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Multiplex biomarker analysis biosensor for detection of hepatitis B virus

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Abstract. In this paper, we report the development of a protein microarray-based biosensor for the detection of the hepatitis B virus (HBV) serological markers using surface plasmon resonance (SPR Printing buffer, protein immobilization time and concentration of the capture protein were optimized systematically to determine the best performance of the biosensor. Under optimal conditions, five hepatitis B markers in 20 μL human serum can be simultaneously detected within 30 minutes, whereas other methods such as ELISA and PCR can detect only one marker within four hours. This platform has been validated by analysis of 35 patients known to have hepatitis B, with 85% agreement between the test platform and analysis by commercial enzyme-linked immunosorbent assay (ELISA) kits. The results demonstrate that the protein microarray with SPR displayed a sensitivity of 0.1 ng mL^{-1} for HBsAg. In addition to high sensitivity, it also shows excellent specificity, reproducibility and stability. This integrated protein microarray technique combined with SPR is a promising candidate for hepatitis B diagnosis with high-throughput.

Keywords: Surface plasmon resonance, protein microarray, hepatitis B virus, label-free, biosensor

1. Introduction

The phenomenon of virus-induced hepatitis becomes increasingly serious in China in recent years. According to the 2011 statistical yearbook of the Republic of China, approximately 1.4 million people were infected with viral hepatitis in 2009, among which approximately 1000 people died of HBV or its related syndrome. Currently, the hepatitis virus is widely spread, with a global distribution. The most common hepatitis viral sub-types include hepatitis A, B, C, D and E. Each is caused by a different hepatitis virus; for example, hepatitis B is caused by the hepatitis B virus (HBV) [1], one of

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the most aggressive viruses threatening public health worldwide [2]. Approximately more than 780,000 people die each year due to the consequences of HBV. Thus, the HBV risk necessitates the development of a rapid, simple and sensitive method for HBV detection to prevent the outbreak of HBV infection.

Currently, along with the development of biological techniques, molecular biology techniques based on gene tests have been established [3-5]. Among them, PCR is the main laboratory diagnostic test for the hepatitis B virus. While robust and sensitive, this method typically requires several sample preparation processes [6]. In recent decades, researchers had been committed to the development of detection technology for HBV, and have established a number of highly sensitive and specific detection techniques [7] such as an enzyme-linked immunosorbent assay (ELISA) [8, 9], electrochemical sensor [10], a molecular beacon-based platform [11], and protein microarray. However, these techniques have inherent limitations [12], such as a labeled tracer requirement and high time comsumption, and thus do not allow real-time detection.

To overcome those limitations, we integrated protein microarray and SPR techniques as an efficient alternative for high-resolution HBV detection. Major advantages of this combination include: (1) a highly parallel assay system, which will provide more information for hepatitis B diagnosis than the single-marker test; (2) a rapid label-free test; (3) intuitionistic results; and (4) no special sample preparation is required for the samples. There are several commercial SPR techniques for HVB diagnosis [13-15]; however, these conventional SPR techniques display several drawbacks in clinical diagnostics such as complicated use, low sensitivity, and high cost.

In this paper, we report a novel method for hepatitis B detection which utilizes SPR as a biosensor platform using HBsAg, HBsAb, HBeAb, HBeAg, and HBcAg as protein probes. We optimized protein probe immobilization conditions including protein probe concentration, immobilization buffer and reaction time. The resulting platform for hepatitis B detection is evaluated in terms of detection limit, specificity, reproducibility and additional parameters; this biosensor platform performance agrees with ELISA. Due to encouraging results, the platform which integrates protein microarray with SPR is a very promising candidate for hepatitis B viral infection detection.

2. Materials and methods

2.1. Materials

The hepatitis B surface antigen (HBsAg), hepatitis B surface antibody (HBsAb), hepatitis B core antigen (HBcAg), hepatitis B core antibody (HBcAb), hepatitis B antigen (HBeAg) and hepatitis B antibody (HBeAb) were obtained from Sciarray (Shenzhen, China). N-ethyl-N-[3-dimethylaminopropy] carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) and 2-aminoethanol hydrochloride were purchased from TCI Development Co., Ltd. (Tokyo, Japan). 12-mercaptododecanoic was obtained from Shanghai Medpep Co., Ltd (Shanghai, China). Serum samples were obtained from HBV infected patients in the Shenzhen Chronic Disease Control Center, who all provided informed consent; samples were kept at -80°C prior to experimental use. Samples were confirmed by commercial ELISA.

2.2. Surface modification for protein microarrays

Bare gold chips used for the preparation of the protein microarray were 18 mm×18 mm glass slides coated with 2 nm Cr and 48 nm Au. These gold chips were gifts from the Science and Technology Research Center (Beijing, China). Before use, the chips were cleaned in a mixture of Milli-Q water, 30% hydrogen peroxide and 25% ammonia for 10 minutes at 60℃, and then washed three times with Milli-Q water and ethanol, respectively. The chips were immersed overnight in 10 mM 12-mercaptododecanoic at room temperature to form a self-assembled monolayer (SAM). After SAM formation on the surface of the chips, the chips were washed with ethanol and water for 5 minutes, respectively.

2.3. Fabrication of protein microarray

Hepatitis B probes were prepared in a printing buffer with serial concentrations (Table 1). Acetate buffer of various pH values (pH = 4.0, 4.5, 5.0, 5.5) was used as the printing buffer. Terminal SAM carboxyl groups were activated with a freshly prepared mixture of EDC (0.2 M) and NHS (0.005 M) for 10 minutes. The chips were then rinsed with Milli-Q water, dried under flowing nitrogen and used immediately for protein microarray printing. The probes were spotted on the activation surface in an array format in various printing buffers; the volume of each spot was 0.4 μL. The schematic of the protein array is displayed in Figure 1(a), in which the numbers represent serial probes according to Table 1; the 0 spot represents the control probe. We fabricated a dual-channel model protein array to detect five biomarkers of HBV via two different methods, as shown in Figure 1(b). This array formation allowed us to simultaneously conduct two detection methods with one chip. After spotting, the printed chips were incubated at room temperature for 15 minutes. The probe-modified sensor chips were immersed in 1.0 M ethanolamine (pH 8.5) for 10 minutes to inactivate the unreacted esters, and then immersed in 1% BSA in phosphate-buffered saline (PBS) for 30 minutes to block the sensor surface.

Table 1

Preparation of probes

Antigen/antibody	Dilution proportion					
HBsAb(pH5.0)	1:12	1:60	1:300	1:1500	1:7500	1:37500
HBsAg(pH 4.5)	1:12	1:60	1:300	1:1500	1:7500	1:37500
HBeAg(pH 5.5)	1:8	1:40	1:200	1:1000	1:5000	1:25000
HBeAb(pH 5.0)	1:8	1:40	1:200	1:1000	1:5000	1:25000
HBcAg(pH 5.0)	1:16	1:80	1:400	1:2000	1:10000	1:50000

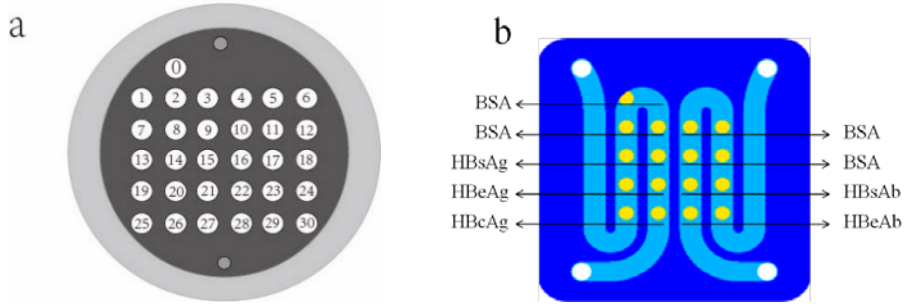

Fig. 1. Models of two protein microarrays. (a) Single-channel protein array; (b) dual-channel protein array.

Table 2

Five HVB serological marker detection methods

Detection target	Detection method	Capture probe	Detection probe
HBsAb	Direct to detect antigen	HBsAg	HBsAg
HBsAg	Direct to detect antibody	HBsAb	HBsAb
HBeAg	Direct to detect antibody	HBeAb	HBeAb
HBeAb	competition immunoassay	HBeAg	HRP-HBeAb
HBcAb	competition immunoassay	HBcAg	HRP-HBcAb

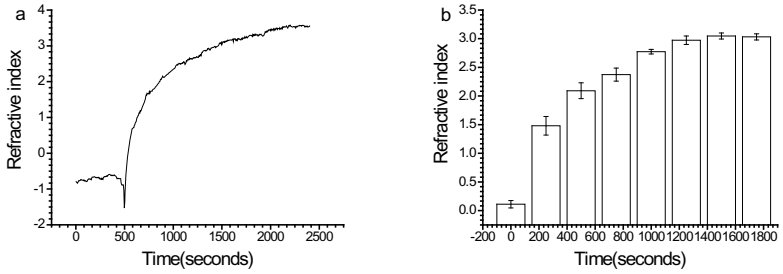

Fig. 2. Kinetics of probe immobilization on the SAM surface. (a) a typical SPR curve for probe immobilization kinetics; (b) SPR response versus incubation time for immobilization of a SAM surface probe (comparison between 1500 s and 1750 s groups was not statistically significant ($p>0.05$), but there was significant difference between the 1250 s and 1750 s groups; an analysis of the data of 1250 s, 1500 s and 1750 s groups shows that the signal had no significant difference; all other groups demonstrated statistically significant differences ($p<0.05$).

2.4. Strategies for the detection of viral antigens and antibodies of HVB

Because HBeAb exhibits cross-reactivity with HBcAb in human serum, we developed a competitive immunoassay to detect HBeAb and HBcAb. In this study, we designed three different antigen–antibody reaction strategies for the direct detection of viral antigens and antibodies of HVB (Table 2).

2.5. SPR measurements

All SPR measurements were performed with an SPR imager from GWC Technologies (Madison, WI). The following buffers and solutions were degassed and filtered through a 0.22 μm filter: (1) running buffer, 10 mM PBS, pH=7.4; (2) deactivation solution, 1 M ethanolamine, pH 8.5; (3) regeneration solution, 0.2 M glycin-HCl, pH 2.5. The probe-modified chip was mounted in the prism-flow cell fixture, and inserted into a GWC SPR system. All SPR experiments were conducted in a PBS buffer at a constant flow rate of 2 μL min^{-1} and at a temperature of 25℃.

2.6. Data acquisition and statistical analysis

Statistical analysis was performed with SPSS software (Statistical Package for the Social Sciences, version 11.5, SPSS, Inc., Chicago, IL). The data was presented as means ± standard deviation (SD). The statistical analysis was carried out by ANOVA followed by LSD; values of $p<0.05$ were considered to indicate statistical significance.

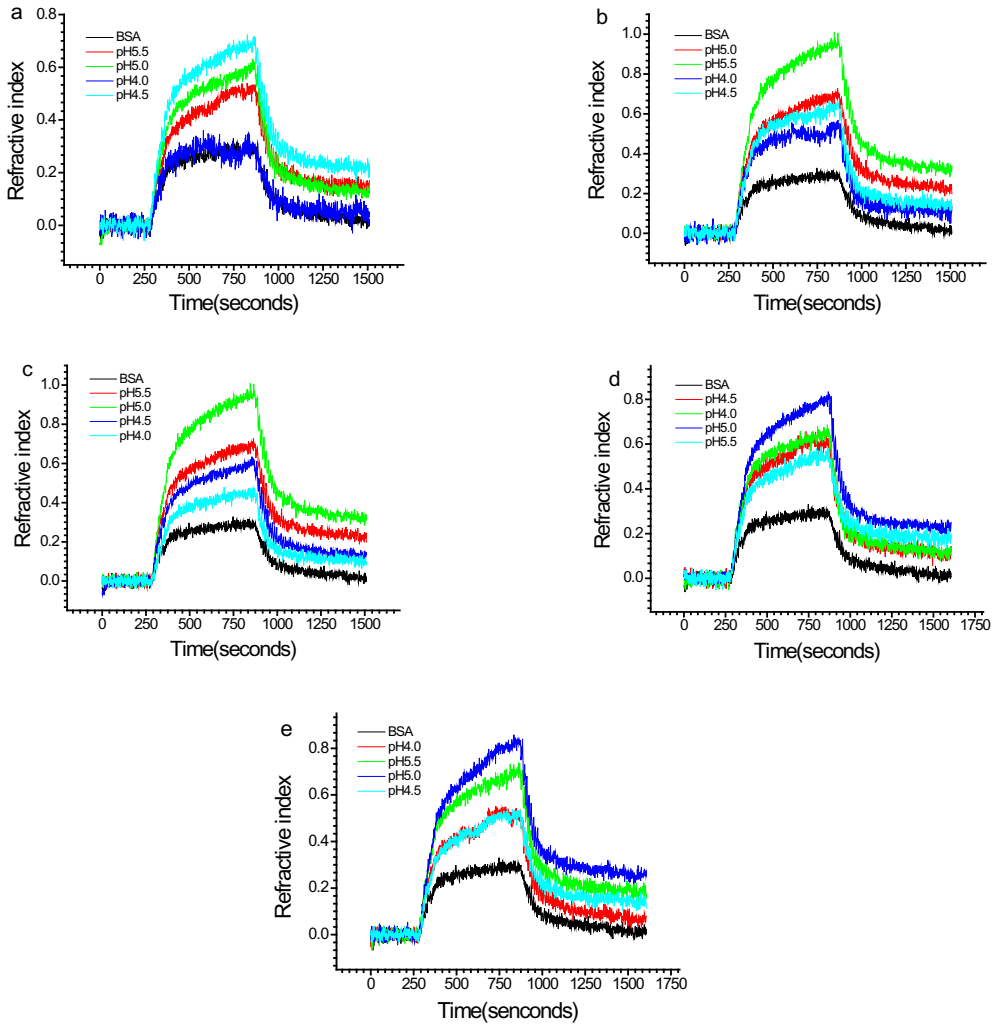

Fig. 3. The optimizal immobilization pH value of antigen/antibody probes: (a) HBeAg, (b) HBsAg, (c) HBsAb, (d) HBcAb, (e) HBeAb. The optimal immobilization pH values of the five capture probes HBsAg, HBsAb, HBeAb, HBeAg, HBcAg were 4.5, 5.0, 5.0, 5.5 and 5.0, respectively.

3. Results

3.1. Optimization of immobilization time

The effect of incubation time on probe immobilization on the SAM surface was studied by pumping 200 μL of probe protein into a flow cell after the chip surface was activated with EDC and NHS solutions. The degree of probe immobilization can be calculated according to the SPR response change value. Figure 2 demonstrates that the degree of probe immobilization increased rapidly during the first 1 minute of incubation, and reached a plateau after 15 minutes. Results of the statistical analysis suggest that an incubation time of 15 minutes is sufficient for probe immobilization.

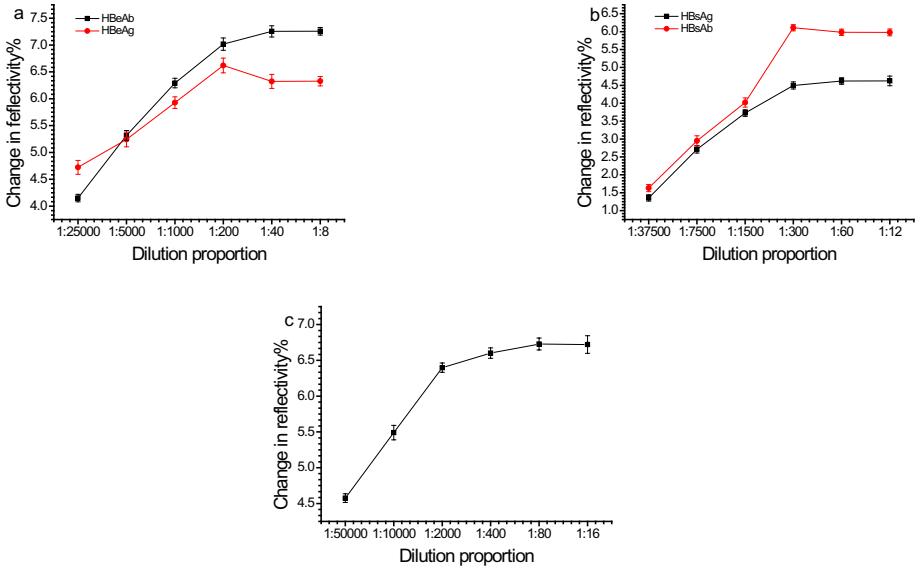

Fig. 4. The optimized concentration of antigen/antibody: (a) HBeAb and HBeAg; (b) HBsAg and HBsAb; (c) HBcAg. The optimal immobilization concentration of probes HBsAg, HBsAb, HBeAb, HBeAg, HBcAg, were 1:300, 1:300, 1:200, 1:200 and 1:400, respectively.

3.2. Optimization of pH value

As the probes were dissolved in printing buffer, the pH value of the printing buffer will significantly affect probe activation. The recognition capacity was used to evaluate the effectiveness of probe immobilization. Probes dissolved in printing buffers of different pH values were immobilized on the activated sensor surface; then 200 μL of reaction mixture was pumped into the flow cell. The interaction process between the probe and the target was monitored in real time using the SPR technique. Figure 3 confirms that each type of probe has a unique optimal pH value for immobilization depending on the probe value. The results indicate that optimal immobilization pH values of the five capture probes HBsAg, HBsAb, HBeAb, HBeAg, HBcAg were 4.5, 5.0, 5.0, 5.5 and 5.0, respectively.

3.3. Optimization of probe concentration

Using the optimal printing buffer and immobilization time, we investigated the effect of probe concentration on the resulting sensor performance. A serial concentration of probes in optimal buffer was printed on the SAM surface. In this study, 200 μL target proteins were applied to investigate performance based on different probe concentrations. Due to the high expense of proteins, the result of this experiment will be useful in the optimization of immobilization to match requirements of high sensitivity and economy. SPR responses for specific interactions as a function of printed probe concentrations are shown in Figure 4. The response change in reflectivity increases with printed protein concentrations of protein dilution ration >1:200. A slight increase occurs when the dilution ratio is lower than 1:200 for HBeAb; this trend was also observed for HBsAg and HBcAg. In contrast, with HBeAg and HBsAb, the signal decreases at higher than optimal concentrations. The optimal

dilution ratios for the five probes HBsAg, HBsAb, HBeAb, HBeAg and HBcAg were 1:300, 1:300, 1:200, 1:200 and 1:400, respectively.

3.4. Reproducibility test

The reproducibility of intra-arrays (three different spots in one chip) and inter-arrays (spots in three different chips) were evaluated by using HBsAg as a model protein. Results indicate that the coefficient variations (CV values) of intra-arrays and inter-arrays were 1.35% and 18.03%, respectively, as shown in Table 3.

3.5. Specificity test

To test the cross-reactivity of antibodies used to detect HBsAg and HBeAg, we demonstrated their specificity using disease sera which were positive for HBsAg and HBeAg. As shown in Figure 5, there is a very clear specific reaction of two HBsAg and HBeAg with their corresponding targets.

3.6. Stability assessment of SPR protein microarray

For reliable results in practical applications, the protein microarray should maintain high biological activity while in storage. Hence, to evaluate the stability of the protein microarray, the chips modified with probes were stored in boxes at 4 ℃. Detection stability tests were performed with the corresponding targets after one week, two weeks, three weeks and four weeks of storage. The protein microarray demonstrated identical signal intensities after four weeks at 4℃, with a CV value of less than 5.16%.

Table 3

Reproducibility of the protein chip

	Test Number			$\bar{x}$	s	CV
	1	2	3			
Inter-arrays	5.4606	6.2359	4.3223	4.3847	0.0590	18.03%
Intra-arrays	4.4512	4.3384	4.3646	5.3396	0.9625	1.35%

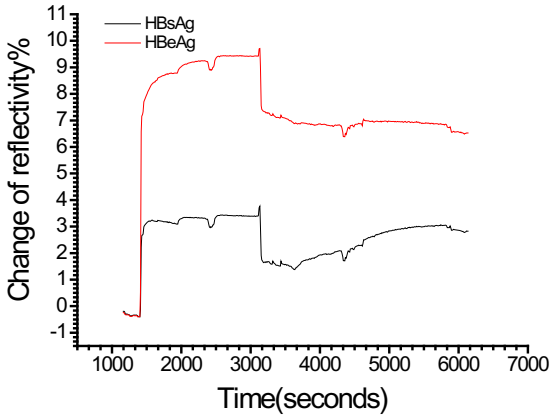

Fig. 5. Typical SPR sensorgrams for specific interaction.

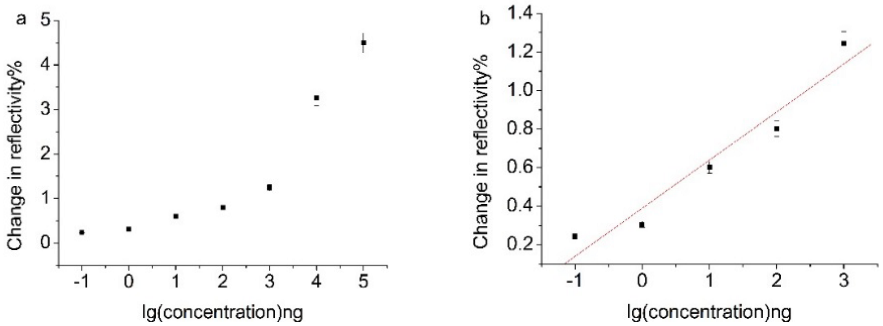

Fig. 6. SPR signal response for detection of different concentrations of HBsAg. (a) SPR response as a function of target concentrations (ranging from 0.1ng to 100 μg mL^{-1}); (b) a linear correlation with wider linear range from 0.1 to 1μg mL^{-1} was achieved.

3.7. Sensitivity and linear range test

To determine the sensitivity of the SPR protein microarray platform developed in this study, we performed SPR experiments using HBsAg as a model protein with serial concentrations (0.1 ng mL^{-1}~100 μg mL^{-1}). As shown in Figure 6, the protein microarray with SPR had a sensitivity of 0.1 ng mL^{-1} for HBsAg, and a linear correlation with a wider linear range from 0.1 to 1μg mL^{-1} (R^2=0.971).

3.8. Evaluation of protein arrays for clinical serum samples

Under optimal conditions, we constructed a platform for a combined protein microarray with SPR for detection of hepatitis B. Thirty-five hepatitis B patients with qualitative results by ELISA were tested using a SPR protein microarray platform. Five types of hepatitis B virus serological detection marker results from the SPR protein microarray were compared to ELISA markers, as shown in Table 4; agreement between the SPR protein microarray and ELISA markers was greater than 85%. Results demonstrate that the detection platform based on SPR and protein microarray is able to successfully detect five hepatitis B markers in the serum of patients in approximately 30 minutes.

Table 4

Comparison of results obtained by SPR protein microarray and ELISA for clinical serum samples

RESULTS	ELISA(n)	SPR(n)
HBsAg＋	35	32
HBsAg－	30	29
HBsAb＋	33	32
HBsAb－	28	26
HBeAg＋	28	27
HBeAg－	30	28
HBeAb＋	11	11
HBeAb－	9	7
HBcAb＋	20	20
HBcAb－	19	19

4. Discussion

Over the past decade, many studies have been conducted on the protein microarray, which is used to detect biological markers [16-18], but only a few reports are available on the protein chip which is used to simultaneously detect five hepatitis B serological markers based on SPR technology. Due to cross-reactions between HBcAb and HBeAb [19], competition testing must be used; on the other hand, labeled analytes are required by traditional test methods so that results of HBsAg, HBeAg and HBsAb tests could be visualized. This method seriously hinders antigen/antibody activity and can cause environmental pollution, both of which have hindered hepatitis B detection chip development.

To overcome those limitations, this study combined SPR technology and a protein microarray to form a new detection technology. This method can not only detect five HBV serological markers in a single chip, but it also uses the direct and competition methods without simultaneous labeling. Optimum protein microarray fabrication and performance must take into consideration the factor of printing buffer, incubation time and probe concentration. We have established an SPR protein microarray platform technology for hepatitis B detection that takes advantage of a high throughput, a simplified process and a short test time. By using this platform, five hepatitis B markers can simultaneously be detected in 20 μL serum in approximately 30minutes, whereas other methods such as ELISA and PCR can detect only one marker in a four-hour period. The agreement between the protein microarray and ELISA results for 35 clinical sample tests was greater than 85%. In addition, the HBsAg detection limit of this platform was 0.1ng mL^{-1}, which meets the clinical standard. Further benefits of this platform have also been demonstrated, particularly with regard to specificity, reproducibility and stability. The protein microarray based on SPR technology is simple, quick and highly sensitive; it is a good prospect for the rapid screening of infections in early diagnosis and clinical applications.

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