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A hybridization chain reaction coupled with gold nanoparticles for allergen gene detection in peanut, soybean and sesame DNAs†

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Food allergy is an abnormal immune response of the immune system to some foods, which has caused great harm to people's health. Therefore, it is particularly important to detect allergens in food. In this article, a hybridization chain reaction (HCR) was coupled with gold nanoparticles (AuNPs) to detect the allergen genes of peanut, soybean and sesame DNAs. Two hairpin probes (H1 and H2) were designed for the allergen target genes of peanut, soybean and sesame DNAs. In the presence of target DNA, the hybridization chain reaction was triggered by it producing long double-stranded DNA (dsDNA) products. In the gold nanoparticle system, long dsDNA couldn't be adsorbed on the surface of AuNPs. When the concentration of salt ions in the solution increased, gold nanoparticles accumulated and led to a decrease of ultraviolet absorption. In the absence of target DNA, no hybridization chain reaction occurred. The hairpin probes could be adsorbed on the surface of AuNPs and no accumulation happened for gold nanoparticles even if the concentration of salt ions in the solution was increased. This method required no enzymes and had a strong specificity, so it was very easy to distinguish target DNA from non-target DNA. The detection limit of three allergens detected by this method was as low as 0.5 nM. The feasibility of this method for the detection of commercial commodities had been demonstrated by the successful detection of the DNAs extracted from commercial commodities, which were treated with extreme thermostable single-stranded binding protein (ET SSB).

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1. Introduction

Food allergy is a global problem, and the occurrence rate continues to increase. The related diseases are on the rise globally.¹ When an allergy occurs, the body's corresponding immunoglobulin binds to the allergen, releasing harmful chemicals that cause allergic symptoms, such as nausea, vomiting, diarrhea, eczema, *etc.* A sharp drop in blood pressure, loss of consciousness, and anaphylactic shock and so on will occur for severe cases.² At present, there is no effective treatment except for avoiding food that may cause allergy, so it is extremely important to detect food allergens. Common food allergens include fish, shrimp, eggs, peanuts,

soybeans, sesame seeds, barley, walnuts, pistachios, almonds, *etc.*³ Among these, the major peanut allergens are Ara h 1, Ara h 2, Ara h 3 and Ara h 6.⁴ Soybean allergens include Gly m Bd 28K, Gly m Bd 30K and Gly m Bd 60K.⁵ There are 2S albumin, 11S globulin and so on for sesame allergens.⁶

Common methods of allergen detection include immunochemical-based detection techniques for allergic proteins, such as enzyme-linked immunosorbent assay (ELISA),⁷ western blotting,⁸ dot immunoblotting,⁹ ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS),¹⁰ *etc.* In addition, there are also DNA detection methods based on nucleic acids, including polymerase chain reaction (PCR),¹¹ rolling circle amplification (RCA),¹² loop-mediated isothermal amplification (LAMP),^{13–15} strand displacement amplification (SDA),¹⁶ recombinase polymerase amplification (RPA),¹⁷ DNA arrays,¹⁸ *etc.* Protein-based detection techniques are cumbersome and require a long detection time. All of these DNA-based tests involve enzymes, which are expensive and require other harsh conditions, such as pH, temperature and ion concentration.^{19,20} However, the HCR, developed by Dirks and Pierce in 2004, does not rely on enzymes and other harsh conditions to detect DNA.²¹ The reaction temperature is 37 °C or lower. The HCR is compatible with other techniques

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and expensive equipment is not needed. The combination of the HCR and some nanomaterials (such as graphene oxide²² and gold nanoparticles²³) makes DNA detection much easier. Zhang *et al.* developed a DNA-stabilized silver nanocluster/graphene oxide-based platform for the sensitive detection of DNA by using the HCR.²⁴ Wang and coworkers used the unique features of SYBR Green I and GO to detect DNA through the HCR.²⁵ Ma *et al.* modified probes on gold nanoparticles that hybridized with target DNA. When the target DNA appeared, it could induce the HCR.²⁶ Liu *et al.* developed a colorimetric method for DNA detection without enzymes by using the HCR and gold nanoparticles.²⁷

In this paper, peanut, soybean and sesame allergen genes were the research objects. The HCR combined with gold nanoparticles was used to detect target DNA without enzymes. Two hairpin probes (H1 and H2) were designed, respectively. The probes were label-free. When the target DNA appeared, it triggered the hybridization chain reaction forming long dsDNA products, which could not be adsorbed on the surface of AuNPs. With the increasing of NaCl concentration, gold nanoparticles accumulated. It turned blue-violet. When there was no target DNA, the hairpin probes would stick to the surface of gold nanoparticles to stop from accumulating. It remained red. This method had a strong specificity and high sensitivity with a mild temperature of 37 °C.

2. Materials and methods

2.1 Reagents and materials

Sodium acetate, sodium chloride, chloroauric acid and sodium citrate were purchased from Sinopharm Chem. Re. Co., Ltd (China). ET SSB was purchased from Haigene Co., Ltd (China). HCR buffer (50 mM CH₃COONa, 1 M NaCl, pH 7.5) was synthesized by our laboratory.²⁸ The gene fragments of Ara h 6,²⁹ Gly m Bd 28K³⁰ and 2S albumin³¹ allergens were selected for peanut, soybean and sesame DNAs, respectively. All oligonucleotides were synthesized by Shanghai Sangon Biotech Co., Ltd (China), whose sequences are shown in Table 1. The synthesized oligonucleotides were placed at −20 °C, heated at 95 °C for 5 minutes before use, then gradually cooled to room temperature, and stored in a refrigerator at 4 °C after 2 hours.³² All other reagents were of analytical purity

and used without further purification. All solutions were prepared with ultrapure water.

2.2 HCR-AuNP detection

1 µL of H1 (10 µM) and 1 µL of H2 were added to 97 µL HCR reaction solution (three times the dilution of the original buffer), and then 1 µL of tested DNA (10 µM) was added into the solution. The total volume of the reaction was 100 µL. After it reacted at 37 °C for 2 h, 5 µL of reaction products was added to 45 µL gold nanoparticle solution (synthesis of AuNPs is in the ESI†) and placed at room temperature for 5 minutes. Ultraviolet-visible absorption was detected at 520 nm using a micro-volume UV-Vis spectrophotometer (SMA4000, Merinton (Beijing) instrument Co., Ltd, Beijing, China), or sodium chloride solution was added to keep the final concentration of 60 mM for 10 minutes for colorimetric detection.

2.3 Agarose gel electrophoresis

1 µL of H1 and 1 µL of H2 were added to 99 µL HCR buffer, respectively. H1 (1 µL) and H2 (1 µL) were added to 98 µL HCR buffer. H1 (1 µL), H2 (1 µL) and target DNA (1 µL) were added to 97 µL HCR buffer. After they reacted at 37 °C for 2 h, 5 µL of DNA samples was mixed with 1 µL 6× loading buffer, and then electrophoresis was carried out with 2% agarose gel in 1× TAE solution. It was performed at 110 V voltage for 30 min, and a gel imaging system (Tanon 3500, Shanghai Tanon Technology Co., Ltd, Shanghai, China) was used to take pictures.

2.4 The extracted dsDNAs were treated with ET SSB

DNAs of peanut, soybean and sesame were extracted by the phenol-chloroform method.¹¹ ET SSB was added to the extracted dsDNA, heated at 91 °C for 6 minutes, and then cooled gradually.^{33,34} It was kept for 1 h at room temperature. The UV-Vis absorption was determined at 260 nm. The HCR could be initiated only when dsDNA was changed into single-stranded DNA (ssDNA) after ET SSB treatment.

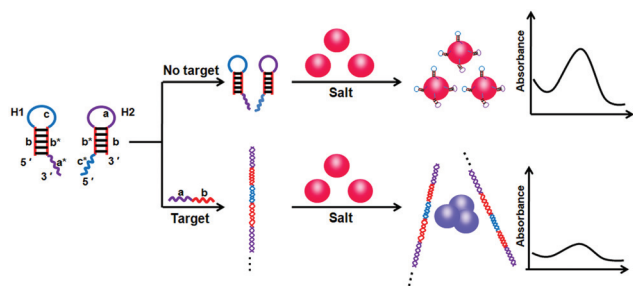
3 Results and discussion

3.1 Principle of the HCR-AuNP detection

It is known that ssDNA can be absorbed on the AuNPs, but dsDNA can't.³⁵ The principle of the HCR-AuNP detection is

Table 1 Peanut, soybean and sesame target DNA and hairpin probes (note: different colors from 5' to 3' are Target: a, b; H1: b, c, b*, and a*; H2: c*, b*, a, and b (see Scheme 1))

DNA name	DNA sequence (5' to 3')
Peanut target	AGCAATGAATGAAGCAAACAAAGCCACACCA
Peanut H1	AAACAAAGCCACACCACTGTTCAAGTTGGTGTGGCTTTGTTTGCTTCATTTCATTGCT
Peanut H2	ACCTGAACAGTGGTGTGGCTTTGTTTAGCAATGAATGAAGCAAACAAAGCCACACCA
Soybean target	ATGATGATGAAGTACGGAAGGAGATTGAA
Soybean H1	CGGAAAGGAGATTGAACCTTAGCCGTATTCATCTCCTTTCCGCTAGTTTCATCATCAT
Soybean H2	TACGGCTAAGTTCAATCTCCTTTCCGATGATGATGAAGTACGGAAGGAGATTGAA
Sesame target	TGAGGAACGTGGACGAGAGGTGCCGCTGTGA
Sesame H1	AGAGGTGCCGCTGTGATATCATACTTTCACAGCGGCACCTCTCGTCCACGTTCTCTCA
Sesame H2	AAGTATGATATCACAGCGGCACCTCTTGAGGAACGTGGACGAGAGGTGCCGCTGTGA



Scheme 1 Principle of the HCR-AuNPs for target DNA detection.

shown in Scheme 1. In the absence of target DNA, the system could not undergo the hybridization chain reaction. The hairpin probes maintained hairpin structures, and their single-stranded parts could be adsorbed on the surface of gold nanoparticles. The gold nanoparticles adsorbed with hairpin probes could not be aggregated in salt solution, showing a dispersed state. The solution remained red, and the absorbance was higher. Target DNA consisted of two parts: a and b. In the presence of target DNA, the hybridization chain reaction was initiated. The complementary pairing of a and b in target DNA and a* and b* in H1 led to the opening of the hairpin structure of H1, which exposed b and c, and then b and c in H1 were hybridized with b* and c* in H2. The exposed a and b of H2 then turned on the hairpin structure of H1, so that long dsDNA target-H1-H2 could be formed by a circular reaction. The formed long double-stranded DNA could not be adsorbed on the gold nanoparticles. The gold nanoparticles accumulated in the salt solution, making the solution appear blue-violet with a lower absorbance.

3.2 HCR-AuNP detection for different combinations of H1, H2 and T

Different combinations of H1, H2 and T (H1, H2, T, H1 + H2, H1 + H2 + T) were selected to explore the feasibility of the HCR-AuNP detection method. The results are shown in Fig. 1. When the reaction system was H1, H2, T and H1 + H2, respectively, they were adsorbed on the surface of gold nanoparticles that were dispersed. The absorbance of H1, H2 or T

was about 0.04, while the absorbance of H1 + H2 was slightly higher, because the number of probes adsorbed on gold nanoparticles in the system was higher than that of H1, H2 or T. When target DNA (T) was added into the H1 + H2 system, the hybridization chain reaction was initiated, forming long dsDNA products. The products could not be adsorbed on the surface of gold nanoparticles. In a certain NaCl solution (60 mM), gold nanoparticles were aggregated and the absorbance would be reduced to about 0.02. It indicated that the target DNA could be detected by this detection method, and the feasibility of the detection method was also confirmed by gel electrophoresis.

3.3 TEM imaging of HCR-AuNP detection

The positive HCR product was added to the gold nanoparticle system. Sodium chloride (60 mM) was added after 5 min, and stabilized for 10 min. After that, the product was fixed on a copper net for transmission electron microscopy (TEM) imaging. As shown in Fig. 2A, the gold nanoparticles were aggregated because the formed dsDNA could not be adsorbed on the gold nanoparticles. The negative HCR sample could be adsorbed on the surface of gold nanoparticles to protect gold nanoparticles from aggregation and presented a dispersed state,²⁷ as shown in Fig. 2B.

3.4 Detection limit of the HCR-AuNPs

Different concentrations of target DNA (50, 30, 10, 8, 6, 4, 2, 1, 0.5, and 0 nM) were set to explore the detection limit of the HCR-AuNPs, as shown in Fig. 3. A1, B1 and C1 were the detection limits of the HCR-AuNPs for peanut, soybean and sesame DNAs, respectively. When the concentration of DNA increased gradually, the fluorescence intensity decreased gradually. A2, B2, and C2 illustrated the relationship between the absorbance ratio $(A_0 - A)/A_0$ and the target DNA concentration (A_0 is the absorbance of non-target DNA and A is the absorbance of target DNA). When the concentration of DNA was 0–10 nM, there was a good linear relationship. The linear regression equations of peanut, soybean and sesame DNAs were $y = 0.0379x + 0.0582$ ($R^2 = 0.9931$), $y = 0.0249x + 0.127$ ($R^2 = 0.9914$), and $y = 0.0309x + 0.997$ ($R^2 = 0.9954$), respectively. The experimental results showed that the detection limit of this method was as low as 0.5 nM, which was lower than Zhang's report (1.18 nM).²⁴ It demonstrated that highly sensitive and label-free detection for peanut, soybean and sesame DNAs could be realized.

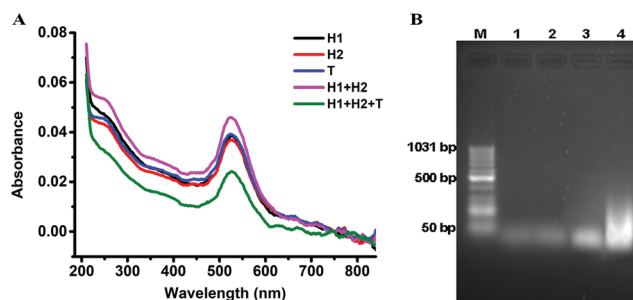


Fig. 1 (A) The absorbances of different combinations of H1, H2 and T were detected by the HCR-AuNPs; (B) gel electrophoresis results. M: DNA marker; lanes 1–4: H1, H2, H1 + H2, and H1 + H2 + target.

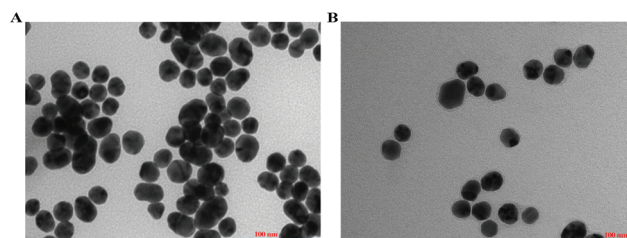


Fig. 2 TEM images of HCR-AuNP positive (A) and negative (B) samples.

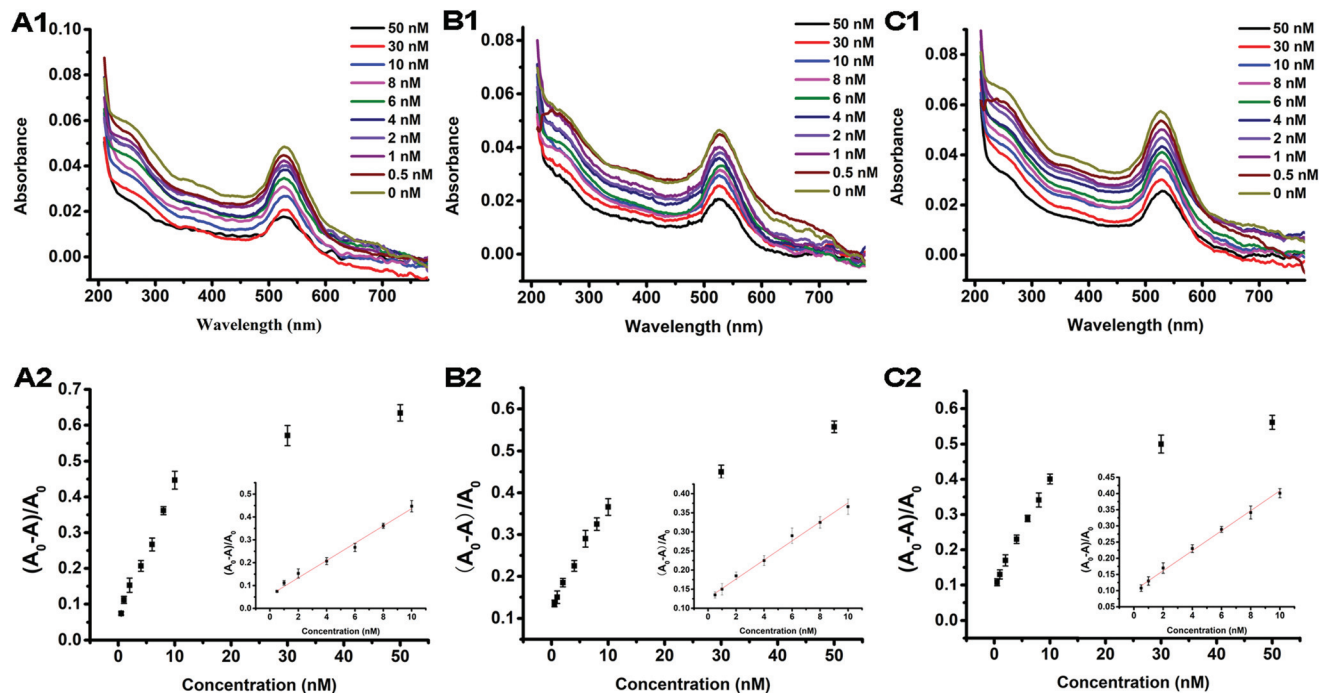


Fig. 3 Detection limit of the HCR-AuNPs for peanut (A), soybean (B) and sesame (C) DNAs.

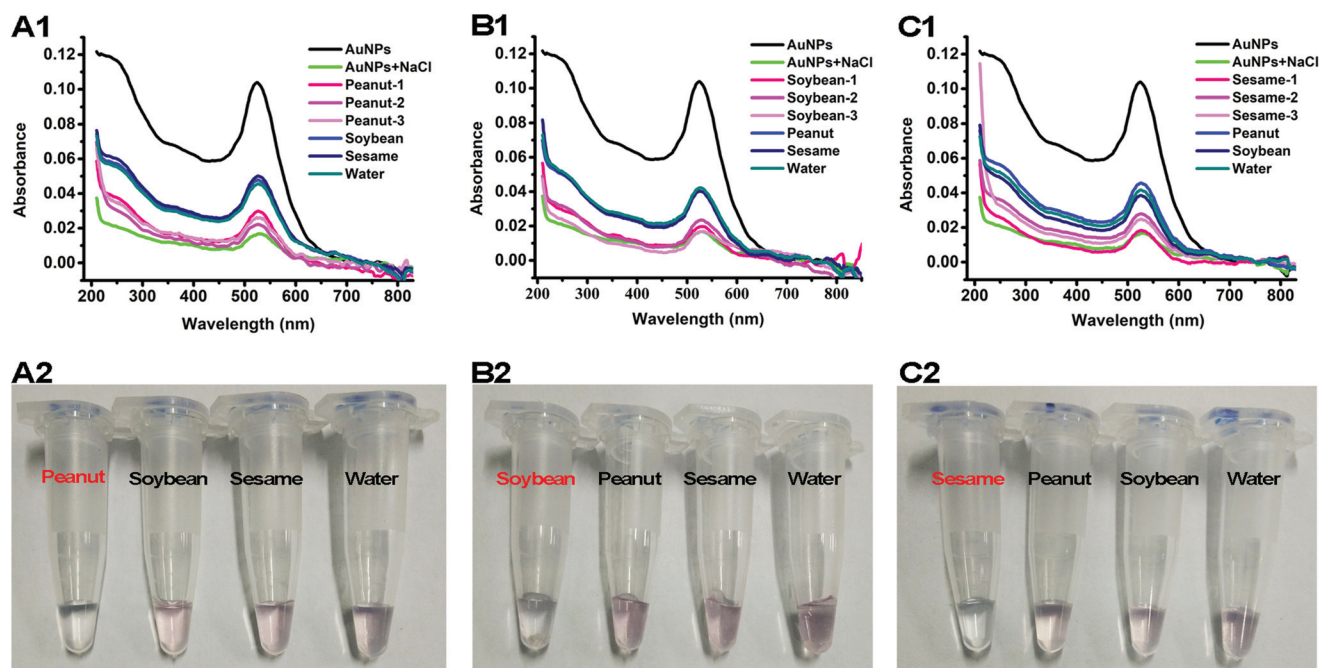


Fig. 4 Detection specificity of the HCR-AuNPs. A1, B1 and C1 are the UV visible absorption detection of peanut, soybean and sesame DNAs, respectively; A2, B2 and C2 are the colorimetric detection of peanut, soybean and sesame DNAs, respectively. The red font is positive, and the black font is negative.

3.5 Detection specificity of the HCR-AuNPs

Three positive and three negative samples were selected to determine the specificity of the HCR-AuNPs. The results are shown in Fig. 4A, B and C. Due to the absence of NaCl solution, the naked gold nanoparticles had the highest absorbance

of about 0.10. NaCl solution (60 mM) was added to gold nanoparticles resulting in the aggregation of gold nanoparticles with an absorbance of about 0.02. In the positive samples, the hairpin probe formed long dsDNA due to the target DNA, and the long dsDNA could not be adsorbed on the surface of gold

nanoparticles. Therefore, gold nanoparticles were aggregated in NaCl solution and had lower absorbances, which were close to the absorbance of AuNPs + NaCl, and the color in the solution was light purple (positive samples in Fig. 4A2, B2 and C2). Due to the absence of the hybridization chain reaction in negative samples, hairpin probes and target DNA would be adsorbed on the surface of gold nanoparticles, which protected gold nanoparticles from aggregation. The absorbances of negative samples were higher than those of positive samples, and the color was red (negative samples in Fig. 4A2, B2 and C2).³⁶

3.6 HCR-AuNP detection for mixed DNA

Two or three different kinds of DNA were selected for mixed DNA detection, and the results are shown in Fig. 5. When two kinds of mixed DNA were detected (Fig. 5A), the target DNA contained in the mixed DNA could trigger the hybridization chain reaction, which had a relatively lower absorbance. The sample without target DNA had a higher absorbance. The same results were obtained when three kinds of mixed DNA were detected (Fig. 5B). It showed that the target DNA could be detected by this method even if the target DNA sample was mixed with other non-target DNA.

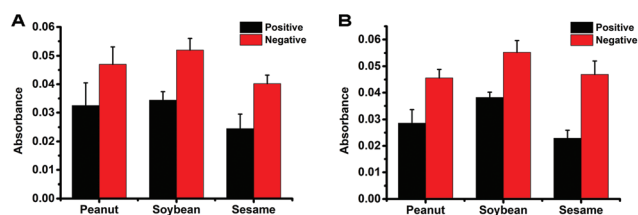


Fig. 5 UV absorption of the HCR-AuNP detection of mixed DNA. A and B represent two and three kinds of mixed DNA tested, respectively. The positive sample contained target DNA, and the negative sample did not contain target DNA.

3.7 HCR-AuNP detection for commercial commodities

Six kinds of biscuits and sweets were selected. After extracting DNA by the phenol–chloroform method, the DNA was treated with ET SSB. Double-stranded DNA changed into ssDNA. Then the HCR-AuNP detection method was used to determine whether the samples contained peanut, soybean or sesame allergens. The results are shown in Table 2. There were peanut allergens in B1, B2, C1, and C2, soybean allergens in B3, B4, C3 and C4, and sesame allergens in B5, B6, C5 and C6. The test results were consistent with the labeling of commodity packaging. In addition, the production line of B2 and B5 commodities had produced commodities containing soybean, and the method had not detected soybean allergens in B2 and B5. Production containing sesame allergens had been produced in C1 and C4 commodity production lines. No sesame allergen had been detected in C1 in this method, but sesame allergen had been detected in C4. The test results showed that the method could detect the products containing the corresponding allergens, but if the production line had produced products containing other allergens, it was difficult to detect other extremely small amounts of allergens.

4. Conclusions

Based on the reaction principle of the HCR, the DNAs of peanut, soybean and sesame were detected by using the adsorption characteristics of gold nanoparticles. With the treatment of ET SSB, dsDNA changed into ssDNA before detection. The results showed that the absorbance of negative samples was relatively higher, while that of positive samples was relatively lower. The detection method could distinguish target DNA from non-target DNA with good specificity, and the detection limit was 0.5 nM. In addition, the operation was relatively simple, and the reaction could take place at only 37 °C,

Table 2 Testing results of marketable commodities (note: “+” means positive. “–” means negative. “B” represents biscuit. “C” represents candy. There were no peanut, soybean and sesame allergens for “nothing” in commodity labeling. “a” signifies that the sample production line had produced products containing soybeans, and “b” signifies that the production line had produced products containing sesame seeds)

Sample	Peanut		Soybean		Sesame		Commodity labeling	Result consistency
	Absorbance	Results	Absorbance	Results	Absorbance	Results		
B1	0.025	+	0.046	–	0.044	–	Peanut	Yes
B2	0.029	+	0.048	–	0.051	–	Peanut	Yes ^a
B3	0.043	–	0.030	+	0.049	–	Soybean	Yes
B4	0.046	–	0.025	+	0.053	–	Soybean	Yes
B5	0.044	–	0.046	–	0.019	+	Sesame	Yes ^a
B6	0.043	–	0.050	–	0.023	+	Sesame	Yes
B7	0.051	–	0.045	–	0.044	–	Nothing	Yes
B8	0.049	–	0.048	–	0.047	–	Nothing	Yes
C1	0.026	+	0.048	–	0.052	–	Peanut	Yes ^b
C2	0.029	+	0.045	–	0.044	–	Peanut	Yes
C3	0.044	–	0.023	+	0.051	–	Soybean	Yes
C4	0.047	–	0.018	+	0.025	+	Soybean	Yes ^b
C5	0.048	–	0.045	–	0.031	+	Sesame	Yes
C6	0.047	–	0.046	–	0.025	+	Sesame	Yes
C7	0.048	–	0.053	–	0.044	–	Nothing	Yes
C8	0.046	–	0.047	–	0.048	–	Nothing	Yes

without the need for other harsh conditions such as enzymes. The method has potential applications in food safety detection, disease diagnosis, environmental monitoring and so on.

Conflicts of interest

The authors declare that there is no conflict of interest.

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