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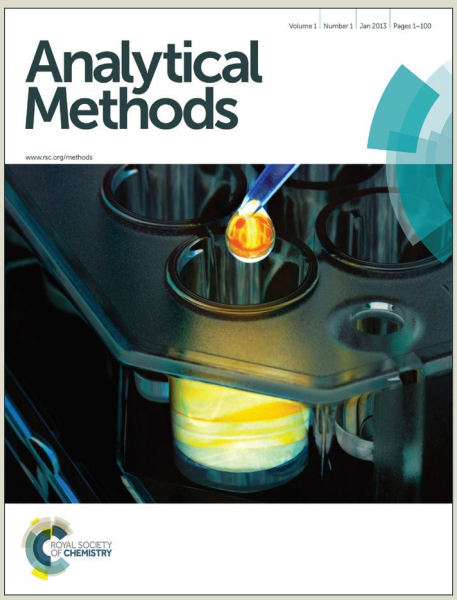

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Development of an immunochromatographic strip for the semi-quantitative and quantitative detection of biotin in milk and milk products

Dezhao Kong, Liqiang Liu, Shanshan Song, Hua Kuang, Chuanlai Xu*

An immunochromatographic strip was developed for the semi-quantitative and quantitative detection of biotin in milk and milk products. This detection system was developed based on a sensitive and specific monoclonal antibody produced in our laboratory. The semi-quantitative results, which were visually obtained in 20 min, revealed that the visual limit of detection was 2.0 μg/100 g with a cut-off value of 8.0 μg/100 g. The quantitative results, which are obtained with a strip scan reader, revealed that the calculated limit of detection was 0.32 μg/100 g with a linear range of 0.65–68 μg/100 g. Biotin content in milk and milk products were determined by the developed immunochromatographic strip, and the results were validated by the microbiological assay method. Our developed immunochromatographic strip system is suitable for the on-site detection and rapid screening of biotin in food samples.

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Introduction

Biotin (coenzyme R, D-biotin, vitamin B7, vitamin H, and W factor),[1] is a water-soluble B vitamin, which participates in multiple metabolic reactions and affects growth and development in living organisms.[2-5] Biotin cannot be synthesized by humans and other mammals; therefore, the vitamin must be obtained from dietary sources such as egg yolk, organ meats, vegetables, and dairy products. Additionally, bacteria present in the large intestine synthesize the vitamin.[6-8] Biotin deficiency, while rare, occurs in subjects with inborn lack of biotin bio-enzyme,[9, 10] on long-term therapy with anticonvulsant agents[11] or parenteral nutrition,[12, 13] with inflammatory bowel disease,[14, 15] or with alcohol addiction[16, 17]. Biotin deficiency causes xerosis cutis, erythra, and hair mats. Based on animal studies, biotin deficiency during pregnancy leads to embryonic growth retardation, congenital malformation, and death.[18-21] In infants, biotin deficiency leads to severe neurological symptoms and sudden infant death syndrome. Therefore, biotin was supplied in many kinds of food, especially the infant foods or some related dietary supplements.[22-25] Biotin determination in fortified or enriched foods such as dietary supplement and infant formula is an important aspect of the food industry.

Currently, biotin can be determined by instrument detection methods[26-29] or microbiological assays.[30-33] While the instrument detection methods are sensitive and accurate, they are expensive, time consuming, and require complex sample pretreatment steps. The microbiological assay, which is the most sensitive, is the standard biotin determination method worldwide. The VitaFast® Vitamin B7 Biotin kit, developed by R-Biopharm Co., Ltd. (Germany), is a microbiological assay with a

detection range of 0.08–0.72 μg biotin/100 g (ml). However, the instability and high price of the test kit limit its applications.

The enzyme-linked immunosorbent assay (ELISA), which is simple, inexpensive, and sensitive, is based on antigen-antibody reactions and is an ideal method for the rapid determination of biotin in food samples.[34] Biotin–Streptavidin System is a newly biological response amplifying system. And this system also be used for the detection of biotin.[35] Additionally, the lateral-flow immunochromatographic strip assay represents a rapid, simple, inexpensive, and instrument-free diagnostic tool[36, 37]. In the immunochromatographic strip assay, semi-quantitative results can be obtained in 5–10 min,[38] while quantitative results can be rapidly obtained with strip scan readers.[39, 40] In this study, we developed and optimized an immunochromatographic strip for the semi-quantitative and quantitative determination of biotin in milk and milk products.

Materials and methods

Chemicals

Biotin N-hydroxysuccinimide ester (Biotin-NHS) and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO, USA). Goat anti-mouse immunoglobulin (IgG) antibody was purchased from Jackson ImmunoResearch Laboratories. Other reagents and chemicals were acquired from the National Pharmaceutical Group Chemical Reagent Co., Ltd. (Shanghai, China).

Nitrocellulose (NC) high-flow-plus membrane (Pura-bind RP) was obtained from Whatman-Xinhua Filter Paper Co. (Hangzhou, China). Sample pad (CB-SB08), polyvinylchloride (PVC) backing card, and absorption pad (SX18) were supplied by Goldbio Tech Co. (Shanghai, China). Conjugated coating antigens (Biotin-BSA) and

specific monoclonal antibodies (anti-Biotin mAb, No. 3C2) were generated in our laboratory.

All buffer solutions were prepared with ultrapure water (Milli-Q purification system, Millipore Co., Bedford, MA, USA). AirJet Quanti 3000^{TM} and BioJet Quanti 3000^{TM} were used as dispensers (XinqidianGene-technology Co. Ltd., Beijing, China), and the strip cutting instrument was CM 4000 (Gene, Shanghai, China). The strip scan reader was provided by Huaan Magnech Bio-Tech Co., Ltd. (Beijing, China).

Preparation of coating antigen and anti-biotin mAb

For the preparation of the immunochromatographic strip, biotin-BSA coating antigen was synthesized in our laboratory. Biotin-NHS was conjugated with BSA by the active ester method. BSA was dissolved in 0.1 M sodium carbonate-bicarbonate buffer (CB, pH 9.6) at 10 mg/ml, and biotin-NHS was dissolved in N, N-dimethyl formamide at 5 mg/ml. First, biotin-NHS was added dropwise into 1 ml BSA, and the mixture was stirred at room temperature (RT) for 2 h. The pH value was adjusted to 7.4. Subsequently, 20 µl of 2 M NH_4Cl was added to the solution and stirred at RT for 10 min. The conjugates were dialyzed against 0.01 M phosphate buffer solution (PBS) for 3 d and subsequently against distilled water for 3 d.

The anti-biotin mAbs were synthesized in our laboratory and purified from ascites via the caprylic acid-ammonium sulfate precipitation method. Indirect competitive ELISA (ic-ELISA) was used for the characterization of mAb which was similar to conventional protocols:[41, 42] The 96-well microwell plates were coated (100 µL/well) with coating antigen at 37°C for 2 h, washed, and blocked with 2% gelatin in CB at 37°C for 2 h. After washing, 50 µL anti-biotin mAb and 50 µL analytes at different concentrations in 0.01 M PBS were added to each well, followed by incubation at 37°C for 30 min. Next, 50 µL horseradish peroxidase (HRP)-labelled goat

anti-mouse IgG was added to each well and incubated at 37°C for 30 min. After three washes, 100 μL 3, 3′, 5, 5′-tetramethylbenzidine (TMB) substrate was added and incubated at 37°C for 15 min. The enzymatic reaction was terminated with 50 μL sulfuric acid (2 M) per well, and the results read using the microplate reader at 450 nm.

The limit of detection (LOD) and 50% inhibition concentration (IC_{50}) values were determined by a standard curve generated by plotting B/B_0 values (where B represents the absorbance at each concentration of biotin and B_0 represents the absorbance in the absence of biotin) on the y axis against biotin concentration on the x axis. The mAb specificity was determined by cross-reactivity (CR):

$$CR\ (\%) = (IC_{50}\ \text{of biotin}/IC_{50}\ \text{of competition analogue}) \times 100\%^{43}$$

Labelling of the mAb with gold nanoparticles (GNPs)

Gold nanoparticles (GNPs) preparation and labelling experiment in our laboratory was as previously described.[44-46] The pH value of the GNP solution was adjusted with 0.1 M K_2CO_3. Purified mAb dissolved in 200 μl of 0.01 M PBS was added dropwise, and the mixture was incubated at RT for 1 h. During this incubation period, mAbs form complexes with GNPs via electrostatic interactions. Subsequently, 1 ml of 0.5% BSA (w/v) dissolved in 0.01 M PBS was slowly added dropwise into the mixture solution, and then incubated at RT for 2 h. BSA was used to block the GNPs and to stabilize the labeled mAb. The solution was centrifuged twice at 7,000 g and washed with 0.02 M PBS (containing 5% sucrose, 1% BSA, and 0.5% PEG 6000, pH 7.4). The resulting precipitate of GNP-labelled mAbs was dissolved in 1 ml gold-labelled re-suspension buffer[47] (0.02 M PBS, 5% sucrose, 1% BSA, 0.02% NaN_3) and stored at 4°C.

Preparation of the immunochromatographic strip

The immunochromatographic strip consisted of four parts: a sample pad, an NC membrane, a PVC backing card, and an absorption pad (Figure 1A). The NC membrane was attached to the middle of the PVC backing card; the sample and absorption pads were attached on both ends of the PVC backing card with a 2-mm overlap in the NC membrane. The sample pad was first immersed in 0.01 M PBS (containing 1% BSA and 0.2% Tween-20) and dried at RT for 4 h to minimize nonspecific binding and matrix interference.[48] Goat anti-mouse IgG was sprayed onto the NC membrane at 1 μl/cm with a membrane dispenser (Xinqidian Gene-Technology Co. Ltd., Beijing, China), resulting in the formation of the control line (C line). The coating antigen was sprayed onto the NC membrane at 1 μl/cm, resulting in the formation of the test line (T line). The NC membrane was dried at 37°C for 30 min. The prepared strip card was cutting onto individual test strips by the cutting instrument (CM 4000) with the width at 3 mm and then stored in a desiccator for further test.

Principle of the immunochromatographic strip assay

Prior to the test, 150 μl of sample solution was mixed with 50 μl of GNP-labelled mAb, allowed to react at RT for 5 min, and added to the sample pad. The solution on the sample pad migrated towards the absorbent pad. The test results were visually obtained in 5 min.

In biotin-negative samples, GNP-labelled mAbs either form a complex with the coating antigens on the T line or are captured by the goat anti-mouse IgG antibodies on the C line (Figure 1B). Therefore, in biotin-negative samples, two red lines emerge on the strip. In biotin-positive samples, GNP-labelled mAbs conjugate with biotin in

the sample solution, limiting the amount of GNP-labelled mAbs that could bind to the coating antigens. Both free GNP-labelled mAbs and GNP-labelled mAbs conjugated with biotin are captured by the goat anti-mouse IgG antibodies on the C line. Therefore, in biotin-positive samples, the T line has a weaker color intensity than in biotin-negative samples, while the C line color intensity is similar between positive and negative samples. Consequently, the color intensity of the T line is inversely proportional to the concentration of biotin in the sample. The C line should always emerge; otherwise, the procedure was incorrectly performed or the strip was poorly assembled.[49]

Characterization of the immunochromatographic strip

The sensitivity of the immunochromatographic strip was determined using a series of samples containing different concentrations of biotin.

For the semi-quantitative assay, results were obtained with the naked eye. The visual limit of detection (vLOD) value was defined as the lowest concentration of biotin that resulted in a definitely weaker T line color intensity than that of the C line color intensity. The cut-off value was the threshold of biotin concentration that resulted in the disappearance of the T line.

For the quantitative assay, the color intensity of the T line was determined with a handheld strip scan reader. T/T_0 values on the y axis (where T represents the optical density value of the T line with the positive sample and T_0 represents the optical density value of the negative sample) were plotted against biotin concentration on the x axis. The calculated limit of detection (cLOD) was defined as the concentration with 10% inhibition of the signal (IC_{10}).

Sample analysis for the immunochromatographic strip assay

Different types of milk and milk products were used for the validation of the developed immunochromatographic strip. Raw milk was obtained from Talent dairy beverage factory (Wuxi, China). Pure milk, infant milk powder, and instant whole cream milk powder were obtained from a local market. The content of biotin in the samples was confirmed by the microbiological assay against the national food safety standard GB 5413.19-2010. In national standard GB 5413.19-2010, the sample was mix with culture medium without biotin and then inoculate with the tested bacterial strain *Lactobacillus plantarum*, ATCC 8014. The growth of microorganisms has a linear relation with the content of target material. Then the content in sample could be calculated based on the absorbance comparative between sample and standard curve.

Raw milk samples (R1, R2, R3, and R4) and pure milk samples (P1, P2, P3, and P4) were centrifuged at 5,000 g for 15 min at 4°C. Following the removal of the lipid layer, the skim milk samples were analyzed by the immunochromatographic strip assay. One gram of infant milk powder samples (I1, I2, I3, and I4) and one gram of instant whole cream milk powder (M1, M2, M3, and M4) were heat-dissolved in 9 ml aqua pura (30 min at 50°C), allowed to cool, and centrifuged at 5,000 g for 15 min at 4°C. The lipid layer was removed, and the skim milk samples were analyzed by the immunochromatographic strip assay.

Results and Discussion

Characterization of coating antigen and mAb

The coating antigens were synthesized in our laboratory. Biotin-NHS was conjugated to BSA at different ratios (BSA:Biotin-NHS; 1:2, 1:4, and 1:8 M:M), and the coating antigens were characterized by sodium dodecyl sulfate polyacrylamide gel

electrophoresis (SDS-PAGE; Figure 2A). The molecular weight (MW) of coating antigens (Biotin-BSA conjugates) were greater than the BSA. Under the SDS-PAGE method, the migration rate of coating antigens were lower than the BSA. So, the position of coating antigens (Biotin-BSA conjugates) was significant different than the BSA and the offset in the position between coating antigens (Biotin-BSA conjugates) and BSA confirmed the success of the experiment. The coating antigen with more Biotin-NHS conjugated to BSA would take lower migration rate, which lead to bigger different of the position on gel.

The standard curve, IC_{50}, LOD, and CR values of mAb were determined by ic-ELISA. The standard curve is shown in Figure 2B. Under optimized conditions, IC_{50} was 0.218 μg/100 g, LOD was 0.038 μg/100 g, and the linear range was 0.096–0.64 μg/100 g. The ic-ELISA results revealed that mAb had no CR with other B vitamins; therefore, the mAb generated in our study was both sensitive and specific for biotin detection.

Optimization of the immunochromatographic strip

The sensitivity of the immunochromatographic strip is mainly affected by the coating antigens and GNP-mAbs. Coating antigens at different ratios (BSA:Biotin-NHS; 1:2, 1:4, 1:8 M:M) were sprayed on the T line at 0.5 mg/ml. The strips were evaluated with 0.01 M PBS containing either 0 or 1.0 μg/100 g biotin. At 1:8 BSA:Biotin-NHS, the coating antigen resulted in a strong color intensity at the T line in the negative samples (Figure 3A). In the positive samples (1.0 μg/100 g biotin), the T line color intensity became weaker, especially at 1:8 BSA:Biotin-NHS. Therefore, 1:8 BSA:Biotin-NHS was selected for further immunochromatographic strip assays as a result of the stronger color intensity of the T line with the negative sample and the

weakest color intensity of the T line with the positive sample.

Coating antigen concentration was optimized. Strips with different coating antigen concentrations (0.25, 0.5, 1, and 2 mg/ml) on the T line were evaluated with 0.01 M PBS containing either 0 or 1.0 μg/100 g biotin. At 0.5, 1, and 2 mg/ml coating antigen, defined T red lines were observed. At 0.25 mg/ml coating antigen, the color intensity of the T line was weak. Significant differences in color intensity between the positive and negative samples were obtained at 0.5 mg/ml coating antigen. Compared to the negative sample, the positive sample had weaker color intensity on the T line, suggesting that a more sensitive detection could be achieved at 0.5 mg/ml coating antigen. Therefore, 0.5 mg/ml coating antigen was selected for the preparation of the T line on immunochromatographic strip (Figure 3B).

GNP-labelled mAb concentration affects immunochromatographic strip sensitivity. GNP-labelled mAb was prepared under optimized conditions, i.e., with 60 μl of 0.1 M K_2CO_3 and 150 μg mAb per 10-ml conjugation system. Each conjugated system (10 ml, pH 9.0) was ultimately dissolved in 1 ml gold-labelled re-suspension buffer. The reaction system (50 μl), which contained different volumes of GNP-labelled mAb (2, 4, 6, and 8 μl), was allowed to react with positive (1.0 μg/100 g) and negative (0 μg/100 g) samples (Figure 3C). At 2 and 4 μl GNP-labelled mAb, the T and C lines had weak color intensities. At 8 μl GNP-labelled mAb, the cut-off value of the strips increased. At 6 μl GNP-labelled mAb, we obtained strong color intensities on both lines with the negative sample and more sensitive detection with the positive sample. Therefore, the optimal system for our developed immunochromatographic strip system consisted of 1:8 BSA:Biotin-NHS, 0.5 mg/ml coating antigen, and 6 μl GNP-labelled mAb.

Analytical characteristics of the immunochromatographic strip

Milk and milk product samples were first tested by the microbiological assay method. Biotin content in each sample was confirmed by the Chinese national food safety standard GB 5413.19-2010. The raw milk samples with the lowest biotin content (1.01 μg/100 g) was selected for the analyses.

Selected raw milk samples were spiked with different concentrations of biotin, resulting in final biotin concentrations of 1.01, 2.0, 4.0, 8.0, 16.0, 32.0, 64.0 μg/100 g raw milk. These samples were analyzed by the immunochromatographic strip assay.

In the semi-quantitative assay, the vLOD and cut-off values in the raw milk sample were 2.0 μg/100 g and 8.0 μg/100 g, respectively (Figure 4). In the quantitative assay, cLOD was 0.32 μg/100 g and the linear range was 0.65–68 μg/100 g (Figure 5).

Sample analysis

All the samples confirmed by the microbiological assay method against the national standard were analyzed by the immunochromatographic strip assay with 0.01 M PBS as the control sample. In the semi-quantitative assay, negative (-), weakly positive (±), and positive (+) results could be obtained. In the quantitative assay, the optical density of the T lines were measured with a strip scan reader and the results were calculated (Table 1).

Based on the semi-quantitative results, there were no positive raw milk or pure milk samples, except for sample P2. Infant milk powder and instant whole cream milk powder samples were biotin-positive, because these samples are generally fortified with the B vitamin. The vLOD and cut-off values of the developed immunochromatographic strip were 2.0 μg/100 g and 8.0 μg/100 g, respectively.

Based on these results, it is difficult to confirm whether the content of biotin in dairy products meets the national standard guidelines.

On the other hand, more sensitive results could be obtained by the quantitative assay. As shown in Table 1, the quantitative assay revealed that biotin concentration in some of the milk products was 77–118 % of the national standard guidelines.

For each type of samples, one sample was randomly chosen (R2, P4, I1, M2) and different concentration of Biotin was spiked into these samples. All the samples were tested by both national standard and immunochromatographic strip assay. The result was shown in Table 2. The recoveries ranging from 68% to 119% based on the national standard. And in liquid samples (R2, P4) the recovery rate was better than the in powder samples (86% to 112% compared with 68% to 119%). This may be caused by the different production process of different products.

Compared to the microbiological assay method, our developed immunochromatographic strip assay was rapid and sensitive for biotin detection in milk and milk products. Even though the microbiological assay method has been developed into a commercial detection kit (VitaFast® Vitamin B7 Biotin kit, R-Biopharm Co., Ltd., Germany), the entire detection process requires 46–50 h and the LOD value is 0.013 μg/100 g (mL) with a linear range of 0.08–0.72 μg/100 g (ml). The detection principle was the same as the national standard. On the other hand, the results from the immunochromatographic test assay can be obtained in 20 min, making it suitable for on-site biotin detection in milk and milk product samples.

Conclusions

A competitive immunochromatographic strip assay was developed for the detection of biotin in foods. The vLOD and cut-off values of our developed method were 2.0

μg/100 g and 8.0 μg/100 g, respectively, in milk samples. Based on the quantitative results, cLOD was 0.32 μg/100 g and the linear range was 0.65–68 μg/100 g. Our developed method was successfully applied to the detection of biotin in milk and milk products. The results were in good agreement with the microbiological assay method. In summary, the immunochromatographic strip assay represents a sensitive and rapid detection method, which is suitable for on-site detection and rapid initial screening of biotin in milk and milk product samples.

Acknowledgements

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Caption:

Fig. 1 Principle of the immunochromatographic strip detection.

Fig. 2 (A) The SDS-PAGE image of Biotin-BSA coating antigens with different ratios, (BSA:Biotin-NHS, M:M); (B) The standared curve of biotin by Ic-ELISA method.

Fig. 3 Optimization of immunochromatographic strip.

Fig. 4 Detection of biotin by immunochromatographic strip.

Fig. 5 The standard curve of biotin in raw milk sample tested by strip scan reader based on the optical density values.

Table 1 Sample analysis by National Standard method and immunochromatographic strip (n=7).

Table 2 Recoveries of Biotin in different samples in analysis by National Standard method and immunochromatographic strip (n=7).

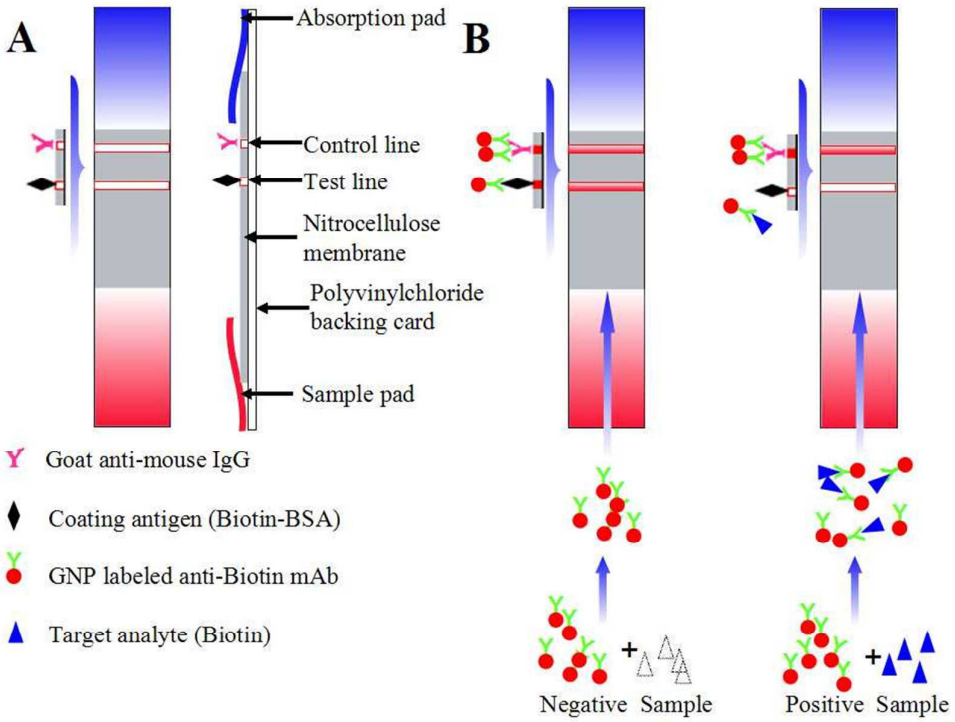

Fig. 1 Principle of the immunochromatographic strip detection: (A) Composition of the immunochromatographic strip. (B) Strip detection with negative sample and positive sample.

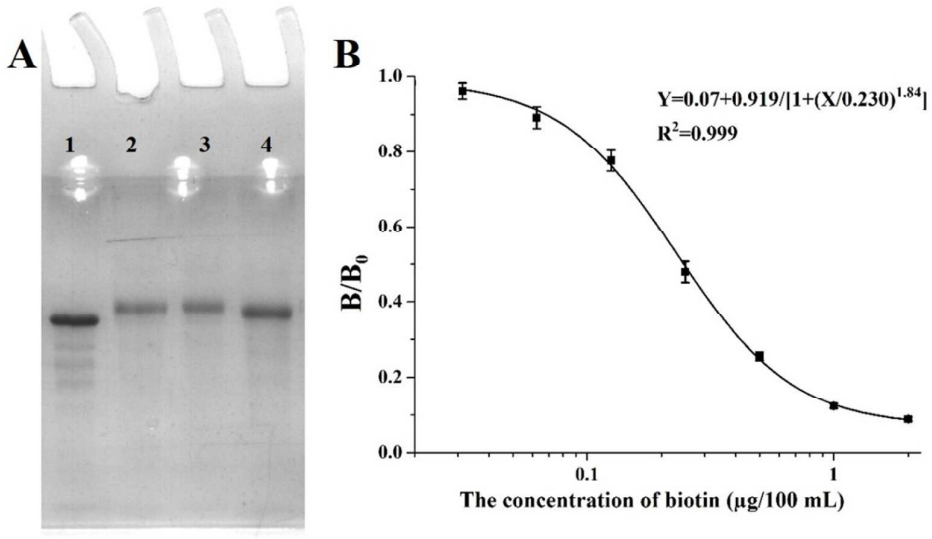

Fig. 2 (A) The SDS-PAGE image of Biotin-BSA coating antigens with different ratios (BSA:Biotin-NHS ,M:M): 1.BSA; 2. Biotin-BSA (1:8); 3. Biotin-BSA (1:4); 4. Biotin-BSA (1:2); (B) The standared curve of Biotin by Ic-ELISA method.

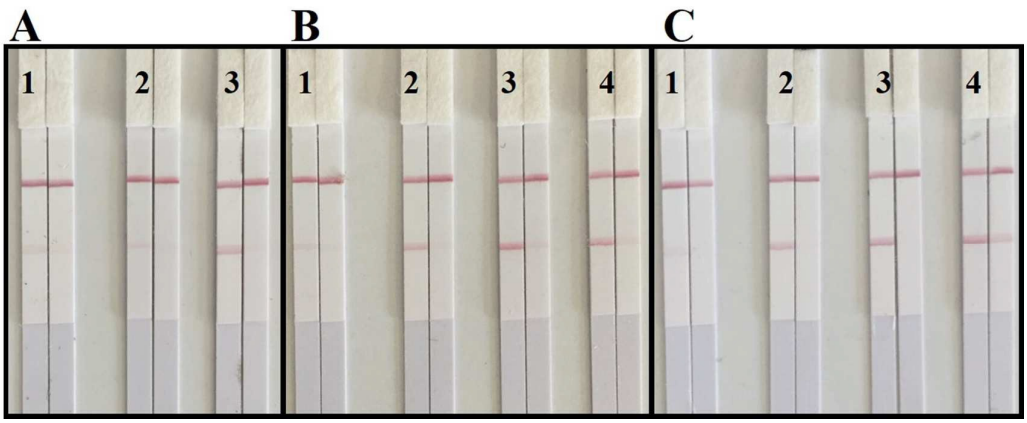

Fig. 3 Optimization of immunochromatographic strip: (A) Strips sprayed coating antigens with different reaction ratios (BSA:Biotin-NHS, M:M): 1. 1:2; 2. 1:4; 3. 1:8. (B) Strips sprayed coating antigens with different concentration: 1. 0.25 mg/ml; 2. 0.5 mg/ml; 3. 1 mg/ml; 4. 2 mg/ml. (C) The dosage of GNP-labeled mAb used for the reaction system: 1. 2 μl; 2. 4 μl; 3. 6 μl; 4. 8 μl.

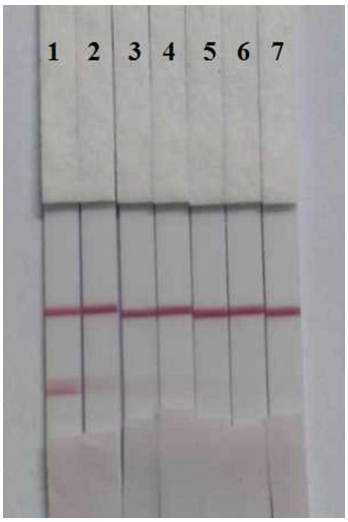

Fig. 4 Detection of biotin by immunochromatographic strip in raw milk sample: 1. 1.01 μg/100 g; 2. 2.0 μg/100 g; 3. 4.0 μg/100 g; 4. 8.0 μg/100 g; 5. 16.0 μg/100 g; 6. 32.0 μg/100 g; 7. 64.0 μg/100 g.

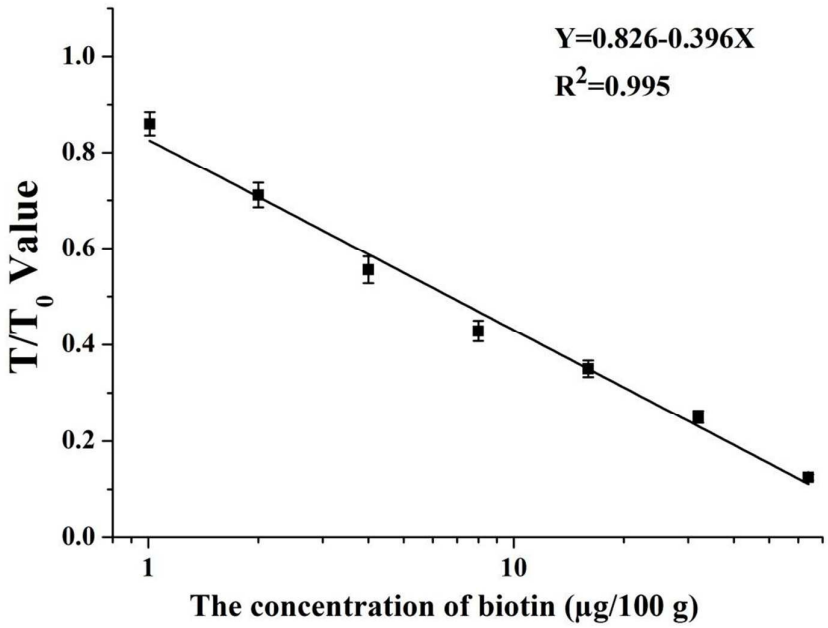

Fig. 5 The standard curve of biotin in raw milk sample tested by strip scan reader based on the optical density values.

Table 1 Sample analysis by National Standard method and immunochromatographic strip (n = 7).

Samples	National Standard method (μg/100 g)	Results by visual observation	Result based on strip scan reader (μg/100 g)
R1	1.37±0.05[a]	-[b]	1.16±0.06
R2	1.01±0.05	-	1.14±0.06
R3	1.12±0.03	-	1.32±0.07
R4	1.42±0.07	-	1.68±0.08
P1	1.52±0.06	-	1.33±0.07
P2	2.25±0.09	±[c]	2.13±0.11
P3	1.59±0.08	-	1.22±0.06
P4	1.24±0.06	-	1.30±0.06
I1	12.4±0.73	+[d]	13.7±0.68
I2	65.3±2.9	+	55.7±2.8
I3	49.8±2.5	+	42.2±2.7
I4	61.1±3.0	+	63.4±3.1
M1	26.3±1.3	+	24.9±1.2
M2	24.3±0.98	+	22.1±1.1
M3	56.4±2.8	+	59.8±3.0
M4	69.1±3.4	+	72.7±3.64

[a] mean value ± standard deviation (n=7)

[b] Negative result. The test line is obviously observed.

[c] Weakly positive result. Light test line is observed.

[d] Positive result. No test line is observed.

Table 2 Recoveries of Biotin in different samples in analysis by National Standard method and immunochromatographic strip (n = 7).

Samples	Spiked concentration (μg/100 g)	National Standard method (μg/100 g)	Result based on strip scan reader (μg/100 g)
R2	0	1.21±0.05[a]	1.32±0.06
	10	10.8±0.81	12.1±0.75
	20	19.6±1.1	19.1±0.84
	40	43.5±1.9	45.2±2.3
P4	0	1.41±0.06	1.45±0.04
	10	11.9±0.62	12.2±0.42
	20	22.4±1.2	19.6±0.92
	40	40.8±2.1	45.5±1.8
I1	0	13.5±0.57	14.2±0.42
	10	24.1±1.1	23.1±0.61
	20	35.2±1.7	40.0±2.3
M2	0	22.8±1.1	18.5±0.78
	10	36.5±1.7	29.4±1.8
	20	49.7±2.8	36.8±2.2

[a] mean value ± standard deviation (n=7)