

Original Article

Establishment of lymphatic filarial specific IgG4 indirect ELISA detection method

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Abstract: Objective: To establish the lymphatic filarial specific IgG4 indirect ELISA detection method and develop the kits. Methods: ELISA and the developed specific IgG4 reagent was used to explore the best way for detecting filarial specific IgG4. Combined with the production process of commercialized enzyme immunoassay kit to develop economical lymphatic filarial specific IgG4 test kit, and to explore the value of the kit in the laboratory. Results: We determined the most optimal detective antigen was Malay adult filarial antigen and the optimal concentration of coating antigen was 1.0 µg/ml. The appropriate serum dilution was 1:20 to 40 and the work titers of specific IgG4 agents was 1: 800. We determined the optimal reaction time for substrates and developed a reproducible and stable detection kit with sensitive and specificity, which was easy to operate. Conclusion: We successfully established the lymphatic filarial specific IgG4 indirect ELISA detection method and developed the kits with good reproducibility and stable result, which should be widely applied.

Keywords: Lymphatic filariasis, specific IgG4, ELISA, kit

Introduction

Enzyme-linked immunosorbent assays (ELISA) is a new technique in immunologic diagnosis. Since its invention in 1960-1970s, ELISA has won worldwide acclaim for fastness, sensitivity and convenience. Now this technique has been successfully applied for immunologic diagnosis of various infectious diseases, parasitic diseases and non-infectious diseases [1]. However, the earlier use of ELISA was hampered by unsatisfactory specificity. During recent years we have witnessed the improvement on techniques and materials, such as preparing coated antigens by genetic engineering method and indirect ELISA using monoclonal antibodies specific for only one antigenic epitope. All these methods aim to improve the specificity. The use of computer-assisted ELISA has promoted practicability and standardization. Since ELISA can detect several hundred or several thousand of samples in a day, it is also used for epidemiological survey of serum samples.

We investigated the optimal detection method for filaria-specific IgG4 antibody based on ELISA and the previous specific IgG4 antibodies. A

filaria-specific IgG4 ELISA kit was prepared with the features of sensitivity, specificity, easily operation and economy. The kit was then used for detecting the serum samples and filter-paper blood samples from filariasis cases, non-filariasis parasitosis cases and healthy population. The sensitivity, specificity and repeatability were compared.

Materials and methods

Experimental animal

Healthy and *Brugia malayi*-infected *Meiiones Unguiculataus* was purchased from National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention.

Reagents

BSA and PNPP were manufactured by Sigma (USA). All other reagents were domestically manufactured and analytically pure.

Blood sample detection

Serum samples from filariasis cases and serum samples and filter-paper blood samples from

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those presenting with microfilaremia were preserved at our laboratory at -40°C .

Negative control serum samples were collected from healthy populations in Penglai County and Changdao County, the non-filariasis endemic regions of Shandong Province in June 1986. All serum samples were subpackaged, freeze-dried and preserved at -40°C .

Sixty and 40 serum samples from healthy personnel as normal control at Shandong Institute of Parasitic Disease Prevention and Control were collected and preserved at -40°C .

Preparation of Brugia malayi adult antigen

Adults were harvested from *Brugia malayi*-infected *Meiiones Unguiculataus* and washed by 0.01 mol/L, pH 7.2 PBS. Following ultrasonic disruption (100 W, 10 min) twice and centrifugation at 6000 rpm for 20 min at 4°C , supernatant was collected as the coating antigen. The protein content was determined as 2.9 mg/ml. The sample was stored at -40°C .

Preparation of microfilarial antigen

Meiiones Unguiculataus seriously infected by *Brugia malayi* was subjected to lavage using normal saline. The cell components in peritoneal fluid were removed by natural deposition. After washing for several times, the pure microfilariae were ultrasonically disrupted at 100 W for 10 min. Then the sample was cold soaked in a fridge at 4°C for 72 h. Centrifugation was performed at 6000 rpm for 15 min at 4°C , and supernatant was collected with protein content determined as 1.26 mg/ml.

ELISA procedures

(1) Coating: The antigen was diluted with 0.05 mol/L pH 9.6 carbonate buffer solution and then coated onto microplate at 100 μl /well. Cell incubation proceeded at 37°C for 2 h, then at 4°C overnight.

(2) Washing: The coating buffer was discarded and the wells were washed with PBST at 300 μl /well. Micro-oscillation for 3 min was repeated for 3 times. The washing liquid was discarded, and absorbent paper was used for drying.

(3) Sealing: Following sealing with 2% BSA at 200 μl /well, cell incubation was carried out at 37°C for 2 h. The washing method was the same as in (2).

(4) Positive serum was added at 100 μl /well with certain dilution, followed cell incubation at 37°C for 2 h. The washing method was the same as in (2).

(5) Diluted specific IgG4 antibodies were added at 100 μl /well, and PBS was added as blank control for cell incubation at 37°C for 2 h. The washing method was the same as in (2).

(6) PNPP substrate solution was added at 100 μl /well for cell incubation away from light for 30 min at 37°C .

(7) Reaction was terminated by adding 2 M NaOH at 100 μl /well. OD405 value was measured using microplate reader.

Screening of coating antigen

The adult antigen and microfilarial antigen were coated onto the plate after dilution. ELISA was performed for 6 adult-positive serum samples. According to the determination of OD405 value, the optimal concentration of coating antigen was determined.

Determination of optimal concentration of coating antigen

The antigen was diluted to 0.1 $\mu\text{g}/\text{ml}$, 0.5 $\mu\text{g}/\text{ml}$, 1.0 $\mu\text{g}/\text{ml}$, 1.5 $\mu\text{g}/\text{ml}$, 2.0 $\mu\text{g}/\text{ml}$, 2.5 $\mu\text{g}/\text{ml}$, 3.0 $\mu\text{g}/\text{ml}$ and 3.5 $\mu\text{g}/\text{ml}$ using 0.05 mol/L pH 9.6 carbonate buffer solution, respectively, before being coated onto the plate at 100 μl /well. Cells were incubated at 37°C for 2 h, and then at 4°C overnight. ELISA procedures were implemented so as to determine the optimal concentration of coating antigen.

Determination of optimal antigen coating conditions

The antigen was diluted to the optimal concentration. Cells were incubated at 37°C for 2 h, at 37°C for 2 h then at 4°C overnight, and at 4°C overnight, respectively, before being coated onto the plate. ELISA procedures were implemented to determine the optimal antigen coating conditions.

Determination of optimal dilution rate of serum

The antigen was diluted to the optimal concentration, and incubated at 37°C for 2 h then at 4°C for overnight. After coating and sealing, the

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plate was washed with PBST for three times. Then negative and positive serum with dilution rate of 1:20, 1:40, 1:60, 1:80 and 1:100 was added, respectively. ELISA procedures were implemented to determine the optimal dilution rate of serum.

Determination of optimal serum reaction time

The antigen was diluted to the optimal concentration. Before coating, incubation was performed at 37°C for 2 h, and then at 4°C for overnight. The plate was sealed and washed with PBST three times. Negative and positive serum with optimal dilution rate was added for cell incubation at 37°C for 30 min, 45 min, 60 min, 75 min, 90 min and 120 min, respectively. Optimal reaction time was determined by ELISA.

Determination of optimal concentration of specific IgG4 antibodies

The antigen was diluted to optimal concentration, and incubated at 37°C then at 4°C for overnight before coating. After sealing, the plate was washed with PBST for three times. Then positive and negative serum with optimal dilution rate was added for incubation. Enzyme-conjugated secondary antibodies diluted at 1:100, 1:200, 1:400, 1:800, 1:1000, 1:1500 and 1:2000 were added. Optimal concentration of specific IgG4 antibodies was determined by ELISA.

Determination of optimal reaction time of specific IgG4 antibodies

The antigen was diluted to the optimal concentration, and incubated at 37°C for 2 h then at 4°C overnight. The antigen was coated onto the plate, and the plate was sealed and washed with PBST three times. Then negative and positive serum with optimal dilution rate was added. Following incubation for optimal duration, specific IgG4 antibodies with optimal dilution rate were added, respectively. The cells were further incubated at 37°C for 30 min, 45 min, 60 min, 75 min, 90 min, 105 min and 120 min, respectively. Optimal reaction time of specific IgG4 antibodies was determined by ELISA.

Optimal reaction time of substrate

Under the optimal conditions determined above, antigen, serum and enzyme-conjugated antibodies were added and incubated with sub-

strate at 37°C for 5 min, 10 min, 15 min, 20 min, 25 min and 30 min, respectively. OD value was measured at 405 nm.

Sensitivity and specificity evaluation of ELISA method

The filaria-positive serum, serum positive for other parasitic diseases and normal serum was detected by ELISA. The sensitivity and specificity were evaluated.

Comparison across the samples

Filter-paper blood samples and serum samples of 2 microfilaremia cases were detected by ELISA (density 7 mf/60 µl and 6 mf/60 µl, respectively). Whatever the dilution rate, negative control had a very low background (lower than 0.07). The positive titer of serum in the same case was higher than that of filter-paper sample by about one dilution rate. To ensure consistency of the positive result and the easy observation of the result, the dilution rate was set as 1:20-40.

Repeatability

Serum samples and filter-paper blood samples from filariasis cases and normal cases were detected using ELISA. The dilution rate was all set as 1:40, with 8 replicate wells.

Stability

Thermal stability test was carried out by treating the antibodies at 37°C in an incubator for 24 h, 48 h, and 72 h, respectively. The positive serum samples from 6 cases were detected by ELISA.

Results

Screening of coating antigen

Adult antigen and microfilarial antigen was coated onto the plate at a certain dilution rate, respectively. The serum from filaria-positive cases was detected by ELISA. It can be seen from **Table 1** that the OD405 of microfilarial antigen was obviously lower than that of adult antigen for the same case. Thus adult antigen was chosen as the coating agent (**Table 1**).

Determination of optimal concentration of coating agent

The antigen was diluted to 0.1 µg/ml, 0.5 µg/ml, 1.0 µg/ml, 1.5 µg/ml, 2.0 µg/ml, 2.5 µg/ml,

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Table 1. Results of specific IgG4 detection using different antigen

Antigen	OD value of positive serum						OD value of negative control					
Adult antigen	0.751	0.936	0.624	1.104	0.847	0.911	0.051	0.062	0.041	0.063	0.054	0.051
Microfilarial antigen	0.157	0.164	0.138	0.182	0.161	0.169	0.047	0.053	0.046	0.057	0.052	0.047

Table 2. Determination of optimal concentration of coating antigen

Antigen concentration	0.10	0.25	0.50	0.75	1.00	1.50	2.00	2.50
Positive serum	0.103	0.214	0.367	0.523	0.775	0.811	0.882	0.908
Negative serum	0.041	0.043	0.052	0.056	0.058	0.064	0.073	0.081
P/N	2.51	4.98	7.06	9.34	13.36	12.67	12.07	11.21

Table 3. Determination of optimal conditions of antigen coating

Coating condition	37°C for 2 h	37°C for 2 h then 4°C overnight	4°C overnight
Positive serum	0.872	1.125	1.021
Negative serum	0.045	0.051	0.049
P/N	19.38	22.06	20.84

Table 4. Determination of optimal dilution rate of serum

Dilution rate	1:20	1:40	1:60	1:80	1:100
Positive serum	2.423	2.316	1.934	1.621	1.256
Negative serum	0.203	0.191	0.145	0.107	0.073
P/N	11.94	12.13	13.32	15.15	17.23

3.0 µg/ml and 3.5 µg/ml using 0.05 mol/L, pH 9.6 carbonate buffer solution, respectively. Then antigen was coated onto the plate at 100 µl/well for cell incubation at 37°C for 2 h, and then at 4°C for overnight. The optimal concentration of coating antigen was determined by ELISA. As shown by the results, P/N was the highest at the concentration of 1.0 µg/ml, which was chosen as the optimal concentration (**Table 2**).

Determination of optimal conditions of antigen coating

Antigen was diluted to optimal concentration and incubated at 37°C for 2 h, at 37°C for 2 h then at 4°C for overnight, and at 4°C for overnight, respectively. Then the antigen was coated onto the plate, and ELISA was carried out to determine the optimal conditions of antigen coating. According to the results, the best effect was obtained by incubation at 37°C for 2 h then at 4°C for overnight. However, the other

two sets of conditions of coating did not affect the detection results. So they were both suitable for choice depending on the specific needs (**Table 3**).

Determination of optimal dilution rate of serum

The antigen was diluted to optimal concentration and incubated at 37°C for 2 h and then at 4°C for overnight before being coated onto the plate. The plate was sealed and washed with PBST for three times. Negative and positive serum with dilution rate of 1:20, 1:40, 1:60, 1:80 and 1:100 were added, respectively. The optimal dilution rate of serum was determined by ELISA. As shown by the results, when the dilution rate was 1:100, the positive serum still had a high OD value. Microfilarial content of blood is usually high during the prevalence of filariasis or at the early stage of prevention and control, so positive titer is high. At this stage, 1:100 dilution of serum can still achieve a good effect. But at the later stage of prevention and control, the microfilarial positive rate and infection intensity are very low. Therefore, the dilution rate of serum was set as 1:20-40 in order to detect low-density microfilaria and filter-paper blood samples (**Tables 4 and 10**).

Determination of optimal reaction time of serum

Antigen was diluted to optimal concentration and incubated at 37°C for 2 h and then at 4°C for overnight. After coating and sealing, the plate was washed with PBST for three times. Negative and positive serum with optimal dilution rate was added for incubation at 37°C for 30 min, 45 min, 60 min, 75 min, 90 min and 120 min, respectively. Optimal reaction time of

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Table 5. Determination of optimal reaction time of serum

Reaction time	30 min	45 min	60 min	75 min	90 min	105 min	120 min
Positive serum	1.013	1.324	1.678	2.012	2.453	2.521	2.630
Negative serum	0.089	0.101	0.118	0.124	0.131	0.143	0.150
P/N	11.57	13.11	14.23	15.14	18.73	17.63	17.53

Table 6. Determining of optimal working titer of specific IgG4

Specific IgG4	1:100	1:200	1:400	1:800	1:1600	1:3200
Positive serum	2.288	2.319	2.446	2.535	2.213	2.011
Negative serum	0.133	0.121	0.110	0.097	0.091	0.087
P/N	17.21	19.33	22.24	26.18	24.32	23.11

Table 7. Determination of optimal reaction time of specific IgG4

Reaction time	30 min	45 min	60 min	75 min	90 min	105 min	120 min
Positive serum	1.632	1.806	2.013	2.312	2.624	2.733	2.801
Negative serum	0.076	0.082	0.087	0.091	0.094	0.118	0.126
P/N	21.47	22.02	23.14	25.41	27.91	23.16	22.23

Table 8. Determination of optimal reaction time of substrate

Reaction time	5 min	10 min	15 min	20 min	25 min	30 min
Positive serum	1.512	1.913	2.135	2.241	2.333	2.697
Negative serum	0.101	0.109	0.115	0.117	0.117	0.121
P/N	14.97	17.55	18.56	19.15	19.94	22.29

Table 9. Result of specific IgG4

Population	Cases detected	Positive	Positive rate (%)
Filariasis	68	64	94.1
Other parasitic diseases	60	0	0
Normal	38	0	0

serum was determined using ELISA. Results showed that P/N was the highest when the optimal reaction time of serum was 90 min (**Table 5**).

Determination of optimal working titer of specific IgG4

Antigen was diluted to optimal concentration and incubated at 37°C for 2 h and then at 4°C for overnight. The plate was coated with antigen, and then sealed and washed with PBST for three times. Negative and positive serum with optimal dilution rate was added and incubated according to optimal reaction time. Specific IgG4 antibodies with the dilution rate of 1:100, 1:200, 1:400, 1:800, 1:1600 and 1:320 were

added, respectively. Optimal working concentration of specific IgG4 was determined by ELISA. According to the result, P/N was the highest at the dilution rate of 1:800 (**Table 6**).

Determination of optimal reaction time of specific IgG4

Antigen was diluted to optimal concentration and incubated at 37°C for 2 h and then at 4°C for overnight. Antigen coating was performed, and the plate was sealed and washed with PBST for three times. Negative and positive serum with optimal dilution rate was added and incubated according to the optimal reaction time. Then specific IgG4 antibodies with optimal working titer were added and incubated at 37°C for 30 min, 45 min, 60 min, 75 min, 90 min and 120 min, respectively. Optimal reaction time of specific IgG4 was determined

by ELISA. It was found that P/N was the highest under the reaction time of 90 min (**Table 7**).

Determination of optimal reaction time of substrate

Under the optimal conditions determined above, the substrate was incubated with antigen, serum and specific IgG4 antibodies at 37°C for 5 min, 10 min, 15 min, 20 min, 25 min and 30 min, respectively. It was observed that P/N was the highest at the substrate reaction time of 30 min (**Table 8**).

Sensitivity and specificity evaluation

The serum samples from cases with filarisis and other parasitic diseases and normal cases were detected by using ELISA. The sensitivity and specificity of the ELISA kit were evaluated. Results showed that the specific IgG4-positive rate was 94.1% among filaria-positive cases. No cross-reactivity was found in cases with other parasitic diseases or in normal cases. Thus the specificity was 100% (**Table 9**).

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Table 10. Comparison of detection results across the samples

Sample	Dilution rate of serum					
	1:20	1:40	1:80	1:160	1:320	1:640
Filaria-positive serum	+++	+++	++	+	+	-
Filaria-positive filter-paper blood sample	+++	++	+	+	-	-
Normal serum	-	-	-	-	-	-
Normal filter-paper blood sample	-	-	-	-	-	-

Comparison of detection results across the samples

Two cases of microfilaremia were detected by ELISA using filter-paper blood samples and serum samples (density 7 mf/60 μ l and 6 mf/60 μ l, respectively). Whatever the dilution rate, the negative control had a very low background (below 0.07). The positive titer of serum was higher by that of filter-paper blood sample by about 1 dilution rate for the same case (**Table 10**). To ensure the consistency of the results and easy observation of the result, the dilution rate was set as 1:20-40.

Repeatability

Serum samples and filter-paper blood samples from filariasis cases and normal cases were all diluted by the rate of 1:40, with 8 replicate wells for each case. The results are shown in **Table 11**. The coefficient of variation (CV) of filaria-positive serum samples and filter-paper blood samples was 6.00% and 4.37%, respectively. CV of normal serum and filter-paper samples was 13.65% and 14.59%, respectively, indicating good intra-batch repeatability (**Table 11**).

Stability

Specific IgG4 antibodies were subjected to thermal stability test at 37°C in an incubator for 24, 48 and 72 h, respectively. Six positive serum samples were detected. The specificity and sensitivity of ELISA kit showed no significant significances (≈ 0.848 , $P > 0.05$) (**Table 12**).

Discussion

ELISA was introduced by Swedean scientists Engvall and Perlman [2] and Dutch scientist Van Weemen BK [3] in 1971. ELISA is a highly sensitive immunologic testing tool that combines the specific antigen-antibody reaction with the enzymatic catalysis of substrate [4].

Initially used for viral and bacterial detection, ELISA has been found to be widely applied in qualitative and quantitative detection of antigens and antibodies since the late 1970s, including some semi-antigens (e.g. drugs, hormones and toxins). Many reports

are known regarding the use of ELISA for the detection of pesticide and veterinary drug residues [5, 6]. The features of accuracy, sensitivity, fastness, specificity and economy facilitate the applications of ELISA in medical and food sciences [7, 8]. ELISA is easy to operate and has a low requirement on sample pretreatment and equipments. Many authorities worldwide give priority to the development of ELISA as one key technique [9, 10].

Antigen-antibody reaction proceeds on polystyrene microtiter plate as the solid-phase carrier for ELISA. The antigens or antibodies bound to the surface of the plate preserve the immunologic activity. The enzyme-conjugated antigens or antibodies not only have immunologic activity, but also enzymatic activity [11]. During testing, the antigens or antibodies bind to the antibodies or antigens on the solid-phase carrier. The antigen-antibody complexes on the solid-phase carrier are washed off. Then the enzyme-conjugated antigens or antibodies are added and bind to the solid-phase carrier by the same reaction. The amount of bound enzymes is in proportion to the amount of antibodies or antigens detected. The substrate added for enzymatic reaction will be catalyzed into colored product, the amount of which is directly proportional to the amount of antibodies or antigens detected. Therefore, the qualitative and quantitative analysis is possible based on the color intensity. The high efficiency of enzymatic catalysis has the effect of magnifying the result of immunologic reaction, thus achieving a high sensitivity. Many factors affect the sensitivity of ELISA [12]. For example, affinity, adsorption performance of ELISA plate, concentration and stability of antigen coating, organic solvent in medium, pH value, time of incubation, non-specificity and cross-reactivity that influence OD [13].

The factors of antigen type, coating concentration and time, dilution rate of serum and reac-

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Table 11. Repeatability test

Sample	Time of repeats								Standard deviation S	Coefficient of variation CV
	1	2	3	4	5	6	7	8		
Filaria-positive serum	0.665	0.035	6.00	0.559	0.554	0.565	0.593	0.603	0.035	6.00
Filaria-positive filter-paper sample	0.479	0.022	4.37	0.494	0.481	0.485	0.546	0.491	0.022	4.37
Normal serum	0.047	0.006	13.65	0.044	0.051	0.061	0.048	0.042	0.006	13.65
Normal filter-paper sample	0.054	0.007	14.59	0.046	0.040	0.051	0.049	0.036	0.007	14.59

Table 12. Thermal stability test

Time	OD405					
	1	2	3	4	5	6
24 h	2.306	2.451	2.033	2.157	2.562	2.117
48 h	2.221	2.308	2.126	2.069	2.369	2.012
72 h	2.264	2.355	2.201	2.108	2.407	2.489

tion time, dilution rate of specific IgG4 antibodies and reaction time, and time of enzyme-substrate reaction were investigated. It was found that antigen type had the greatest impact on the sensitivity and specificity of ELISA, followed by the concentration of coating antigen. A too high antigen concentration would lead to intensive interaction between the antigen molecules, causing the protein molecules to be multilayered. Such proteins can be easily washed off, thus increasing non-specificity. Under a low antigen concentration, the surface of the solid-phase carrier may not be completely occupied by antigens, which also leads to non-specificity [14]. The prepared kit showed higher sensitivity to adults than to microfilariae [15]. Titration was carried out using the adult antigens. When the concentration of coating antigen was 1.0 µg/ml, the specificity was good. When some vacant sites are created under a low concentration of coating antigen, the sealing liquid can fill these sites and prevent readsorption of the disturbing substances. Therefore, plate sealing is necessary for ELISA, but improper operation may increase the background for negative control. We applied the commercialized sealing liquid for 1 h, and the vacant sites were effectively sealed.

In indirect ELISA, the dilution rate of serum affects the specificity and sensitivity of ELISA [16]. An inappropriate dilution of serum may lead to non-specific reaction given the complex protein composition of serum. Thus it is important to optimize the dilute rate of serum to minimize non-specificity [17]. When the dilution rate

was 1:100 for negative and positive serum, the background was low and P/N was higher. For high-titer serum in the endemic stage, 1:100 dilution was sufficient to achieve a good detection. But this dilution rate can lead to missed diagnosis when the microfilarial density is extremely low after over 50 years of disease prevention and control. So we set the dilution rate to be 1:20-40.

Antigen type, serum, specific IgG4 antibodies and color developer were optimized. Efforts were made for standardization by referring to the technique used for commercialized ELISA kit. The stability of the prepared kit was tested. Detections were performed for serum samples and filter-paper blood samples from both filariasis cases and normal controls using the prepared kit. The coefficient of variation (CV) for serum samples and filter-paper blood samples was 6.00% and 4.37% in filariasis cases, respectively, while that for normal controls was 13.65% and 14.59%, respectively. The result indicated good intra-batch repeatability.

During thermal stability test, the sensitivity and specificity of specific IgG4 ELISA kit did not change significantly after treatment under 37°C for 2 h, 48 h and 72 h. The quality guarantee period was 7 days under room temperature and over 6 months at -4°C. The short-term storage under room temperature will not affect the test result, the experiment showed good reproducibility and the result was easy to determine. Especially by optimization of reagents and experimental conditions, it was convenient to operate, and can be detected with a blood filter paper. It was to facilitate to collect, transport and store, which was suitable applied for field and grass-roots, and should be widely applied.

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Disclosure of conflict of interest

None.

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