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Detection of transgenic rice line TT51-1 in processed foods using conventional PCR, real-time PCR, and droplet digital PCR

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ABSTRACT

To assess the effects of food processing on the detection and quantification of transgenic rice TT51-1 in processed food by polymerase chain reaction (PCR) technology, we monitored the presence of TT51-1 components in rice crackers at different processing stages using conventional PCR, quantitative real-time PCR (qPCR), and droplet digital PCR (ddPCR) with standard or validated primers and probes. In conventional PCR, relatively longer amplification targets, such as the *Bacillus thuringiensis* (Bt) gene (301 bp) and the event-specific target (274 bp), were barely detected in baked, fried or microwaved samples. In qPCR, the amplification fluorescence signal was detected in boiled, dried, baked, and microwaved samples, but barely observed in fried samples. Conventional PCR with the same primers used in qPCR detected the corresponding shorter targets in all samples. The conventional PCR results were mainly consistent with the results of qPCR. The results indicate that food processing directly affects the detection of transgenic components, and suggest that relatively shorter fragments should be selected as the amplification targets for this type of analysis. We established qPCR and duplex ddPCR methods for quantifying TT51-1. The results of an orthogonal experiment indicated that the optimal conditions for TT51-1/PLD duplex ddPCR were 500/250 nM of primers/probe combined with 58 °C annealing temperature. Both methods were feasible for quantitative detection of TT51-1 in processed samples, with duplex ddPCR being a more attractive method for detecting transgenic components in processed food due to its stability, accuracy, PCR inhibitor resistance, and the lack of a need for reference materials.

1. Introduction

In recent years, the number of genetically modified (GM) crops has rapidly increased. According to The International Service for the Acquisition of Agri-biotech Applications (ISAAA, 2017), the global area of GM crops increased from 11.0 million hectares in 1997 to 189.8 million hectares in 2017. Currently, the dominant cultivated GM crops are soybean, maize, cotton, and canola. Rice is one of the most important crops, but the development of GM rice has not been as rapid as for the above four crops. At present, only a few herbicide-resistant or insect-resistant events in GM rice have been created for commercial application. Herbicide-resistant rice varieties LL62, LL06, and LL601 are approved for release in the United States and Canada (Babekova, Funk, Pecoraro, Engel, & Busch, 2009), and a type of insect-resistant rice is only allowed for semi-commercial planting in Iran (James, 2007).

China mainly grows rice and accounts for approximately 20% of the global rice production (Wang, Chen, Xu, Dai, & Shen, 2015). Extensive research toward the development of GM rice has been performed in China over the past 20 years. Various GM rice strains with new traits have been studied, including those with insect-, herbicide-, and disease-resistance; improved nutrient values; drought and salt resistance; and other traits (Bajaj & Mohanty, 2005). Despite expanding research on GM rice, no GM rice has yet been approved for commercial planting in China due to concerns regarding the potential biosafety and environmental risks, as well as various ethical concerns. Among all of the researched transgenic rice strains, TT51-1 with insect resistance is the most well-known event. For this transgenic rice, a hybrid Cry1Ab/Ac

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gene was introduced into the rice genome of Minghui 63 using a biolistic method and regulated by the rice actin 1 gene promoter and nopaline synthase gene (*nos*) terminator (Tu et al., 1998). Previous studies demonstrated that TT51-1 meets the insect-resistance requirements for commercial application (Tu et al., 2000). The Chinese government has issued biosafety certificates for TT51-1 (Wang et al., 2015). The US Food and Drug Administration (FDA) identified no safety or regulatory issues with food derived from TT51-1, and considers TT51-1 as safe as conventional rice varieties (FDA, 2017). TT51-1 thus shows great potential for rice production and may be entered into the food chain.

DNA-based detection of GM components in food, especially highly processed food, such as by PCR technology, remains challenging. Currently, conventional PCR and quantitative real-time PCR (qPCR) are widely used for detecting and quantifying GM ingredients in processed foods (Engel, Moreano, Ehlert, & Busch, 2006). Processed food undergoes several physical treatments, chemical changes, and/or biologic reactions, factors that could affect DNA degradation or extraction, which affect the detection of GM components by PCR-based methods (Fumière, Dubois, Baeten, Holst, & Berben, 2006). We previously reported that thermal processes, especially severe thermal treatment, directly affect the detection of GM components in three transgenic rice strains by conventional PCR (Wang et al., 2015). In some samples treated with high temperatures, such as baking at 200 °C for 30 min, detection of the target segment was difficult. Costa et al. used conventional PCR to monitor transgenic soybeans during the process of refining soybean oil, and failed to detect the transgenic components in fully refined soybean oil (Costa, Mafra, Amaral, & Oliveira, 2010). Fernandes et al. utilized conventional PCR and qPCR to assess the degradation, amplification, and quantification of GM DNA in the process of making various breads. They observed that DNA was degraded in all parts of different bread samples, the target DNA was easily detected in all samples, and that, while the quantification of GMO was feasible, the quantities were considerably underestimated in some samples (Fernandes, Oliveira, & Mafra, 2013). Considering the limits of conventional PCR and qPCR for detecting and quantifying GM components in food, digital PCR (dPCR), a novel PCR technology, has been utilized to monitor the presence of GM components in food (Demeke, Holigroski, Eng, & Xing, 2016; Morisset, Štebih, Milavec, Gruden, & Žel, 2013; Zhu et al., 2016). For dPCR, the PCR mixture first undergoes limiting dilution and is distributed into a very large number of independent partitions such that each partition ideally contains 0 or 1 copies of the target DNA, and then after end-point PCR amplification, the absolute copies of the target DNA in the original sample are calculated using Poisson statistics. dPCR is considered a powerful alternative method for identifying and quantifying GM components in food.

In this study, we evaluated the ability of current PCR technologies, including conventional PCR, qPCR, and droplet dPCR (ddPCR), to detect TT51-1 in processed food, and assessed the effects of different food processing methods on these tests (study overview is provided in Fig. S1). A traditional rice food, rice crackers, with 5% or 1% TT51-1 was made, and samples obtained at different processing stages were tested. Moreover, standard or validated PCR methods are often used in routine testing, so all the primers and probes used in the three PCR methods were derived from standard or validated methods.

2. Material and methods

2.1. Materials

TT51-1 seed flour was kindly provided by Shanghai Jiaotong University (Shanghai, China). Minghui 63 seeds were collected by our laboratory (Hangzhou, China). Sucrose, potato starch, and salt were purchased from a local market (Hangzhou, China). All materials except for the TT51-1 samples were checked by PCR to ensure that none of the items contained any transgenic components.

2.2. Preparation of rice crackers

Rice flour containing 5% or 1% (w/w) of the TT51-1 event was generated by mixing TT51-1 seed flour with Minghui 63 seed flour in a DFT-100 mill (Linda Machinery Co., LTD, Zhejiang, China). The rice crackers were produced according to previous reports (Keeratipibul, Luangsakul, & Lertsatchayarn, 2008; Song et al., 2011) with some modifications as follows: 99 g rice flour (containing 5% or 1% the TT51-1 event) was mixed with 1 g salt, 2 g sucrose, and 5 g potato starch. After sufficient mixing, the mixture was cooked with the appropriate amount of water in a pressure cooker (170 KPa, 115 °C for 20 min). The cooked dough was cooled and kneaded, and then allowed to stand at room temperature for 2 days. Small circular pieces approximately 3.0 cm in diameter and 0.5 cm thick were cut from the hard cake. The small circular cakes were dried at 80 °C for 5 h, then baked (200 °C, 5 min), fried (180 °C, 2 min), or microwaved (700 W, 1 min) to produce the final rice crackers. Baking, frying, and microwaving were performed independently, and not in succession. In this study, samples were collected after the boiling, drying, baking, frying, or microwaving steps.

2.3. DNA extraction

The cetyltrimethylammonium bromide (CTAB) method was used to extract DNA from all samples according to a previous report (Mäde, Degner, & Grohmann, 2006) with some modifications. For each sample (200 mg), 0.8 mL lysis buffer I (117 g/L NaCl, 20 g/L CTAB, 0.2 M Tris–HCl, 50 mM EDTA, pH 8.0), 0.4 mL lysis buffer II (50 g/L sodium N-lauroylsarcosine), and 20 μL proteinase K (50 mg/mL) were added to a 2-mL microcentrifuge tube; for the fried samples, an extra 0.5 mL of hexane was added. The samples were incubated at 65 °C for 1 h with occasional stirring. The samples were centrifuged at 12 000 g for 20 min and the supernatant was collected in a new microcentrifuge tube, and then an equal volume of phenol/chloroform (1:1) was added, followed by gentle blending and centrifugation at 12 000 g for 15 min. The upper phase was then transferred into a new microcentrifuge tube and mixed with an equal volume of chloroform/isoamylalcohol (24:1). Following centrifugation (12 000 g for 10 min), the supernatant was transferred into a new microcentrifuge tube. This step was repeated 2–3 times until the supernatant was clean and clear. The supernatant was combined with 0.6 vol of isopropanol and 0.1 vol of acetic acid (3 M, pH 5.2) and incubated at −20 °C for 1 h. The mixture was centrifuged at 12 000 g for 10 min. The supernatant was discarded and the pellet washed with 800 μL ethanol (70% v/v). After centrifugation, the supernatant was carefully discarded by pipetting, the pellet was dried, and the DNA was dissolved in 100 μL of 0.1 × Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 7.0). All the extracts were kept at −20 °C until further analysis.

The quantity and purity of the extracts were assessed by measuring the absorbance at 260 and 280 nm in a spectrophotometer (Ultrospec 1100 Pro; GE Healthcare, USA). The purity of the extracted DNA was evaluated using the absorbance ratio of 260 and 280 nm (A_{260nm}/A_{280nm}), and the DNA concentration was determined by measuring the absorbance at 260 nm (1 absorbance unit corresponds to 50 μg/mL of dsDNA). The integrity of the extracted DNA was analyzed by electrophoresis in a 1.0% agarose gel carried out in 1 × Tris-borate-EDTA buffer for 40 min at 120 V and stained with GelRed solution.

The DNA purity and yields of all samples extracted using the CTAB method are shown in Table S1, and the results of agarose gel electrophoresis of all extracted DNA are presented in Fig. S2.

2.4. Primers and probes

The detection strategies and schematic diagram of TT51-1 are presented in Fig. 1. There are currently four different PCR detection approaches – screening, gene-specific, construct-specific, and event-specific approaches – for monitoring TT51-1. Conventional PCR and qPCR

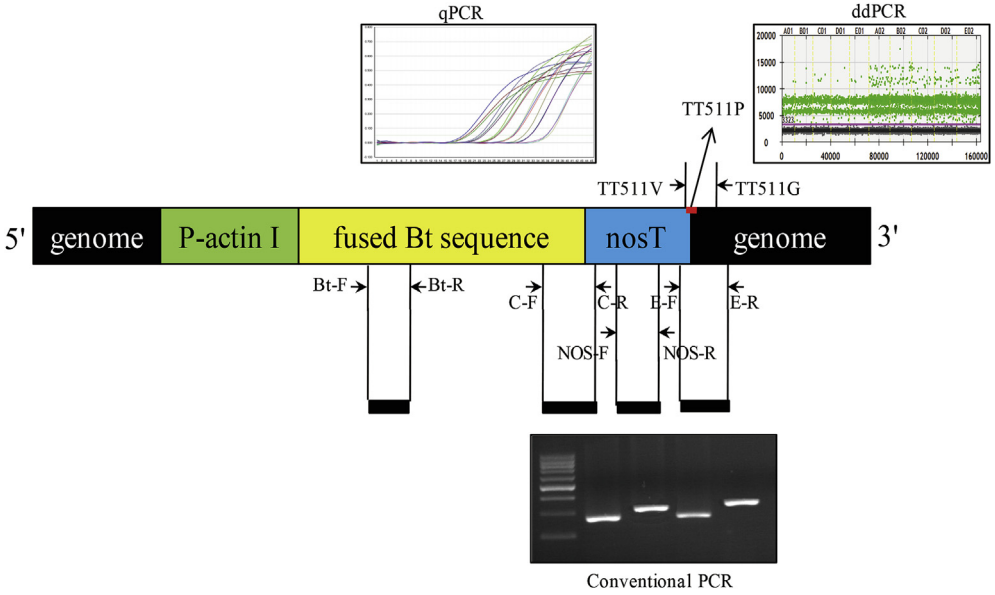

Fig. 1. Schematic diagram of the strategy for detecting TT51-1 in processed foods with conventional PCR, qPCR, and duplex ddPCR.

Table 1
Primers and probes used in the study.

Target	Name	Sequence (5–3)	Amplicon/bp	Ref.
Qualitative detection using conventional PCR				
Rice *SPS* gene	SPS-F	TTGCGCCTGAACGGATAT	277	Jiang et al. (2009)
	SPS-R	GGAGAAGCACTGGACGAGG		
T-nos	NOS-F	GAATCCTGTTGCCGGTCTTG	180	Jin et al. (2007)
	NOS-R	TTATCCTAGTTTGCGCGCTA		
Fused Bt	Bt-F	GAAGGTTTGAGCAATCTCTAC	301	Jin et al. (2007)
	Bt-R	CGATCAGCCTAGTAAGGTCGT		
Bt sequence/T-nos	C-F	GCAGGAGTGATTATCGACAGATTC	147	Mäde et al. (2006)
	C-R	AAGACCGGCAACAGGATTCA		
pFHBT1/rice genome	E-F	AGCAGAACTTTAACCCCCGAA	274	Lu et al. (2009)
	E-R	AGAGCCTCGTTGGATTTCTTACAT		
Qualitative detection using qPCR				
Rice *SPS* gene	SPS-2F	TTGCGCCTGAACGGATAT	81	Jin et al. (2007)
	SPS-2R	CGGTTGATCTTTTCGGGATG		
	SPS-P [a]	TCCGAGCCGTCCGTGCGTC		
T-nos	NOS-2F	CATGTAATGCATGACGTTATTTATG	84	Waiblinger, Ernst, Anderson, and Pietsch (2008)
	NOS-2R	TTGTTTTCTATCGCGTATTAAATGT		
	NOS-P [a]	ATGGGTTTTTATGATTAGAGTCCCGCAA		
Fused Bt	Bt-2F	GGGAAATGCGTATTCAATTCAAC	73	Jin et al. (2007)
	Bt-2R	TTCTGGACTGCGAACAATGG		
	Bt-P [a]	ACATGAACAGCGCCTTGACCACAGC		
pFHBT1/rice genome	TT511V	AGAGACTGGTGATTTCAGCGGG	120	Gang et al. (2010)
	TT511G	GCGTCCAGAAGGAAAAGGAATA		
	TT511P[b]	ATCTGCCCCAGCACTCGTCCG		
Quantitative detection using qPCR and ddPCR				
PLD gene	KVM159	TGGTGAGCGTTTTGCAGTCT	64	Gang et al. (2010)
	KVM160	CTGATCCACTAGCAGGAGGTCC		
	TM013[a]	TGTTGTGCTGCCAATGTGGCCTG		
pFHBT1/rice genome	TT511V	AGAGACTGGTGATTTCAGCGGG	120	Gang et al. (2010)
	TT511G	GCGTCCAGAAGGAAAAGGAATA		
	TT511P[b]	ATCTGCCCCAGCACTCGTCCG		

[a] The probe was labeled with 5-carboxyfluorescein (FAM) and 3-black hole quencher (BHQ1).
[b] The probe was labeled with 5-hexachlorofluorescein (HEX) and BHQ1.

were used for qualitative detection of TT51-1 based on different elements of TT51-1. For quantitative detection of TT51-1, primers and probes located at integration junction regions were used for event-specific detection of the TT51-1 event using qPCR and ddPCR. For qualitative detection, the rice *sucrose-phosphate synthase* (*SPS*) gene was used as the endogenous reference gene, and for quantitative detection, the *phospholipase D family* (*PLD*) gene was used as the endogenous reference gene. All the primers and probes were synthesized by Invitrogen Biotechnology Co., Ltd. (Shanghai, China) according to previous reports (Ding et al., 2004; Gang et al., 2010; Jiang et al., 2009; Jin et al., 2007; Lu et al., 2009; Mäde et al., 2006). Detailed information of all primers and probes is provided in Table 1.

2.5. Conventional PCR

Conventional PCR was performed in a 25-μL volume with 10 × PCR buffer, 200 μM dNTP, 0.5 μM of each primer, 1.25 U Taq DNA polymerase (TaKaRa Biotechnology Co.), and 50–100 ng DNA template. All

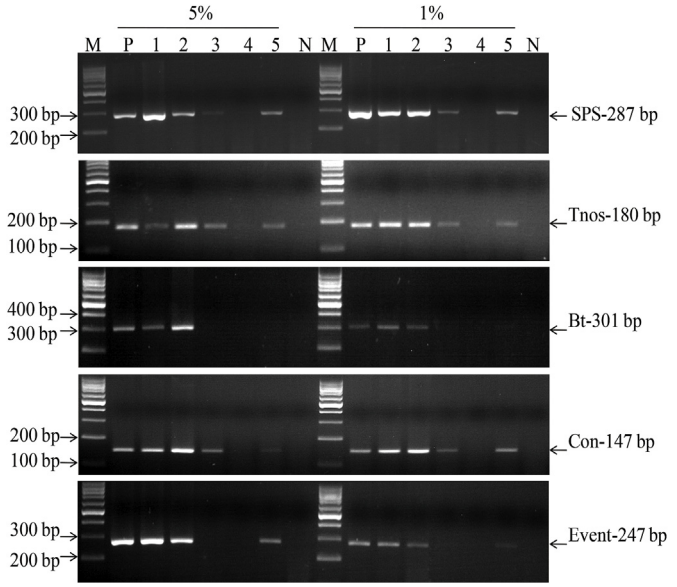

Fig. 2. Conventional PCR detected the TT51-1 event at the 5% and 1% level in processed foods with different target locations of the event. Lane M, 100-bp ladder; lane P, untreated; lane N, blank control; lane 1, boiled; 2, dried; 3, baked; 4, fried; and 5, microwaved.

PCR programs comprised a 95 °C/5 min denaturation step, followed by 35 cycles of 94 °C/30 s, 58 °C/30 s, and 72 °C/30 s, with a final elongation step of 72 °C/7 min. To confirm the sequence of each target, the conventional PCR amplicon was purified and sequenced by Sangon Biotech (Shanghai, China). For agarose gel electrophoresis analysis, 8 μL of each PCR reaction mixture was run on a 2% gel in Tris-borate-EDTA buffer (45 mM Tris, 45 mM borate, 1 mM EDTA, pH 8.0) with GelRed dye at 120 V for 1.5 h. After completing the electrophoresis, the gel was observed under UV light and the results were recorded photographically.

2.6. Real-time PCR

Real-time PCR (25 μL volume) was performed using 12.5 μL 2 × TaqMan Universal Mastermix (Applied Biosystems), 2 μL template (containing 20 ng genomic DNA), 400 nM of each primer, and 200 nM probe. The real-time PCR assays were performed on an ABI 7500 Real-time Detection System (Applied Biosystems) with the following conditions: an initial denaturation step (95 °C/5 min), 45 cycles at 95 °C/10 s and 60 °C/60 s. To establish a standard curve, we used six serially diluted DNAs (47, 4.7, 0.47, 0.047, and 0.0047 ng/reaction) extracted from TT51-1. DNA samples for quantification were tested against the standard curve. The results of each PCR assay were collected and processed using ABI sequence Detection Software version 1.4 (Applied Biosystems). The relative GMO content was calculated as: (mean amount of TT51-1)/(mean amount of PLD gene) × 100.

2.7. Droplet digital PCR

Duplex droplet digital PCR (Duplex ddPCR) was established using a pair of primers for the TT51-1 event, a pair of primers for the endogenous PLD gene, and two probes for the TT51-1 event and PLD gene, respectively. Briefly, the final concentrations of the PLD and TT51-1 primers and probes were added to 10 μL of 2 × ddPCR supermix (Bio-Rad, USA), and the final volume was adjusted with water to 19 μL. Then, 1 μL of template DNA (containing 20 ng genomic DNA) was added. A total of 20 μL of this mixture was placed into a cell of a BioRad DG8™ cartridge and 70 μL of droplet generator oil was added to this well. The cartridge was placed into a QX200 droplet generator (Bio-Rad) to generate the droplets. The droplets were transferred to a 96-well PCR plate. After heat-sealing with a foil seal, the PCR plate was placed in a 7500 Real-time PCR system and amplified with the following cycling conditions: 95 °C for 10 min, 40 cycles of 94 °C for 10 s and the designated temperature (55, 58, 60, 62, 64, or 68 °C) for 60 s for annealing and extension, 10 min at 98 °C for reaction termination, and cooled to 4 °C. Following amplification, the plate was placed into the QX200 droplet reader (Bio-Rad) for data analysis. PLD with the 5-carboxyfluorescein (FAM) signal was monitored in channel 1, and TT51-1 with the 5-hexachlorofluorescein (HEX) signal was measured in channel 2. Only samples with a minimum of 10 000 droplets were accepted and considered applicable for subsequent analysis. The copy number of each target was provided by the Quantasoft software (version 1.6.6.0320, Bio-Rad) using Poisson statistics. The relative GMO content was calculated as: (mean copy number of TT51-1)/(mean copy number of PLD gene) × 100.

2.8. Optimization of duplex droplet digital PCR

An orthogonal experiment was performed to select the optimal concentration of the primer/probe for the TT51-1 event and PLD gene, and the optimal reaction temperature for the duplex ddPCR. Four reactions (I-IV) with different final concentrations of primer/probe for the TT51-1 event and PLD gene were performed at different annealing temperatures (55, 58, 60, 62, 64, and 68 °C). DNA extracts (10 ng) from 100% TT51-1 material were prepared for each assay. The different concentration ratios of the primer/probe were 125/125 nM (I), 250/250 nM (II), 500/250 nM (III), and 500/500 nM (IV) for the TT51-1 event and PLD gene, respectively. More detailed information of the four reaction systems are provided in Table S2. The optimal experimental conditions for the primer and probe concentrations and annealing temperature were defined as those with the highest amplification efficiency and the highest droplet separation.

3. Results and discussion

3.1. Qualitative detection by conventional PCR and qPCR

Fig. 2 shows the results of conventional PCR, amplifying the rice endogenous *SPS* gene and the different target locations in the TT51-1 event, in various processed foods containing 5% and 1% transgenic rice TT51-1. For all assays, the expected PCR amplification bands were observed in all untreated samples, and not found in any of the blank controls, validating the conventional PCR system. PCR amplification detected the endogenous *SPS* gene in processed food samples containing 5% and 1% transgenic rice TT51-1 that were boiled, dried, bakd, and/or microwaved, but not in the fried samples. For the *nos* gene and construct-specific detection, the expected 180-bp fragments of the *nos* gene and 147-bp fragments of the construct-specific target were detected in the boiled, dried, baked, and microwaved samples, but not in the fried samples. For the *Bacillus thuringiensis* (Bt) gene, the expected 301-bp fragments were only observed in the boiled and dried samples. For event-specific detection of TT51-1, the 274-bp fragments were detected in boiled, dried, and microwaved samples, but not in the baked or fried samples.

Fig. 3 shows the results of qualitative detection of TT51-1 by qPCR. For the *SPS* gene (81 bp), *nos* gene (84 bp) and event-specific target (120 bp), the amplification curves were observed in the boiled, dried, baked, and microwaved samples, but not in the fried samples. For the Bt gene (73 bp), the amplification curves were observed in all treated samples, including boiled, dried, baked, microwaved, and fried samples.

The detection results indicated that the qPCR methods were more suitable than standard or validated conventional PCR for qualitative detection of TT51-1 in processed food. When using conventional PCR methods to monitor TT51-1 in highly processed food, it is important to

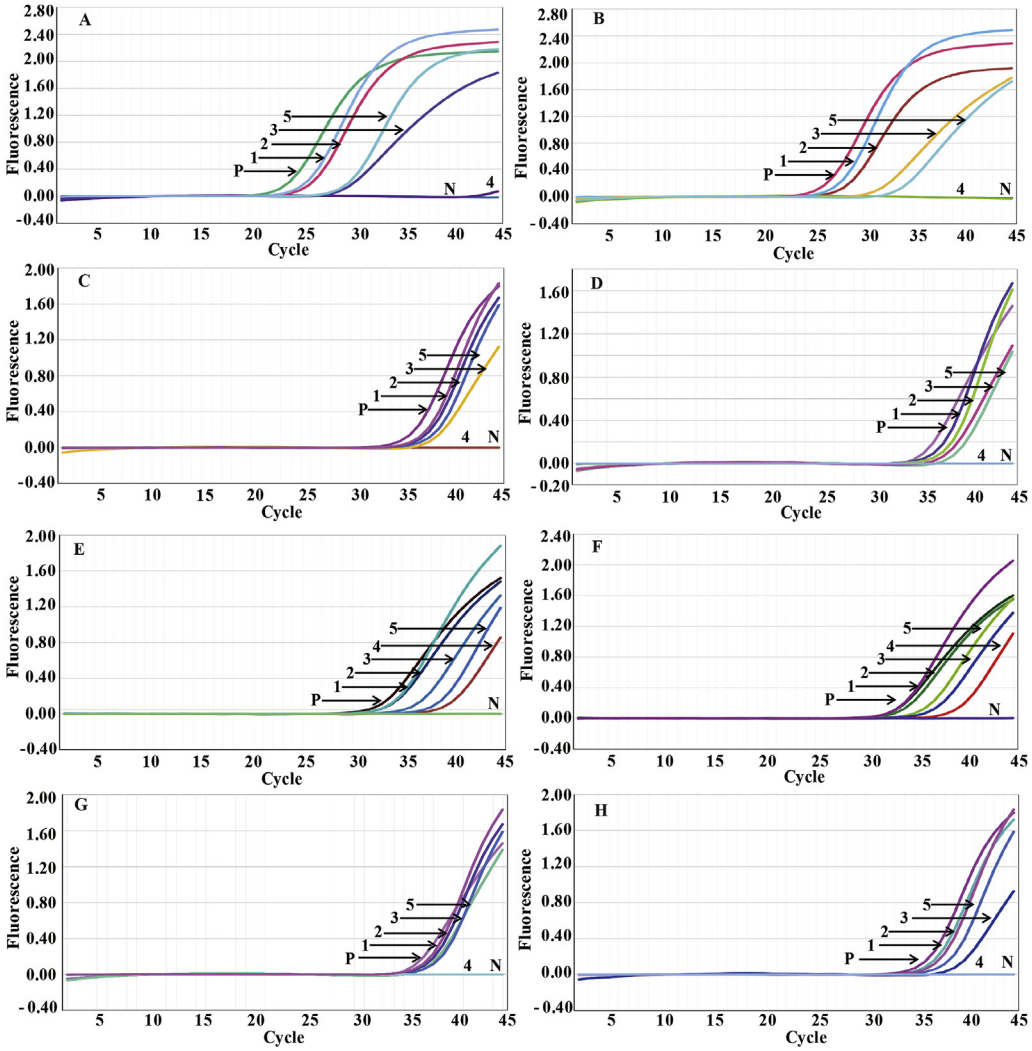

Fig. 3. qPCR detected the TT51-1 event at the 5% and 1% level in processed foods with different target locations of the event. (A), 5% samples using SPS (81 bp); (B),1% samples using SPS (81 bp); (C), 5% samples using the *nos* gene (84 bp); (D), 1% samples using the *nos* gene (84 bp); (E), 5% samples using the Bt gene (73 bp); (F), 1% samples using the Bt gene (73 bp); (G), 5% samples using the event-specific target region (120 bp); (H), 1% samples using the event-specific target region (120 bp). P, untreated; N, blank control; 1, boiled; 2, dried; 3, baked; 4, fried; and 5, microwaved.

be aware of the possibility of false-negative results, especially when using long DNA fragments as detection targets, such as the Bt gene (301 bp) and the event-specific target (274 bp). The qPCR methods may be better for detecting TT51-1 in processed food for two reasons. First, the qPCR methods introduced TaqMan probes into amplification systems, making fluorescence signal detection more specific and sensitive. Second, the detection targets of the qPCR methods are relatively shorter than those of conventional PCR methods. Smaller DNA fragments in processed food are more stable than longer fragments. Therefore, we used conventional PCR with the same primers used in qPCR to detect corresponding shorter targets in all samples. These conventional PCR results were mainly consistent with the results of qPCR. For SPS (81 bp), *nos* gene (84 bp), and the event-specific target region (120 bp), the corresponding amplicons were observed in the boiled, dried, baked, and microwaved samples, but not in the fried samples (Fig. S3). With the Bt gene (73 bp), the expected amplification bands were obtained in all treated samples, but the bands were faint in fried samples (Fig. S3). These results indicated that shorter fragments are more stable and could be more easily detected in processed food, consistent with previous reports (Gryson, 2010; Wang, X., Chen, Xu, Dai, & Shen, 2015; Yoshimura et al., 2005). Therefore, for detection of TT51-1 in processed foods using standard or validated PCR methods, we suggest using the qPCR methods. In laboratories unable to perform qPCR, the primers used in qPCR methods could be used in conventional PCR to detect shorter fragments.

Two other factors might also contribute to the poor performance of conventional PCR and qPCR on the fried samples. One is that DNA is seriously damaged during the frying process (see the results of agarose gel electrophoresis of DNA extracted from fried samples, Fig. S2), making it difficult to amplify the DNA fragments from fried samples, as reported previously (Costa et al., 2010; Gryson et al., 2002; Song et al., 2011). The second potential factor is poor DNA extraction efficiency from the fried samples because DNA, particularly short DNA fragments, was lost during the extraction process and the fried samples likely contained high levels of PCR inhibitors. Previous studies reported that it is difficult to extract high-quality DNA from samples containing oil or other substances that are co-extracted with the DNA and inhibit PCR (Arun, Yilmaz, & Muratoglu, 2013; Elsanhoty, Ramadan, & Jany, 2011; Kakihara, Matsufuji, Chino, & Yamagata, 2007). Further, detection of the event-specific target region (120 bp) using ddPCR also failed in the fried samples (data not shown), so subsequent quantitative analysis based on the event-specific target (120 bp) using qPCR and ddPCR was not conducted on the fried samples.

Based on the above results and previous reports, we propose the following recommendations for the detection of GM ingredients in processed products based on PCR methods. First, relatively shorter

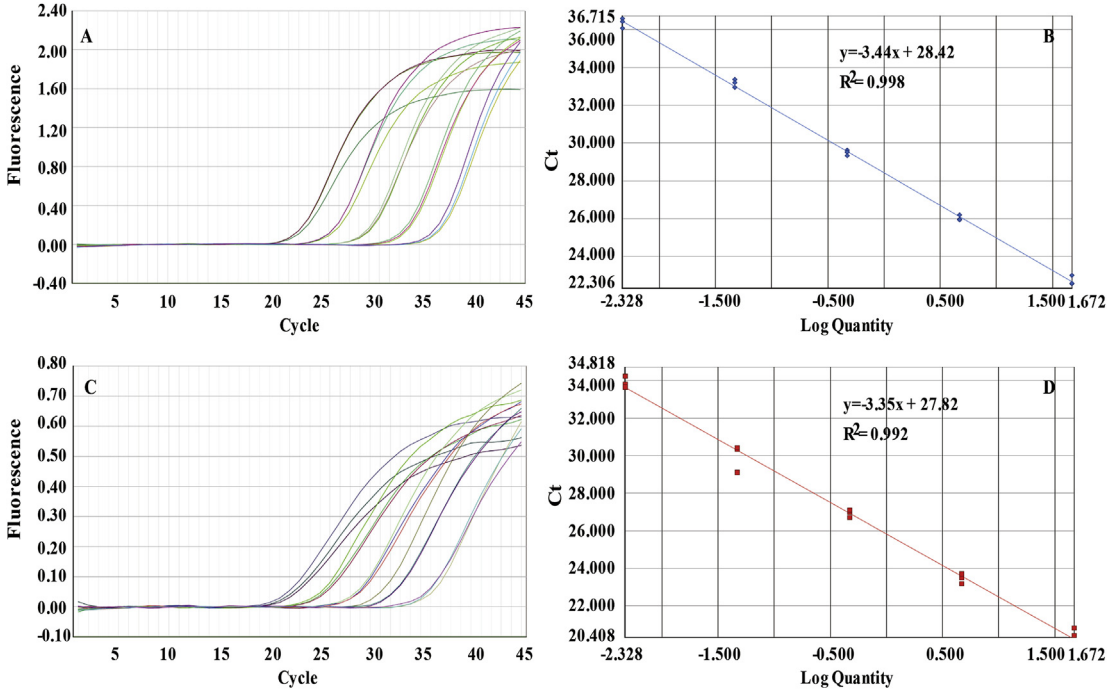

Fig. 4. Amplification and standard curves for the event-specific quantitative PCR method using gradient-diluted TT51-1 genomic DNA as the template. Amplification graph for the TT51-1 event-specific assay (A). Standard curve for the TT51-1 event-specific assay (B). Amplification graph for the PLD gene assay (C). Standard curve for the PLD gene assay (D). The rice genome in each dilution was 47, 4.7, 0.47, 0.047, and 0.0047 ng/reaction, respectively.

fragments should be selected as the amplification targets because they are more stable than the longer fragments in processed products. In addition, according to our previous report, the amplification efficiency of the selected target should also be evaluated. Moreover, appropriate and efficient DNA extraction methods should be developed to deal with complex substances and processed products to obtain high-quality DNA.

3.2. Establishment of qPCR for quantitative detection

Fig. 4 A–D shows the amplification plots and standard curves of the TT51-1 events and endogenous PLD gene. According to the slope of each standard curve derived from the TT51-1 genomic DNA dilution series, the PCR efficiency of the TT51-1 event-specific and endogenous PLD quantitative systems was 95.29% and 98.84%, respectively. The squared correlation coefficient (R^2) values of the endogenous TT51-1 events and PLD gene were 0.998 and 0.992, respectively. The template quantity correlated with the threshold cycle (Ct) values at $R^2 > 0.98$, which is the minimal accepted requirement for assay suitability (Marchesi et al., 2015; Taverniers, Van Bockstaele, & De Loose, 2004).

Further, the repeatability of the above real-time PCR system was tested with serial dilutions in three parallel assays, as described above. The relative SD (RSD) values of the real-time PCR for TT51-1 and PLD ranged from 0.50% to 2.32% (Table 2), calculated from three parallel assays. All the above data demonstrated that the quantitative qPCR assays were stable and reliable for quantifying TT51-1 DNA with a template ranging from 0.0047 ng to 47 ng. In this range, as little as 0.023% of TT51-1 DNA could be detected and quantified using a total of 20 ng rice genomic DNA as the template. Thus, the qPCR method completely meets the requirements for quantifying all the samples used in this study containing 5% or 1% TT51-1.

3.3. Establishment of duplex ddPCR

The concentration of primers and probes, and the annealing temperature play a critical role in duplex ddPCR (Dalmira et al., 2016). An orthogonal experiment was performed to select the optimal combination of the concentration of the primers and probe for the TT51-1 event and PLD gene, and the annealing temperature. The optimization results are shown in Fig. 5. Among all four reaction systems (I-IV) with different concentrations of primers/probe for TT51-1 and PLD, compared at the same annealing temperature, the lowest fluorescence amplitude of PLD or TT51-1 was observed in reaction system I with the lowest concentration of primers/probe. This indicates that the amount of primers/probe in reaction system I was insufficient to obtain a high fluorescence amplitude in the TT51-1/PLD duplex ddPCR. In the other reaction systems (II-IV), the fluorescence amplitude of PLD or TT51-1 was enhanced with an increase in the primers/probe concentration, but the fluorescence amplitude was not significantly different between the three reaction systems at the same annealing temperature. In all reaction systems, however, the fluorescence amplitude of both PLD and TT51-1 decreased with an increase in the annealing temperature. Further, according to the copy values of PLD and TT51-1 detected in the duplex ddPCR under different conditions (Table S3), the annealing temperature had the greatest influence on the performance of TT51-1/PLD duplex ddPCR. In reaction system I, even with a low concentration of primers/probe, the final calculated copy values of TT51-1 and PLD were acceptable at a suitable annealing temperature, e.g., 55 °C or 58 °C (Table S3). Further, in all four reaction systems (I-IV), the fluorescence amplitude drastically decreased at higher annealing temperatures, such as at 64 °C or 68 °C. The TT51-1 target was more susceptible to temperature and no positive droplets were detected at some higher annealing temperatures, indicating that the higher annealing temperature was detrimental to PLD, and especially for TT51-1, leading to lower amplification efficiency, lower fluorescence amplitude, and lower resolution, therefore providing an underestimation of the copy number. To obtain higher droplet resolution and available copy number, reaction system III combined with a 58 °C annealing temperature was determined to be the optimal condition for TT51-1/PLD duplex ddPCR. Two-dimensional (2D) plots generated from the TT51-1/PLD duplex ddPCR with reaction system III by amplifying at different annealing temperatures are shown in Fig. 6. According to these results, with

Table 2
The results of qPCR and duplex ddPCR using dilution DNA series (0.0047 ng – 47 ng).

DNA amount (ng)	Target	Ct value of RT-PCR				RT-PCR	Copy value of duplex ddPCR				ddPCR
		Parallel 1	Parallel 2	Parallel 3	Mean of Ct values	RSD(%)	Parallel 1	Parallel 2	Parallel 3	Mean of copy values	RSD(%)
47	TT51-1	23.00	22.56	22.58	22.71	1.10	14564	16985	12789	14779.3	14.25
	PLD	21.91	22.42	22.82	22.38	2.04	15895	17452	12258	15201.7	17.53
4.70	TT51-1	26.20	25.968	25.94	26.04	0.55	2189	1723	1964	1958.7	11.89
	PLD	25.18	25.48	25.70	25.45	1.03	1537	1839	2379	1918.3	22.24
0.47	TT51-1	29.62	29.52	29.33	29.49	0.50	183	159	174	172.0	7.05
	PLD	28.69	29.00	29.11	28.94	0.74	176	187	168	177.0	5.38
0.047	TT51-1	33.36	32.94	33.19	33.17	0.64	22	19	28	23.0	19.92
	PLD	31.11	32.36	32.42	31.96	2.32	19	23	26	22.7	15.49
0.0047	TT51-1	36.58	36.43	36.07	36.36	0.72	5.2	3.9	4.7	4.6	14.25
	PLD	36.24	35.81	35.65	35.90	0.85	4.9	5.9	4.2	5.0	17.09

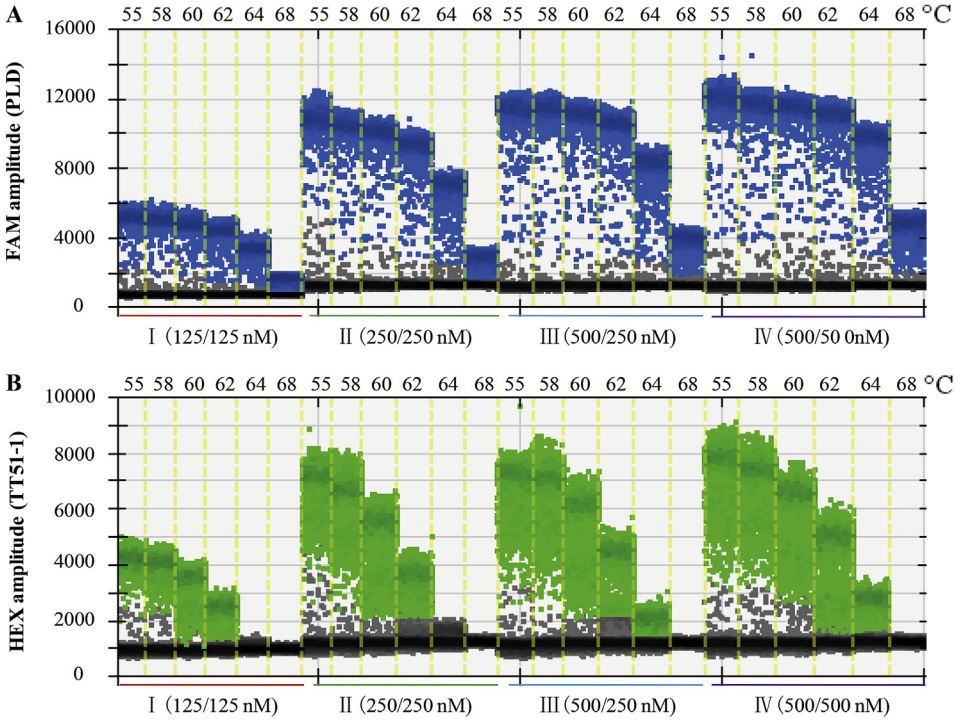

Fig. 5. One-dimensional (1D) plots generated from the optimization of the TT51-1/PLD duplex ddPCR system, with different concentrations of primers/probe (I-IV) at different annealing temperatures (55–68 °C). Droplet colors indicate which target was amplified: PLD (blue), TT51-1 (green), and no amplification (gray). The x-axis shows the fluorescence amplitude corresponding to the FAM fluorophore (PLD, plot A) and the HEX fluorophore (TT51-1, plot B). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

reaction system III and a 58 °C annealing temperature, the TT51-1/PLD duplex ddPCR had the highest droplet resolution, and droplet resolution decreased with an increase in the annealing temperature.

Furthermore, the TT51-1 genomic DNA dilution series (0.0047 ng–47 ng) used in qPCR was employed as templates in the established TT51-1/PLD duplex ddPCR to evaluate its performance range. The copy values of TT51-1 and PLD as well as the RSD values of each target copy value are presented in Table 2. The linear relation of the template amount and copy value for TT51 (R^2: 0.999) and PLD (R^2: 0.999) was generated based on these data (Fig. S4). This indicated that the established TT51-1/PLD duplex ddPCR was also applicable for quantifying the samples containing 5% or 1% TT51-1 used in this study.

3.4. Quantitative detection by qPCR and duplex ddPCR

The qPCR and ddPCR results for event-specific quantitative analysis of diverse processed foods containing 5% or 1% TT51-1 are shown in Table 3. For qPCR detection, the bias was 0.53%–13.67%, and the RSD was 2.25%–6.80%. For ddPCR detection, the bias was 0.67%–9.00%, and the RSD was 1.88%–6.25%. According to the performance requirements for analytical methods for GMO testing (Marchesi et al., 2015), the detection results of both the qPCR and ddPCR were acceptable and accurate, establishing that qPCR and ddPCR are suitable for detecting and quantifying GM ingredients in some processed foods. Compared with qPCR, however, ddPCR was more stable and feasible. First, the range of the bias and RSD of the qPCR was generally larger than those of ddPCR, especially for samples undergoing treatments such as baking. Under this condition, the bias and RSD of the qPCR results for the 5% TT51-1 sample were 13.67% and 6.11%, respectively, and for the 1% TT51-1 sample, 13.00% and 3.98%, respectively. The bias and RSD of the ddPCR results, however, were 5.00% and 4.50% for the 5% TT51-1 sample, respectively, and 9.00% and 3.81% for the 1% TT51-1 sample, respectively. The bias and RSD of qPCR results obtained from the two samples were greater for qPCR than for ddPCR, indicating that, compared with qPCR, ddPCR is more stable and accurate for quantifying the GM ingredients in highly processed products. This is due to the peculiarity of ddPCR, which has tens of thousands of separate droplets with the final value calculated according to the endpoint amplification result from all of the droplets by Poisson distribution (Morisset et al., 2013; Sykes et al., 1992). Therefore, compared with qPCR, the detection results of ddPCR are not as susceptible to amplification efficiency (Iwobi, Gerdes, Busch, & Pecoraro, 2016). Processed products usually contain many PCR inhibitors that affect the efficiency of PCR (Gryson, 2010). Furthermore, when using qPCR for quantitative

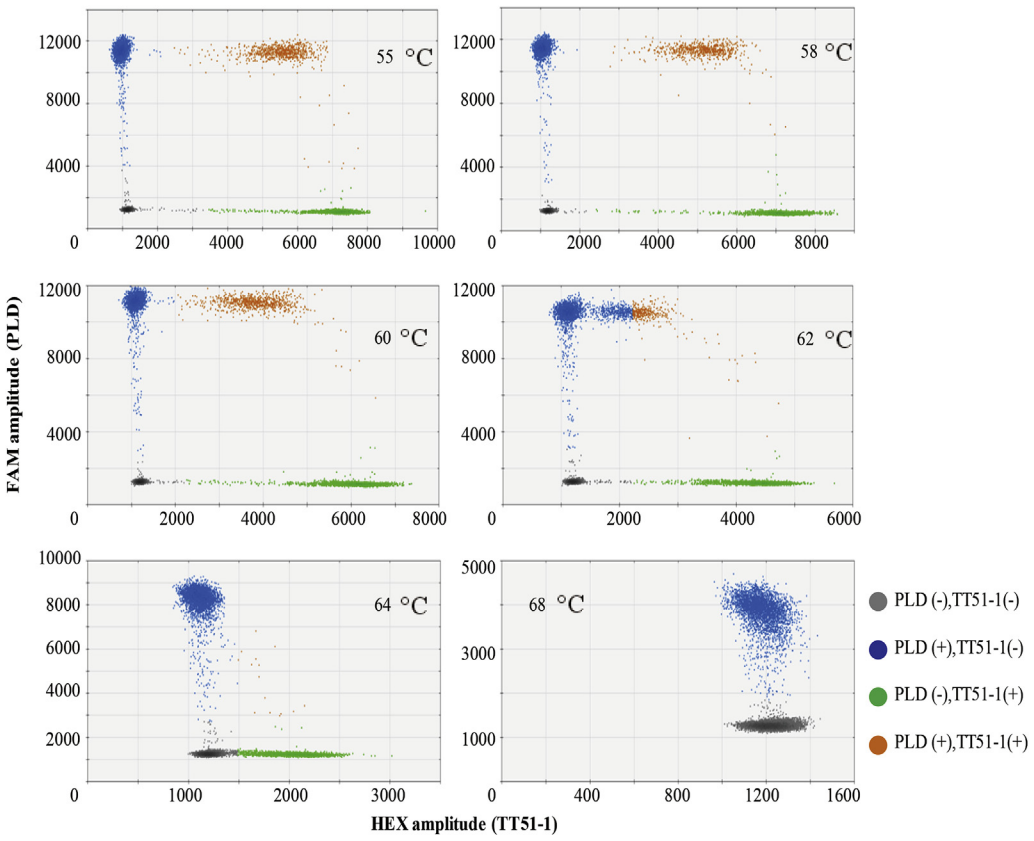

Fig. 6. Two-dimensional (2D) plots created during the optimization of the TT51-1/PLD duplex ddPCR assay. Representative plots for each of the six evaluated temperatures (55–68 °C) are shown. Droplet colors indicate which target was amplified: PLD (blue), TT51-1 (green), neither (gray), or both (orange). The x-axis shows the fluorescence amplitude corresponding to the HEX fluorophore (TT51-1), and the y-axis represents the fluorescence amplitude corresponding to the FAM fluorophore (PLD). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

analysis, reference materials, which are expensive and sometimes difficult to obtain, must be used to establish a standard curve (Debode, Marien, Janssen, & Berben, 2010; Shimizu et al., 2008; Wang, Chen, Xu, Wang, & Shen, 2014). In addition, potential differences between the reference materials and tested samples also affect the accuracy and precision of the final quantitative result (Scholtens et al., 2010; Sivaganesan, Seifring, Varma, Haugland, & Shanks, 2008).

In summary, we compared three PCR methods – conventional PCR, qPCR, and duplex ddPCR – to evaluate their ability to detect GM ingredients in processed foodstuff. Conventional PCR and qPCR are used as predominant screening methods to monitor the presence of GM ingredients in routine analysis. In this study, various targets of TT51-1 combined with rice endogenous genes were used in conventional PCR and qPCR to detect TT51-1 in diverse processed foods with different GM contents. Shorter amplification targets with high amplification efficiency should be used for detecting GM ingredients in highly processed foods using PCR methods. Further, more attention should be focused on developing DNA extraction methods for different processed foods to obtain high-quality DNA for downstream PCR analysis. In addition, qPCR and duplex ddPCR were established and their feasibility for accurate quantitative detection of TT51-1 in all of the samples was compared. The results demonstrated that both methods are feasible for quantitative detection of TT51-1 in processed samples. Compared with qPCR, ddPCR is more stable, accurate, and tolerant to PCR inhibitors. Furthermore, unlike qPCR, ddPCR requires no reference materials for establishing a standard curve to measure the GM content. These features demonstrate that duplex ddPCR can be a powerful tool for identifying and detecting GM gradients in processed food. In this study, an orthogonal assay was also performed to select the optimum system from different combinations of primers/probe concentrations and annealing temperatures. Our results indicate that the annealing temperature plays a critical role in the performance of TT51-1/PLD duplex ddPCR. This optimization strategy for developing duplex ddPCR provides valuable insight for future studies.

Table 3
The results of qPCR and duplex ddPCR event-specific quantitative detection of TT51-1 in various processed samples.

Samples	qPCR detection						Duplex ddPCR detection					
	Three parallels			Mean	Bias (%)	RSD (%)	Three parallels			Mean	Bias(%)	RSD (%)
Untreated (5%)	4.86	4.8	5.42	5.03	0.53	6.80	5.34	4.82	4.94	5.03	0.67	5.41
Boiled (5%)	4.97	4.91	5.23	5.04	0.73	3.38	4.92	4.85	5.41	5.06	1.20	6.03
Dried (5%)	4.93	4.59	4.79	4.77	4.60	3.58	4.87	4.63	5.24	4.91	1.73	6.25
Baked (5%)	4.62	4.19	4.14	4.32	13.67	6.11	4.68	4.99	4.58	4.75	5.00	4.50
Microwaved (5%)	4.81	4.36	4.9	4.69	6.20	6.17	4.76	4.93	4.95	4.88	2.40	2.14
Untreated (1%)	0.96	0.98	1.04	0.99	0.67	4.19	0.98	1.05	1.01	1.01	1.33	3.46
Boiled (1%)	0.93	0.98	0.94	0.95	5.00	2.78	0.96	0.97	0.91	0.95	5.33	3.39
Dried (1%)	0.92	0.95	0.91	0.93	7.33	2.25	0.92	0.97	0.91	0.93	6.67	3.44
Baked (1%)	0.89	0.83	0.89	0.87	13.00	3.98	0.89	0.89	0.95	0.91	9.00	3.81
Microwaved (1%)	0.89	0.95	0.91	0.92	8.33	3.33	0.94	0.91	0.91	0.92	8.00	1.88

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Notes

The authors declare no competing financial interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2018.11.032.

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