

Rapid detection of wheat components in starch products using the Proofman-LMTIA method

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Abstract

For rapid detection of wheat components in starch products, this study developed the analysis method based on the ladder-shape melting temperature isothermal amplification (LMTIA) coupled with proofreading enzyme-mediated probe cleavage (Proofman). With the internal transcribed spacer (ITS) gene of *Triticum aestivum* L. (wheat) as the target, specific primers and the Proofman probe were designed, the reaction temperature was optimized, and the sensitivity and the specificity of the Proofman-LMTIA method were determined. With the results indicating that the optimal reaction temperature of this Proofman-LMTIA method was 65°C, it can specifically detect wheat components rather than seven other species; the sensitivity was 10 fg (femtograms) of the genomic deoxyribonucleic acids (gDNAs) of *Triticum aestivum* L. and the detection limit was 0.1% of the artificially adulterated wheat starch. The Proofman-LMTIA reaction could be completed in 20 minutes, and 1 of 12 commercial starch products was found to have adulterated wheat starch. In conclusion, the established Proofman-LMTIA method was suitable, or rapid and accurate, for the detection of wheat components in starch products.

Keywords: wheat starch; Proofman probe; ladder-shape melting temperature isothermal amplification

Introduction

As the most extensively cultivated crop globally (Luo *et al.*, 2023), wheat (*Triticum aestivum* L.), a crop of the grass family, is known for its remarkable adaptability (Guo *et al.*, 2024) that serves as the staple food for around 35% of the world's population. More than two-thirds of the wheat has been processed into a variety of

food products for humans (Ulrike *et al.*, 2021). Twenty percent of the human body's daily requirement for energy and protein were provided by wheat (Peter *et al.*, 2023), a product abundant in carbohydrates, proteins, dietary fiber, and a range of vitamins as well as essential minerals such as calcium and iron (Brittany *et al.*, 2020). Due to its significant economic value and the increasing global demand, wheat-based foods have been increasingly

adulterated with low-priced starches or wheat starch mixed with expensive starch products; this false labeling has become widespread (Ankara *et al.*, 2023; He *et al.*, 2023; Salvatore *et al.*, 2020; Zhou *et al.*, 2022). This adulteration not only infringes on the rights and interests of consumers but also impacts a market with a fair competition environment. Hence, implementation of stringent standards is essential where they govern the incorporation of wheat starch into high-price commercial starch products, facilitating effective monitoring of any potential adulteration.

In recent years, spectroscopy-based detection technologies have emerged as common methods for identifying the source of starch, frequently due to their elimination of sample pretreatment requirements (Ayvaz *et al.*, 2021; Liu *et al.*, 2023; Marquez *et al.*, 2016; Pastor *et al.*, 2019). However, these methodologies require specialized equipment and skilled personnel, which limit them mainly to laboratory application due to the complexity of data analysis. Another category of detection methods are based on deoxyribonucleic acid (DNA) analysis, such as polymerase chain reaction (PCR), real-time PCR, and droplet digital PCR, which are widely used for the detection of starch products due to their high specificity and sensitivity (Carloni *et al.*, 2017; Chenet *et al.*, 2020; Caterina *et al.*, 2021; Lüthy *et al.*, 2001). But as these PCR-based methods require a complicated thermal cycler, a range of nucleic acid isothermal amplification techniques have been developed, such as the loop-mediated isothermal amplification (LAMP) (Notomi *et al.*, 2000), rolling circle amplification (RCA) (Murakami *et al.*, 2009), cross priming amplification (CPA) (Xu *et al.*, 2012), and so on and so forth. Any of these techniques can exponentially amplify the target sequence of DNA at constant temperature. Among these techniques, LAMP stands out as a particularly promising method for biological analysis due to its simplicity, high specificity, and high sensitivity (Glökler *et al.*, 2021); however, the technique is subject to high false positive rate (Cai *et al.*, 2009), and the detection of wheat components in commercial starch with the LAMP method or the ladder-shape melting temperature isothermal amplification (LMTIA) method has not currently been reported. Hence the necessity to develop a simple, rapid, sensitive, and cost-effective method for starch identification.

Based on the LAMP technology, a novel method known as LMTIA was developed in 2021 (Wang *et al.*, 2021), characterized by its rapidity, simplicity, and cost-effectiveness. Notably, the primer design is more straightforward compared to the LAMP methods, requiring only one pair of primers or two pairs of nested primers. The incidences of false positives and nonspecific amplification were significantly reduced while at the same time enhancing both sensitivity and specificity (Cui *et al.*,

2024). The amplification time for the target sequence is only 20 minutes. In contrast to the PCR techniques, this method operates at constant temperature rather than cycling temperature; it solely relies on the Bst (*Bacillus stearothermophilus*) DNA polymerase to produce a single-stranded DNA template. Additionally, the reagent cost per test is less than USD 2.0. This technique was applicable across various domains, including food safety and medical diagnostics. The studies of the LMTIA technique have been reported on detection of cassava components in sweet potato (Zhang *et al.*, 2022), identification of soybeans in edible oil samples (Gu *et al.*, 2023), detection of African swine fever virus (Wang *et al.*, 2022), and authentication of meat products (Wang *et al.*, 2022). The enzymatic-mediated probe cleavage (Proofman) probes (Ding *et al.*, 2021) are used to improve the detection speed and throughput of the LMTIA methods. The Proofman-LMTIA methods have been effectively applied to dual detection of sweet potato and corn (Cui *et al.*, 2024), rapid discrimination of *Panax quinquefolium* and *Panax ginseng* (Zhang *et al.*, 2023), and rapid distinguishing of *Bupleurum scorzoniferifolium* Willd and *Bupleurum chinense* (Liu *et al.*, 2024).

The Proofman-LMTIA methods (mentioned above) have been widely used in the identification of food, pathogenic bacteria, and Chinese medicinal materials. However, the detection of wheat components in commercial starch with the Proofman-LMTIA method has not currently been reported, and the objective of this study was to establish the Proofman-LMTIA method for rapid detection of wheat components in commercial starch products.

Materials and Methods

Materials

Wheat starch and lotus root starch were purchased from Pangdonglai Supermarket, Ltd. in Xuchang, Henan Province, China, and potato, pea, cassava, sweet potato, mung bean, maize, and 12 commercial starch products were purchased from Xiyunmeng Supermarket, Ltd. in Xuchang, Henan Province, China.

Target sequence selection and Proofman-LMTIA primer design

According to the study by Wang *et al.* in 2021, the target sequence selected had the ladder-shape melting temperature curves and the GC (guanine-cytosine) content ranging from 40% to 80%, and was of high specificity (Wang *et al.*, 2021). The internal transcribed spacer (ITS) sequence of *Triticum aestivum* L. was obtained from the GenBank database, the target sequence was selected with the Oligo 7 software (Molecular Biology Insights, Inc.

Colorado Springs, CO, USA), and the LMTIA primers and probes were designed with the online software Primer3Plus (<https://www.primer3plus.com>).

DNA extraction

The purchased starch products were in powder form, the samples for specificity determination were fresh plants, and the 12 commercial samples were starch noodles. The genomic deoxyribonucleic acids (gDNAs) were extracted from wheat, sweet potato, maize, cassava, potato, lotus, pea, and green bean with the Plant Genomic DNA Extraction Kit (Beibei Biotech Co. Ltd., Zhengzhou, China). The content and purity of gDNAs were analyzed with the NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Temperature optimization of the Proofman-LMTIA reaction

With the extracted gDNAs, the temperature of the LMTIA reaction was optimized. The Proofman-LMTIA reaction temperature was optimized using 10 µL reaction system (Table 1). The reaction mixture was overlaid with 20 µL of liquid paraffin to avoid aerosol contamination. The diethylpyrocarbonate-treated water (DEPC H₂O) (deoxyribonucleases/ribonucleases [DNase/RNase] free) was used as negative control and the gDNAs from wheat were diluted to 1 ng/µL as positive control. The reaction system was heated at 61°C, 63°C, 65°C, and 67°C in the Gentier 96E Automatic Medical Real-Time PCR Analysis System (Xian Tianlong Technology Co., LTD, Xi'an, Shanxi Province, China), (Yao *et al.*, 2024), respectively, and the fluorescent signals were collected 40 times with 30-second intervals. The experiment was repeated twice and parallel tests conducted in each experiment.

Specificity determination of the Proofman-LMTIA method

The specificity was determined at optimized temperature. The gDNAs from wheat starch and seven other common starches, including potato, pea, cassava, sweet potato, mung bean, maize, and lotus, were used to determine the specificity of the Proofman-LMTIA method.

The amounts of gDNAs templates were 2 µL (1 ng/µL) per reaction; these reaction mixtures were heated at optimized temperature for 20 minutes. The experiment was repeated twice and parallel tests conducted in each experiment.

Sensitivity determination of the Proofman-LMTIA method

The gDNAs extracted from *Triticum aestivum* L. was diluted in a 10-fold series to six gradient concentrations of 1 ng/µL, 100 pg/µL, 10 pg/µL, 1 pg/µL, 100 fg/µL, and 10 fg/µL for determining the sensitivity of the Proofman-LMTIA method. The positive controls were the 1 ng/µL gDNAs of *Triticum aestivum* L. and the negative controls were DEPC H₂O. The reaction mixtures were heated at optimized temperature for 20 minutes. The experiment was repeated twice and parallel tests conducted in each experiment.

Detection limit determination of the Proofman-LMTIA method

To determine the detection limit of the Proofman-LMTIA method, wheat starch was mixed with maize starch at percentages (weight by weight [w/w]) of 20, 10, 5, 1, and 0.1, respectively. The gDNAs were extracted using the Plant Genomic DNA Extraction Kit as described in the DNA extraction. Positive controls were 1 ng/µL gDNAs of *Triticum aestivum* L. and negative controls were DEPC H₂O. The reaction mixtures were heated at optimized temperature for 20 minutes. The experiment was repeated twice and parallel tests conducted in each experiment.

Commercial starch product test

A total of 12 commercial starch products were tested for wheat components and ground into powder with a high-speed grinder. The gDNAs were extracted using the Plant Genomic DNA Extraction Kit as described in Section DNA extraction. The reaction mixtures were heated at optimized temperature for 20 minutes. The 1 ng/µL gDNAs of *Triticumaestivum* L. were used as positive controls, while the DEPC H₂O was used as negative controls, and the gDNAs extracted from the commercial products were used as samples to be tested. The experiment was repeated twice and parallel tests conducted in each experiment.

Table 1. The Proofman-LMTIA reaction system.

Reagents	Added amount (10 µL)
5 × LMTIA mix	2 µL
Bst	0.40 µL
F (100 µM)	0.16 µL
B (100 µM)	0.16 µL
LF (100 µM)	0.04 µL
Probe (100 µM)	0.1 µL
DEPC H ₂ O	Add to 8 µL
Positive control	2 µL
Negative control	2 µL

Results

LMTIA primers and probe design

With the ITS gene of *Triticumaestivum* L. as the target, the specific 75-nt (nucleotide) sequence with the ladder-shaped melting temperature curve was selected with the Oligo 7 software (Molecular Biology Insights, Inc. Colorado Springs, CO, USA) (Figure 1), and the LMTIA primers of wheat was designed with the online software

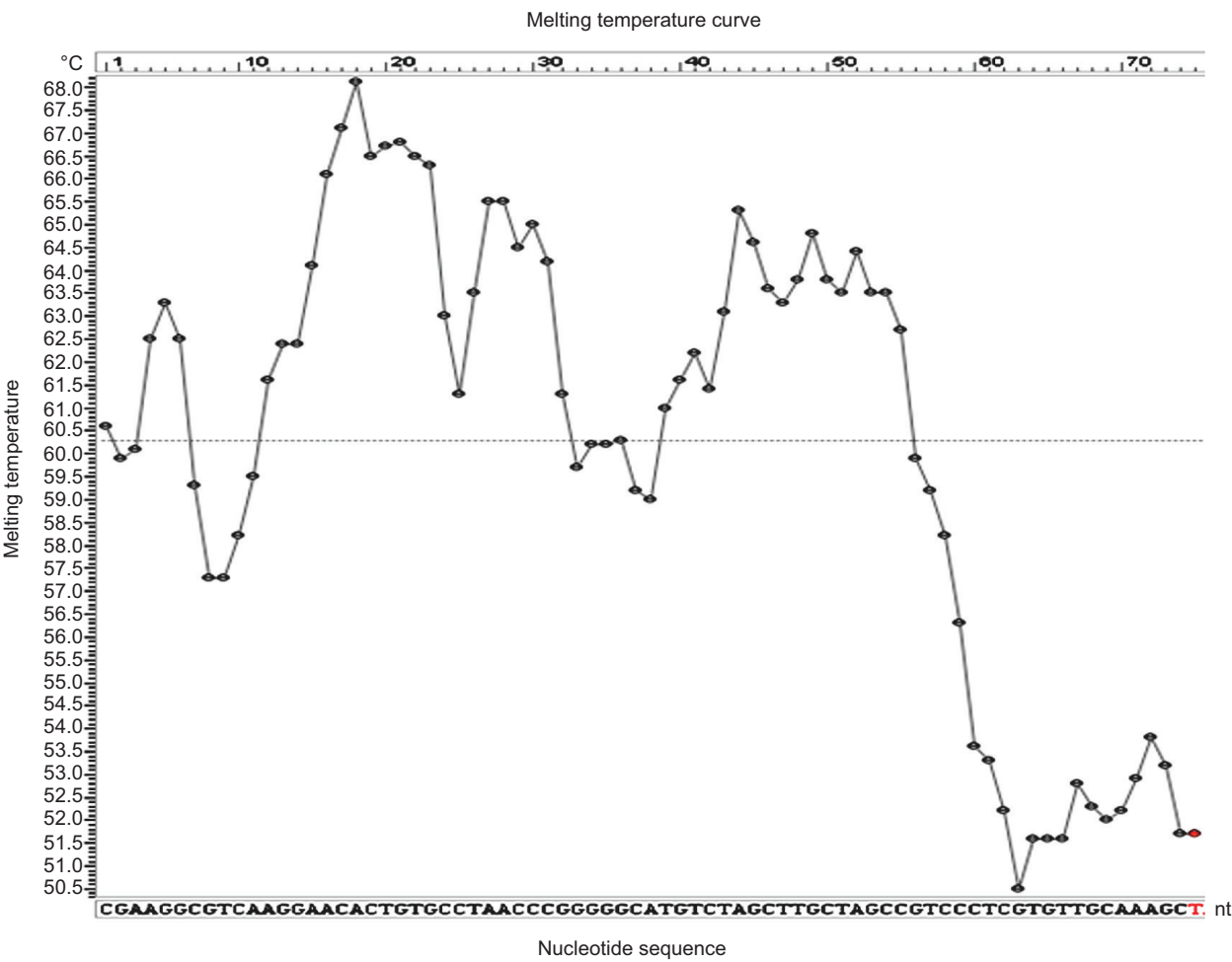


Figure 1. The target sequence with the ladder-shape melting temperature curve for detection of wheat components with the Proofman-LMTIA method.

Primer3Plus (<https://www.primer3plus.com>). Upon the loop primer forward (LF) sequence, the Proofman probe with a quencher was labeled at the end of the 5' nucleotide and a fluorophore was labeled at the end of the 3' mismatch nucleotide. The sequences of primers and probes are shown in Table 2.

DNA extraction

The concentrations of the gDNAs extracted from wheat, sweet potato, maize, cassava, potato, lotus root,

pea, mung bean, and commercial starch products were higher than 1 ng/μL, with their A260/A280 ratios falling between 1.8 and 2.0 (Table 3).

Temperature optimization of the Proofman-LMTIA reaction

The Proofman-LMTIA system was prepared in the PCR reaction tube; 20 μL liquid paraffin was added to avoid aerosol pollution. The Proofman-LMTIA reaction systems were heated at 61°C, 63°C, 65°C, and 67°C, respectively. The result shows that all the reactions with gDNAs

Table 2. The sequences of the primers, the Proofman probe, and the target for detection of wheat components with the Proofman-LMTIA method.

Primers and probes	Sequence (5' to 3')
XM-F	5'-AGCAAGCTAGACTTTTCGAAGGCGTCAAGGAACACT-3'
XM-B	5'-TAGCTTGCTAGCTTTTAGCTTTGCAACACGAGGGA-3'
XM-LF	5'-ATGCCCCCGGGTTAG-3'
XM-LF Probe	5'BHQ2-ATGCCCCCGGGTTAA-3'6-FAM
XM: Wheat	CGAAGGCGTCAAGGAACACTGCCTAACCCGGGGGCATGTCTAGCTTGCTAGCCGTCCTCGTGTGCAAAGCT

Table 3. The concentration and purity of the extracted gDNAs.

No.	Samples	Concentration (ng/mL)	Purity (A260/A280)
1	Wheat	26	1.89
2	Sweet potato	56	1.95
3	Maize	36	1.89
4	Cassava	21.9	1.96
5	Potato	98	1.87
6	Lotus	18.6	1.81
7	Pea	36.2	1.89
8	Mung bean	57	1.82

of *Triticum aestivum* L. were positive ($Ct\ 11.92 \pm 0.30$ at 61°C , $Ct\ 11.46 \pm 0.07$ at 63°C , $Ct\ 11.9 \pm 0.02$ at 65°C , $Ct\ 20.29 \pm 0.6$ at 67°C) and all the reaction with DEPC H_2O were negative; however, the amplification efficiency and the repeatability were the highest at 65°C , so the optimal temperature was 65°C (Figure 2).

Specificity determination of the Proofman-LMTIA method

To verify the that the designed LMTIA primers and Proofman probe were specific to the gDNAs of *Triticum aestivum* L. at 65°C , the gDNAs from potato, pea, cassava, sweet potato, mung bean, maize, and lotus were

used for determination with DEPC H_2O as the negative control. The results showed that the reactions with the gDNAs of *Triticum aestivum* L. were tested to be positive ($Ct\ 9.29 \pm 0.12$), and the reactions with the gDNAs from potato, pea, cassava, sweet potato, green bean, maize, and lotus as well as DEPC H_2O had no amplification, which indicated that the established Proofman-LMTIA method was of high specificity (Figure 3).

Sensitivity determination of the Proofman-LMTIA method

The 1 ng/ μL , 100 pg/ μL , 10 pg/ μL , 1 pg/ μL , 100 fg/ μL , and 10 fg/ μL gDNAs of *Triticum aestivum* L. were used for the sensitivity determination of the established Proofman-LMTIA method. The result shows that statistical analysis for each concentration reaction system were: $Ct\ 10.09 \pm 0.02$ of 1 ng/ μL , $Ct\ 11.30 \pm 0.07$ of 100 pg/ μL , $Ct\ 11.68 \pm 0.09$ of 10 pg/ μL , $Ct\ 14.78 \pm 0.06$ of 1 pg/ μL , $Ct\ 15.35 \pm 0.21$ of 100 fg/ μL , and $Ct\ 18.01 \pm 0.84$ of 10 fg/ μL ; all negative controls were negative (Figure 4). Hence, the sensitivity was 10 fg gDNAs of *Triticum aestivum* L., indicating that the established Proofman-LMTIA method was of high sensitivity.

Detection limit determination of the Proofman-LMTIA method

The gDNAs extracted from maize starch adulterated with 20%, 10%, 5%, 1%, and 0.1% (w/w) wheat starch were used for the detection limit determination, and the DEPC H_2O

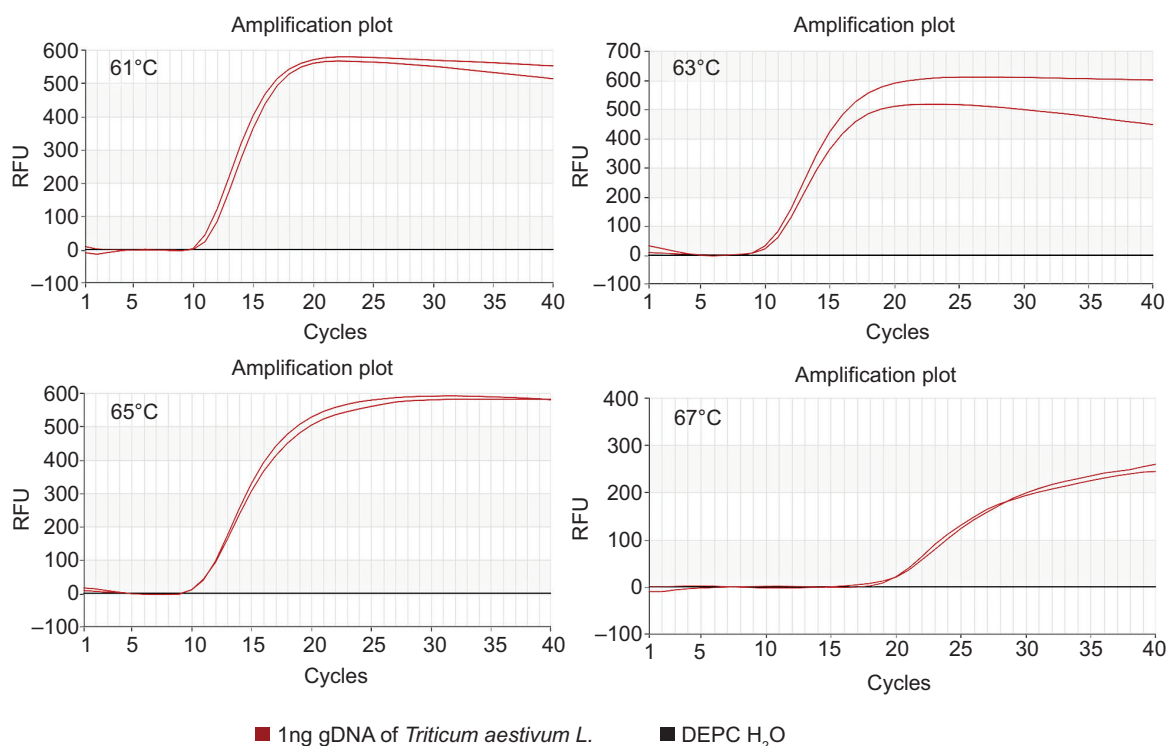


Figure 2. Temperature optimization of the Proofman-LMTIA reaction for detection of wheat components. The amplification plots of Proofman-LMTIA reactions at different temperatures (61°C , 63°C , 65°C , 67°C); positive control: gDNAs of *Triticum aestivum* L.; negative control: DEPC H_2O .

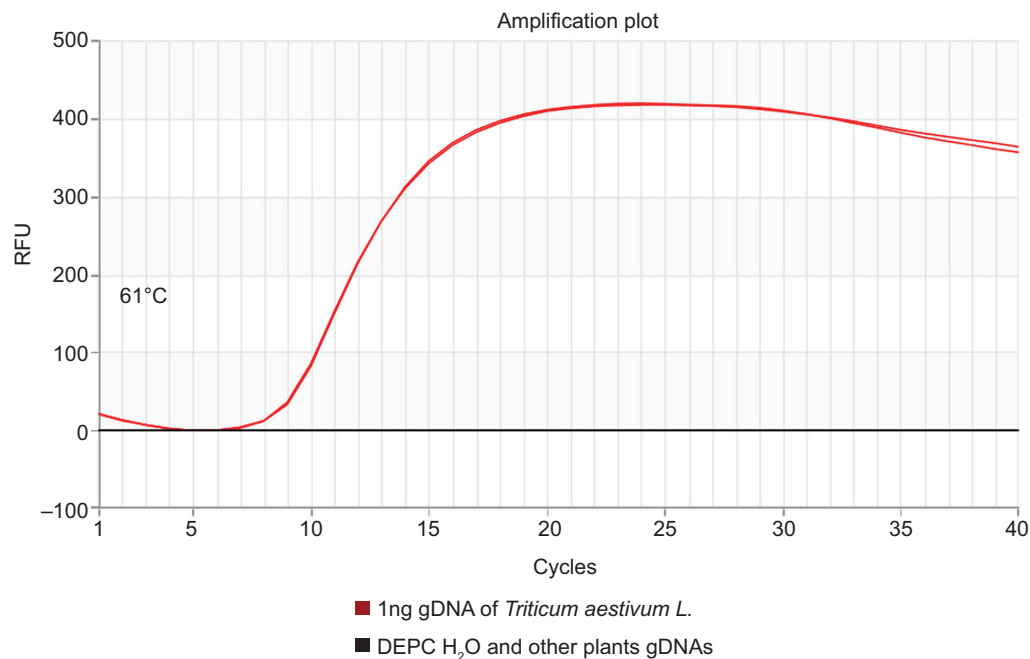


Figure 3. The specificity determination of the Proofman-LMTIA method for detection of *Triticumaestivum* L. gDNA. Others included the gDNAs from potato, pea, cassava, sweet potato, green bean, maize, and lotus.

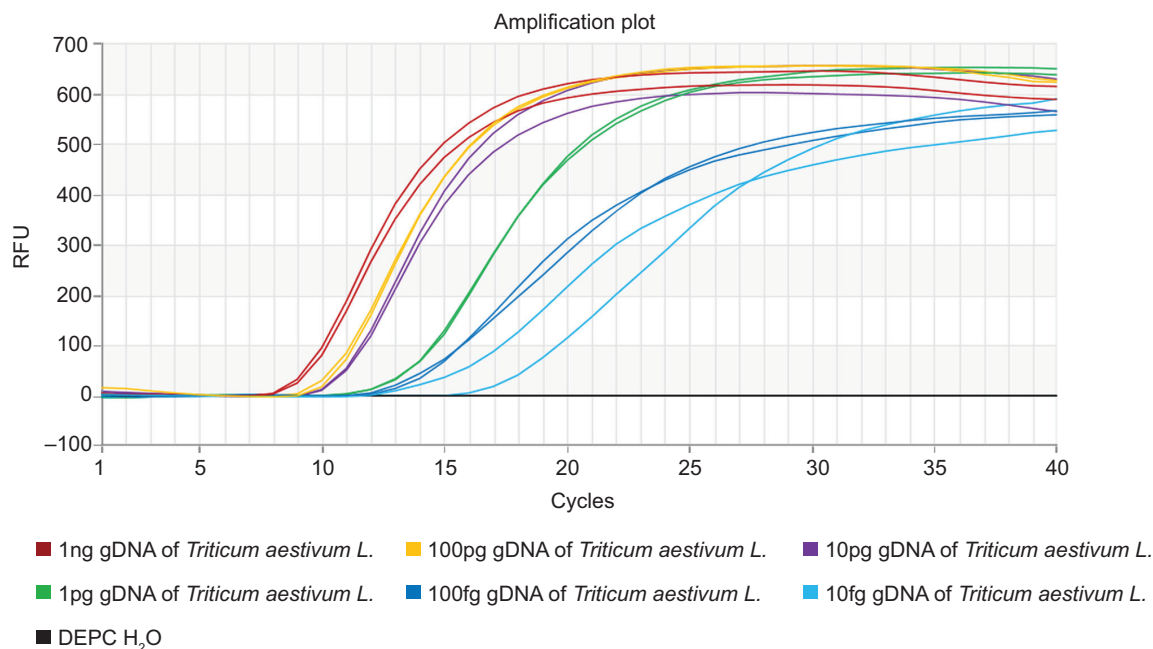


Figure 4. The sensitivity of the Proofman-LMTIA method determined with serial 10-fold dilution of *Triticumaestivum* L. gDNA.

was used at the negative controls. Statistical analysis of each adulteration ratio was: Ct 11.16 ± 0.10 of 20%, Ct 13.35 ± 0.07 of 10%, Ct 13.83 ± 0.25 of 5%, Ct 14.68 ± 0.26 of 1%, and Ct 18.97 ± 0.01 of 0.1%; all negative controls were negative (Figure 5). Consequently, the detection limit of the established Proofman-LMTIA method was 0.1%.

Commercial starch product test

In order to validate the practical application value of the Proofman-LMTIA method, 12 commercial noodle products were tested for wheat components; the shape of these products are shown in Figure 6. The results of wheat component detection in the commercial starch products

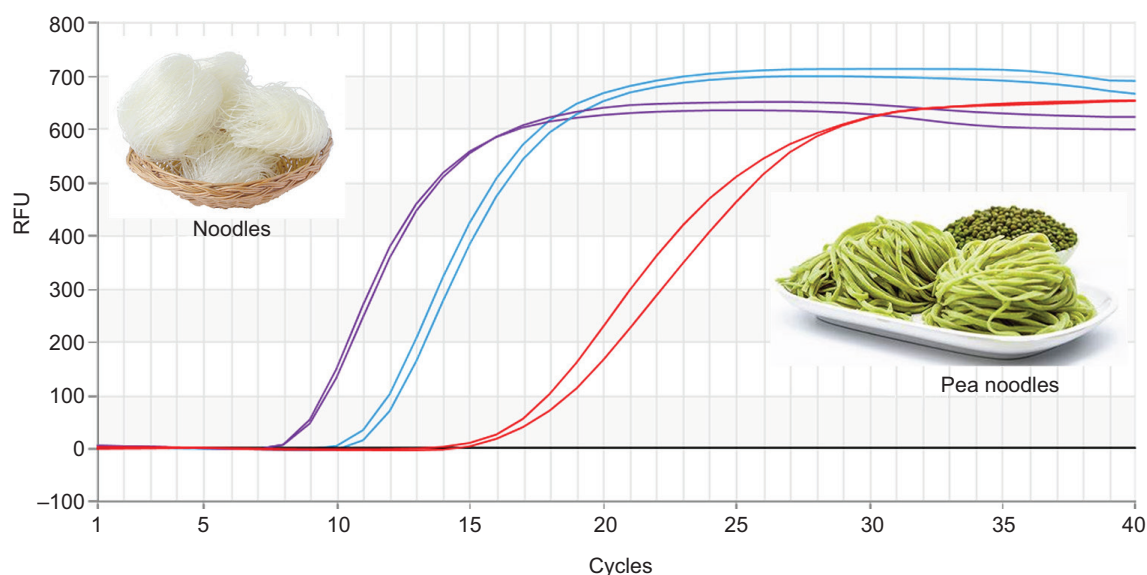


Figure 5. The detection limit of the Proofman-LMTIA method for detection of wheat components adulterated into the commercial starch product.

were detailedly summarized in Table 4 and Figure 6. The reactions of the positive controls, No 4 sample, and No 8 sample were wheat components positive (positive sample: Ct 9.62 ± 0.16 ; No 8 sample: Ct 12.24 ± 0.23 ; No 4 sample: Ct 18.75 ± 1.05), whereas the reactions of the other samples and negative controls were wheat components negative. The results indicated that No 4 and No 8 samples contained wheat components, whereas the other samples did not contain any. The label of No 4 sample did not indicate any wheat components, whereas the label of No 8 sample indicated the wheat components. It was confirmed that the established Proofman-LMTIA method could effectively validate the presence of wheat

components in commercial starch products, which possessed high practical value.

Discussion

Food adulteration has become an important food safety issue around the world. Economically motivated food adulteration has an impact on consumers' right to know; it also reduces the overall quality of food. At present, the economically motivated food adulteration is common in high-value products tainted with low-value products, namely the high-price products were adulterated with the low-price products (Kaldeli *et al.*, 2024). The identification of starch adulteration currently is a challenge (a large number of researchers had paid attention to starch authentication). Vis-NIR spectroscopic and chemometric models were used for detecting premium green banana flour contaminated with wheat different levels (Ndlovu *et al.*, 2021). Fourier transform near-infrared and mid-infrared (FT-NIR, FT-MIR) spectroscopy and chemometric analysis were used to detect milk powder adulterated with corn starch and wheat flour (Edwin *et al.*, 2024). Raman spectroscopy and one-class support vector machine were used to detect adulterants in cassava starch; cassava starch samples were modified with adulterants ranging from 0.5 to 50%, such as wheat flour, sodium bicarbonate, and others. This method resulted in the possibility of detecting adulterations over 2% (Cardoso *et al.*, 2021). Schulze established the primer probe systems for real-time quantitative fluorescent PCR (real-time PCR) and droplet digital PCR (ddPCR), which were used for detection and validation of cereals such

Table 4. The commercial starch products tested with the Proofman-LMTIA method.

No.	Commercial samples	Labeling ingredients	Wheat
1	Maize starch: 1	Maize	--
2	Potato starch: 2	Potato	--
3	Sweet potato starch: 3	Sweet potato	--
4	Pea noodles	Pea	++
5	Lotus starch: 1	Lotus	--
6	Lotus starch: 2	Lotus	--
7	Lotus starch: 3	Lotus	--
8	Noodles: 1	Wheat	++
9	Noodles: 2	Pea, maize	--
10	Noodles: 3	Pea, mung bean	--
11	Noodles: 4	Sweet potato	--
12	Noodles: 5	Potato	--

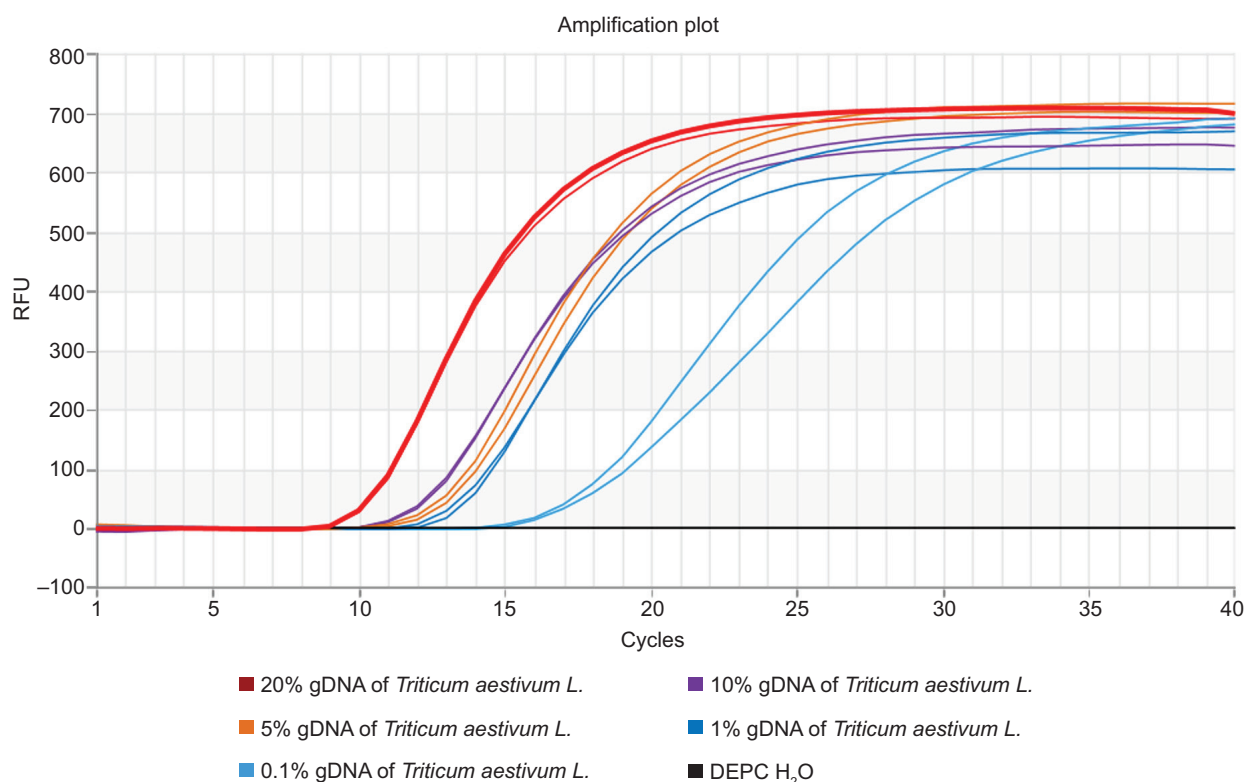


Figure 6. Detection of the commercial starch products with the Proofman-LMTIA method.

as bread wheat, durum wheat, rye, and barley. ddPCR could correctly detect proportions of 50%, 60%, and 90% wholemeal rye flour in a mixture of wholemeal common wheat flour (Schulze *et al.*, 2021).

Although these methods have shown the capability for detection of illegal adulteration in various starch products, they have some disadvantages such as expensive equipment, complex instrument operation and data analysis, long reaction time, and many kinds of reagents used; therefore, the need to establish a simple, rapid, sensitive, and economical technology for starch product identification. The Proofman-LMTIA method has been used in many fields for rapid detection. Compared with FT-NIR, FT-MIR, Roman spectroscopy, real-time PCR, and ddPCR, the Proofman-LMTIA method is simple, and the amplification is completed in 20 minutes at constant temperature without the need for complex and precise equipment and data analysis. In this study, the Proofman-LMTIA method for detection of wheat components was established. When the specificity of the Proofman-LMTIA method was verified with several common starches (potato, cassava, mung bean, sweet potato, lotus root powder, and corn), the sensitivity of the Proofman-LMTIA method was 10 fg/ μ L gDNAs of *Triticum aestivum* L. and the detection limit of the Proofman-LMTIA method was as low as 0.1%.

To verify the application prospects of the Proofman-LMTIA method, we tested six commercial starch products and six noodle products from different regions. Their labels indicated that only No 8 sample contained wheat components and the other 11 samples did not contain any. However, the test results showed that No 4 sample contained wheat components, an indication of adulteration with wheat starch; the established Proofman-LMTIA method can detect such fraud.

In this study, the Proofman-LMTIA technique was found to be suitable for the detection of wheat components in commercial starch products. Beyond the application in the detection of starch products, the Proofman-LMTIA technique was also utilized for the identification of other food fraud, such as meat product (Wang *et al.*, 2022), honey (Yao *et al.*, 2024), and identification of Chinese medicinal materials (Zhang *et al.*, 2023). In summary, the Proofman-LMTIA system is a sensitive, specific, quick, simple, and an economic method. The popularization of this method could effectively identify the adulteration of commercial starch, protect consumers, and contribute to food safety.

Conclusions

In this study, Proofman-LMTIA method has been established for rapid detection of wheat component in starch.

The LMTIA primers and Proofman probe were designed for the target sequence of wheat gene with high specificity. The sensitivity of the Proofman-LMTIA assay at 10 fg/uL gDNAs of *Triticum aestivum* L. was proven at the optimal temperature of 65°C, where the whole amplification could be finished within 20 minutes. Additionally, the cost is very low, with the reagent cost per test being less than USD 2.0. Summarily, the Proofman-LMTIA methodology has been successfully established, notable for its rapid, simple, economic, specific, and sensitive in the detection of wheat component in starch. The popularization of this Proofman-LMTIA technique should improve the quality control of wheat component in starch products, provide guidelines for the authentication of wheat ingredients, and supervision of food market. The Proofman-LMTIA technology not only identifies wheat component in starch, but can also be used to certify the origin of a wide range of plants.

Data Availability Statement

The data used to support this study's findings are available from the corresponding author upon request.

Author Contributions

Conceptualization: D.W. and Y.W.; methodology: D.W.; software: W.Y.; validation: D.W. and D.Z.; formal analysis, D.W. and Y.W.; investigation, C.S., B.Y., and S.G.; resources, D.W. and X.Z.; data curation, D.W. and F.X.; writing—original draft preparation: D.Z.; writing—review and editing: D.W.; project administration: D.W.; funding acquisition: D.W. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

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