Original Article

Study on the Simultaneously Quantitative Detection for β-Lactoglobulin and Lactoferrin of Cow Milk by Using Protein Chip Technique*

YIN Ji Yong, HUO Jun Sheng#, MA Xin Xin, SUN Jing, and HUANG Jian

National Institute for Nutrition and Health, Chinese Center for Disease Control and Prevention, Beijing 100050, China

Abstract

Objective To research a protein chip method which can simultaneously quantitative detect β-Lactoglobulin (β-L) and Lactoferrin (Lf) at one time.

Methods Protein chip printer was used to print both anti-β-L antibodies and anti-Lf antibodies on each block of protein chip. And then an improved sandwich detection method was applied while the other two detecting antibodies for the two antigens were added in the block after they were mixed. The detection conditions of the quantitative detection for simultaneous measurement of β-L and Lf with protein chip were optimized and evaluated. Based on these detected conditions, two standard curves of the two proteins were simultaneously established on one protein chip. Finally, the new detection method was evaluated by using the analysis of precision and accuracy.

Results By comparison experiment, mouse monoclonal antibodies of the two antigens were chosen as the printing probe. The concentrations of β-L and Lf probes were 0.5 mg/mL and 0.5 mg/mL, respectively, while the titers of detection antibodies both of β-L and Lf were 1:2,000. Intra- and inter-assay variability was between 4.88% and 38.33% for all tests. The regression coefficients of protein chip comparing with ELISA for β-L and Lf were better than 0.734, and both of the two regression coefficients were statistically significant (r = 0.734, t = 2.644, P = 0.038; and r = 0.774, t = 2.998, P = 0.024).

Conclusion A protein chip method of simultaneously quantitative detection for β-L and Lf has been established and this method is worthy in further application.

Key words: Protein chip; Simultaneously; Quantitative detection; β-Lactoglobulin; Lactoferrin

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INTRODUCTION

There is high nutrition value in cow milk, and World Health Organization (WHO) has chosen it as an evaluating indicator of life level. β-Lactoglobulin (β-L) and Lactoferrin (Lf) are the main components of cow milk, and they can reflect the nutrition value of cow milk at some extent[1]. In proteins of cow milk, β-L is 50% of whey protein, and it is 12% of total protein, and its average concentration is 2-4 g/L in cow milk. β-L is consisted of 162 amino acid residues which included

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#Correspondence should be addressed to HUO Jun Sheng, Professor, Doctor, Tel: 86-0-13501152782, E-mail: jshuo@263.net.cn

Biographical note of the first author: YIN Ji Yong, male, born in 1975, Doctor, Associate Professor, majoring in nutrition and food safety.

4 cysteine residues formed disulfide bond, and its molecular weight is 18 kD. All of these characters ensure the stability of β-L and decide β-L can be a mark protein for distinguishing true and false of cow milk[2-3]. On the other hand, Lf is consisted of 700 amino acid residues and its molecular weight is 80 kD. Its concentration in bovine coloctrum and in general milk are 0.8 g/L and 0.02-0.4 g/L, respectively[4]. Lf can combines with iron ion and improves the absorption and utilization of iron ion for human body, besides, it also has series of effects for human body health including antibacterial, antivirus and immunoregulation, and so on[5]. These advantages ensure Lf can be chosen as mark protein for evaluating nutrition value of cow milk.

In the past years, there are many detection methods can detect β-L and Lf[6], while all of these methods have a common disadvantage, they can't simultaneously present the results of β-L and Lf in one detection. This would increase the system error among the different detection methods and then would affect the accuracy of data analysis. Besides, practical production of dairy needs simultaneous, quick, high throughput method to enhance detection efficiency and identify the quality of cow milk. Unfortunately, these traditional methods couldn't satisfy these requirements. At present, although a method once simultaneously detected β-L and Lf[7], its sensibility and specificity would be able to be further enhanced if sandwich method were chose in its method and aldehyde-based three-dimensional substrate is used as coating material.

Protein chip is a new immunology detected technique and being widely applied in many fields of research and production. This technique adopts aldehyde-based three-dimensional substrate and immunology principle between antigen and antibody to specially detect the antigen or antibody of sample, and it can simultaneously, quickly, highly throughput detect various proteins in one detection. Therefore, it not only can identify the quality of cow milk but also can increase detection efficiency. Besides, because it can simultaneously detect various proteins, the accuracy of this technique is higher than that of other single detection and it can reduce the system error among different methods. In recent years, this technique has penetrated into many researches and obtained many achievements[8-11]. However, few studies have reported measurement of nutrition biomarkers in cow milk with this technology. β-L and Lf, as marker protein of nutrition value and quality of cow milk status, need to be measured quantitatively, quickly, and simultaneously, while current techniques are inadequate. The protein chip method, which adopted aldehyde-based three-dimensional substrate and sandwich immunology method, is potentially suitable technique for using in measurement of nutrition value and quality of cow milk. Therefore, the primary aim of this study is to establish and evaluate a protein chip platform, which adopted aldehyde-based three-dimensional substrate and sandwich immunology method, so as to simultaneously measure β-L and Lf.

MATERIALS AND METHODS

Materials

This study involved the materials including NaH_2PO_4, Na_2HPO_4, NaCl, KCl, Tween20, printing buffer (code 440015, Capital Bio Corporation), mouse monoclonal anti-β-L antibody, rabbit polyclonal anti-β-L antibody, mouse monoclonal anti-Lf antibody, rabbit polyclonal anti-Lf antibody (Huada Corp.), goat polyclonal antibody IgG-Cy3-linked (code 111-165-003, 115-165-003, Jackson Immuno Research), ELISA kit (β-Lg Kit: SEB023Bo, Cloud-CloneCorp; Lf Kit: SEA780Bo, Cloud-Clone Corp.), protein chip (Polymer Slide-G, Capital Bio Corporation), printer (Personal Arrayer™ 16 Protein Chip Spotter, Capital Bio Corporation), and scanners (LuxScan™10K Chip Scanner, Capital Bio Corporation). Control samples of β-L (L0130, SIGMA Ltd.) and Lf (L9507, SIGMA Ltd.) were used as standards for the calibration curves.

Procedure of Protein Chip Basic

The antibody probes of β-L and Lf, the positive control (non-specificity rabbit IgG), the negative control (PBS pH 7.2) were successively printed in each of 12 blocks on the PSG chip. After all of probes were immobilized (at 37 °C for 16-18 h), the intermixed antigens were added in each block and were incubated (30 μL/block, 37 °C incubation, 1 h), and the chip was washed with 1‰ PBST wash buffer (3 min/time, 5 times) after it was incubated 1 h. Afterwards, the intermixed detection antibodies of β-L and Lf were added in each block and incubated (30 μL/block, 37 °C incubation, 1 h), and the chip was washed like last washing step. Finally, the goat antibody coupled to Cy3 was added in each block and incubated (30 μL/block, 37 °C incubation, 1 h), and after the chip was washed, it was scanned so as

to obtain detection images and result value by using laser scan.

Optimizing the Monoclonal Antibodies of β-L and Lf

There were three mouse monoclonal antibodies (mAb) for β-L (7#, 28#, 66#) and Lf (1#, 35#, 75#), respectively. And the optimal one of the three monoclonal antibody should be adopted by optimizing experiment and be applied in the protein chip of sandwich mode. The three mouse mAbs of β-L (7#, 28#, 66#) and Lf (1#, 35#, 75#), respectively, were successively printed in each of 12 blocks on the PSG chip, and all of these concentrations were 0.5 mg/mL. There were two standard concentrations (high 2 μg/mL and low 0.2 μg/mL) of β-L and Lf, respectively, were used in this optimizing experiment. PBS was applied as negative control. And the titers of detection antibody for β-L and Lf were 1:1,000. The titer of goat anti-rabbit antibody was 1:600. The judgment criterion was there was a simple dose-response relation between the concentration of antigen and signal value, and there was the largest signal of high concentration protein, at the same time, the background noise signal was relative lower.

Optimizing the Kind of β-L and Lf Probes

The experimental procedures were the same as the basic procedure of protein chip. When mouse monoclonal antibody (mAb) was chosen as printing probe, rabbit polyclonal antibody (pAb) was used as detection antibody; while rabbit pAb was chosen as printing probe, the mouse mAb was used as detection antibody so as to optimize the best probe for every antigen protein. Both mouse mAb of β-L and Lf were printed as probes in Block 1-Block 4, and both rabbit pAb of β-L and Lf were printed as probes in Block 5-Block 8. Rabbit pAb of β-L were used as detection antibodies in Block 1 and Block 2, and rabbit pAbs of Lf were used as detection antibodies in Block 3 and Block 4. Meanwhile, mouse mAbs of β-L were used as detection antibodies in Block 5 and Block 6, and mouse mAbs of Lf were used as detection antibodies in Block 7 and Block 8. The β-L antigens were added in Block 1 and Block 5. The Lf antigens were added in Block 3 and Block 7. The PBS was added in other blocks as negative sample. The concentrations of the probes were 0.5 mg/mL for β-L and Lf, whether the mouse mAbs were chosen as probes or the rabbit pAbs were chosen as probes. Both of the concentrations of antigen β-L and antigen Lf were 2 μg/mL, and the negative control was PBS. Both of the titers of the detection antibodies were 1:1,000. Both of the titers for goat anti-mouse and goat anti-rabbit antibody were 1:600. The judgment criterion was the difference value between positive sample and negative sample should be largest, at the same time, the value of negative sample should be lowest.

Optimizing the Concentrations of the Probes, the Detection Antibodies of β-L and Lf

The experiment procedures were the same as the basic procedure of protein chip. All of the blocks were implemented one dilution (1:600) of the second antibodies. The concentrations of the probes and the detection antibodies of β-L and Lf were optimized by using the crisscross serial-dilution analysis. Different concentrations were used to print probes of β-L (1, 0.5, 0.25 mg/mL) and Lf (1, 0.5, 0.25 mg/mL) in each block. Four concentrations of antigens were chosen to add in different block, respectively: high concentration (β-L 2 μg/mL, Lf 1 μg/mL), middle concentration (β-L 1 μg/mL, Lf 0.1 μg/mL), low concentration (β-L 0.2 μg/mL,Lf 0.02 μg/mL) and NC (PBS). Then, three titers of detection antibodies were added in corresponding block: high (β-L 1:1,000, Lf 1:2,000), medium (β-L 1:2,000, Lf 1:6,000) and low (β-L 1:4,000, Lf 1:9,000). In the optimizing, every antigen could be detected by 9 pairs of antibodies. The optimization criteria were that: (1) the signal value was unsaturated (the difference between signal value and background value < 65,535) at high antigen concentrations, (2) the signal value of low antigen concentrations had difference of statistical significance with that obtained from NC. At the same time, the background signal of the chip had to be low.

Homogeneity of the Spots of the β-L and Lf Probes

The free Cy3 (1:100,000) were added in mouse mAbs of β-L and Lf, respectively, and then they were printed in 462 spots in different block by contacted printing. Both of concentrations of the probes of β-L and Lf were 0.5 mg/mL. The chip was directly scanned after probes were printed. The first stable section of these signal spot was chosen to decide pre-spot number and largest spot number.

Lower Limit of Detection and Biologic Limit of Detection

The experiment procedures were the same as the basic procedure of protein chip. Different concentrations were used to print probes of β-L (0.5

mg/mL) and Lf (0.5 mg/mL) in each block. The mixed antigens were diluted from high concentrations (β-L: 33.52 ng/mL, Lf: 14.38 ng/mL) to low concentrations (β-L: 2.09 ng/mL, Lf: 0.89 ng/mL) by using 1:2 serial dilutions. The PBS was used as the NC. In the mixed detection antibodies, both of the dilutions of two detection antibodies for β-L and Lf were 1:2,000. The dilution of the second antibodies was 1:600. The same operation was repeated 12 times for each concentration. Based on the results, the lower limit of detection (LLD) of the method was obtained from the corresponded concentration of the 2SD above the mean of signal when NC was used, at this time, the corresponded concentration of the mean was regarded as zero concentration. Finally, the biologic limit of detection (BLD) was found by selecting the minimal concentration of mixed antigens that corresponded signal mean level subtracted 2SD value can be just higher than the signal level at LLD.

Establishing the S-Curve Models and Standard Curve for β-L and Lf

The experiment procedures were the same as the basic procedure of protein chip. The same concentrations (0.5 mg/mL) were used to print probes of β-L and Lf in each block. The mixed antigens were diluted from the highest concentrations (β-L 10.725 μg/mL, Lf 1.382 μg/mL) to the lowest concentrations (β-L 67.03 ng/mL, Lf 14.4 ng/mL) by using 1:2 serials dilutions. The S-shaped curves of two antigens were respectively determined as the relationship between the concentration and signal value of antigens. And then, depended on the found straight line ranges, 5 mixed standard points in the line range of the two antigens, respectively, were chosen: β-L 4,290 ng/mL, Lf 230.38 ng/mL; β-L 2,145 ng/mL, Lf 184.3 ng/mL; β-L 1072.5 ng/mL, Lf 57.6 ng/mL; β-L 402.2 ng/mL, Lf 28.8 ng/mL; β-L 134 ng/mL, Lf 14.4 ng/mL, and PBS was chosen as the NC. In the mixed detection antibodies, both of the titers of the detection antibodies for β-L and Lf were 1:2,000. The dilution of the second antibody was 1:600. The mean levels of these standard points were calculated and used as the basis to obtain standard curve and model of the two antigens, respectively. Finally, standard curves and models were established for β-L and Lf based on the relation between the antigen concentration and the detection signal level.

Precision

Three cow's milks of unknown concentrations were tested 8 times on one protein chip at one experiment to assess intra-assay precision. Another three cow's milks of unknown concentrations were tested in 8 separate assays to assess inter-assay precision, and the every sample was repeated twice in every assay.

Recovery

Three pairs of mixed standards points of β-L and Lf (β-L 1492.2 ng/mL, Lf 120.7 ng/mL; β-L 746 ng/mL, Lf 60.4 ng/mL; β-L 373 ng/mL, Lf 30.2 ng/mL) were obtained from one mixed initial concentration (β-L 2984.4 ng/mL, Lf 241.5 ng/mL) by using 1:2 serial dilution, and then the dilution recovery rates of the detection method were calculated based on above data and results of detection.

On the other hand, mixed standards of initial concentration (β-L 67,150 ng/mL, Lf 6,520 ng/mL) was added in mixed standards of three pairs of β-L and Lf (β-L 895.3 ng/mL, Lf 90.6 ng/mL; β-L 596.9 ng/mL, Lf 60.4 ng/mL; β-L 426.4 ng/mL, Lf 45.3 ng/mL), respectively, and the added ratio was 1% volume of 30 μL, and then the recovery rates of the detection method were calculated.

Application Studies

For testing performance of this new method, 8 samples were analyzed by using traditional methods (ELISA Kit) and protein chip, respectively. The commercial ELISA Kits of β-L and Lf from Clone Cloud were used as a reference to reflect the performance of protein chip. The correlation and consistency between the 2 methods were calculated by the correlation analysis and paired-comparisons *t* test. And the correlation efficiency of the two methods were calculated and tested by *t* testing.

Calculations and Statistics

All statistical analyses were done with Excel 2003 (Microsoft) and SPSS13.0. The standard curves and models were made with Excel 2003 (Microsoft). The *t* test (α = 0.05) was used to choose mouse mAb and probe. The correlation analysis and paired-comparisons *t* test (α = 0.05) were used to compare the difference between the protein chip and the traditional method.

RESULTS

Optimizing the Monoclonal Antibodies of β-L and Lf

The results showed that all of the three mouse

monoclonal antibodies (7#, 28#, 66#) of β-L could combine with protein chip, and all of them could combine with β-L antigen and could appear the dose-response relation. And the same results could be found in the three mouse monoclonal antibodies (1#, 35#, 75#) of Lf. These phenomenon indicated all of these antibodies had bioactivity on protein chip. As the judgment criterion of this experiment, the 66# mouse monoclonal antibody of β-L and 75# mouse monoclonal antibody of Lf could simultaneously satisfy the requirement of high concentration antigen and background noise at one experiment. Therefore, 66# mAb of β-L and 75# mAb of Lf were chosen as one antibody of antibody pair for β-L and Lf, respectively (Table 1, Table 2).

Optimizing the Kind of β-L and Lf Probes

Both the mouse mAb and rabbit pAb o fβ-L and Lf were able to be attached to the chip. The signal values of mouse mAb as probes for both of β-L and Lf were significantly higher than that of rabbit pAbs as probes for both of them ($t_{β\text{-}L}$ = 58.643, t_{Lf} = 3.217, $P_{β\text{-}L}$ < 0.0001, P_{Lf} = 0.032). And for negative sample, the signal values of mouse mAb as probes for both of β-L and Lf were significantly lower than that of rabbit pAbs as probes ($t_{β\text{-}L}$ = -79.141, t_{Lf} = 51.993, $P_{β\text{-}L}$ < 0.0001, P_{Lf} < 0.0001). At the same time, the difference of positive antigen and negative sample when mouse mAb was used as probe was obviously larger than that when pAbs was used as probe (Table 3, Table 4).

Optimizing the Concentrations of the Probes, the Detection Antibodies of β-L and Lf

The optimal concentrations of the probes were 0.5 mg/mL for β-L and Lf, respectively. And the optimal titer of the detection antibodies were 1:2,000 for β-L and Lf, respectively (Table 5, Table 6).

Table 1. The Comparison of the Signal Value of Three Mouse Monoclonal Antibodies of β-L Reacting with β-L (n = 3, $\bar{x} \pm s$)

Group	Mouse mAb 7# of β-L	Mouse mAb 28# of β-L	Mouse mAb 66# of β-L
β-L (High concentration)	35,328 ± 4,244[a,b]	22,607 ± 677	23,734 ± 3,306
β-L (Low concentration)	5,659 ± 694	3,475 ± 178	7,408 ± 1,225
Negative control group	3,587 ± 293[b]	1,429 ± 192[b]	690 ± 169

Note. [a]Compared with mouse mAb 28# of β-L, P < 0.05; [b]Compared with mouse mAb 66# of β-L, P < 0.05.

Table 2. The Comparison of the Signal Value of Three Mouse Monoclonal Antibodies of Lf Reacting with Lf (n = 3, $\bar{x} \pm s$)

Group	Mouse mAb 1# of Lf	Mouse mAb 35# of Lf	Mouse mAb 75# of Lf
Lf (High concentration)	57,769 ± 30	52,606 ± 9,013	57,676 ± 29
Lf (Low concentration)	64,097 ± 35	33,595 ± 6,652	36,375 ± 4,476
Negative control group	3,537 ± 483	880 ± 515	634 ± 168[a,b]

Note. [a]Compared with mouse mAb 1# of Lf, P < 0.05; [b]Compared with mouse mAb 35# of Lf, P < 0.05.

Table 3. The Comparison of the Signal Value of β-L for Different Probe (n = 3, $\bar{x} \pm s$)

Antigen	Probe	Detection Antibody	Probe	Detection Antibody
	Mouse mAb of β-L	Rabbit pAbs of β-L	Rabbit pAbs of β-L	Mouse mAb of β-L
β-L	64,677 ± 6[*]		46,339 ± 542	
PBS	1,874 ± 242[*]		34,369 ± 669	
Difference	62,803		11,970	

Note. [*]Compared with the probe of Rabbit pAbs of β-Lg, $t_{β\text{-}L}$ = 58.643, t_{PBS} = -79.141, $P_{β\text{-}L}$ < 0.0001, P_{PBS} < 0.0001.

Table 4. The Comparison of the Signal Value of Lf for Different Probe (n = 3, $\bar{x} \pm s$)

Antigen	Probe	Detection Antibody	Probe	Detection Antibody
	Mouse mAb of Lf	Rabbit pAbs of Lf	Rabbit pAbs of Lf	Mouse mAb of Lf
Lf	60,400 ± 4,456[*]		50,830 ± 2,585	
PBS	6,722 ± 701[*]		34,889 ± 624	
Difference	53,668		15,942	

Note. [*]Compared with the probe of Rabbit pAbs of Lf, t_{Lf} = 3.217, t_{PBS} = -51.993, P_{Lf} < 0.0001, P_{PBS} < 0.0001.

Homogeneity of the Spots of the β-L and Lf Probes

The result of scan and the analysis of spot homogeneity revealed that the first stable interval of β-L 66# was from 44th spot to 92th spot and the first stable interval of Lf 75# was from 47th spot to 90th spot (Figure 1). Therefore, the preprinted number should choose 46 spots and the largest number of single printing should not be more than 44 spots. These data would be used as the parameter to print mouse mAbs of β-L and Lf.

Table 5. The Concentration Choosing of Probe and Detection Antibody for β-L by Chessboard Titration Experiment

Detection Antibody Titer	Concentration of Probe (1 mg/mL) *vs.* Different Concentration of Antigen (μg/mL)				Concentration of Probe (0.5 mg/mL) *vs.* Different Concentration of Antigen (μg/mL)				Concentration of Probe (0.25 mg/mL) *vs.* Different Concentration of Antigen (μg/mL)			
	2	1	0.2	PBS	2	1	0.2	PBS	2	1	0.2	PBS
1:1,000	64,620	47,911	17,931	1,722	64,724	41,252	9,308	1,092	25,731	15,651	4,404	241
1:2,000	65,001	43,752	12,388	1,127	**65,018**	**29,163**	**6,555**	**850**	23,663	13,246	3,889	252
1:4,000	44,332	30,778	9,273	1,299	39,583	18,538	5,441	809	13,818	8,699	2,825	417

Table 6. The Concentration Choosing of Probe and Detection Antibody for Lf by Chessboard Titration Experiment

Detection Antibody Titer	Concentration of Probe (1 mg/mL) *vs.* Different Concentration of Antigen (μg/mL)				Concentration of Probe (0.5 mg/mL) *vs.* Different Concentration of Antigen (μg/mL)				Concentration of Probe (0.25 mg/mL) *vs.* Different Concentration of Antigen (μg/mL)			
	1	0.1	0.02	PBS	1	0.1	0.02	PBS	1	0.1	0.02	PBS
1:2,000	61,175	35,623	17,801	10,129	**61,254**	**28,084**	**11,372**	**3,542**	61,005	18,065	6,884	1,608
1:6,000	57,066	19,515	14,358	9,177	40,772	12,863	6,062	3,507	35,408	9,123	4,751	1,967
1:9,000	36,248	17,179	16,279	9,477	32,579	15,260	7,207	3,597	23,186	7,976	5,046	2,214

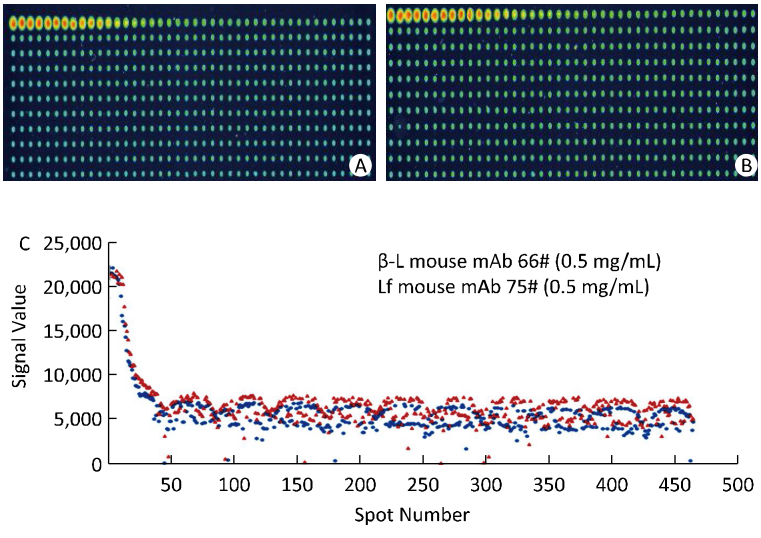

Figure 1. This figure showed the test for homogeneity during spotting of the β-L 66# probe (0.5 mg/mL) and Lf 75# probe (0.5 mg/mL). The (A) image was for β-L 66# probes and the (B) image was for Lf 75# probes. The (C) image showed the trend of homogeneity during spotting of the β-L 66# probe and Lf 75# probe.

Lower Limit of Detection and Biologic Limit of Detection

The LLD of β-L 66# and Lf 75# was 5.4 ng/mL and 0.96 ng/mL, respectively. The BLD of β-L 66# and Lf 75# was 33.52 ng/mL and 3.60 ng/mL, respectively (Table 7 and Table 8).

Establishing the S-curve Models and Standard Curve for β-L and Lf

In the images A and B of Figure 2, the results showed that the signal value of β-L antigen started to enter platform stage from 4290.00 ng/mL and the linearity range of β-L between signal value and concentration was from 134.06 to 4290.00 ng/mL. On the other hand, in the images C and D of Figure 2, the signal value of Lf antigen started to enter platform stage from 460.75 ng/mL and the linearity range of Lf between signal value and concentration was from 14.40 to 460.75 ng/mL.

As above data, the standard curves of β-L and Lf were established, respectively (Figure 3). And the standard equations of β-L and Lf were $y_{\beta\text{-L}} = 1.0577\ x + 156.07$ and $y_{\text{Lf}} = 20.126\ x + 81.544$, respectively. And the results of F-test revealed that both of the two equations were statistically significant ($F_{\beta\text{-L}} = 804.069$, $P_{\beta\text{-L}} < 0.0001$, $F_{\text{Lf}} = 5431.103$, $P_{\text{Lf}} < 0.0001$).

Table 7. The BLD of β-L on Protein Chip (n = 12)

Signal Value	Concentration (ng/mL)					
	33.52	16.73	8.37	4.18	2.09	0
$\bar{x}$	408.00	101.21	69.83	51.25	32.13	0
s	79.29	81.65	32.23	29.78	65.48	32.91
$\bar{x}$-2s	249.42	-62.01	5.38	-8.30	-98.83	
Compared with the signal value mean of LLD	**> 2 × 32.91**	< 2 × 32.91				

Table 8. The BLD of Lf on Protein Chip (n = 12)

Signal Value	Concentration (ng/mL)					
	14.38	7.19	**3.60**	1.80	0.90	0
$\bar{x}$	2264.04	1139.20	731.38	350.00	218.96	0
s	546.21	580.25	259.42	84.09	206.45	98.02
$\bar{x}$-2s	1171.63	-21.28	212.53	181.80	-193.94	
Compared with the signal value mean of LLD			**> 2 × 98.02**	< 2 × 98.02		

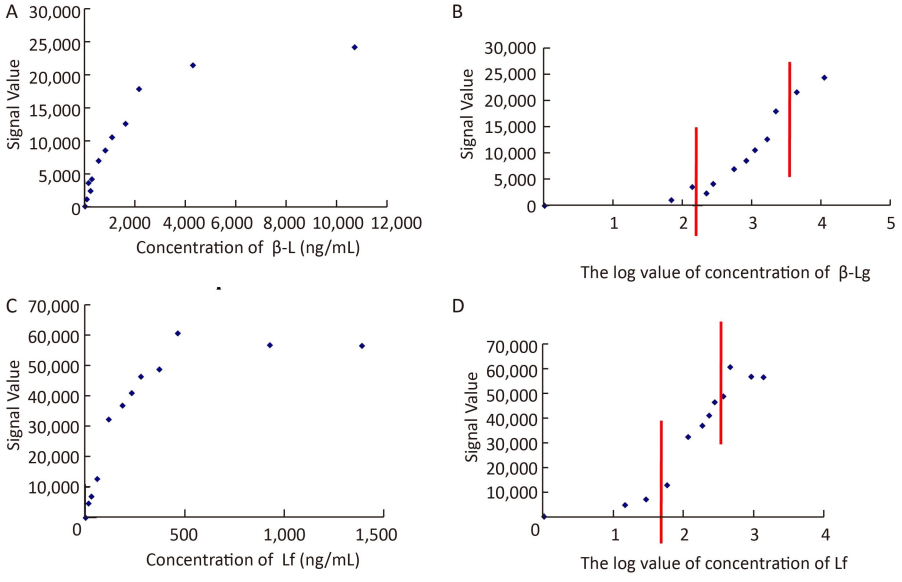

Figure 2. The S-curve and linear relationship between concentration and signal value for β-L and Lf, respectively.

Precision

The results of intra-assay precision and the inter-assay precision can be checked through Table 9 and Table 10. The intra-array precision range was 4.88%-14.18% for Lf, and it was 7.64%-11.61% for β-L. And all of these precisions were not more than 15% for both β-L and Lf. The inter-array precision range was 24.74%-38.33% for Lf, and it was 14.87%-33.48% for β-L. As a whole, the precision of both the intra-assay and the inter-assay of β-L was better than that of Lf.

Recovery

The results of dilution recovery revealed that the dilution recovery rates both of β-L and Lf could approach 100% when the dilutions of the two antigens were between the second standard point and the third standard point (Table 11).

The results of adding standard recovery revealed that the range of recovery rate was 83.71% -128.52% for Lf and its mean was 110.73%, while the

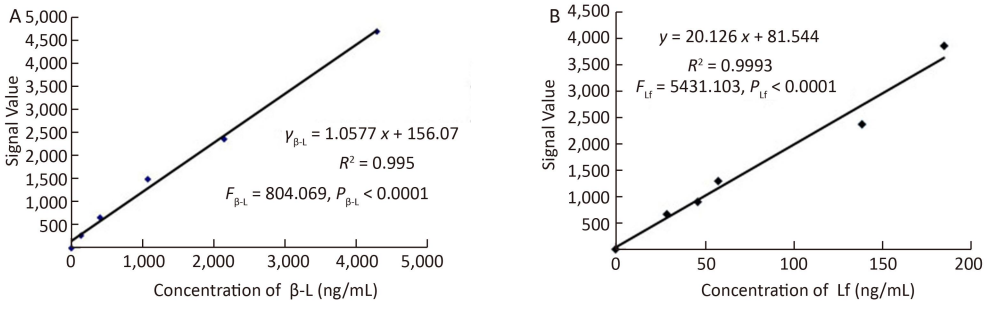

Figure 3. The standard curves between concentration and signal value for β-L and Lf, respectively.

Table 9. The Intra-assay Precision of β-L and Lf by Simultaneous Detection with Protein Chip (n = 8)

Sample	I	II	III
β-L intra-assay			
$\bar{x}$ (ng/mL)	1465.69	850.90	978.88
s (ng/mL)	112.03	98.77	81.57
CV (%)	7.64	11.61	8.33
Lf intra-assay			
$\bar{x}$ (ng/mL)	217.64	234.06	230.05
s (ng/mL)	30.85	24.61	11.23
CV (%)	14.18	10.51	4.88

Table 10. The Inter-assay Precision of β-L and Lf by Simultaneous Detection with Protein Chip (d = 8, n = 2)

Sample	I	II	III
β-L inter-assay			
$\bar{x}$ (ng/mL)	1136.31	459.20	947.27
s (ng/mL)	168.92	153.74	217.97
CV(%)	14.87	33.48	23.01
Lf inter-assay			
$\bar{x}$ (ng/mL)	198.59	189.27	216.0
s (ng/mL)	76.12	61.43	53.45
CV (%)	38.33	32.46	24.74

range of recovery rate was 86.11%-107.27% for β-L and its mean was 95.17%. Therefore, the recovery rate of β-L was better than that of Lf when they were simultaneously detected (Table 12).

Application Studies

The results of paired t-test showed that the difference of β-L between protein chip and ELISA kit was not significant (t = 1.290, P = 0.238), and the difference of Lf between the two methods also was not significant (t = 1.925, P = 0.096) (Table 13). The correlation coefficients between the protein chip and the ELISA kit were obtained for β-L (r = 0.734) and for Lf (r = 0.774), respectively. And t-test results of the two correlation coefficients showed that both of the two coefficients were statistically significant ($t_{\beta\text{-L}}$ 2.644, $P_{\beta\text{-L}}$ = 0.038; t_{Lf} = 2.998, P_{Lf} = 0.024) (Figure 4). Therefore, the correlations both of β-L and Lf between the two methods were statistically significant.

Table 11. The Dilution Recovery of β-L and Lf (n = 3)

	Detected Concentration (ng/mL)	Theoretical Concentration (ng/mL)	Dilution Recovery Rate (%)
β-L	1681.45	1492.20	112.68
	726.04	746.10	97.32
	188.04	373.05	50.41
Lf	142.18	120.73	117.76
	64.30	60.37	106.52
	43.69	30.18	144.76

Table 12. The Adding Standard Recovery of β-L and Lf (n = 3)

	Sample (ng/mL)	Standard (ng)	Detected Result (ng/mL)	Adding Standard Recovery Rate (%)
β-L	740.53	335.75	1345.71	92.13
	654.37	335.75	1361.07	107.27
	438.88	335.75	1007.04	86.11
Lf	111.85	32.60	164.77	83.71
	77.08	32.60	153.75	119.95
	36.73	32.60	119.34	128.52

Table 13. The Comparison of Protein Chip and ELISA for Detecting β-L and Lf (n = 8, $\bar{x} \pm s$)

	Protein Chip	Elisa	Difference
β-L (μg/mL)	112.94 ± 55.9	90.45 ± 72.6	22.49 ± 49.39
Lf (μg/mL)	107.41 ± 31.6	92.69 ± 32.8	14.73 ± 21.65

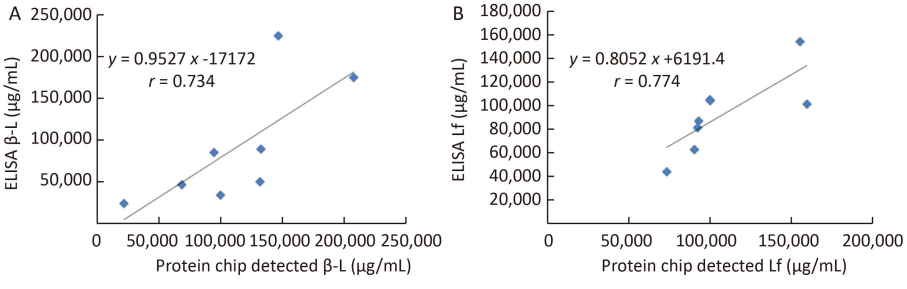

Figure 4. The correlation analysis of protein chip and ELISA for β-L (A) and Lf (B).

DISCUSSION

We have successfully established a detection platform for simultaneously measuring β-L and Lf. The basic process of this method was that print antibody probes on the protein chip, respectively, and then successively added mixed antigens, mixed detection antibodies and second antibodies with Cy3 in sequence. The signal intensity of Cy3 was positively proportional with the amount of antigen in sample. After the relevant research conditions were optimized and confirmed, the detection performances of the protein chip were further evaluated by using some indicators, such as precision, recovery rate and practical application.

In recently, although a method once achieved simultaneous detection for β-L and Lf[7], it is totally different with our platform. For coating material, it chose 96 well plate that is a kind of polystyrene material, and its coating ability was weaker than our coating material that was aldehyde-based three-dimensional substrate. For detection method, it was based on the competitive immunological reaction while our method was sandwich method. It is truism that sandwich method has more sensitivity and specificity than competitive immunological method. From the comparison between their result about detection limits (50 ng/mL β-L and 30 ng/mL of Lf) and our LLD (5.4 ng/mL β-L and 0.96 ng/mL of Lf), we also can find all of our results were better than competitive immunological method. Besides, antibody also is a influenced factor for specificity of detection, and all of antibody in their method were polyclonal antibodies (goat polyclonal antibody and donkey polyclonal antibody) while we used mouse monoclonal antibody to combine with aldehyde-based three-dimensional substrate. Finally, their method chose AgNPs as signal material while our method adopted Cy3. The different signal material also could lead to different sensitivity and specificity. Therefore, protein chip in our research for β-L and Lf also is the first report in a certain degree. And it also has potential as an alternative analytical tool to detect β-L and Lf in one detection.

As we know, β-L exists genetic polymorphism (variant A and B of beta-lactoglobulin). At the beginning of the research, we once taken into account this problem. While after we checked out relevant documents[12-13], we chose the whole native β-L which included of variant A and variant B as antigen. Because although β-L has two variants (A and B), these variants differ by only two amino-acid substitutions: 64^{th} [Asp(A) → Gly(B)], which is fully exposed to the solvent, and 118^{th} [Val(A) → Ala(B)], immersed in the hydrophobic core of the protein. And the two substitutions, 64^{th} [Asp(A) → Gly(B)] and 118^{th} [Val(A) → Ala(B)], wouldn't produce different antibody. So the whole native β-L which included of variant A and variant B was chosen to produce antibody. This means, in our platform, the mouse monoclonal antibody of β-L can combine both of variant A and variant B when native β-L was used as antigen, or when milk was detected. Therefore, our detection result includes variant A and variant B, and our result aims at whole native beta-lactoglobulin and does not distinguish the two variants. Besides, the β-L ELISA Kit of commercial antibody company (Cloud-Clone Corp) also aimed at the whole native β-L that included of variant A and variant B. Therefore, the object of the two methods was consistent, and our results were competent for the results of ELSIA Kit (Cloud-Clone Corp).

In our research, there were positive control (non-specificity rabbit IgG) and negative control (PBS pH 7.2) to ensure the quality of detection in each block. The positive control reflected the whole progress of the experiment, and if its signal was lower than 10,000, the positive results of the protein chip itself was unstable and the result was unreliable. The negative control reflected the non-specific reaction and cleanliness of printing needle, and the signal value of negative control will be more than 2,000 if there was non-specific reaction or there were residue of antibody probe in printing needle.

A modified sandwich method was applied in this detection platform so as to capture the relatively bigger antigen molecular in cow milk. In addition, the series performances including stability, luminance, and ground noise of the Cy3 were better than that of other fluorescent dyes, therefore, the second antibody with Cy3 was chosen in this platform.

For the platform, the first and foremost key point was which antibody should be chosen as the printing probe. In the experiment, all of antibodies for β-L and Lf (three mouse mAb and one rabbit pAb for every antigen) could combined the protein chip. However, only when the mouse mAb 66# for β-L and mouse mAb 75# for Lf were printed as probe, the signal value achieved best effect for β-L and Lf, respectively. And in this condition, the difference between the high concentration antigen and negative value was largest for both β-L and Lf. Therefore, it could ensure the enough detectable

space for antigen so as to achieve the feasibility of simultaneous detection for two antigen. Besides, in this condition, the difference between the lowest concentration antigen and negative value also was largest for β-L and Lf, respectively. Therefore, this condition ensured the LLD and BLD could achieve smallest for β-L and Lf, respectively. As above analysis, mouse mAb 66# for β-L and mouse mAb 75# for Lf were chosen as the probe of β-L and Lf, respectively.

In different documents, different researcher chose different number of pre-spotting to ensure the printing effect of probe. Jiang[14] suggested the number should be 10, while Yin[15] once suggested it should be 40 for both of SF and sTfR as his experiment result. The main reason was the viscosity of various probe was different, and the pre-spotting number was directly proportional to the viscosity of protein. In this research, the first stable interval of β-L 66# was from 44^{th} spot to 92^{th} spot and the first stable interval of Lf 75# was from 47^{th} spot to 90^{th} spot. Therefore, for simultaneously printing two probes in one block, the 46 spots were chosen as the number of pre-spotting in this platform and the single printing number should not exceed 44 spots.

The LLD and BLD of β-L were 5.4 ng/mL and 33.52 ng/mL, respectively. And the relevant results of Lf were 0.96 ng/mL and 3.59 ng/mL, respectively. All of these indicators were lower than that of ELISA. Therefore, the new platform could found more β-L and Lf, even the concentrations of β-L and Lf were lower, when it was used to identify the quality of cow milk.

In the experiment of standard curve, the ranges of two standard curves could cover the two antigens in one sample as one dilution. In cow milk sample, the concentrations of β-L and Lf were 2-4 mg/mL and 0.02-0.40 mg/mL, respectively. In the two standard curves, there were 5 pairs of standard points were chosen to establish standard curves for β-L and Lf, respectively. In these standard points, the standard antigens were diluted from the highest concentrations (β-L 10.725 μg/mL, Lf 1.382 μg/mL) to the lowest concentrations (β-L 67.03 ng/mL, Lf 14.4 ng/mL) by using 1:2 serials dilutions. Therefore, when the dilution ratio was approximate 1:400 for anyone sample, the post-dilution concentrations both of β-L and Lf would not exceed the ranges of the two standard curve. The two standard curves which were established by using 5 pairs of standard points were quite competent for simultaneously detecting two antigen proteins in one sample in one detection.

The results of precision revealed that intra-precision of this platform could meet the basic requirement for detection of β-L and Lf, while the inter-precision of this platform were lower than that of ELISA kit. The reasons of error might come from the unstably operation and unskilled technique of experimenter among different times. In fact, this operator was a undergraduate and all of her experiment times was not more than 100 hours that mean her experience was not enough. Therefore, the stability of this platform, especially was inter-precision, might be enhanced after the experimenter obtained more experiences and skills. The results of dilution recovery revealed that there was better dilution recovery rate in higher interval of the standard curve for both of β-L and Lf, and this result indicated that the detection result would be more stable and more accurate if cow milk sample was diluted in the higher part of standard curves in further practice. In the application study, the ELISA kit was used to compare with protein chip because there was not Golden standard in biological detection field. However, the comparison still had practical significance and it could indicate real situation. As comparisons of many times, the correlation coefficients between protein chip and ELISA kit for β-L and Lf were $r = 0.734$ and $r = 0.774$, respectively. Although they weren't above 0.95, the two correlation coefficients still were statistically significant and both of the two relationships were high correlation. Therefore, the protein chip was one kind of equivalent technique with ELISA kit. While based on the cautious viewpoint, we should not suggest to replace the ELISA kit by using this method, because there was not golden standard to access the final effect of the two methods.

To evaluate the quality of milk with a simple, quick and high throughput method has been a trend in the research of milk field. The study aimed at establishing and optimizing a new detecting platform for simultaneously measuring main protein of milk. As above experiments, the new detecting platform for simultaneously measuring β-L and Lf has been established and it has been able to meet basic requirement of laboratory although it still need be improved in further practice. On the other hand, with the increasing of requirement of grasping whole information about all milk proteins components, since all of them are regulated by mammary gland molecular machinery, the proteomic technology of

milk protein components would obtain more achievement and development. As one part of proteomic technology, the detection of protein chip for milk protein also would got more achievement. Therefore, this research just is a start point but not terminal point in the field, and we would enlarge, step by step, the detection number of milk proteins in further research so as to realize the systematic, general, fast, real time and accuracy detection and evaluation for all of milk proteins included of casein, α-Lactoalbumin, and so on.

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