Original Article

Study on The Method of Quantitative Analysis of Serum Ferritin and Soluble Transferrin Receptor with Protein Microarray Technology*

YIN Ji Yong, SUN Jing, HUANG Jian, LI Wen Xian, and HUO Jun Sheng#

Institute for Nutrition and Food Safety, Chinese Center for Disease Control and Prevention, Beijing 100050, China

Abstract

Objective To establish and evaluate a protein microarray method for combined measurement of serum ferritin (SF) and soluble transferrin receptor (sTfR).

Methods Microarrayer was used to print both anti-SF antibodies I and anti-sTfR antibodies I on each protein microarray. Anti-SF antibodies II and anti-sTfR antibodies II were used as detection antibodies and goat antibodies coupled to Cy3 were used as antibodies III. The detection conditions of the quantitative analysis method for simultaneous measurement of SF and sTfR with protein microarray were optimized and evaluated. The protein microarray was compared with commercially available traditional tests with 26 serum samples.

Results By comparison experiment, mouse monoclonal antibodies were chosen as the probes and contact printing was chosen as the printing method. The concentrations of SF and sTfR probes were 0.5 mg/mL and 0.5 mg/mL respectively, while those of SF and sTfR detection antibodies were 5 μg/mL and 0.36 μg/mL respectively. Intra- and inter-assay variability was between 3.26% and 18.38% for all tests. The regression coefficients comparing protein microarray with traditional test assays were better than 0.81 for SF and sTfR.

Conclusion The present study has established a protein microarray method for combined measurement of SF and sTfR.

Key words: Protein microarray; Optimization; Combined measurement conditions; Serum ferritin; Soluble transferrin receptor.

Biomed Environ Sci, 2012; 25(4):430-439 doi: 10.3967/0895-3988.2012.04.008 ISSN:0895-3988

www.besjournal.com(full text) CN: 11-2816/Q

INTRODUCTION

Iron deficiency (ID) affects approximately 2 billion population globally[1]. It is considered one of the most serious nutrition issues. Sufficient data have been accumulated to show that ID reduces the physical activity in adults and impairs the brain development in children. To identify the populations at risk for the deficiency, there is an urgent need for simple and reliable methods to assess iron status. Serum ferritin (SF) and soluble transferrin receptor (sTfR) have been widely used as iron indicators in recent years[2]. SF and sTfR correlate numerically with body iron stores. Measuring SF and sTfR allows us to evaluate body iron storage and to determine whether this storage

*The study was funded by the 863 Program entitled as "The research and exploration of nutrition fortified food for improving growth and development (2010AA023004)",and performed by the Trace Elements Nutrition Key Laboratory of the Ministry of Health.

#Correspondence should be addressed to: HUO Jun Sheng, Researcher, and Ph.D, Tel: 86-10-83132383; E-mail: jshuo@263.net.cn

Biographical note of the first author: YIN Ji Yong, male, born in 1975, Ph.D, lecturer, majoring in detection, nutriology, and immunology.

Received: September 8, 2011; Accepted: May 4, 2012

is adequate that was previously tested by bone marrow staining or other less precise method i.e. defining iron deficiency with the serum content[3].

Enzyme-linked immunosorbent assay (ELISA), immunoturbidimetry (ITA), and radioimmunoassay (RIA) are currently used as the major detection methods for SF and sTfR. However, none of these techniques can measure SF and sTfR simultaneously to be able to reduce cost and volume of blood samples. In contrast, protein microarray is recognized as a high throughput analytical method using multi-target proteins. This technique may be used to measure SF and sTfR simultaneously, and is therefore extremely valuable for iron status studies.

In recent years, the technology of protein microarray has been continuously improved and its application has penetrated into more scientific areas[4-7]. However, few studies have reported measurement of nutrition biomarkers with this technology. SF and sTfR, as biomarkers of iron status, need to be measured quantitatively, quickly, and simultaneously, for which current techniques are inadequate. The protein microarray method is potentially suitable for use in measurement and survey of iron status and other nutritional status in both clinical and population studies. Therefore, the primary aim of this study is to establish and evaluate a protein microarray method for combined measurement of SF and sTfR.

MATERIALS AND METHODS

Materials

This study involves the materials including NaH_2PO_4, Na_2HPO_4, NaCl, KCl, Tween20, bovine serum albumin (BSA), Skim milk powder, printing buffer (code 440015, CapitalBio Corporation), mouse monoclonal anti-ferritin antibody (code ab10060, abcam), rabbit polyclonal anti-ferritin antibody (code ab7332), mouse monoclonal anti-sTfR antibody (code ab38168, abcam), rabbit polyclonal anti-sTfR antibody (code NB100–62443, Novus), goat polyclonal antibody IgG-Cy3-linked (code PA43002, PA43004, GE Healthcare; code 611-104-122, ROCKLAND), immunoturbidimetry kit (Roche Laboratories), protein microarray (Polymer Slide-G, CapitalBio Corporation), printer (PersonalArrayer™ 16 Microarray Spotter, CapitalBio Corporation), and scanners (LuxScan™ 10K Microarray Scanner, CapitalBio Corporation). Serum control samples of SF (code 30-AF15, Fitzgerald) and sTfR (code 05–52172, ARP-1) were used as standards for the calibration curves.

Methods

Procedure of Protein Microarray Basic The antibody probes of SF and sTfR, the quality control (Cy3-coupled BSA), the positive control (non-specificity rabbit IgG), the negative control (3% BSA) and the blank control (PBS pH7.2) were successively printed in each of 12 blocks on the PSG microarray. After they were immobilized (at 37 °C for 16 h) and blocked (by adding 3% BSA, 40 μL/block, at 37 °C for 30 min), the protein microarray was washed in 1‰ PBST wash buffer (5 min/time, 3 times). And then, the antigens were added and incubated (30 μL/block, 37 °C incubation, 1 h), and the microarray was washed with 1‰ PBST wash buffer (5 min/time, 5 times). Afterwards, the detection antibodies were added and incubated (30 μL/block, 37 °C incubation, 1 h), and the microarray was washed with 1‰ PBST wash buffer (5 min/time, 5 times). Finally, the goat antibody coupled to Cy3 was added and incubated (30 μL/block, 37 °C incubation, 1 h), and the microarray was washed with 1‰ PBST wash buffer (5 min/time, 5 times) and scanned to obtain images and result value by laser scan.

Optimizing the Kind of SF and sTfR Probes The experimental procedures were the same as the basic procedure of protein microarray. Both mouse monoclonal antibodies (mAb) of SF and sTfR were printed as probes in Block1, 2, 3, and Block4, and both rabbit polyclonal antibodies (pAb) of SF and sTfR were printed as probes in Block 5, 6, 7, and Block 8. Rabbit pAbs of SF were used as detection antibodies in Block 1 and Block 2, and rabbit pAbs of sTfR were used as detection antibodies in Block 3 and Block 4. Mouse mAbs of SF were used as detection antibodies in Block 5 and Block 6, and mouse mAbs of sTfR were used as detection antibodies in Block 7 and Block 8. The SF antigens were added in Block 1 and Block 5. The sTfR antigens were added in Block 3 and Block 7. Three percent BSA were added in other blocks. The concentrations of the probes were 1 mg/mL for the mouse mAbs of SF, 1 mg/mL for the mouse mAbs of sTfR, 1 mg/mL for the rabbit pAbs of SF and 0.09 mg/mL for the rabbit pAbs of sTfR. The concentrations of the antigens of SF and sTfR were 2 μg/mL and 5 μg/mL respectively and the negative control was 3% BSA. The concentrations of the detection antibodies were 0.5 μg/mL for the rabbit pAbs of SF, 0.18 μg/mL for the rabbit pAbs of sTfR, 2 μg/mL for the mouse mAbs of SF and 2 μg/mL for the mouse mAbs of sTfR. The

dilution of both the goat anti-mouse and goat anti-rabbit antibody was 1:1000.

Optimizing the Printing Method Mouse mAbs of SF and sTfR were printed respectively by contact printing and non-contact ink jet printing on different microarray. The concentration of the mouse mAbs of SF and sTfR using both printing methods was 1 mg/mL. The subsequent steps were the same as in the procedure of protein microarray basic. The concentrations of the SF and sTfR antigens were 2 μg/mL and 5 μg/mL respectively. The negative control (NC) was 3%BSA. The concentrations of the SF and sTfR detection antibodies were 0.5 μg/mL and 0.18 μg/mL respectively. The dilution of goat anti-rabbit antibodies was 1:1000.

Homogeneity of the Spots of the SF and sTfR Probes Mouse mAbs of SF and sTfR were printed in 400 spots in each of blocks by contact printing. The concentrations of the SF and sTfR probes used were 0.5 mg/mL and 0.5 mg/mL respectively. The microarray was directly scanned after it was printed.

Optimizing the Concentrations of the Probes, the Detection Antibodies of SF and sTfR The experiment procedures were the same as the basic procedure of protein microarray. All of the blocks were divided into two groups to receive different dilutions of the second antibodies. The concentrations of the probes and the detection antibodies of SF and sTfR were optimized by using the crisscross serial-dilution analysis in each group. Different concentrations were used to print probes of SF (0.5, 0.25, 0.125 mg/mL) and sTfR (0.5, 0.25, 0.125 mg/mL) in each block. Three concentrations of antigens were chosen: high concentration (SF 2 μg/mL, sTfR 10 μg/mL), low concentration (SF 5 ng/mL, sTfR 25 ng/mL) and NC (3% BSA). Then, three concentrations of detection antibodies were added: high (SF 10 μg/mL, sTfR 1.8 μg/mL), medium (SF 5 μg/mL, sTfR 0.36 μg/mL) and low (SF 1 μg/mL, sTfR 0.18 μg/mL). The optimization criteria were that: (1) the signal reading was unsaturated (signal value -background value <65535) at high antigen concentrations, (2) the signal reading at low antigen concentrations had difference of statistical significance from that obtained with NC. At the same time, the background reading of the microarray had to be low.

Optimizing the Kind of Blocking Reagent and Second Antibody The experiment procedures were the same as the basic procedure of protein microarray. The twelve blocks on the microarray were divided into four groups of 3 blocks each. Different concentrations were used to print probes of SF (0.5 mg/mL) and sTfR (0.5 mg/mL) in each block. The high concentration of mixed antigens (SF 512.5 ng/mL, sTfR 2500 ng/mL), the low concentration of mixed antigen (SF 5 ng/mL, sTfR 10 ng/mL) and the NC (3% BSA) were added into one of the three blocks in every group separately The first and the second groups used 3% BSA as the blocking reagent. The third and the fourth groups used 3% skim milk powder as the blocking reagent. The first and the third groups used second antibodies made by GE Healthcare. The second and the fourth groups used second antibodies made by ROCKLAND. In the mixed detection antibodies, the concentrations of the SF and sTfR detection antibodies were 5 μg/mL and 0.36 μg/mL respectively. The dilutions of goat anti-rabbit antibodies were 1:1000 (GE Healthcare) and 1:41000 (ROCKLAND instruction manual). The optimization criteria were that: (1) the signal reading was unsaturated (signal value -background value <65535) at high antigen concentrations, (2) the signal reading at low antigen concentrations had difference of statistical significance from that with NC, (3) the difference between high concentration antigen and NC was as large as possible. The final optimized conditions were selected based on detection results for both SF and sTfR.

Lower Limit of Detection and Biologic Limit of Detection The experiment procedures were the same as the basic procedure of protein microarray. Different concentrations were used to print probes of SF (0.5 mg/mL) and sTfR (0.5 mg/mL) in each block. The mixed antigens were diluted from high concentrations (SF: 16.0 ng/mL, sTfR: 6.25 ng/mL) to low concentrations (SF: 2.0 ng/mL, 0.78 ng/mL) by using 1:2 serial dilutions. Three percent BSA was used as the NC. In the mixed detection antibodies the concentrations of the SF and sTfR detection antibodies were 5 μg/mL and 0.36 μg/mL respectively. The dilution of the second antibodies was 1:1000. The same procedure was repeated 12 times for each concentration. Based on the results, the lower limit of detection (LLD) of the method was the concentration that corresponded to mean 3SD above zero when the NC was used. Finally, the biologic limit of detection (BLD) was found by selecting the minimal concentration of mixed antigens that gave a mean signal level-with 3SD value greater than the signal level at LLD.

Establishing the SF and sTfR Models and Standard Curve The experiment procedures were the same as the basic procedure of protein microarray. Different concentrations were used to print probes of SF (0.5 mg/mL) and sTfR (0.5 mg/mL) in each block.

Five concentrations of mixed antigens were chosen: SF 512.5 ng/mL, sTfR 625 ng/mL; SF 128.125 ng/mL, sTfR 312.5 ng/mL; SF 32.03 ng/mL, sTfR 156.25 ng/mL; SF 16.0 ng/mL, sTfR 78.13 ng/mL; SF 2.0 ng/mL, sTfR 19.53 ng/mL, and 3% BSA was chosen as the NC. In the mixed detection antibodies, the concentrations of the SF and sTfR detection antibodies were 5 μg/mL and 0.36 μg/mL respectively. The dilution of the second antibody was 1:1000. We calculated the mean levels and used these levels as the basis to obtain their standard curve and model respectively. Standard curves and models were established for SF and sTfR based on the relation between the antigen concentration and the detection signal level.

Precision Three samples of known concentrations were tested 18 times on one protein microarray to assess intra-assay precision. Another three samples of known concentrations were tested in 10 separate assays to assess inter-assay precision, and for every sample the assay was repeated twice.

Recovery The recovery of SF and sTfR spiked to three mixed levels (SF: 32.03 ng/mL, sTfR: 39.06 ng/mL; SF: 64.06 ng/mL, sTfR: 78.13 ng/mL; SF: 128.13 ng/mL, sTfR: 156.25 ng/mL) in three samples.

Application Studies To test this new method, 26 serum specimens were analyzed with traditional methods and protein microarray respectively. The commercial immunoturbidimetry SF and sTfR kits from Roche Laboratories were used as a reference to compare the protein microarray results. The correlation and agreement between the 2 methods were calculated by the correlation analysis and paired -comparisons *t* test.

Calculations and Statistics All analyses were done with Excel 2003 (Microsoft) and SPSS13.0. The standard curves and models were made with Curve expert 1.3. A two-sample *t*-test was used to compare the two group samples measured and to test the difference between low concentration antigen and NC within the experiment that optimized the kind of blocking reagent and second antibody ($\alpha=0.05$). A factorial experimental design was used to optimize the kind of blocking reagent and second antibody ($\alpha=0.05$). The correlation analysis and paired -comparisons *t* test ($\alpha=0.01$) was used to compare the difference between the protein microarray and the traditional method.

RESULTS

Optimizing the Kind of SF and sTfR Probes

Both the mouse mAb and rabbit pAb of SF and sTfR were able to be attached to the microarray. The differences between SF antigen and the NC of SF were similar for different SF probes. However, when rabbit pAbs of sTfR were used as probe, the difference between high concentration sTfR and the NC of sTfR was too low to satisfy the requirement of detection for low concentration sTfR (Table 1).

Optimizing the Printing Method

Contact printing was better than non-contact ink-jet printing for both SF and sTfR because the contact printing could increase the detection range between the high concentration antigen and the NC (Table 2). Besides, the signal images made by no-contact ink jet printing were inferior to those obtained from contact printing (Figure 1).

Homogeneity of the Spots of the SF and sTfR Probes

Between the 40th spot and the 200th spot, the spots of the SF and sTfR probes were basically homogeneous (Figure 2). After the 200th spot, the signal intensity of the SF probe obviously decreased; however, the signal intensity of the sTfR probe remained unchanged until the 240th spot. Therefore, pre-spotting 40 spots was necessary to ensure good results for both SF and sTfR probes in preparing protein microarray. The number of spots should be controlled in 160 spots after each sampling.

Table 1. Detection Value of Different Antibodies as Probe for SF and sTfR ($\bar{x} \pm s$)

Probe	The Mouse Antibody of SF as the Detection Antibody		The Mouse Antibody of sTfR as the Detection Antibody		Probe	The Rabbit Antibody of SF as the Detection Antibody		The Rabbit Antibody of sTfR as the Detection Antibody	
	3% BSA	SF	3% BSA	sTfR		3% BSA	SF	3% BSA	sTfR
Rabbit antibody of SF	23010±942	35689±1965	—	—	Mouse antibody of SF	14086±1021	29357±1216	—	—
Rabbit antibody of sTfR	—	—	12127±1244	14318±974	Mouse antibody of sTfR	—	—	6360±329	18309±479
Difference	—	12679	—	2191	—	—	15271	—	11949

Table 2. Detection Value of Different Print Methods ($\bar{x} \pm s$)

Probe	Non-contact Ink Jet Printing				Contact Printing			
	The Rabbit Antibody of SF as the Detection Antibody		The Rabbit Antibody of sTfR as the Detection Antibody		The Rabbit Antibody of SF as the Detection Antibody		The Rabbit Antibody of sTfR as the Detection Antibody	
	3% BSA	SF	3% BSA	sTfR	3% BSA	SF	3% BSA	sTfR
Mouse antibody of SF	274±20	2230±136	—	—	14086±1021	29357±1216	—	—
Mouse antibody of sTfR	—	—	145±40	2321±174	—	—	6360±329	18309±479
Difference	—	1956	—	2176	—	15271	—	11949

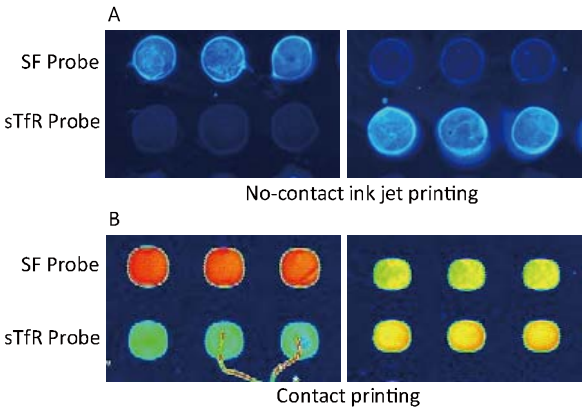

Figure 1. Each box represents one block. Both SF probe and sTfR probe were printed in each block. The two blocks in the first row (A) reflected the detection results of no-contact ink jet printing and the two blocks in the second row (B) reflected the detection results of contact printing. The blocks in the left column were detection signal images to SF antigens. The blocks in the right column were detection signal images to sTfR antigen. Within each block, the first row reflected the detection results of SF antigens, and the second row reflected the detection results of sTfR antigens.

Optimizing the Concentrations of the Probes, the Detection Antibodies of SF and sTfR

The optimal concentration was 0.5 mg/mL for the SF probe and 5 μg/mL for the SF detection antibody. The optimal concentration was 0.5 mg/mL for the sTfR probe and 0.36 μg/mL for the sTfR detection antibody.

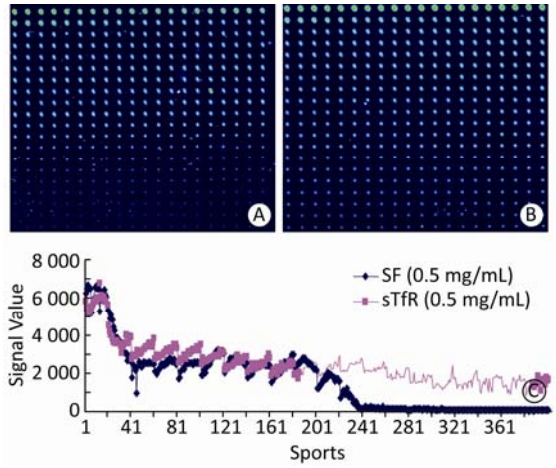

Figure 2. This figure showed the test for homogeneity during spotting of the SF and sTfR probes. The A image was for SF probes and the B image for sTfR probes. The C image showed the trend of homogeneity during spotting of the SF and sTfR probes.

Optimizing the Kind of Blocking Reagents and the Second Antibodies

There were interactions between the second antibodies and blocking reagents and these interactions appeared in high and low concentration SF groups and the NC group of SF ($F_{SF\ high}$=8.443, $P_{SF\ high}$=0.02; $F_{SF\ low}$=224.383, $P_{SF\ low}$=0.000; $F_{SF\ N}$=23.244, $P_{SF\ N}$=0.001). Therefore, the detection values from the interaction between the second antibody made by GE and the 3% BSA were the best for SF. There were also interactions between the second antibodies and the blocking reagents, which appeared in high, low concentration sTfR group and the NC group of sTfR respectively ($F_{sTfR\ high}$ =26.719,

$P_{sTfR\ high}$=0.001; $F_{sTfR\ low}$ =159.363, $P_{sTfR\ low}$ =0.000; $F_{sTfR\ N}$=22.638, $P_{sTfR\ N}$=0.001). Therefore, the detection value, in the high and low concentration sTfR groups and the NC group, from the interaction between the second antibody made by GE and the 3% skim milk power was the best. The desired difference between low concentration sTfR and NC was achieved by using the second antibodies made by GE and 3% skim milk power, but the same conditions showed no statistically significant difference between low concentration SF and NC (t=0.581, P=0.592).

Only when the second antibodies (GE) and 3% BSA were used, the differences between low concentration SF and NC as well as those between low concentration sTfR and NC, were both statistically significant (Table 3). In addition, when the second antibodies made by ROCKLAND and 3% BSA were used, detection signal values tended to be low for both SF and sTfR.

Table 3. Comparing the Means for Different Detection Items by Interaction between Different Second Antibodies and Blocking Buffers ($\bar{x} \pm s$, n=3)

Second Antibody	Blocking Buffer	Signal Value Mean					
		High SF	Low SF	NC of SF	High sTfR	Low sTfR	NC of sTfR
GE	3% BSA	13 898±3028	1 854±12 [a]	876±33	4 404±740	432±86 [b]	86±12
	3% skim milk powder	17 786±1662	549±38	536±6	6 932±86	534±37[c]	35±8 [c]
ROCKLAND	3% BSA	4 324±352	291±17	113±14	8 16±208	144±7[c]	46±12
	3% skim milk powder	2 382±150	68±7	55±11	1 029±57	138±3[c]	48±4

Note. NC: negative control. [a]: compared with the NC group of SF for second antibody made by GE and 3% BSA, t=4.801, P=0.039; [b]: compared with the NC group of SF for second antibody made by GE and 3% BSA, t=6.921, P=0.018; [c]: compared with the NC group of sTfR for second antibody made by GE and 3% BSA, t=6.195, P=0.003.

Lower Limit of Detection and Biologic Limit of Detection

The LLD of SF was 0.788 ng/mL and the LLD of sTfR was 0.446 ng/mL. The BLD of SF was 2 ng/mL and the BLD of sTfR was 1.56 ng/mL.

Establishing the SF and sTfR Models and Standard Curves

By choosing the best fitting model using Curve expert 1.3, the correlation coefficients of the standard curves of SF and sTfR were always higher than 0.97 (Figure 3). Differences between duplicate measurements on the same microarray were kept<5%. The coefficient of determination of the standard curve of SF was 0.977 and the best fitting model for the protein microarray method was:

$$y = -6.49 + 0.014x$$

The coefficient of determination of the standard curve of sTfR was 0.994 and the best fitting model for the protein microarray method was:

$$y = 13.94 - 0.005x + 4.38E\text{-}007x^2$$

Precision

The results of intra-assay precision and the inter-assay precision can be checked through Table 4 and Table 5. The intra-array precision range was between 5.03% and 8.88%, and all of these precisions were not more than 10% for both SF and sTfR. The inter-array precision range was between 7.35% and 18.38%, and all of these precisions were not more than 20% for both SF and sTfR. As a whole, the precision of both the intra-assay and the inter-assay of sTfR was better than SF.

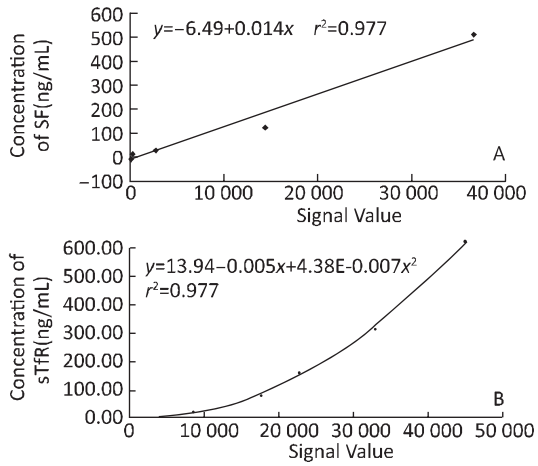

Figure 3. Standard curve of the SF antigen (A) and sTfR antigen (B). The standard curve of SF antigen was made by using serial dilutions of purified SF (2-512.5 ng/mL). The standard curve of sTfR was made by using serial dilutions of purified sTfR (19.53-625 ng/mL). Each point represents the mean of triplicate detection.

Table 4. Intra-Array Precision for the Protein Microarray for Three Samples

Sample	*n*	SF			sTfR		
		M	SD	CV(%)	M	SD	CV(%)
I	18	11.18	0.97	8.70	65.30	4.06	6.20
II	18	52.11	3.82	7.33	83.80	7.40	8.88
III	18	22.60	1.89	8.39	64.92	3.26	5.03

Table 5. Inter-array Precision for the Protein Microarray for Three Samples

Sample	*n*	SF			sTfR		
		M	SD	CV%	M	SD	CV%
I	5	12.98		13.42	76.24	5.61	7.35
II	5	29.90		18.38	67.86	8.18	12.05
III	5	1.48		10.77	65.40	8.37	12.80

Recovery

The recovery can be examined by Table 6. The recovery of 3 samples was 70.76%-125.21% for SF and 74.32%-116.15% for sTfR. The lowest mean recovery rate was 87.26% on SF and the best mean recovery rate was 98.17% on sTfR. As a whole, the recovery of sTfR was better than SF when they were simultaneously detected.

Application Studies

The paired-comparisons of t-test results for 26 serum samples showed that there were no significant difference between protein microarray and commercial immunoturbidimetry for detecting SF (P=0.012) and sTfR (P=0.014) (Table 7). The best correlations between the protein microarray and the traditional methods were obtained for SF (r=0.868) and the better correlation was obtained for sTfR (r=0.815) (Figure 4).

Table 6. Recovery for SF Standard and sTfR Standard (%)

Sample	SF			sTfR		
	I	II	III	I	II	III
Low	79.85	70.76	125.21	74.32	99.26	81.26
Recovery rate Medium	110.80	110.42	107.47	116.15	101.13	106.29
High	97.02	80.61	120.23	104.04	106.70	85.62
Mean recovery rate	95.89	87.26	117.63	98.17	102.37	91.06
Proportional system error	4.11	12.74	17.63	1.83	2.37	8.94

Table 7. The Comparison of Protein Microarray and Immunoturbidimetry Methods for SF and sTfR ($\bar{x} \pm s$, α=0.01)

	Protein Microarray	Immunotur Bidimetry	Difference
SF (ng/mL)	94.74±44.9	82.31±47.7	12.430±23.9
sTfR (μg /mL)	2.01±0.4	2.22±0.7	-0.202±0.4

DISCUSSION

In this paper, we established and optimized the detection conditions of the protein microarray method to simultaneously measure SF and sTfR. The principle of this method was to print a protein microarray with antibodies that "captured" the antigens. Antigens, detection antibodies and the Cy3-coupled second antibodies were successively added. The protein microarray was washed before each application. The Cy3 signal intensity was directly proportional to the amount of antigen in the sample. After the series of basic research conditions was confirmed, we evaluated the practical applicability of the protein microarray in terms of its precision, recovery and practical application.

In each block, the detection results were controlled by the quality control (Cy3-coupled BSA), the positive control (non-specificity rabbit IgG), the negative control (3% BSA) and the blank control (PBS pH7.2). The Cy3-coupled BSA controlled the quality of the protein microarray itself, and its detection signal should be bright. If the signal values of Cy3-coupled BSA were lower than 2000, the quality of the protein microarray itself was unstable and the result was unreliable. The positive control controlled the progress of the whole experiment operation and its result should be bright if the progress is normal.

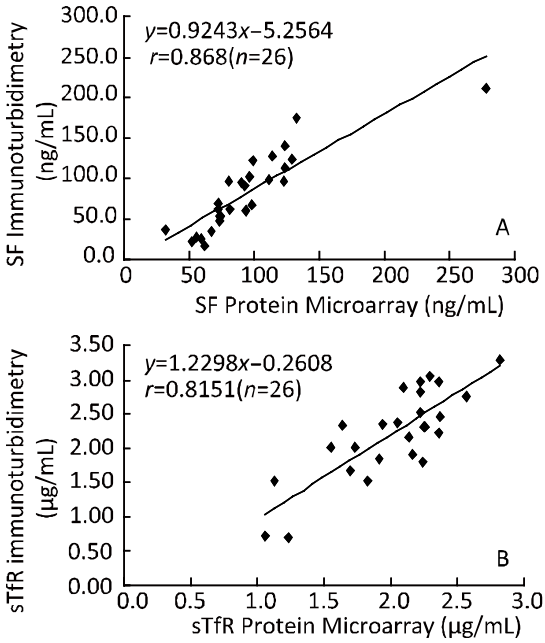

Figure 4. The correlation analysis of protein microarray method and immunoturbidimetry method for SF (A) and sTfR (B) (Roche, immunoturbidimetry kit).

The negative control controlled the non-specific reaction and its result should be dark if there is no non-specific reaction in the whole experiment process. The blank control controlled the print operation and its result should be dark if the cleanliness was enough after every print antibody probe.

In this study, the sandwich method was chosen as the operation method based on the basic standard: When the molecule is a whole protein molecule in nature , the sandwich method can more efficiently detect the measurement indicators than other methods[8]. The molecules of the SF and sTfR are whole bigger molecules in nature; therefore, this study chose the sandwich method as the detection method. In addition, Cy3 was chosen as the detecting method because it produced more luminance, sound stability and low ground noise than other fluorescent dyes such as fluorescein isothiocyanate, dansyl chloride and so on[9].

In this study, the first and foremost key point was which antibody should be chosen as the probe. We tested a mouse mAb and a rabbit pAb respectively and separately, which were printed on the protein microarray. Both mouse mAb and rabbit pAb of SF and sTfR were able to be attached to the microarray. However, the mouse mAb as a probe was better than the rabbit pAb for sTfR, as it caused greater difference between high concentration antigens and the NC, which could improve the measurement range and sensitivity. Besides, because our objective was to detect SF and sTfR simultaneously, the mouse mAb as probe was more appropriate than the rabbit pAb for both SF and sTfR. The difference between different sTfR antibodies might have been chiefly owing to the fact that the original concentration of our purchased sTfR rabbit pAb (0.18 mg/mL) was lower than the sTfR mouse pAb (2 mg/mL). After the original antibody was diluted by using printing buffer at a ratio of 1:1, the rabbit pAb had a concentration of 0.09 mg/mL only. These low concentrations led to lower ability of association with sTfR antigens.

In terms of the printing method, different articles[10-13] offered different recommendations. The differences mainly depend on the kind of the microarray used and the characteristics of the printer. Usually, a membrane carrier is more applicable to non-contact printing, and glass substrate carrier is more applicable to the contact printing. In this study, the glass substrate was chosen as the carrier. Moreover, the printer made by CapitalBio Corporation was better suited to contact printing than to non-contact printing. These previous observations were confirmed by our experimental results. Therefore, contact printing was used in this study.

Some researchers suggested[14] that the number of the pre-spotting should be 10 to ensure the basic printing conditions of protein microarray. However, we were only able to achieve homogeneity between the 40th and the 200th spots of probe in our experiment. The main reason was that protein viscosity was different among proteins. The number of pre-spotting needed to grow with decrease of protein viscosity. Neither of our probes had high viscosity. The number of the pre-spotting also depends on the printing method. Usually, the number of pre-spotting in contact printing is higher than the number in non-contact printing. Therefore, pre-spotting 40 spots was used as the basic printing condition to ensure good results for both SF and sTfR. After the 200th spot, the signal intensity of the sTfR probe remained unchanged until the 300th spot, but our objective was to detect SF and sTfR simultaneously. Therefore, the range of homogeneity of the spots of the SF and sTfR probes was chosen from the 40th to the 200th and the

number of spots was controlled in 160 spots after every sampling.

For the choice of the second antibody and blocking reagent, the second antibodies made by GE and 3% skim milk powder satisfied the detection of sTfR, but they led to lack of statistically significant difference between the low concentration of SF and the NC of SF. This result would have meant loss of detection specificity at low concentration of SF. When the second antibodies made by ROCKLAND and 3% BSA were used, the narrower detection range would lead to lower sensitivity. Since simultaneous detection of SF and sTfR was the ultimate objective, the second antibodies made by GE and the 3% BSA were chosen to be the optimal condition for the detection system.

The LLD of SF was 0.788 ng/mL and the BLD of SF was 2 ng/mL. The SF normal range was from 12 ng/mL to 200 ng/mL[15] in serum sample. However, the LLD and the BLD of sTfR were lower compared to SF, with a higher normal range. Therefore, the dilution of serum should be controlled between 1:6 and 1:8. As the volume added in every block was 30 uL, it would be possible to use 5 μL or less of serum to measure both SF and sTfR. Thus capillary blood sampling can also be used. This advantage is important for the application of this method, because capillary blood sampling is usually better accepted culturally and easier to perform, especially for children.

The LLD of the protein microarray for SF and sTfR were comparable to the LLD of acknowledged ELISA method. The LLD of the protein microarray for SF was 0.788 ng/mL (or 0.023 ng per block) and the value was approximate to the result from ELISA (2 ng/mL, or 0.4 ng per tube)[16]. The LLD of the protein microarray for sTfR was 0.446 ng/mL (or 13.38 pg per block) and it was lower than the result from ELISA (0.5 nmol/L, with the molecular weight of sTfR being 85KD)[17].

Curve Expert 1.3 provides several different models to calculate the standard curve. Given consideration to the physiological ranges of both SF and sTfR, the best fitting model was chosen to draw standard curves for SF and sTfR respectively, but a linear regression for SF and a semi logarithm for sTfR gave similar results. Based on these standard curves, signal levels were found to be directly proportional to the quantity of standard antigens.

The comparable results of LLD between protein microarray and acknowledged ELISA and the establishment of standard models demonstrated that the detection conditions of protein microarray for simultaneous quantitative detection of SF and sTfR has been verified. These results of intra-assay and inter-assay precision, recovery and application studies showed that the protein microarray can be used to measure serum samples in further research.

To evaluate people's nutritional status with a simple, quick, and high throughput method and a less volume of samples has been a main objective in the development of nutritional assessment. This study aimed at establishing and optimizing a new detection method to simultaneously measure multiple biomarkers of nutrition. The protein microarray method developed in this study may allow a new measurement approach for nutritional status in the future.

ACKNOWLEDGEMENTS

We thank SHE Xin Shu for critical reading of the manuscript, Prof. KANG Xi Xiong, LIU Zhi Zhong, ZHANG Guo Jun and CHEN Yan for their technology support, Prof. HE Wei, LIU Qiong Min and NIE Yan Li for their technology consultation.

REFERENCES

1. WHO/UNICEF/UNU (1998) Iron Deficiency Anemia: Prevention Assessment and Control, Report of a Joint WHO/UNICEF/UNU Consultation. World Health Organization, Geneva, Switzerland.
2. Cook JD, Flowers CH, Skikne BS. The quantitative assessment of body iron. Blood, 2003; 9, 3359-64.
3. Juergen GE, John EE, Chrestine MP, et al. Combined meas- urement of ferritin, soluble transferrin receptor, retinol binding protein, and C-reactive protein by an inexpensive, sensitive, and simple sandwich Enzyme-linked immunosorbent assay technique. Nutr, 2004; 11, 3127-32.
4. MacBeath G, Schreiber SL. Printing proteins as microarrays for high throughput function determination. Science, 2000; 5485, 1760-3.
5. Zhou H, Roy S, Schulman H, et al. Solution and chip arrays in protein profiling. Trends Biotechnology, 2001; 10 Supply, 534-9.
6. Lee YS, Mrksich M Protein chips: from concept to practice. Trends Biotechnology, 2002; 12 Supply, S14-8.
7. Kodadek T. Development of protein-detecting microarrays and related devices. Trends Biochem Sci, 2002; 6, 295-300.
8. ZHANG J, GAO Z, ZHOU HL.(2005). Protein microarray techn- ology. Bio-chip Technology. (CHEN ZHONGBIN), pp.126-7. Chemistry Industry Press, Beijing. (In Chinese)
9. ZHANG J, GAO Z, ZHOU HL. (2005). Protein microarray techn- ology. Bio-chip Technology. (CHEN ZHONGBIN), pp.123-4. Chemistry Industry Press, Beijing. (In Chinese)

10.Stears RL, Martinsky T, Schena M. Trends in microarray analysis. Nat Med, 2003; 9, 140-5.
11.Newman JD, Turnera PF. Ink-jet printing for the fabrication of amperometric glucose biosensors. Anal Chim Acta, 1992; 1, 13-17.
12.Blanchard AP, Kaiser RJ, Hood LE. High-density oligonucleotide arrays. Biosens Bio electr, 1996; 11, 687-90.
13.Lemmo AV, Fisher JT. Geysen HM, et al. Characterization of an inkjet chemical microdispenser for combinatorial library synthesis. Anal Chem, 1997; 4, 543-51.
14.Jiang L, Guo Z, Chen Z, et al. Optimization of the conditions for protein chip preparation. J First Mil Med Univ, 2004; 11, 1230-2.
15.WHO/UNICEF/UNU (2001). Iron Deficiency Anemia: Prevention Assessment and Control, A guide for programme managers. World Health Organization, Geneva, Switzerland.
16.Revenant MC ".Sandwich" enzyme immunoassay for serum ferritin with polypropylene test tubes as the solid phase. Clin Chem, 1983; 4, 681-3.
17.Allen J, Backstrom KR, Cooper JA, et al. Measurement of soluble transferrin receptor in serum of healthy adults. Clin Chem, 1998; 1, 35-9.