminating redundant method-development efforts. Because the study operates at the molecular level, its outputs will also support refuge management and resistance stewardship strategies as these crops move toward commercial release, thereby minimising their environmental footprint.

Methods

Preparation of the plant materials

The seeds of Zhonghuang 6106 soybean were provided by the Institute of Crop Sciences, Chinese Academy of Agricultural Sciences. Three hundred healthy, plump, and uniform seeds were individually sown in seedling trays and cultivated in a controlled environment chamber until they reached the V3 growth stage. Leaves of uniform developmental stage and position were then harvested from each plant, immediately flash-frozen in liquid nitrogen, and stored at − 80 °C for subsequent analyses.

The grinding process was performed using a Retsch MM 400 grinding system. Approximately 100 mg of each leaf was placed into a 5-cm-diameter stainless-steel grinding jar, pre-cooled in liquid nitrogen for 2–3 min, and then ground at 20 Hz for 90 s. During grinding the jars remained tightly sealed to prevent cross-contamination. The resulting powder was then transferred to sterile 1.5 mL microcentrifuge tubes and stored at −70 °C until genomic DNA extraction.

Zhonghuang 10, used as the recipient for genetic transformation, was provided by the Institute of Crop Sciences, Chinese Academy of Agricultural Sciences. Genomic DNA was extracted from this line using the same protocol as for Zhonghuang 6106.

Target Genes	Sequences (5'–3')	Amplicon Size (bp)
Lectin	QF: CACCTTTCTCGCACCAATTGACA QR: TCAAACTCAACAGCGACGAC QP: HEX- CCACAAACACATGCAGGTTATCTTGG -TAMRA	102
Zhonghuang6106	ZH2-5-QF3: ACCTTCTGGCTCCTTCAAACAC ZH2-5-QR2: CTAGAGCAGCTTGAGCTTGGATC ZH2-5-P2: FAM- AACTGAAGGCGGGAAACGACAAT -BHQ1	80

Table 3. Primers and probes used for the Zhonghuang 6106 soybean analysis.

Extraction and evaluation of genomic DNA

The Plant Maxi Kit (DNeasy, Qiagen, Düsseldorf, Germany) was employed to isolate high-quality nucleic acids. The integrity of the genomic DNA was assessed using 1% agarose gel electrophoresis. Genomic DNA integrity was assessed by 1% (w/v) agarose gel electrophoresis, and purity was evaluated by UV absorbance at 230, 260 and 280 nm with a NanoDrop™ 1000 spectrophotometer (Thermo Fisher, Wilmington, DE, USA). Concentrations were determined fluorometrically using a Quantus™ Fluorometer (Promega, Madison, WI, USA). DNA was considered sufficiently pure when (i) electrophoresis revealed a single, high-molecular-weight band without smearing, and (ii) absorbance ratios were 1.8–2.0 for A_{260}/A_{280} and > 2.0 for A_{260}/A_{230}. Samples failing to meet these criteria were re-extracted.

PCR test

The exogenous gene sequence of Zhonghuang 6106 was provided by the Institute of Crop Science, Chinese Academy of Agricultural Sciences. It is inserted into chromosome 17 of soybean, at a site containing a complete expression cassette with two copies of the *G2-EPSPS* gene and one copy of the *GAT* gene. The exogenous gene fragments are connected by soybean genomic DNA segments. Based on the 5' flanking sequence of the exogenous insert, primers and probes for Zhonghuang 6106 were designed using Primer Express 3.0 software. The primers and probe specific to the soybean taxon were adopted from established methods[28,29]. For fluorescent labelling, FAM and HEX were used as 5′ reporter dyes, while BHQ1 and TAMRA were employed as 3′ quenchers (Table 3). The primers and probes were synthesized by Sangon Biotech (Shanghai, China).

The dPCR reaction mixture consisted of 10 µL of 2×dPCR Supermix for Probes (Bio-Rad, Hercules, CA, USA), 1 µL of each primer (final concentration 0.5 µM), 1 µL of probe (final concentration 0.25 µM), 2 µL of DNA template, and 5 µL nuclease-free water, in a total volume of 20 µL. Amplification was performed at a constant ramp rate of 2.0 °C·s^{-1}, with an initial denaturation at 95 °C for 5 min, followed by 45 cycles of 95 °C for 30 s and 60 °C for 1 min, and a final enzyme deactivation step at 98 °C for 10 min. Fluorescent signals from the amplified droplets were read on a QX100 Droplet Reader (Bio-Rad) and analyzed using QuantaSoft version1.7.4.917.

Homogeneity assessment

Fifteen tubes were randomly selected from 300 samples of transgenic soybean Zhonghuang 6106 genomic DNA and individually labeled. Three subsamples were withdrawn from each tube-top, middle, and bottom—and the copy numbers of the target sequence, the internal standard gene, and their ratio were measured for each subsample using dPCR. Systematic differences among subsamples were assessed by one-way analysis of variance (ANOVA, *F*-test). Homogeneity was confirmed when the F-ratio did not exceed the critical value.

The homogeneity assessment of gDNA CRMs was conducted based on their characteristic values, while the uncertainty evaluation was performed in accordance with ISO Guide 35:2017.

Stability assessment

Short-term stability assessment: To evaluate the short-term stability of the gDNA CRMs–which may be compromised by temperature fluctuations during transport–we selected four temperature conditions: −20 °C, 4 °C, 25 °C, and 37 °C. For each temperature condition, three vials were randomly selected at six time points (0, 1, 3, 5, 7, and 10 days); each vial was analysed in triplicate ($N = 3$, $n = 3$). The short-term stability was assessed by dPCR quantification of the target and internal-standard gene sequences.

Long-term stability assessment: To simulate real-world storage conditions, the long-term stability of the gDNA CRMs was evaluated at −20 °C. At each of five time points (0, 1, 2, 4 and 6 months), three vials were randomly selected and each vial was analysed in triplicate ($N = 3$, $n = 3$). The copy numbers of the transgene-specific sequence and the internal-standard gene were determined by dPCR at every sampling time.

The short-term and long-term stability of the gDNA CRMs were assessed using their characteristic values, and the associated uncertainties were evaluated in accordance with ISO Guide 35:2017.

Collaborative validation of laboratories

The dPCR characterization of the gDNA CRMs for Zhonghuang 6106 was conducted in eight qualified GMO detection laboratories, each recognized as an authoritative nucleic acid quantitative measurement facility in China. These laboratories received aliquots from the same batch, shipped under low-temperature conditions to ensure they remained frozen upon arrival. A standardized technical protocol was employed to ensure operational consistency across all sites, yielding eight independent data sets. Raw dPCR results were then exported and statistically analysed by the CRM provider in accordance with ISO Guide 35:2017.

Data availability

No datasets were generated or analysed during the current study.

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Author contributions

Y.W. and Q.L. conceptualization, X.Z. methodology, X.Q. software, C.W. and N.L. validation, R.L. and X.Q. formal analysis, Y.W. and Q.L. investigation, X.Z. resources, C.W. writing—original draft preparation, R.L. and

X.Z. writing—review and editing, X.Q. visualization, Y.W. supervision, All authors reviewed the manuscript.

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The authors declare no competing interests.

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