

Development of Sandwich ELISA and Immunochromatographic Strip for the Detection of Peanut Allergen Ara h 2

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Abstract In order to develop highly sensitive immunoassays for detecting the major peanut allergen, Ara h 2, we prepared six monoclonal antibodies (mAb) with high affinity to Ara h 2. Using mAb 5 as a capture antibody and HRP-labeled mAb 1 as a detection antibody, a sandwich enzyme-linked immunoassay (ELISA) was established with a limit of detection (LOD) of 0.02 ng/mL. An immunochromatographic strip based on these two mAbs was also established with a LOD of 1 ng/mL. Both tests showed high specificity in cross-reactivity experiments. Sample analyses indicated that these two immunoassays were feasible, accurate, and highly sensitive for measuring allergen Ara h 2 of food.

Keywords Peanut allergen · Ara h 2 · Monoclonal antibody · Sandwich ELISA · Immunochromatographic strip

Introduction

The broad use of peanuts in foods has led to increasing concerns about allergic reactions to peanuts, which can be severe, prevalent, and tend to persist into adulthood (Maloney et al. 2006; Sicherer et al. 1999; Sicherer et al. 2003). In over 90 % of individuals who suffer from peanut allergies, recognition of the major allergens Ara h 1 and Ara h 2 in peanuts by serum IgE has been implicated (Viquez et al. 2003). Ara h 2, a 17–19-kD doublet glycoprotein, accounts for 5.9–9.3 % of total

peanut protein, which is second only to 12–16 % of Ara h 1; other peanut allergens represent molecules, a very low content in peanuts (Koppelman et al. 2001). Therefore, efforts to develop an assay to specifically measure Ara h 1 or Ara h 2 would be contributed to judging the products containing peanut or not (Palmer et al. 2005; Radosavljevic et al. 2010). Moreover, the assay for a specific allergen would not only be useful for screening for peanut but also useful for studying threshold doses of exposure and conducting risk assessment since the results could be accurately quantified. The mechanisms of peanut allergy also would be better understood owing to the definition of the components being measured (Pomes et al. 2003).

Recently, immunoassay technologies have been widely applied to detect peanut allergens in food products because they are convenient, quick, and sensitive (Holzhauser et al. 2002; Kiening et al. 2005; Peng et al. 2013; Stephan and Vieths 2004). Accordingly, ELISA methods based on monoclonal antibodies to test for the presence of peanut allergen Ara h 1 in food have been well developed (Peng et al. 2013; Pomes et al. 2003). Kun-Mei Ji et al. developed a gold immunochromatography assay for the rapid detection of Ara h 1 (Ji et al. 2011). In addition, Several PCR methods are available for the detection of traces of peanut in food (Lopez-Calleja et al. 2013; Stephan and Vieths 2004). Stephan and Vieths (2004) described a PCR-ELISA based on Ara h 2 gene with a limit of detection (LOD) of 2 ppm. Lopez-Calleja et al. (2013) reported a TaqMan real-time PCR method using Ara h 2 primer with 10-ppm LOD. However, few studies have described the detection of peanut allergen by measuring Ara h 2 or provided a method to quantitatively measure Ara h 2. As a significant predictor of clinical reactivity to peanut, studies of the detection of Ara h 2 are warranted (Bublin and Breiteneder 2014; Dang et al. 2012; Klemans et al. 2013). Herein, for the first time, a sandwich ELISA

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and an immunochromatographic strip method based on two monoclonal antibodies are established for the detection of the major peanut allergen, Ara h 2.

Experiments

Materials and Instruments

Ara h 2 standards (ST-AH2) and Ara h 1 standard (ST-AH1) were obtained from INDOOR Biotechnologies (Charlottesville, VA, USA). Complete and incomplete Freund's adjuvant, bovine serum albumin (BSA), horseradish peroxidase (HRP), and goat antimouse immunoglobulin conjugated to HRP were purchased from Sigma-Aldrich (St. Louis, MO, USA). The 3, 3',5,5'-Tetramethylbenzidine (TMB) substrate and HRP were obtained from Aladdin Chemistry Co. (Shanghai, China). Nitrocellulose high-flow plus membranes (Pura-bind RP) were from Whatman-Xinhua Filter Paper Co. (Hangzhou, China). The glass fiber membrane (CB-SB08), polyvinylchloride (PVC), and absorbance pad (SX18) were purchased from Goldbio Tech Co. (Shanghai, China). All cell fusion reagents were obtained from Sunshine Biotechnology Co. (Nanjing, China). Other reagents were purchased from the National Pharmaceutical Group Chemical Reagent Co. (Shanghai, China). BALB/c mice obtained from the Shanghai Laboratory Animal Center (Shanghai, China) were used in immunization procedures. A microplate reader (Thermo Labsystems; Chicago, IL, USA) was used to measure optical density (OD).

A total of seven types of commercial food products, including peanut kernels, seaweed-flavored peanut beans, Astick peanut crispy rolls, lizkitchenlife, M&M's cho-peanut beans, cashew nuts, and walnuts, were obtained from the Vanguard Market in Wuxi, China.

The buffers and solutions used in this study were (1) PBS: 0.01-M phosphate-buffered saline (PBS), pH 7.4; (2) coating buffer: 0.05-M carbonate-buffered saline, pH 9.6; (3) blocking buffer: coating buffer containing 0.2 % (w/v) gelatin; (4) washing buffer: 0.01-M PBS (pH 7.4) containing 0.2 % (v/v) Tween 20; (5) antibody dilution buffer: 0.01-M PBS containing 0.1 % (w/v) gelatin and 0.05 % (v/v) Tween-20; (6) TMB substrate solution: 5:1 (v/v) mixture of substrate buffer (mixture of 18 mL of 30 % H₂O₂ and 100 mL of 0.1-M citrate phosphate buffer) and TMB solution (60-mg TMB dissolved in 100-mL glycol); and (7) stop solution: 2-M sulfuric acid.

Peanut Protein Extraction and Ara h 2 Purification

Crude protein extract was obtained as described previously (Peng et al. 2013). Briefly, fresh peanuts were ground to powder and defatted using petroleum ether and then were

extracted using 0.01-M PBS overnight at 4 °C. After centrifugation, supernatants were precipitated with ammonium sulfate and purified with ion exchange chromatography. The separated components containing abundant native Ara h 2 were confirmed by SDS-PAGE and stored at −20 °C for further assays.

Preparation of Ara h 2 mAbs

The mAbs to Ara h 2 were obtained by immunizing female BALB/c mice with purified Ara h 2 as an immunogen (Peng et al. 2014). Briefly, 100-μL purified Ara h 2 (1 mg/mL) was emulsified with the same volume of Freund's complete adjuvant. The emulsion was injected subcutaneously into 8-week-old female BALB/c mice. A total of three booster immunizations per month were performed to administer 50-μg peanut proteins in Freund's incomplete adjuvant. The spleen of the mouse exhibiting the highest affinity to Ara h 2 by indirect ELISA was fused with SP2/0 myeloma cells after intraperitoneally injecting 25-μg immunogen. Hybridoma cells were cultured in 96-well plates using HAT solution as culture medium. Then, 7 days after fusion, culture supernatants were screened by indirect ELISA. Hybridoma cells exhibiting high affinity to Ara h 2 were subcloned by limiting dilution using HT solution as culture medium. After repeating these steps three times, the hybridoma cells were collected and intraperitoneally injected into paraffin-primed BALB/c mice. Ascites were extracted to produce mAb after 8 days.

Purification and Labeling of mAb

The mAb was purified by n-caprylic-ammonium sulfate precipitation. Briefly, 33-μL n-caprylic was dropped into 1-mL ascites mixed with 1-mL sodium acetate solution (pH 4.5) for 30 min with gentle shaking at room temperature (RT). Supernatants were filtered using a 0.22-μm filter membrane after centrifugation. Equal volume-saturated ammonium sulfate was added dropwise to the supernatants, which were kept standing overnight. After centrifugation, the precipitate was resuspended with 0.01-M PBS and dialyzed for 3 days. The mAb concentration was determined using an ultraviolet spectrophotometer. The mAb was labeled with HRP using the modified sodium periodate method, as described previously (Kuang et al. 2013). Briefly, HRP was oxidized with 0.06-M NaIO₄ for 30 min at 4 °C and was terminated by adding 0.16-M glycol for 30 min. The purified mAb was added dropwise into the mixture, and 0.05-M carbonate buffer (pH 10.0) was added to maintain the system at pH 9.0. After 16 h, the reaction was stopped by NaBH₄, and then, an equal volume-saturated (NH₄)₂SO₄ was added to obtain a stable antibody. After incubating overnight, the mixture was centrifuged, and

the precipitate was resuspended with 0.01-M PBS. The HRP-labeled mAb was obtained after dialyzing for 3 days.

Indirect ELISA

A 96-well microplate was coated with 100 μ L/well of 0.2- μ g/mL ST-AH2 in coating buffer at 4 °C overnight. Plates were blocked with 200 μ L/well of blocking buffer for 2 h at 37 °C after washing three times with washing buffer. After washing, 100 μ L/well of cell supernatant, mouse serum, or mAb diluted with antibody dilution buffer was added and incubated for 30 min at 37 °C. After washing, 100 μ L/well of HRP-labeled goat antimouse immunoglobulin (1:3000 v/v) was added and incubated for 30 min at 37 °C. After washing, 100 μ L/well of TMB substrate was added, and 15 min later, the reaction was stopped by adding 50 μ L/well of 2-M sulfuric acid. The absorbance of each well was read using a microplate reader at 450 nm (Le et al. 2013; Li et al. 2014).

Sandwich ELISA

A 96-well microplate was coated with 100 μ L/well of anti-Ara h 2 mAb (2 μ g/mL) overnight at 4 °C. After washing, 200 μ L/well of blocking buffer was added for incubation for 2 h at 37 °C. After washing, 100 μ L/well of standard solution or sample extract solution was added for 1 h at 37 °C. After washing, 100 μ L/well of HRP-labeled anti-Ara h 1 mAb (1:800 v/v) was added for 1 h at 37 °C. After washing, 100 μ L/well of TMB substrate was added for 15 min at 37 °C. Then, the reaction was stopped by adding 2-M sulfuric acid. Absorbance was measured using a microplate reader at OD₄₅₀ (Feng et al. 2013; Peng et al. 2014).

Sandwich ELISA Optimization

A chalkboard method was designed to obtain an optimal concentration of capture antibody and detection antibody in the sandwich ELISA. Briefly, plates were coated with capture mAb diluted at 10, 5, 2.5, and 1.25 μ g/mL, respectively. After blocking, 100- μ L 25-ng/mL ST-AH2 and 100- μ L 0.01-M PBS were added as positive and negative controls, respectively. Subsequently, detection antibody was added at serial dilutions ranging from 0.625 ng/mL to 5 μ g/mL. After coloration and termination, as described previously, the highest OD₄₅₀ ratio of positive and negative controls (P/N value) was regarded to be the optimal combination.

An Immunochromatographic Strip for Detecting Ara h 2

An immunochromatographic strip was developed according to a method reported previously (Lee et al. 2006; Liu et al.

2014a, b). Colloidal gold nanoparticles (GNPs) with a 20-nm size were obtained using the sodium citrate reduction method (Xing et al. 2013). The pH of 1-mL colloidal gold solution was adjusted to 6.0 using 4- μ L 0.1-M K₂CO₃ (Xing et al. 2014a, b). Subsequently, 150- μ L 0.2-mg/mL mAb 1 was added into the GNP solution (1 mL). After incubation for 30 min at room temperature, 50 μ L of 10 % BSA solution was added to block the GNP surface. The mixture was centrifuged at 12,000 rpm for 20 min, and the precipitate was resuspended in PBS containing 2 % (w/v) BSA, 2 % (w/v) sucrose, and 0.02 % (w/v) sodium azide.

The strip was assembled as shown in Fig. 5a. The colloidal gold-labeled mAb 1 was sprayed onto the glass fiber membrane as a sample pad. The mAb 5 (4 mg/mL) and goat antimouse IgG antibody were dispersed on the nitrocellulose membrane as the test (T) and control (C) lines. After drying 2 h at 37 °C, the integrated strips were stored in a desiccator for later use. Upon testing, samples were dropped on the sample pad where positive samples would be captured by GNP-labeled mAb 1. Along with the liquid flow to the test and control lines by capillary forces, the captured antibody complex proceeds to interact with mAb 5 coated on the test line and goat antimouse antibody coated on the control line. When both T line and C line became colored, it was considered to be positive. If only C line was colored, it was considered to be negative. No lines colored indicated an invalid test that should be repeated with a new strip.

The sensitivity of the test strip was determined by testing serially diluted Ara h 2 standard solutions.

Spiking Experiments, Specific Evaluations, and Tests with Processed Food

Nestle fibre diet of corn milk for breakfast which contains 9 % protein, 88 % carbohydrate, and 3 % fat was selected for spiking experiments. This sample that did not contain peanuts was diluted ten times with 0.01-M PBS and then was spiked with ST-AH2 at concentrations of 0.2, 1, and 2 ng/mL. The concentration of Ara h 2 in the spiked sample was measured by sandwich ELISA.

The cross-reactivity assay was carried out by detecting cashew nuts, walnuts, BSA, egg albumin (OVA), soy proteins, and pea proteins. Other food samples from a local supermarket were prepared to determine the practicability, as previously described. Briefly, 1-g solid sample was ground in a grinder and defatted using petroleum ether. The precipitate was then extracted with 10-mL extraction solution (0.01-M PBS) by stirring overnight at room temperature. After centrifugation at 8000 rpm for 10 min, supernatants were diluted serially with 0.01-M PBS and were measured using either sandwich ELISA or strips.

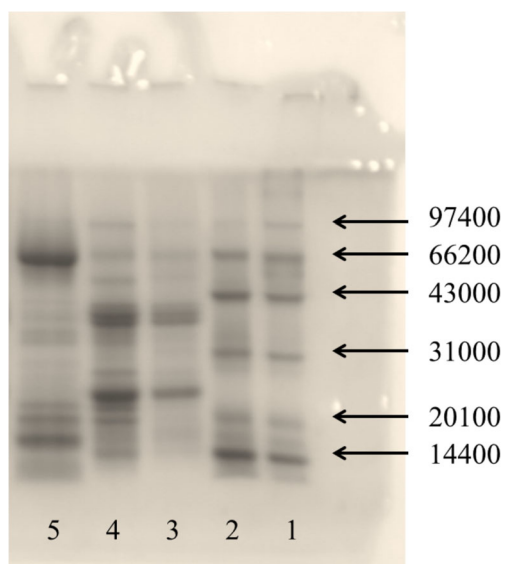


Fig. 1 SDS-PAGE of 40, 60, and 80 % saturated ammonium sulfate component. *Lanes 1 and 2*, MW standards; *lane 3*, 40 % component; *lane 4*, 60 % component; and *lane 5*, 80 % component

Results and Discussion

Production of Ara h 2 mAb

Peanut protein extract precipitated by 80 % saturation with ammonium sulfate revealed bands between 17 and 19 kDa, which were greater than those revealed with 40 % or 60 % saturation (Fig. 1). After further purification to 80 % saturated ammonium sulfate using ion exchange chromatography, two main types of eluate were collected and assayed by SDS-PAGE (Figs. 2 and 3). Figure 3 shows that the final eluate had expected bands between 17 and 19 kDa, which indicated that this eluate might contain a large amount of native Ara h 2,

Fig. 2 Ion exchange chromatography of 80 % saturated ammonium sulfate component

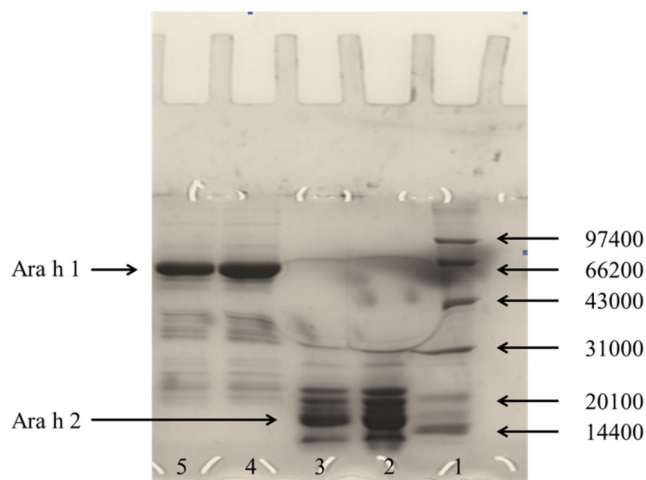
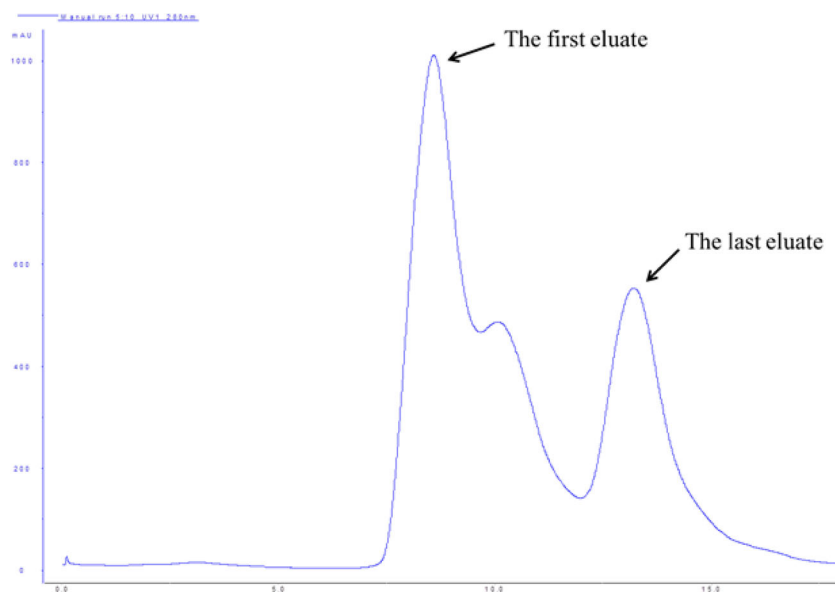


Fig. 3 SDS-PAGE of two main kinds of eluate. *Lane 1*, MW standards; *lanes 2 and 3*, the last eluate; *lanes 4 and 5*, the first eluate

and it was therefore selected as an immunogen to simulate immune responses in mice. Mouse serum was assayed by indirect ELISA after the fourth immunization. The mouse with the highest titer was selected for cell fusion. A total of 12 strongly positive wells that showed anti-Ara h 2 reactivity were obtained from the initial screening via indirect ELISA. Additionally, after three generations of cloning, six stable hybridoma cell lines, termed mAb 1–mAb 6, were selected for mAb production. HRP-labeled mAb 1–mAb 6 were obtained after these mAbs were labeled separately with HRP.

Pairwise Assay

As shown in Table 1, 36 combinations were obtained using mAb 1–mAb 6 as a capture antibody and HRP-labeled mAb 1–mAb 6 as a detection antibody for sandwich ELISA.

Aliquots of 100- μ L standard solution (25-ng/mL ST-AH2) were added as positive controls, and aliquots of 100- μ L standard diluted solution (0.01-M PBS) were added as negative controls for this sandwich ELISA. The highest OD₄₅₀ ratio of the positive and negative controls (P/N value) was obtained using mAb 5 as a capture antibody and HRP-labeled mAb 1 as a detection antibody. Consequently, this combination was selected for use in further experiments.

Sandwich ELISA Optimization

As shown in Table 2, the highest P/N value was obtained when the concentration of mAb 5 was 2.5 μ g/mL and the concentration of HRP-labeled mAb 1 was 1.25 μ g/mL.

Development of a Sandwich ELISA for Ara h 2

The sandwich ELISA was constructed using mAb 5 at 2.5 μ g/mL as a capture antibody and HRP-labeled mAb 1 at 1.25 μ g/mL as a detection antibody. Standard curves for the detection of Ara h 2 were established with double-gradient dilutions from 0.006 to 25-ng/mL ST-AH2 (Fig. 4). The LOD calculated based on the average baseline value plus $3 \times$ SD was 0.02 ng/mL. Peanut is composed of 22–30 % protein and 44–56 % oil, and Ara h 2 as a major allergen accounts for 5.9–9.3 % of total peanut protein (Bublin and Breiteneder 2014). Therefore, after conversion, the LOD of ELISA assay was between 0.7 and 1.7 μ g peanut/kg of product.

Evaluation of the Ara h 2 Test Strip

Ara h 2 standards were diluted to concentrations of 0, 0.5, 1, 2, , and 8 ng/mL in 0.01-M PBS (pH 7.4). As shown in Fig. 5b, the T line produced by 1 ng/mL was clearly distinguishable, while the line obtained by 0.5 ng/mL was difficult to discriminate from the background. Therefore, the sensitivity of the strip was found to be 1 ng/mL. Likewise, the LOD of the strip could also be expressed to be between 35.8 and 77.1 μ g peanut/kg of product.

Table 1 P/N value of 36 combinations in pairwise analysis

Detection antibody	Capture antibody					
	mAb 1	mAb 2	mAb 3	mAb 4	mAb 5	mAb 6
HRP-mAb 1		7.07	3.25	1.24	19.26	1.29
HRP-mAb 2	1.70		0.89	0.89	1.31	0.94
HRP-mAb 3	1.21	1.03		0.98	1.00	1.00
HRP-mAb 4	1.80	2.65	1.41		18.70	1.08
HRP-mAb 5	14.85	1.44	1.18	2.72		2.85
HRP-mAb 6	1.30	1.57	1.16	1.11	6.42	

Table 2 P/N value of different concentration of capture antibody and detection antibody in optimization analysis

	mAb 5				
	P/N value	10 μ g/mL	5 μ g/mL	2.5 μ g/mL	1.25 μ g/mL
HRP-labeled mAb 1	5 μ g/mL	15.09	18.23	17.38	10.53
	2.5 μ g/mL	19.76	20.60	18.51	13.11
	1.25 μ g/mL	21.07	20.85	22.64	12.18
	0.625 μ g/mL	16.61	15.62	12.37	10.11

Detection of Ara h 2 in Food Using the Sandwich ELISA and Strip Methods

Food samples were measured using the newly developed sandwich ELISA and strip methods. As shown in Table 3, peanut kernel showed the highest content of Ara h 2, which reached 806 μ g/g. Lizkitchenlife had the lowest value of 19 μ g/mL, followed by 36 μ g/mL for seaweed-flavored peanut bean, 125 μ g/mL for Astick peanut crispy rolls, and 194 μ g/mL for M&M's cho-peanut bean. By contrast, other samples, including cashew nuts, walnuts, BSA, egg albumin (OVA), soy proteins, and pea proteins, were below the LOD, indicating that the assay had very low cross-reactivity (data not shown). Because of the effect of complex food matrix and processing practices, the efficiency of protein extraction and recognition by antibodies were predicted to be greatly reduced (Khuda et al. 2012; Schmitt et al. 2009; Wen et al. 2007). For this reason, the Ara h 2 contents that we detected in processed foods using the optimized sandwich ELISA were lower than the predicted values. However, the two enzyme immunoassays that we developed still satisfy the requirements for a rapid screening method for peanut allergens because of their high sensitivities and specificities.

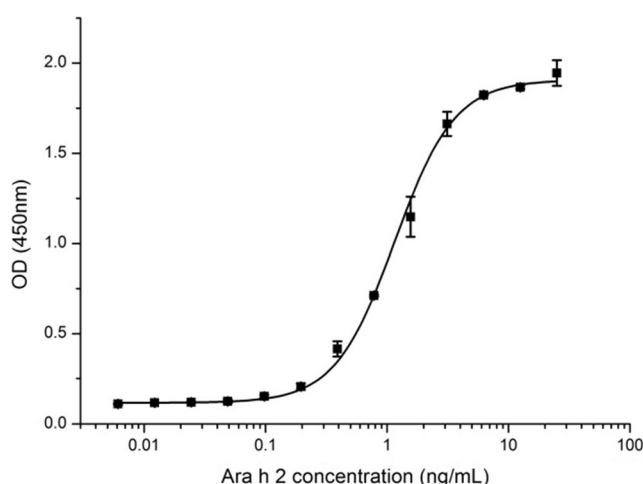


Fig. 4 Calibration curve of the optimized sandwich ELISA

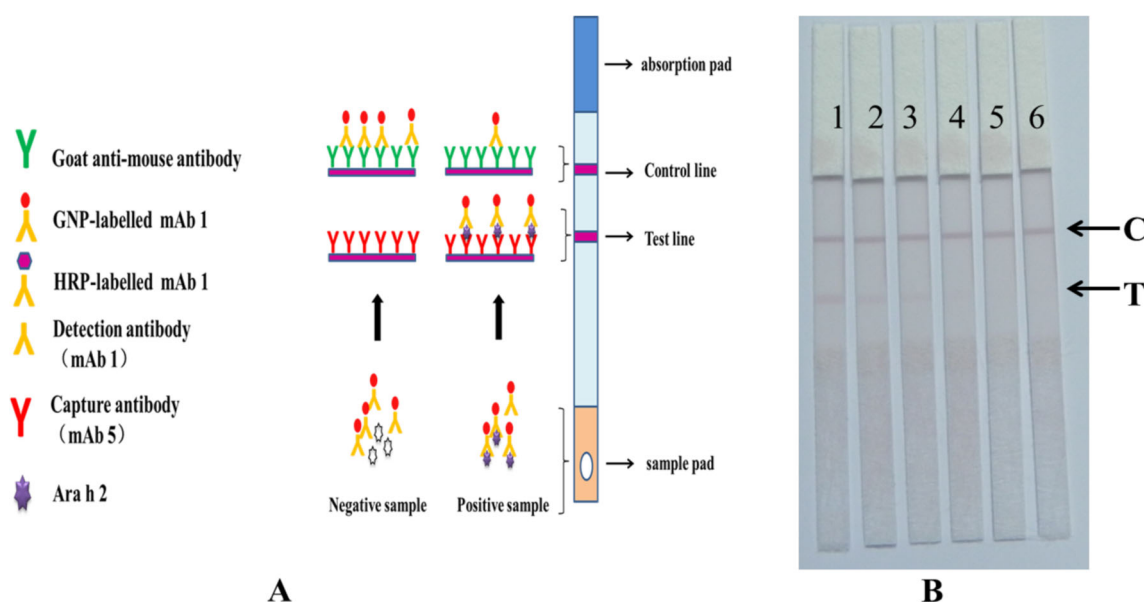


Fig. 5 **a** Schematic of a gold immunochromatography strip based on mAb. **b** Detection of Ara h 2 with the developed strips. 1, 8 ng/mL; 2, 4 ng/mL; 3, 2 ng/mL; 4, 1 ng/mL; 5, 0.5 ng/mL; 6, 0 ng/mL

Recovery

As shown in Table 4, intra-assay recovery ranged from 87 to 90 %, and inter-assay recovery ranged from 81 to 95 %. The intra- and inter-assay coefficients of variation ranged from 1.4 to 8.8 % and from 1.1 to 13.5 %, respectively.

Conclusion

In conclusion, six high-affinity mAbs that recognized Ara h 2 were obtained using purified Ara h 2 as an immunogen. Using mAb 5 as a capture antibody and HRP-labeled mAb 1 as a detection antibody, a highly sensitive sandwich ELISA was developed to detect Ara h 2 in peanuts and peanut products. The LOD of the assay was 0.02 ng/mL, whereas some real-time PCR methods were on the Ara h 2 gene with detection limit of ppm level (Lopez-Calleja et al. 2013; Stephan and

Vieths 2004). These newly developed assays also showed very low cross-reactivity to cashew nuts, walnuts, BSA, egg albumin (OVA), soy proteins, and pea proteins. Our recovery and validation study findings further established that the assay provides an accurate and stable method for detecting Ara h 2 in foods. Furthermore, a gold immunochromatography assay based on these two mAbs was also established with an LOD of 1 ng/mL. Both the sandwich ELISA and strip assays described in this study were effective tools for detecting peanut allergens. The sandwich ELISA had lower LOD and could yield quantitative results after reading with a microplate reader. Positive or negative results of the strip assay, which could estimate whether a product contained peanut allergens or not, were visible to the naked eye. However, generating quantitative results required a specific strip reader (Xing et al. 2014a, b). The whole test process for a strip required only 10 min without any apparatus, which was much simpler and faster than ELISA. These attributes made it more suitable as a self-assessment tool for customers without specialized skills and for the large amount of food sample testing that takes place in custom checks. Peanuts are widely used for the preparation of many

Table 3 Detection of Ara h 2 in processed foods by the optimized sandwich ELISA

Processed foods	ELISA test	Strip test
Peanut kernels	806 ^a	Positive
Seaweed-flavored peanut beans	36 ^a	Positive
Astick peanut crispy rolls	125 ^a	Positive
lizkitchenlife	19 ^a	Positive
M&M's cho-peanut beans	194 ^a	Positive
Cashew nuts	Negative	Negative
Walnuts	Negative	Negative

^a Means Ara h 2/food (μg/g)

Table 4 Recovery of Ara h 2 from spiked milk

Spiked level (ng/mL)	Intra-assay (n=6)		Inter-assay (n=6)	
	Mean ± SD (ng/mL)	Recoveries (%)	Mean ± SD (ng/mL)	Recoveries (%)
0.2	0.18±0.005	90.00	0.19±0.017	95.00
1	0.89±0.072	89.00	0.81±0.11	81.00
2	1.74±0.025	87.00	1.83±0.02	91.50

foods, which represents a potential threat to peanut allergy sufferers. Therefore, the development of a highly sensitive and convenient sandwich ELISA and an immunochromatography strip method to measure Ara h 2 could estimate whether or not products contained peanuts. Furthermore, it yielded two new important quantitative tools to monitor Ara h 2 content.

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Conflict of Interest Juan Peng declares that she has no conflict of interests. Shanshan Song declares that she got grant from Jiangsu Province (BE2013613). Liqiang Liu declares that he got grant from Jiangsu Province (BE2013611) and MOF(201310128). Hua Kuang declares that she got grant from MOST (2012BAD29B05). Chuanlai Xu declares that he got grant from MOST(2012BAK08B01) and MOE(201310135). This article does not contain any studies with human subjects. All animal studies were carried out under the guidance of animal welfare committee of Jiangnan University.

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