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# A Real-time Fluorescent Quantitative PCR Method for Detection of Genetically Modified Maize MON88017

**Jun SONG** *, **Dong WANG**

Analytical and Testing Center, Sichuan Academy of Agricultural Sciences, Chengdu 610066, China

**Abstract** In order to improve the standardized technical system of quantitative analyses for genetically modified organisms (GMOs) and protect China's bio-safety and reduce ecological risk, we establish a quantitative detection method for the genetically modified (GM) maize MON88017 using real-time fluorescent quantitative PCR. Meanwhile, the method is evaluated by several methodological indicators such as specificity, sensitivity, accuracy and uncertainty of measurement. The results show that the method has strong specificity in analysis of genetically modified maize MON88017. The mean value (1.54%) repeatedly measured for 29 times with the relative deviation of 2.7% was close to the real value (1.50%) and the variation coefficient of the measured value was 0.1. The tested recovery rate is 100% and the uncertainty of measurement is 0.096. 5 copies of the MON88017 molecular fragment can be detected at 97.5% confidence level. Consequently, the quantitative detection method established in this paper for the GM maize MON88017 has fairly high specificity, accuracy and sensitivity and this technology established in this paper can provide good technical support for the safety supervision of genetically modified organisms in China.

**Key words** GM maize MON88017, Quantitative detection method, Evaluation method

## 1 Introduction

With the large-scale cultivation of genetically modified crops, the safety of genetically modified plant itself and its potential threat to human health and environment has become drawn the widespread public concern[1]. In recent years, China's rice exported to the EU has been found to exceed the threshold content of genetically modified components provided by the EU[2]. In the international trade of agricultural products, limited by the trade barriers set up by the importing country using quantitative detection technology for genetically modified ingredients, seriously affecting the export of agricultural products in China.

Therefore, the effective, rapid, accurate and quantitative analysis of genetically modified components in agricultural products has become an urgent problem to be solved at present. Up to now, China has promulgated 101 GMO safety testing technical standards, including 41 agricultural genetically modified component testing standards, 39 environmental safety testing technical standards and 21 commodity inspection industry technical standards for GMO detection. The establishment of quantitative analysis technology for related genetically modified organisms and products is particularly urgent.

Based on DNA content of agricultural products, QRT-PCR (Quantitative Real Time Polymerase Chain Reaction) is now the main method to quantitatively analyze the content of genetically modified components in agricultural products using the ratio of copies of specific genetically modified components to copies of standard genes within species. In addition to the use of a pair of chemically synthetic specific oligonucleotide polymer molecules, the QRT-PCR method[3-7] adds a specific oligonucleotide polymer molecule marked with fluorescent chemical groups matching DNA template to the PCR reaction system. In the PCR reaction, the PCR reaction product is continuously accumulated, and the fluorescence signal intensity is also proportionally increased.

When the fluorescence signal exceeds the set threshold value, the fluorescent signal can be detected. In the logarithmic product growth period, there is a linear relationship between the $C_t$ (Cycle of Threshold) of each DNA template and the logarithm of starting copy number of this template. Using the known standard matrix material of starting copy number or standard plasmid molecule, the standard curve can be drawn.

By importing the $C_t$ value obtained from the unknown samples through analytical instrument into the standard curve, we can calculate the starting copy number of endogenous gene and genetically modified components in this sample, and the calculated percentage of target nucleic acid copy number is just the relative content of genetically modified components. MON88017, developed by Monsanto, is the genetically modified maize with insect resistance and herbicide tolerance characteristics.

In 2011, EFSA (European Food Safety Authority) published a risk assessment report on genetically modified maize MON88017 in the European market, and considered that MON88017 was unlikely to have adverse effects on the environment except for the resistance evolution of Cry3Bb1 protein contained in maize to coleopteran pests[8].

In late 2013, China approved the import of genetically modified maize MON88017 as raw processing material, and the importing was valid until December 31, 2016. Currently, there have been relatively sophisticated qualitative methods for the detection of MON88017[9-10], but the quantitative analysis method is sel-

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* Corresponding author. E-mail: scnuwd@163.com

dom reported[10].

According to the flanking DNA sequence inserted by MON88017 foreign gene into the receptor maize genome, this paper establishes a quantitative analysis method with high sensitivity for genetically modified maize MON88017 and its derivatives, in order to provide an accurate quantitative analysis technique for the quantitative analysis of China's genetically modified organisms and products.

## 2 Materials and methods

**2.1 Materials** 100% genetically modified maize MON88017 powder was used for standard curve preparation; 1.5% genetically modified maize MON88017 powder (100% genetically modified maize MON88017 powder and 0% genetically modified maize powder were evenly prepared in this laboratory based on mass percentage of 1.5%) was used as test sample.

**2.2 Methods**

**2.2.1** Reagents and instruments. The core reagents of the experiment included genomic DNA isolation and purification reagent and probe type (TaqMan hydrolysis probe) real-time polymerase chain reaction reagent (Real Time Polymerase Chain Reaction (PCR) Master Mix), purchased from TIANGEN TianGen Biochemical Technology (Beijing) Co., Ltd.

The key instruments used in the experiment included ultra trace spectrophotometer (Nanodrop 1000, Thermo Fisher Scientific Corporation) and real-time fluorescence PCR system (Real Time PCR System 7500, Applied Biosystem Corporation, USA), which were used to test the quality of genomic DNA isolated and purified from genetically modified maize MON88017, amplify maize zSS Ⅱ b endogenous gene and MON88017 transformation event DNA fragment (Sequence of Transformation Event MON88017).

**2.2.2** Isolation and purification of DNA. 0.1 g of 100% genetically modified maize MON88017 powder and 1.5% genetically modified maize MON88017 powder were weighed, respectively. According to the instructions of plant genomic DNA purification kit [TIANGEN, TIANGEN TianGen Biochemical Technology (Beijing) Co., Ltd.], the genomic DNA of genetically modified maize MON88017 was isolated and purified.

**2.2.3 Primer and probe design** In accordance with national standards GB19495.4-2004[11], the primers and probes were synthesized and the maize internal standard gene zSSⅡb was amplified.

According to the flanking sequence of genetically modified maize MON88017 (GI:397146042), the Primer Express3.0 (Applera Corporation, US) software was used to design genetic crossing expression regulation element (CaMV35S) and specific primer and probe of maize genome (Table 1), and amplify MON88017 molecular fragment.

zSS II b and MON88017 primer and probe designed in this test were chemically synthesized by Sangon Biotech. The synthesis purity was HPLC grade, and probe 5' and 3' ends were labeled FAM (6-carboxy-fluorescein) and TAMRA (6-carboxy-tetramethylrhodamine), respectively.

**Table 1 The primers and probes designed in this study**

| Gene | The primer and probe sequences | Product size (bp, base pairs) |
|---|---|---|
| zSS II b | 5'-CTCCCAATCCTTTGACATCTGC-3' | |
| | 5'-TCGATTTCTCTCTTGGTGACAGG-3' | |
| | 5'-FAM-AGCAAAGTCAGAGCGCTGCAATGCA-TAMRA-3' | 151 bp |
| MON88017 molecular fragment | 5'-CGCTAGCAGCTCTCCTCCAA-3' | |
| | 5'-CCGGACATGAAGCCATTTACA-3' | |
| | 5'-FAM- CTTTTTTGCCGGAGTATGACGGTGACG-TAMARA-3' | 97 bp |

**2.2.4** Standard curve preparation and reaction system. The genomic DNA of 100% MON88017 was diluted into 5 concentration gradients in accordance with 1: 5: 100, 20, 4, 0.8, 0.16 ng/μL; the genomic DNA concentration of 1.5% MON88017 was diluted to 50 ng/μL. 3 μL of the above DNA dilution was added to the 25 μL reaction system: TaqMan Master Mix (2×) 12.5 μL, upstream and downstream primer (10 μmoL/L) 1 μl, probe (10 μmoL/L) 0.5 μL, replenishment to 25 μL.

Three parallel reactions were made for each concentration gradient DNA dilution and 29 times repeated analysis was conducted on the test sample (1.5%). The prepared reaction system was placed on the 7500 fluorescent quantitative PCR instrument, and the following program was run: first running 95° denaturation DNA template, 10 min; then running 45 thermal cycles (95° denaturation, 15 s; 59° annealing, 60 s.

**2.2.5** Method specificity, sensitivity, precision and uncertainty of measurement. To test the specificity of the established method, except that the designed primers and probes were compared in the databases (EMBL, GenBank, Patent, *etc.*), the designed primers and probes were also used to detect genetically modified maize TC1507, NK603, MON863, MON810, 3272 powder samples, as well as genetically modified soybean, cotton, rape, sugar beet seed powder samples so as to determine the specificity of the method.

According to the maize gene size ($2.5 \times 10^9$ base pairs), it was calculated that 3 μL of 100% MON88017 maize DNA solution (50 ng/μL) contained 50000 MON88017 molecular fragments, and after 1:10 gradient dilution for 4 times, 5 gradient copies of MON88017 molecular fragment were 50000, 5000, 500, 50, 5, respectively. With the DNA of 5 gradient copies as template, the real-time amplification was conducted to determine the sensitivity of the method.

The sample containing 1.5% genetically modified components was repeatedly detected for 29 times, and then coefficient of varia-

tion and recovery rate of these measurement data were calculated to evaluate the precision of the method. The coefficient of variation and recovery rate were calculated as follows:

coefficient of variation ($CV$) = standard deviation ($S$)/arithmetic average ($X$)

recovery rate (%) = $C_1/C_2 \times 100\%$

where $C_1$ is the average measured value of genetically modified sample components and $C_2$ is the true value of genetically modified sample components.

The uncertainty of measurement of this experiment was calculated as follows:

$$U = 2 \times \sqrt{0.0481^2 + (0.1829 \times C)^2}$$ [12],

where $U$ is the expanded uncertainty; $C$ is the measured value of sample.

## 3 Results and analysis

**3.1 Method specificity and fitted standard curve** The primers and probes designed in this experiment were used for DNA analysis of genetically modified maize MON88017, TC1507, NK603, MON863, MON810, 3272 powder samples, as well as genetically modified soybean, cotton, rape, sugar beet seed powder samples (Fig. 1), and only the signal of MON88017 could be detected, indicating that the primers and probes of MON88017 molecular fragment designed for quantitative analysis had very strong specificity for MON88017.

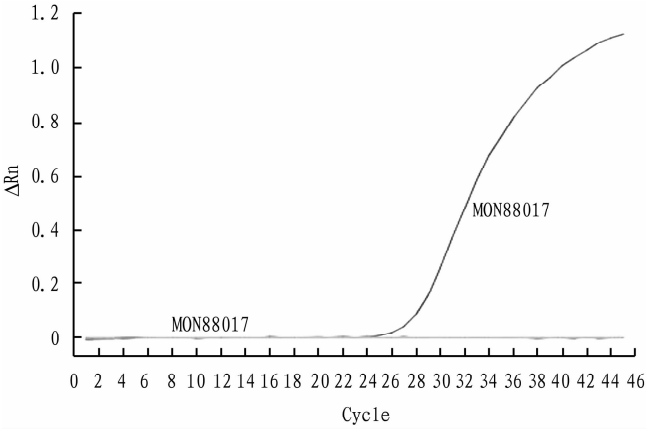


**Fig. 1 Method specificity**

According to the least square method and 7500 software (Applied Biosystem Corporation, USA), the standard curve was fitted (Fig. 2-3). The standard curve equation of genetically modified maize MON88017 was $Y = -3.25X + 32.63$ (Fig. 2), the correlation coefficient $R^2$ was 0.999, and the amplification efficiency was 103.18%. The standard curve equation of maize endogenous gene zSS II b was $Y = -3.28X + 31.63$ (Fig. 3), the correlation coefficient $R^2$ was 0.997, and the amplification efficiency was 99.73%.

The results showed that the amplification efficiency of endogenous gene (zSS II b) and exogenous gene (59122 molecular fragment) in this test was greater than 90%, and the correlation coefficient $R^2$ was ≥98%, indicating that the amplification efficiency of the primers and probes designed in this test was high, and there was a good linear relationship among the established standard curve equation dataset.

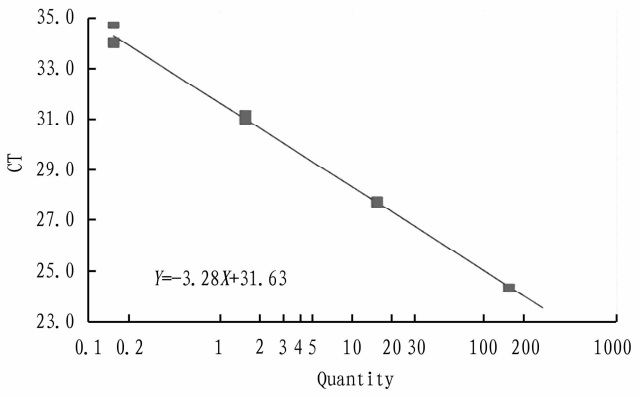


**Fig. 2 zSS II b gene standard curve and standard curve equation**

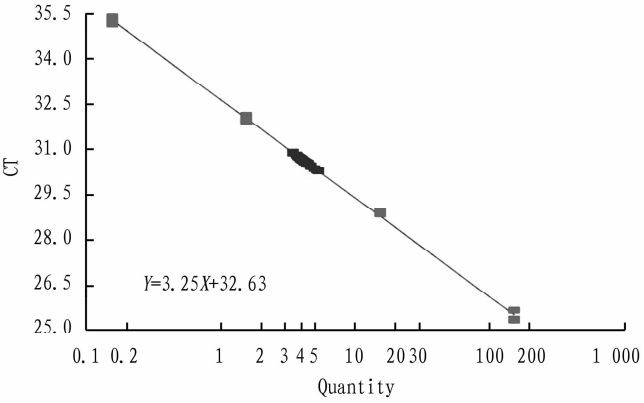


**Fig. 3 Standard curve of MON88017 molecular fragment and standard curve equation**

**3.2 Method sensitivity and precision** 50000, 5000, 500, 50, 5 copies MON88017 molecular fragments were used as templates, and the real-time analysis results showed that this method could detect 5 copies MON88017 molecular fragment (Fig. 4 and Table 2). To verify the reliability of genetically modified maize MON88017 quantitative LOD, this study conducted 40 times repeated detection on 5 copies MON88017 molecular fragments, and

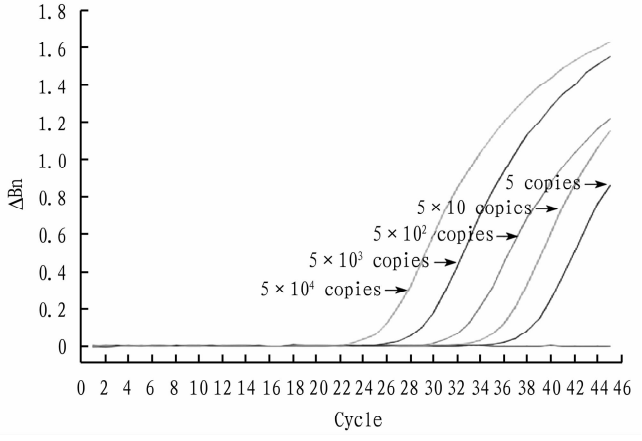


**Fig. 4 Limit of detection of 5 copies MON88017 molecular fragments**

the results showed that 39 times could detect 5 copies MON88017 molecular fragment (Table 3).

Thus, the experimental results showed that at the 97.5% confidence level, the quantitative detection method limit of genetically modified maize MON88017 in this paper was 5 copies. The method established in this test was used to detect the test sample containing 1.50% MON88017 for 29 times, and the results showed that in the test sample, the relative content of MON88017 was 1.24% – 1.86%, and the average relative content was 1.54% (Table 4), close to the real content of 1.50%.

The relative deviation between the measured value and the true value was 2.7%. The coefficient of variation of 29 times measurement results was 0.1104 and the recovery rate was 100%, indicating that this analysis method was of good accuracy and reproducibility.

**Table 2 Method sensitivity analysis results**

| Copy number | $C_t$ values | | | Average $C_t$ values |
|---|---|---|---|---|
| | 1 | 2 | 3 | |
| $5 \times 10^4$ | 26.13 | 26.04 | 25.91 | 26.03 |
| $5 \times 10^3$ | 29.13 | 29.12 | 29.16 | 29.13 |
| $5 \times 10^2$ | 32.88 | 32.68 | 32.63 | 32.73 |
| $5 \times 10^1$ | 35.46 | 35.87 | 35.91 | 35.75 |
| $5 \times 10^0$ | 38.56 | 38.53 | 39.45 | 38.85 |

**Table 3 Ct values for limit of detection of the new method at 97.5% confidence level**

| Repeated test | $C_t$ values |
|---|---|
| 1 | 38.30135 |
| 2 | 38.22563 |
| 3 | 39.14856 |
| 4 | 36.62408 |
| 5 | 39.20158 |
| 6 | 38.10036 |
| 7 | 39.35188 |
| 8 | 37.86245 |
| 9 | 37.77774 |
| 10 | 36.87487 |
| 11 | 39.18285 |
| 12 | 39.14905 |
| 13 | 35.56659 |
| 14 | 36.16182 |
| 15 | 37.10299 |
| 16 | 37.29623 |
| 17 | 37.90170 |
| 18 | 38.01934 |
| 19 | 36.72259 |
| 20 | 37.18439 |
| 21 | 38.80519 |
| 22 | 36.95868 |
| 23 | 39.82681 |
| 24 | 37.97231 |
| 25 | 40.14629 |
| 26 | 37.59507 |
| 27 | 39.12237 |
| 28 | 37.78530 |
| 29 | 36.65046 |
| 30 | 37.22565 |
| 31 | 39.26849 |
| 32 | 38.33007 |
| 33 | 38.21866 |

(to be continued)

(continued)

| Repeated test | $C_t$ values |
|---|---|
| 34 | 37.19111 |
| 35 | 38.58676 |
| 36 | 37.08962 |
| 37 | Undetermined |
| 38 | 38.14819 |
| 39 | 39.12046 |
| 40 | 36.91719 |
| 16 | 38.30135 |
| 17 | 38.22563 |
| 18 | 39.14856 |
| 19 | 36.62408 |
| 20 | 39.20158 |
| 21 | 38.10036 |
| 22 | 39.35188 |
| 23 | 37.86245 |
| 24 | 37.77774 |
| 25 | 36.87487 |
| 26 | 39.18285 |
| 27 | 39.14905 |
| 28 | 35.56659 |
| 29 | 36.16182 |
| 30 | 37.10299 |
| 31 | 37.29623 |
| 32 | 37.90170 |
| 33 | 38.01934 |
| 34 | 36.72259 |
| 35 | 37.18439 |
| 36 | 38.80519 |
| 37 | 36.95868 |
| 38 | 39.82681 |
| 39 | 37.97231 |
| 40 | 40.14629 |
| Mean | 37.97156 |

**Table 4 Quantitative detection results of MON88017 event specific fragment in testing samples** (1.50%)

| Number of test repetitions | zSS II b $C_t$ values | zSS II b absolute content//ng | MON88017 fragment $C_t$ values | MON88017 fragment absolute content//ng | MON88017 fragment relative content//% |
|---|---|---|---|---|---|
| 1 | 23.249 | 368.538 | 30.430 | 6.562 | 1.781 |
| 2 | 23.248 | 368.803 | 30.502 | 6.234 | 1.690 |
| 3 | 23.214 | 377.623 | 30.553 | 6.011 | 1.592 |
| 4 | 23.267 | 363.979 | 30.823 | 4.957 | 1.362 |
| 5 | 23.213 | 377.975 | 30.553 | 6.011 | 1.590 |
| 6 | 23.230 | 373.341 | 30.351 | 6.945 | 1.860 |
| 7 | 23.076 | 415.349 | 30.262 | 7.402 | 1.782 |
| 8 | 23.317 | 351.593 | 30.594 | 5.838 | 1.660 |
| 9 | 23.177 | 387.479 | 30.389 | 6.759 | 1.744 |
| 10 | 23.023 | 430.686 | 30.291 | 7.247 | 1.683 |
| 11 | 22.958 | 450.601 | 30.486 | 6.305 | 1.399 |
| 12 | 22.929 | 459.639 | 30.428 | 6.572 | 1.430 |
| 13 | 23.000 | 437.726 | 30.426 | 6.581 | 1.504 |
| 14 | 23.003 | 436.880 | 30.388 | 6.762 | 1.548 |
| 15 | 23.075 | 415.690 | 30.465 | 6.400 | 1.540 |
| 16 | 22.938 | 456.690 | 30.643 | 5.638 | 1.235 |
| 17 | 23.011 | 434.372 | 30.385 | 6.778 | 1.560 |
| 18 | 22.936 | 457.397 | 30.604 | 5.797 | 1.267 |
| 19 | 23.177 | 387.282 | 30.614 | 5.754 | 1.486 |
| 20 | 23.184 | 385.553 | 30.424 | 6.592 | 1.710 |
| 21 | 23.059 | 420.262 | 30.487 | 6.303 | 1.500 |
| 22 | 22.999 | 437.852 | 30.463 | 6.411 | 1.464 |
| 23 | 23.197 | 382.083 | 30.668 | 5.538 | 1.449 |
| 24 | 23.360 | 341.313 | 30.562 | 5.973 | 1.750 |
| 25 | 23.198 | 381.709 | 30.654 | 5.592 | 1.465 |
| 26 | 23.034 | 427.590 | 30.710 | 5.374 | 1.257 |
| 27 | 23.179 | 386.772 | 30.606 | 5.789 | 1.497 |
| 28 | 23.351 | 343.594 | 30.911 | 4.656 | 1.355 |
| 29 | 23.249 | 368.538 | 30.430 | 6.562 | 1.781 |
| Mean | 23.129 | 402.085 | 30.524 | 6.171 | 1.541 |

**3.3 Uncertainty of measurement** The uncertainty of measurement is calculated as follows:

$$U = 2 \times \sqrt{0.0481^2 \times (0.1829 \times C)^2}$$

It was calculated that the uncertainty of measurement of MON88017 by 29 times quantitative analysis was 0.096, less than 10%, and the measurement value distribution range was narrow [(1.54 ± 0.096)%], indicating that the quantitative analysis method could well detect the genetically modified maize MON88017.

## 4 Discussions

China currently implements zero tolerance qualitative identification system on the genetically modified agricultural products, and this identification system lacks sufficient scientificity, which is not conducive to the healthy industrial development of genetically modified organisms. European Union (identification threshold of 0.9%), Japan (5%) and South Korea (3%) adopt a quantitative labeling system[13-14], and only when the GMO content in the product is higher than the threshold value will there be a need to identify the GMO content.

The fluorescent quantitative PCR method is mainly used for the international quantitative analysis of genetically modified components. Based on the quantitative PCR principle, this paper designed the primers and probes for quantitative analysis of genetically modified maize MON88017, and established a real-time quantitative PCR detection method with high sensitivity for genetically modified maize MON88017. From the analysis of practice, this paper also used the designed primers and probes to further analyze other genetically modified maize strains resistant to herbicide and insect, but did not detect the MON88017 molecular fragment, fully demonstrating that the quantitative analysis method for genetically modified maize MON88017 had high specificity.

In practice, the PCR amplification efficiency was in the range of 90% to 110%, the average value of corresponding standard curve slope was −3.6 to −3.1, and the $C_t$ value of test sample was in the range of 18 to 30 cycles, so that the reliable measurement results could be obtained[15]. Using the quantitative PCR detection method for genetically modified maize MON88017, the slope of the standard curve prepared was −3.57, the amplification efficiency was 90.56% and the correlation coefficient $R^2$ was 0.983. The detection results (1.54%) obtained by this standard curve were close to the true value (1.50%) of MON88017 content

in sample.

The relative deviation (2.7%) between the measured value and the true value was less than 10%, and the recovery rate of test sample was 100%, fully suggesting that the measurement results of the present method had high accuracy and good reproducibility. At the high confidence level (97.5%), the method established in this paper could detect 5 copies MON88017 molecular fragments, with the sensitivity much higher than that of 17 to 30 copies reported by Yuan Lei *et al.*[10], indicating that this method had high sensitivity.

Theoretically, 1 copy DNA molecule could be detected by PCR, but in practice, due to low uniformity of DNA macromolecule solution, trace liquid transfer and other problems, 5 copies MON88017 molecular fragments could not be detected at the 100% confidence level in the experiment. By repeated measurements of the same sample 29 times, the measured value was 1.24% to 1.86%, and the coefficient of variation was 0.1, showing that the measurement by this method had high precision.

The uncertainty of measurement is a parameter associated with measurement results, for characterizing the dispersibility of the measured values[16]. The uncertainty of measurement (U) was 0.096, less than 10%, and the measured value was 1.34% to 1.64%, indicating that the detection quality of this method was high. Therefore, the real-time quantitative PCR analysis method for genetically modified maize MON88017 was a high sensitivity quantitative analysis technique for genetically modified organisms and products, which could provide good technical support for the safety supervision of genetically modified organisms in China.

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