



Analytical Methods

Detection of peanut (*Arachis hypogaea*) allergen by Real-time PCR method with internal amplification controlWen-Ju Zhang¹, Qin Cai¹, Xiao Guan, Qin Chen^{*}

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ARTICLE INFO

Article history:

Received 20 January 2014

Received in revised form 23 June 2014

Accepted 16 November 2014

Available online 22 November 2014

Keywords:

Peanut (*Arachis hypogaea*)

Allergen

Ara h 1

Real-time PCR

Internal amplification control (IAC)

ABSTRACT

Specific primer sets were designed based on the DNA sequence of Ara h 1, one of the major peanut (*Arachis hypogaea*) allergens, and a competitive internal amplification control (IAC) was designed by compound primer technology. By choosing 314 copies/PCR as the IAC dosage, a Real-time PCR method with IAC was established for detecting peanut allergen Ara h 1 DNA. The method showed high specificity with a detection limit of 0.005% peanut. A series of commercial food products with/without peanut components were tested. Among these products, the peanut allergen Ara h 1 DNA could be detected in 12 products labelled containing peanut ingredients, in two without a declaration of peanut and one labelled that was produced in a facility that produced peanut-containing foods. This indicates that the method is highly sensitive for the detection of peanut ingredients in foods.

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1. Introduction

Hypersensitivity reaction to dietary substances from peanuts is a public health problem, even a trace amount of peanut can trigger severe allergic reactions. The symptoms of peanut allergies varied from mild urticaria to life-threatening anaphylactic shock. Indeed, several fatal cases caused by peanut allergies have already been reported (Bock, Muñoz-Furlong, & Sampson, 2001, 2007; Zhao-Yun, 2001). Generally, peanut allergies do not disappear with age (Sampson, 2004). There is currently no effective therapy for peanut allergies (Kun-Mei, Jia-Jie, Hai-Yan, Xiao-Yu, & Zhi-Gang, 2010). Avoiding potential peanut-containing foods is the only way for sufferers to avoid being harmed. Therefore, establishing a reliable method for the detection of peanut allergens is very important.

Real-time PCR technique is for DNA detection and quantitation, which monitors the accumulation of fluorescent signal produced during the amplification of a DNA template in real time. In recent years, it has been widely used in the detection of food allergens, such as celery (Fuchs, Cichna-Markl, & Hocheegger, 2012), crustacean (Herrero, Vieites, & Espiñeira, 2012) and peanut (López-Calleja et al., 2013). In Real-time assay, the presence of impurities and other inhibitors that are present during DNA extraction may lead to the false-negative results, which mean originally positive samples were detected as negative. False-negative results may

result in fatalities in peanut allergy sufferers who inadvertently consume contaminated products (Xiao-Hua & Xian-Ming, 2010). Adding an IAC sequence to the PCR reaction system is one of the ways to improve the reliability of PCR results. In this study, internal amplification control (IAC), an artificial DNA sequence was constructed and co-amplified simultaneously with target gene. PCR combined with IAC have been widely used in detecting foodborne pathogens, such as *Vibrio parahaemolyticus* (Xiao-Hua et al., 2010) and *Salmonella* (Xian-Long et al., 2011). However, this method has not been reported in food allergens detection. Given this, we established a Real-time PCR with IAC method, and applied for detecting peanut allergen Ara h 1 DNA in the commercial food products.

2. Materials and methods

2.1. Samples preparation and pre-treatment

All samples used in this study were purchased from local supermarket and pre-treated. Among them, Peanut (*Arachis hypogaea*), sorghum (*Sorghum bicolor*), corn (*Zea mays*), barley (*Hordeum vulgare*), wheat (*Triticum aestivum*), buckwheat (*Fagopyrum esculentum*), oat (*Avena sativa*), rice (*Oryza sativa*), millet (*Setaria italica*), soybean (*Glycine max*), peas (*Pisum sativum*), sesame (*Sesamum indicum*), celery (*Apium graveolens*), tomato (*Solanum lycopersicum*), apple (*Malus domestica*), pear (*Pyrus bretschneideri*), shrimp (*Palinuridae*), crab (*Liocarcinus marmoreus*), oyster (*Crassostrea gigas*), atlantic salmon (*Salmo salar*), 8 kinds of biscuits, 2 kinds of powders, 2 kinds of candies, and 2 kinds of nuts were grinded

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for DNA extraction and stored at 4 °C. Liquid samples, including cow's milk and 6 kinds of drinks were used for DNA extraction without pre-treatment.

2.2. DNA extraction

DNA from peanut (including peanut with wheat powder as matrix), sorghum, corn, barley, wheat, buckwheat, oat, rice, millet, soybean, peas, celery, tomato, apple, pear and were extracted by the modified CTAB (Cetyl Trimethyl Ammonium Bromide) method (Qin et al., 2009; Moller, Bahnweg, Sandermann, & Geiger, 1992). 0.2 g samples were grinded and incubated in 800 μ L TES (100 mM Tris, pH 8.0, 10 mM EDTA, 2% SDS), including 50 μ g Proteinase K at 60 °C for 1 h. Salt concentration was adjusted to 1.4 M with 5 M NaCl, 1/10 volume 10% CTAB (cetyltrimethylammonium bromide) was added, incubating for 30 min at 65 °C, and centrifuged for 10 min. Supernatant was transferred to a new tube, equal volume phenol–chloroform–isoamyl alcohol (phenol: chloroform: isoamyl alcohol, 25:24:1, v/v) was added and centrifuged. Supernatant was transferred to a fresh tube, equal volume SEVAG (chloroform: isoamyl alcohol, 24:1, v/v) was added and centrifuged. Supernatant was transferred to a fresh tube, 3 μ L RNase (10 mg/mL) was added and the sample was incubated at 37 °C for 30 min. 0.7 volume isopropanol was added to precipitate DNA, sample was placed at –20 °C for approx 60 min, and centrifuged. Supernatant was removed and pellets were washed twice with cold 70% ethanol, dried in ambient, and dissolved in about 50 μ L ddH₂O. The DNA were stored at –20 °C. DNA was extracted from cow's milk using the method described previously (Qing-Jin, Zhi-Rui, Wen-Ju, & Qin, 2012). The Plant Genomic DNA Kit (TIANGEN, Beijing, China) was used to extract DNA from sesame exactly according to the manufacturer's instructions. The QIAGEN DNeasy® Blood and Tissue Kit (QIAGEN, MD, USA) was used to extract DNA from shrimp, crab, oyster and atlantic salmon exactly according to the manufacturer's instructions. The GMO food DNA Extraction Kit (TIANGEN, Beijing, China) was used to extract DNA from various commercial food products exactly according to the manufacturer's instructions. The DNA extractions were repeated 3 times and mixed for the following experiment. DNA concentrations (ng/ μ L) were determined spectrophotometrically using a NanoDrop™ 1000 spectrophotometer (Thermo Scientific, MA, USA).

2.3. Primers

Primer sets Ara h 1-F/R were designed using the Primer Premier 5.0 (Premier, CA) based on the Ara h 1 gene (AF432231) from peanut. Sequences of specific primer sets 18S rRNA-F/R based on the nuclear 18S rRNA gene were derived from previous research (Shu-Ya, Hua-Jie, Yi-Ning, Ni, & Fu-Wei, 2012). The primers FSL-F/R were designed based on the gene encoding FSL R2-561 (NC017546) gene from *Listeria monocytogenes*. All primers were synthesized by Sangon Biotech (Shanghai, China). The sequences of all primers are listed as follows, Ara h 1-F, CTGGAACCTTGAACTCGT, Ara h 1-R, GTTGATGGCTACTGGATGA, 18S rRNA-F, TCTGCCCTATCAACTTTCGATGGTA, 18S rRNA-R, AATTGCGCGCCTCTGCCTTCCTT, FSL-F, TAGCCAACCGATGTTCTGTATC, FSL-R, CATCATTTAGCGTGACTTTCTTTCA.

2.4. Conventional PCR

The concentrations of the reagents in the final volume of 20 μ L were: 2 μ L 10 $\times$ Buffer (Mg²⁺ plus) (Takara Biotechnology, Dalian, China), 3.2 μ L dNTP (2.5 mM) (Beijing ComWin Biotech, Beijing, China), 0.2 μ L rTaq (5 U/ μ L) (Takara Biotechnology, Dalian, China), 1 μ L primers (each 3 μ M), 2 μ L DNA template, 11.6 μ L double distilled water. The reaction was run on an iCycler® Thermal Cycler

(Bio-Rad, USA) and the temperatures applied were as follow: 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 52 °C for 30 s (FSL-F/R), 54 °C for 30 s (18S rRNA-F/R), 59 °C for 30 s (Ara h 1-F/R and IAC-F/R) and 72 °C for 25 s; followed by one step at 72 °C for 10 min. The PCR products were electrophoresed on a 1.5% agarose gel in 1 $\times$ TAE and analysed with Tanon-3500 Gel Image System (Tanon Science and Technology, Shanghai, China).

2.5. Real-time PCR

The Real-time PCR reaction was performed in a final volume of 20 μ L, containing 10 μ L 2 $\times$ SYBR® Green Real-time PCR Master Mix (Toyobo, Japan), 1 μ L primers (each 3 μ M), 50 ng DNA template, IAC (optimised concentration), double distilled water up to 20 μ L. Application was carried out in a CFX96 Real-time PCR System (Bio-Rad, USA), using the following PCR programme: initial denaturation at 95 °C for 30 s; 40 cycles consisting of denaturation at 95 °C for 10 s, annealing at 59 °C for 15 s, extension at 72 °C for 25 s, data acquisition of the signal took place once every 0.5 °C, continuous 5 s during annealing and elongation of each cycle from 55 °C to 95 °C.

2.6. IAC construction

The IAC primer sets IAC-F/R (IAC-F, CTGGAACCTTGAACTCGTAGCCAAC, IAC-R, GTTGATGGCTACTGGATGACATCATT) were formed by connecting part sequences of the primers FSL-F/R to 3' end of Ara h 1-F/R primer pairs and synthesized by Sangon Biotech (Shanghai, China). While the IAC construction was carried out according to the compound primer technique (Siebert & Larrick, 1993), the scheme was showed in Fig. 1A. Firstly, a 351 bp amplicon was obtained when *Listeria monocytogenes* DNA was amplified based on FSL-F/R primer sets (Fig. 1B). Secondly, the 351 bp amplicon and IAC-F/R primer sets were used as template and primers to generate a 389 bp product (Fig. 1C). Thirdly, the obtained 389 bp fragment was ligated into the pMD18-T Simple Vector (Takara Biotechnology, Dalian, China), and transformed into *Escherichia coli* DH5 α (TIANGEN, Beijing, China), then positive clones were selected by a PCR screening with the vector specific M13-F/R primers. Finally, IAC-plasmid purification was carried out using the TIANprep Mini Plasmid Kit (TIANGEN, Beijing, China) according to the manufacturer's instructions and sequenced by Sangon Biotech (Shanghai, China). The copy numbers of IAC-plasmid could be calculated as follows: $N = (m/M) \times 6.02 \times 10^{23}$, where N equals to the copy numbers of IAC (copies), m equals to the mass of IAC (g), M equals to the molecular weight of IAC (1.92×10^6 g/mol).

3. Results

3.1. Amplification of specific primers based on 18S rRNA

Specific 18S rRNA-F/R primers were used to amplify various DNA samples from peanut and other samples. DNA extracted from all the plants and animal species yielded amplicons with the same length (approximately 137 bp), indicating that these DNA samples were all available for PCR amplification.

3.2. Sensitivity of the Real-time PCR method without IAC

The DNA from peanut powder serially diluted with wheat powder were extracted and amplified with primers Ara h 1-F/R. The result was shown in Fig. 2. 0.005% peanut or above was capable to be detected, while no signal was observed below 0.005%.

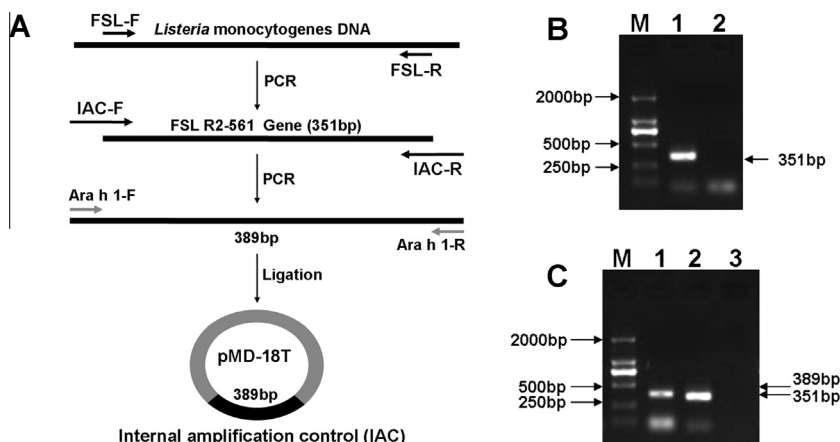


Fig. 1. IAC construction. (A) The scheme of IAC construction; (B) the amplification of a part of FSL R2-561 gene (351 bp). M. DM 2000 marker, 1–2: *Listeria monocytogenes*, negative control; (C) the amplification of 389 bp sequence. M. DM 2000 marker, 1–3: *Listeria monocytogenes*, 351 bp amplicon, negative control.

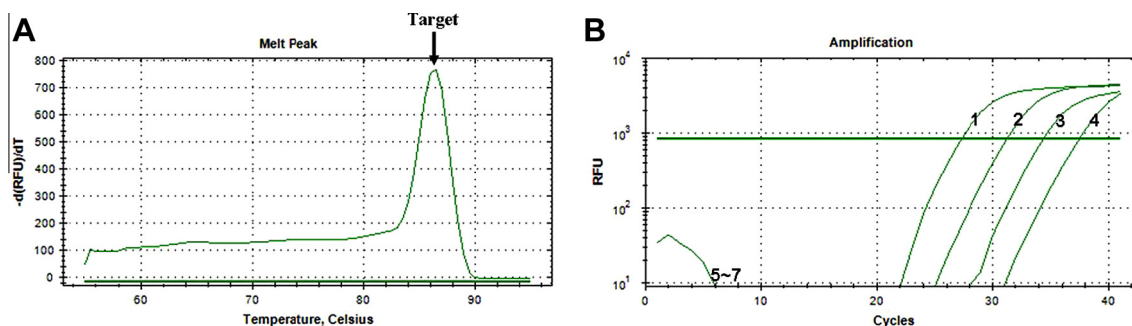


Fig. 2. The amplification of Ara h 1 fragment. (A) The melt curve of Ara h 1 fragment; (B) the sensitivity of PCR method without IAC. 1–7: 5%, 0.5%, 0.05%, 0.005%, 0.0005%, 0.00005% peanut and negative control, respectively.

3.3. Effect of IAC on the Real-time PCR detection sensitivity

3.3.1. The detection limit of IAC

IAC-plasmid was 10-fold serially diluted from 1.57×10^5 copies/PCR to 1.57 copies/PCR, which was used as template and with Ara h 1-F/R primer sets, and the IAC detection limit was tested. The results were shown in Fig. 3A and B. No signal was generated when IAC template was below 1.57×10^2 copies/PCR, while IAC signal was detected when IAC template was 1.57×10^2 copies/PCR or above. As a result, 1.57×10^2 copies/PCR was determined as the detection limit of IAC.

3.3.2. The detection sensitivity of the Real-time PCR method with IAC

Three different amounts of IAC (4-fold, 2-fold, 1-fold of the detection limit of IAC, that is 628, 314, 157 copies/PCR, respectively) were added to the Real-time PCR reactions with DNA from 0.005% peanut sample and the detection sensitivity was tested. Both target and IAC signal were detected, when IAC was 314 copies/PCR; either IAC or target signal was detected at 628 or 157 copies/PCR IAC, respectively (Fig. 3C). In a conclusion, 314 copies/PCR IAC was determined as the optimal amount for the detection of peanut allergen Ara h 1 by Real-time PCR method with IAC, and the detection sensitivity of the Real-time PCR with IAC was still 0.005% peanut, suggesting IAC not altering the detection sensitivity of PCR method.

3.4. Specificity of Real-time PCR method with IAC

The specificity of Real-time PCR assay was verified using 50 ng DNA from 15 plant species (sorghum, corn, barley, wheat,

buckwheat, oat, rice, millet, soybean, peas, sesame, celery, tomato, apple and pear) and 5 animal species (shrimp, crab, oyster, atlantic salmon and cow's milk). The results from wheat, celery and shrimp were shown in Fig. 4. Only peanut DNA template yielded detectable both target and IAC amplicons, whereas those non-peanut DNA templates only generated the IAC signal, indicating the high specificity of the PCR method with IAC.

3.5. Application of Real-time PCR method with IAC

20 Commercial food products from different brands with different label declarations were analysed by using the Real-time PCR method with IAC. 9 products contain peanut as one of the ingredients, 3 with trace amount of peanut, 2 sharing production line with peanut products, 1 with dairy products and 5 were unlabelled. The results are listed in Table 1. Peanut DNA could be detected in 12 products labelled as “containing” or “may contain” peanut, while one of the products that was produced in a facility that produced peanut-containing food and two unlabelled were also declared to contain peanut. Among all the samples, the IAC signal from Powder-1 and Drink-4 could not be detected in the first round Real-time PCR assay (Fig. 5A). When these two DNA samples were 10-fold diluted, both IAC signal, a positive peanut signal in “Drink-4”, and a negative peanut signal in “Powder-1” were detected simultaneously (Fig. 5B).

4. Discussion

Currently, enzyme-linked immunosorbent assay (ELISA) is one of the most commonly used methods by the food industry and

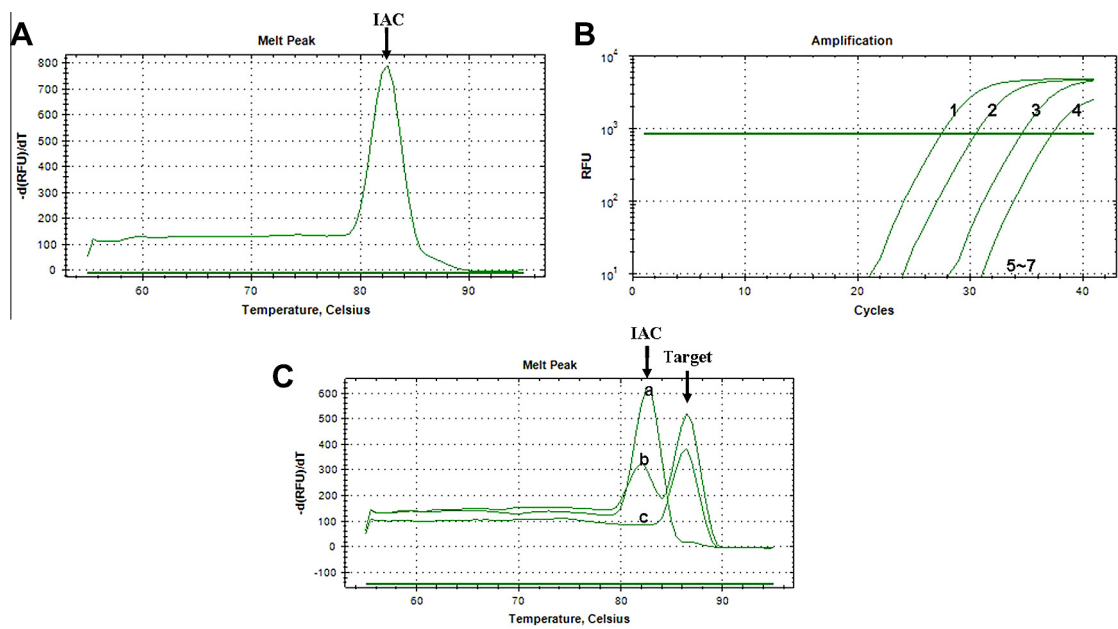


Fig. 3. The establishment of PCR system with IAC. (A) The melt curve of IAC; (B) the detection limit of IAC. 1–7: 1.57×10^5 , 1.57×10^4 , 1.57×10^3 , 1.57×10^2 , 15.7, 1.57 (copies/PCR) and negative control, respectively. (C) Effect of IAC on Real-time PCR method. (a–c) 628, 314 and 157 (copies/PCR), respectively.

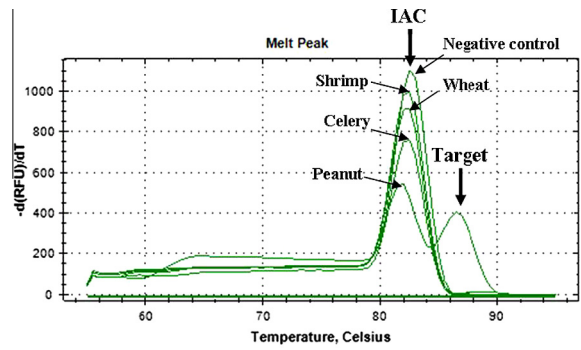


Fig. 4. The specificity of the Real-time PCR method with IAC.

official food control agencies to assess food allergens, such as peanut allergen Ara h 1 (Besler, 2001). It has been reported that a Sandwich ELISA can be used for detection of potential trace amount of peanut (*A. hypogaea*) allergens in processed foods (Stephan & Vieths, 2004). However, the immunochemical method was based on the specific antigen–antibody reaction, which may fail to detect the allergens covered by the food matrix. On the other hand, commercial food processes often involve heat treatment, which denatures food proteins, thereby altering protein tertiary structure and thus affect antigen–antibody reactions (Hird, Lloyd, Goodier, Brown, & Reece, 2003). Compared to protein-based methods, DNA-based methods provide another possibility for food allergens detection. There are several relative methods have been reported in the literature (Hirao, Watanabe, Temmei, Hiramoto, & Kato, 2009; Hird et al., 2003; López-Calleja et al., 2013; Rossi et al., 2006; Scaravelli, Brohée, Marchelli, & van Hengel, 2008; Sforza, Scaravelli, Corradini, & Marchelli, 2005; Stephan & Vieths, 2004; Watanabe et al., 2006). However, PCR methods can be affected even inhibited by several factors. Complex food ingredients and the presence of impurities during DNA samples preparation, may lead to the ineffective PCR reactions. Due to the reaction error, the positive sample might be detected as negative, which is defined as false-negative (Xiao-Hua & Xian-Ming, 2010).

Table 1
Application of the Real-time PCR method with IAC.

Classification	Commercial food product	Allergen information	IAC	Peanut ingredients
Biscuits	Biscuit-1	Unlabelled	+	–
	Biscuit-2	Unlabelled	+	+
	Biscuit-3	Production line ^a	+	–
	Biscuit-4	Production line	+	+
	Biscuit-5	May contain	+	+
	Biscuit-6	May contain	+	+
	Biscuit-7	Contain	+	+
	Biscuit-8	Contain	+	+
Powders	Powder-1	Unlabelled	+ ^b	–
	Powder-2	Contain	+	+
Drinks	Drink-1	Unlabelled	+	–
	Drink-2	Unlabelled	+	+
	Drink-3	Unlabelled	+	–
	Drink-4	May contain	+ ^b	+
	Drink-5	Contain	+	+
	Drink-6	Contain	+	+
Candies	Candy-1	Contain	–	+
	Candy-2	Contain	+	+
Nuts	Nut-1	Contain	–	+
	Nut-2	Contain	–	+

“+” Indicates a melt peak at the corresponding position, “–” indicates no melt peak at the corresponding position.

^a The peanut products had been processed on its production line.
^b The amplicons could not be detected in the first round assay by using original DNA samples. After the DNA samples were 10-fold diluted, the IAC signals could be detected.

Adding an IAC to the PCR reaction is one of the effective ways to show false-negative results (Xiao-Hua & Xian-Ming, 2010; Jing, 2010). The European Standardization Committee (CEN), in collaboration with International Standard Organization (ISO) has proposed a general guideline for PCR testing of food-borne pathogens that requires presence of IAC in the reaction mixture (European Standardization Committee & International Standard Organization, 2005). The Real-time PCR method with IAC for the detection of *Salmonella enterica* (Jing et al., 2010) from food

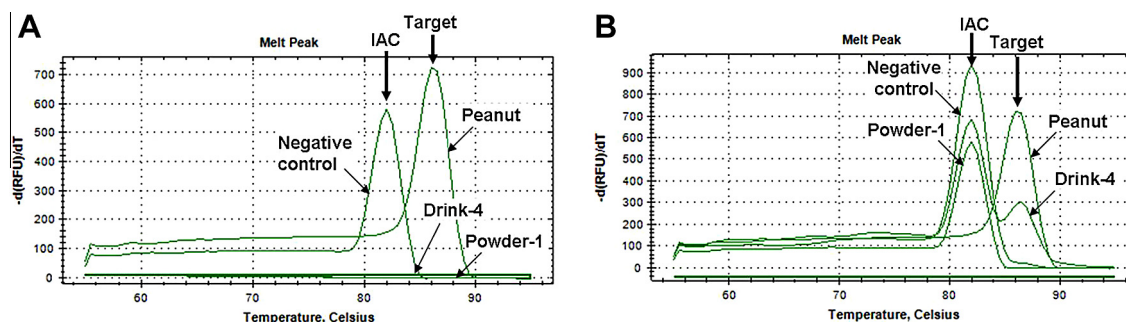


Fig. 5. Indication of PCR inhibition by IAC. The original DNA samples were used in (A), and 10-fold diluted DNA samples were used in (B).

suggested that false-negative results can be found effectively. In a conclusion, IAC can enhance the reliability of PCR methods for detecting target genes in food products.

There are two critical parameters in constructing a competitive IAC. First, low homology between the target gene and IAC is required to avoid being cross-linked between complementary strands. In present study, a part of the FSL R2-561 gene from *Listeria monocytogenes*, which have no homology with the Ara h 1 gene from peanut, was designed as IAC; Second, the size of the IAC should be larger than the target to ensure the competitive edge of the latter. In a PCR reaction, simultaneous amplification of two different DNA fragments flanked by the same primer sets, smaller amplification products is kinetically favored over larger amplicons (Hoorfar et al., 2004; Xian-Long et al., 2011). So, the IAC was designed 200 bp larger than the target, which was amplified preferentially. Due to the competition between the target and IAC, IAC signal may be failed in detection, while the target DNA signal can be detected, which is a valid positive result (Hoorfar et al., 2004). In the cases of Candy-1, Nut-1, and Nut-2 (Table 1), the target DNA signals were detected and the detection of IAC signal was failed.

When adding IAC to a PCR reaction, the amount of IAC is also critical for the detection limit. The competitive IAC can reduce the PCR amplification efficiency and thereby result in a lower detection limit. Thus, establishing an amount of IAC for PCR reaction is the most critical parameter (Hoorfar et al., 2004). Abundant amount of IAC will compete with the target DNA and abolish the target signal, which may cause false-negative results. In addition, if used at high concentration, the IAC might not detect weak inhibition, which could cause false-negative results if the target is present in extremely low concentrations (Rosenstrauss, Wang, Chang, DeBonville, & Spadaro, 1998). So, the lowest reproducible amount of IAC should be optimised. In this study, 314 copies/PCR IAC was effective for detecting both target and IAC signals, and the detection limit was not affected.

By using the Real-time PCR method with IAC, 20 commercial food products were tested. The peanut allergen Ara h 1 DNA could be detected in 9 products with peanut labels, 2 without peanut declarations (Biscuit-2, Drink-2) and 1 labelled that was produced in a facility that produced peanut-containing products (Biscuit-4), suggesting that the Real-time PCR method with IAC can effectively detect trace amounts or potential peanut ingredient in food. Particularly, there were neither IAC nor target signal detected in two samples (Powder-1, Drink-4), suggesting the PCR reaction was inhibited and the presence of impurities during DNA sample preparation may be responsible for the invalid results. To eliminate the effect of inhibitors in the PCR reaction, two ways can be applied, the dilution of template DNA and further purifying the sample DNA. In this study, when DNA samples were 10-fold diluted, Powder-1 showed a negative result and Drink-4 showed a positive result, which were consistent with the labels (Table 1). These

results suggested that the Real-time PCR method with IAC could effectively detect the trace amount of peanut allergen Ara h 1 DNA, and the false-negative results might occur due to the competition between the target DNA and IAC. In conclusion, the Real-time PCR method with IAC can enhance the reliability of PCR methods and be applied into universal detection of food allergens.

Acknowledgements

The authors wish to thank Dr. Zhan-min Liu from Shanghai University for his kindly providing the *Listeria monocytogenes* DNA and the primers for construction of IAC. This study is supported by the National Natural Science Foundation of China (No. 31201306) and the project of Food Science Discipline Construction of Shanghai University.

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