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High-Throughout Simultaneous Detection of Vitamin B_{12} and Folate using Intrinsic Factor and Folate Binding Protein

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The research was to establish high-throughout simultaneous detection of vitamin B_{12} and folate correspondingly using intrinsic factor and folate binding protein. The influence of linker selection, experiment temperature, sample dilution solution pH, coating time, serum pretreatment, incubation time and concentration were evaluated for further experiment. Standard curve was established. ECL signal of 21℃ was higher than that of 37℃. At 21℃, ECL signal of pH5.5 was higher than that of pH7.3 for linker1 detecting vitamin B_{12} and linker10 detecting folate. Higher protein incubation concentration (FBP 25μg/L, IF 1.33μg/L) had significantly higher signal than the other incubation concentration (FBP 7.5μg/L, IF 0.4μg/L). Incubation 0.5h had higher signal than incubation 1h. Coating 0.5h was chosen as the suitable coating time. R-squares of standard curve were 0.991 for vitamin B_{12} and 0.993 for folate using the first pretreatment method. A simultaneous detection method of vitamin B_{12} and folate was initially established. 12uL was enough for the volume of detection serum, detection time was as quickly as 2-3 hours, and the method was convenient and high-throughout. This study provides technical support for solving the bottleneck problems existing in human nutrition detection, and might contribute to the accurate nutrition development of human.

Keywords: simultaneous detection; high-throughout; folate; vitamin B_{12}

1. INTRODUCTION

The folate and vitamin B_{12} belong to water-soluble B vitamins, and are important to health of human. Vitamin B_{12} serves in multiple metabolic reaction and affects human growth and cell development including red blood formation, nervous system function and DNA synthesis and regulation[1-4]. Vitamin B_{12} deficiencies in human could occur in diseases such as pernicious anemia, nerve degeneration and cardiovascular disease[1,5]. Folate plays an important role in preventing cardiovascular disease, neural tube defects, and is required in cellular functions like cell replication,

growth, and amino acid biosynthesis[6]. Folate deficiency is related to hyperhomocysteinemia that is an established independent risk factor for vascular disease, stroke[7]. Increased intakes of vitamins such as vitamin B_{12}, folate and vitamin B_6 have been shown to decrease significantly plasma homocysteine levels[7].

The microbiological assay method is the most common analysis methods of vitamin B_{12} and folate[1]. The microbiological assay method is effective and could achieve low detection limits (low ng/L, pM range), but it requires specific microorganisms, is limited in the time-consuming defect, and has the potential for unspecific growth because of vitamin B_{12}/folate fragments or other constituents in samples [1,8]. In addition, the detection of vitamin B_{12} and folate uses instrumental methods, like radioisotope labeling, chemiluminescence(CL), electrochemiluminescence(ECL), high-performance liquid chromatography/tandem mass spectrometry(HPLC/MS-MS)[1,9-13]. Although the instrumental detection assays are accurate and sensitive methods, they're time-consuming, expensive and professional operator-requiring[1]. Enzyme-linked immunosorbent assays (ELISAs) offer relatively low-cost and high-throughput analyses, and don't significantly require sample pretreatment[8]. Traditional immunoassays require antibodies respectively specific to folate and vitamin B_{12}. One potential option to obviate the current assay limitations is to use intrinsic factor for vitamin B_{12} detecting and folate binding protein for folate detecting rather than antibodies in high-throughout assays.

Simultaneous detection of vitamins attracts researchers' attention, as the development of nutrition and social economy. HPLC and radioimmunoassay methods for simultaneous detection vitamins were already reported [14-15]. But both two detect methods have defects: especially HPLC is not cost-effective for detecting large numbers of samples due to the cost of instruments, maintenance and skilled analysts[8]; radioimmunoassay methods have defects of radiation hazard. So the simultaneous detection of vitamins might be solved by enzyme-linked immunosorbent assay (ELISA) and electrochemiluminesecence immunoassay (ECLIA), while related papers about simultaneous detection of vitamins by ELISA and ECLIA haven't been reported. Sample volume was reduced by simultaneous detection of vitamins, and experiment time was also reduced. So simultaneous detection of vitamins had significant effects in little precious samples especially those from infants, and besides the methods were easy to promote using because of timesaving.

In this work, high-throughout simultaneous detection of vitamin B_{12} and folate correspondingly using intrinsic factor and folate binding protein was researched, and the following factors -incubation temperature, sample dilution solution pH, incubation and coating time- were considered to optimize the experiment method. The success of the method described provides further information that the similar method like intrinsic factor-based and folate binding protein-based approach combining solid microassay might serve as a platform technology for multi-vitamin simultaneous detection development.

2. EXPERIMENTAL SECTION

Ruthenium-labeled intrinsic factor (IF), biotin-labeled vitamin B_{12}, ruthenium-labeled folate binding protein (FBP), biotin-labeled folate were purchased from Roche (Mannheim, Germany). U-PLEX Development Pack (4-Assay SECTOR Plate MSD), linker1, 3, 8, 10, stop solution, read buffer were purchased from Meso Scale Discovery-MSD (Maryland, America). MESO QuickPlex SQ 120 from

MSD (Maryland, America) was used to detect the ECL signal. Standard Reference Material (SRM) 1950 was purchased from National Institute of Standard Technology.

Bovine serum albumin (BSA), phosphate buffer solution with Tween-20 (PBST) and Phosphate Buffered Saline (PBS, pH 7.2~7.4) were purchased from Solabio (Beijing, China). Dithiothreitol and Sodium 2-Mercaptoethanesulfonate were purchased from Roche (Mannheim, Germany). Ultrapure water was got from pure water filter of MERCK MILLIPORE (Darmstadt, Germany). Micropipettors were purchased from Eppendorf (Hamburg, Germany). Centrifuge tubes were purchased from Corning (Newyork, USA).

Linker, experiment temperature and sample dilution solution pH selection assays. As was shown in figure1 (partly from 4-Assay SECTOR Plate MSD instruction), there were ten combining sites on one well of 96-well U-Plex plate. U-Plex linkers were specific to combining sites. The sites/linkers were named site/linker 1, 2, 3, 4, 5, 6, 7, 8, 9, 10. We selected two suitable sites/linkers for simultaneously detecting folate and vitamin B_{12} from sites/linkers1, 3, 8 and 10.

Figure 1.10 combining sites specific to 10 linkers on one well. The sites/linkers were named site/linker 1, 2, 3, 4, 5, 6, 7, 8, 9, 10. We selected two suitable sites/linkers for simultaneously detecting folate and vitamin B_{12} from sites/linkers1, 3, 8 and 10.

The detection results were influenced by experiment temperature and sample dilution solution pH. The MSD detection was finished between 20-26℃ as shown in the instruction, while in general, 37℃ was the suitable binding temperature of folate and folate binding protein, as well as vitamin B_{12} and intrinsic factor. Then the different detection temperatures were compared, and the suitable temperature was selected for further assay. The pH values (pH7.3 and pH5.5) of sample dilution solution were compared, and the suitable pH value of sample solution was selected for further assay.

Biotin-labeled folate was incubated respectively with linkers 1, 3, 8 or 10 for 30min, and stop solution was respectively added into the four mixture solutions for 30min. Folate/linker1/stop solution, folate/linker3/stop solution, folate/linker8/stop solution, folate/linker10/stop solution and stop solution were mixed as the ratio of 1:1:1:1:6. Vitamin B_{12} experiment was conducted as the same steps. 50uL folate or vitamin B_{12} solution was respectively added on the wells of the U-PLEX plate and the plate was coated for 30min on 37℃ or 21℃. Then every well was washed 3 times by PBST. FBP was diluted to 25μg/L with 1%BSA PBS (pH7.3 or pH5.5), and was added on the wells coated by folate. IF was diluted to 1.33μg/L with 1%BSA PBS (pH7.3 or pH5.5), and was added on the wells coated by vitamin B_{12}. The wells were incubated for 30min as the temperature 37℃ or 21℃. And then the wells were washed 3 times, read buffer was added and the plate was read by MESO QuickPlex SQ 120.

Coating time, incubation time and concentration assay. The detection results were influenced by coating time, incubation time and incubation concentration. The MSD coating time was one hour as the instruction, while in general, 0.5 hour was generally used as coating time. Then the coating time (1h or 0.5h) was compared. The incubation time (0.5h or 1h) was compared. Two kinds of binding protein incubation concentration (FBP 25μg/L, IF 1.33μg/L or FBP 7.5μg/L, IF 0.4μg/L) were compared.

Biotin-labeled folate was incubated with linker 10 for 30min, and stop solution was added and incubated for 30min. Biotin-labeled vitamin B_{12} was incubated with linker 1 for 30min, and then stop solution was added and incubated for 30min. Folate/linker10/stop solution, vitamin B_{12}/linker1/stop solution and stop solution were mixed as the ratio of 1:1:8. 50uL folate and vitamin B_{12} mixture solution was coated on the wells of the U-PLEX plate for 0.5h or 1h at 21℃. The plate was washed three times. 100ng/mL folate and 100ng/mL vitamin B_{12} (both were solved in 1%BSA PBS of pH5.5) were mixed to 50ng/mL standard solution as the ratio of 1:1. FBP, IF and 50ng/mL standard solution were mixed as the ratio of 1:1:1, at that time, FBP was diluted to 25μg/L, and IF was diluted to 1.33μg/L. FBP, IF and 0ng/mL standard solution (1%BSA PBS) were mixed as the ratio of 1:1:1, at that time, FBP was diluted to 25μg/L, and IF was diluted to 1.33μg/L. FBP, IF, 50ng/mL standard solutions, and 1%BSA PBS (pH5.5) were mixed as the ratio of 1:1:1:7, at that time, FBP was diluted to 7.5μg/L, and IF was diluted to 0.4μg/L. FBP, IF, 0ng/mL standard solutions, and 1%BSA PBS (pH5.5) were mixed as the ratio of 1:1:1:7, at that time, FBP was diluted to 7.5μg/L, and IF was diluted to 0.4μg/L. Incubation time was 0.5h or 1h. The plate was washed three times. Read buffer was added and the plate was read by MESO QuickPlex SQ 120.

Competitive assay for folate and vitamin B_{12}. Biotinylated vitamin B_{12} was incubated with linker 1 (containing streptavidin) for 30min, and biotinylated folate was incubated with linker10 (containing streptavidin) for 30min. Then the above two solutions were respectively reacted with stop solution for 30min. Vitamin B_{12}/linker1/stop solution, folate/linker10/stop solution and stop solution were mixed as the ratio of 1:1:8. 50uL mixture solution per well were coated on the U-PLEX plate for 30min. At that time, vitamin B_{12} was coated on site1, and folate was coated on site10. Wash the plate 3 times using 0.5‰ PBST.

Serum/standard solution, ruthenium-labeled intrinsic factor and folate binding protein were mixed as the ratio of 1:1:1. 50uL mixture solution above were added into the wells of the plate, and vitamin B_{12}/folate in serum/standard solution was combined with intrinsic factor/folate binding protein, competing with vitamin B_{12}/folate coated on the well of the plate. After 30min incubation, the plate was washed 3 times, read buffer was added and the plate was read by MESO QuickPlex SQ 120.

Serum pretreatment. Vitamin B_{12} and folate were released from their respective binder in serum by alkaline solutions containing sodium hydroxide at pH12-13[15]. SRM1950 was is intended to represent “normal” human plasma, and could also be used for comparison of measurement technologies. Vitamin B_{12} standard solution, folate standard solution (in pH5.5 PBS containing 1%BSA) and SRM1950 was processed as the following two methods: the first one, the solution was combined with NaOH solution (pH=1) as the ratio of 9:1, then 1/9 volume HCl of the mixture solution was added, dithiothreitol and sodium 2-Mercaptoethanesulfonate were added into the solution; the second one, the solution was combined with NaOH solution (pH=1) as the ratio of 99:1, then 1/9 volume HCl of the

mixture solution was added, dithiothreitol and sodium 2-Mercaptoethanesulfonate were added into the solution.

Curve fitting. Curves are fitted using a 4-parameter logistic algorithm in competitive ELISA [16] using the software in MESO QuickPlex SQ 120, and the curves are not linear.

3. RESULTS AND DISCUSSION

Experiment temperature, pH and linker selection assay. As was shown in figure 2, ECL signals of 21℃were higher than that of 37℃for folate (linker1) and vitamin B_{12} (linker10). At 21℃, linker 10 had the higher signals than other linkers for detecting folate, linker 1 had the higher signals than other linkers for detecting vitamin B_{12}. At 21℃, ECL signals of pH5.5 were higher than those of pH7.3 for linker1 detecting vitamin B_{12} and linker10 detecting folate.

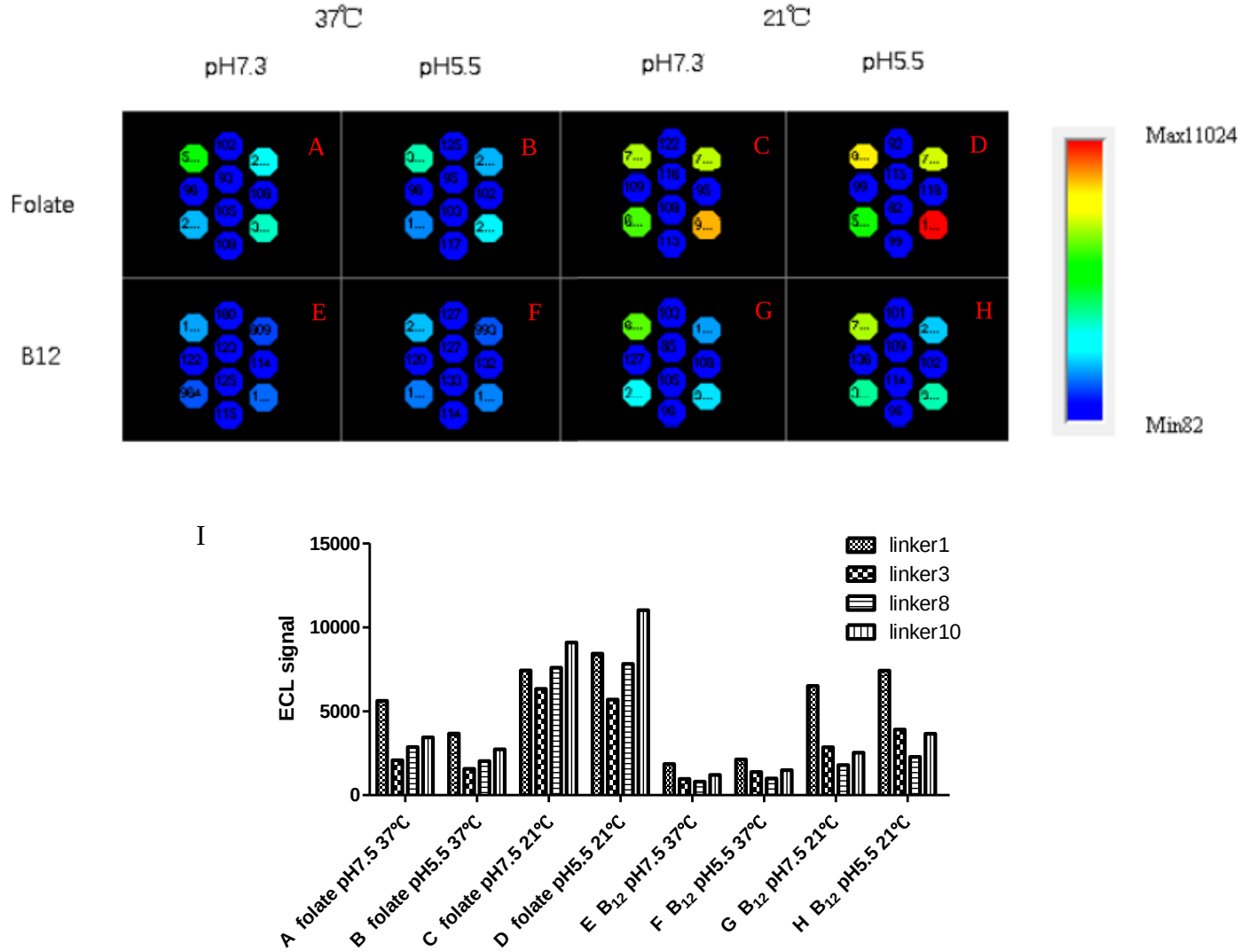

Figure 2. Experiment temperature, pH and linker selection assay. Biotin-labeled folate was respectively incubated with linkers 1, 3, 8 or 10 for 30min, and stop solution was respectively added and incubated for 30min. Folate/linker1/stop solution, folate/linker3/stop solution, folate/linker8/stop solution, folate/linker10/stop solution and stop solution were mixed as the ratio of 1:1:1:1:6.

Vitamin B_{12} detection was conducted as the same steps. 50uL folate solution were coated on the A/B/C/D well of the U-PLEX plate for 30min (A and B wells for folate detecting were incubated on 37℃; C and D wells for folate detecting were incubated on 21℃); 50uL vitamin B_{12} solution was coated on the E/F/G/H well of the plate for 30min (E and F wells for vitamin B_{12} detecting were incubated on 37℃; G and H wells for vitamin B_{12} detecting were incubated on 21℃). FBP was diluted to 25μg/L with 1%BSA PBS (pH7.3 or pH5.5), pH7.3 processed solution was added to A and C wells, and pH5.5 processed solution was added to B and D wells. IF was diluted to 1.33μg/L with 1%BSA PBS (pH7.3 or pH5.5), pH7.3 processed solution was added to E and G wells, and pH5.5 processed solution was added to F and H wells. A, B, E and F wells were incubated at 37℃; C, D, G and H wells were incubated at 21℃. Read buffer was added and the plate was read by MESO QuickPlex SQ 120. Picture "I" showed the detecting value of every site on every well (A, B, C, D, E, F, G, or H well).

Coating time, incubating time and concentration assay: As was shown in figure 3 and table 1, Coating 1h had higher signals than coating 0.5h for detecting folate; while, coating 0.5h had higher signals than coating 1h for detecting vitamin B_{12}. As was shown in figure3 and table1, coating 0.5h still had high signals for detecting folate, while, coating 1h had low signals for detecting vitamin B_{12}. Coating 0.5h was chosen as the suitable coating time.

Incubating 0.5h had higher signals than incubating 1h and the experiment was time-saving.

Obviously, higher protein incubation concentration (FBP 25μg/L, IF 1.33μg/L) had significantly higher signals than other incubation concentration (FBP 7.5μg/L, IF 0.4μg/L). 25μg/L FBP and 1.33μg/L IF were chosen for further experiment, molecular weight of FBP and IF was respectively 29.3kD (kg/mol) and 46.47kD (kg/mol), so molar concentration of FBP and IF was respectively 0.853pmol/L and 0.029pmol/L. Concentration of biotinylated folate and vitamin B_{12} was respectively 0.486 and 0.714 μg/L, molecular weight of folate and vitamin B_{12} was respectively 441 and 1355, and molar concentration of folate and vitamin B_{12} was about 1.102 pmol/L and 0.527 pmol/L respectively. The binding ratio of folate and folate binding protein in this experiment was 1.29:1, the binding ratio of vitamin B_{12} and intrinsic factor in the experiment was 18.17:1.

For further experiment, 0.5h was chosen as coating time; 0.5h was chosen as the suitable incubation time; 25μg/L FBP and 1.33μg/L IF were chosen as the suitable incubation concentration. Through the results, the whole experiment time was about 2-3 hours.

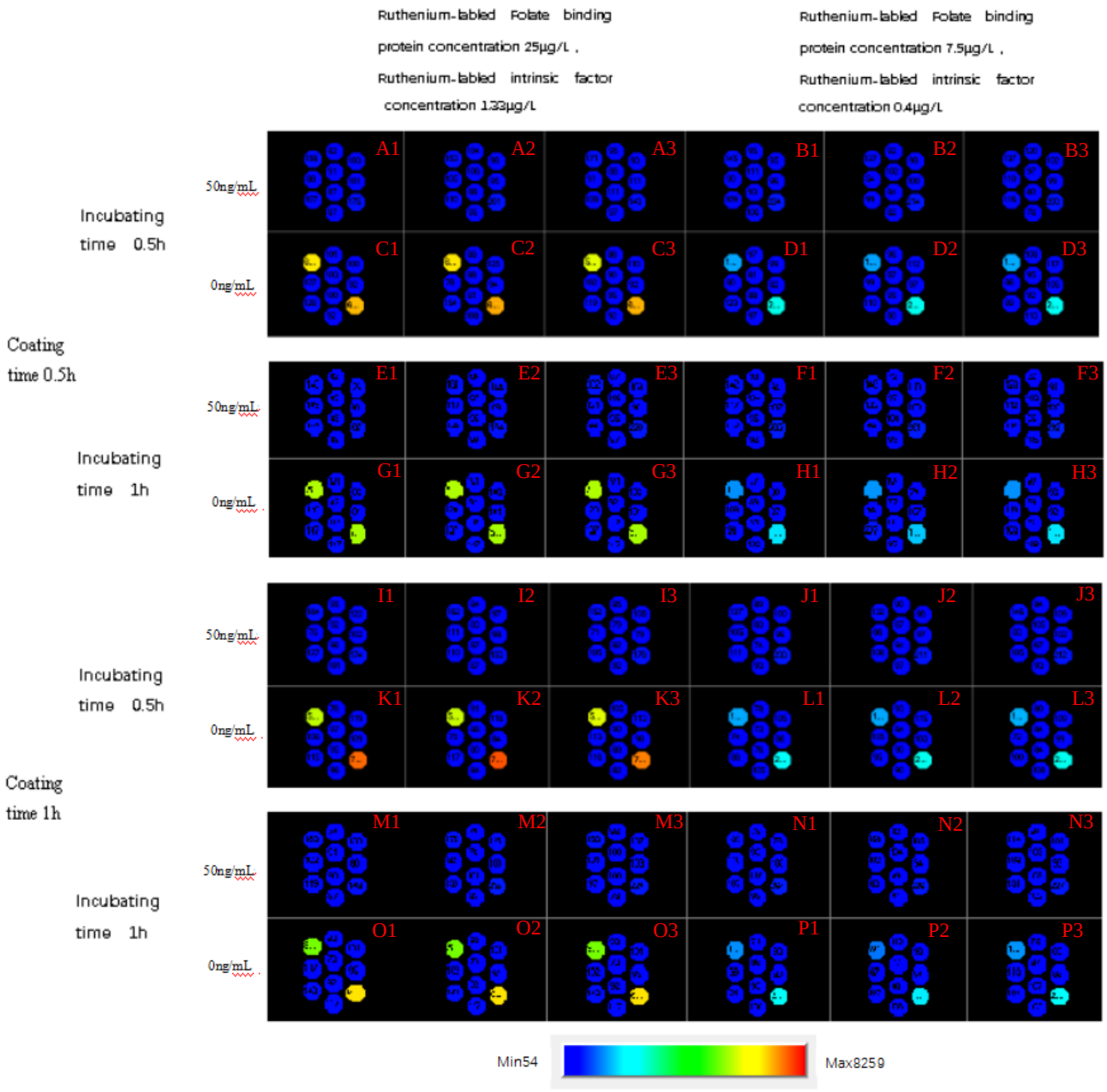

Figure 3. Coating time, incubating time and concentration assay for simultaneous detection of folate and vitamin B_{12}. A1-A3, coating time 0.5h, incubating time 0.5h, 50ng/mL folate and vitamin B_{12} standard solution, FBP concentration 25μg/L, IF concentration 1.33μg/L; B1-B3, coating time 0.5h, incubating time 0.5h, 50ng/mL folate and vitamin B_{12} standard solution, FBP concentration 7.5μg/L, IF concentration 0.4μg/L; C1-C3, coating time 0.5h, incubating time 0.5h, 0ng/mL folate and vitamin B_{12} standard solution, FBP concentration 25μg/L, IF concentration 1.33μg/L; D1-D3, coating time 0.5h, incubating time 0.5h, 0ng/mL folate and vitamin B_{12} standard solution, FBP concentration 7.5μg/L, IF concentration 0.4μg/L; E1-E3, coating time 0.5h, incubating time 1h, 50ng/mL folate and vitamin B_{12} standard solution, FBP concentration 25μg/L, IF concentration 1.33μg/L; F1-F3, coating time 0.5h, incubating time 1h, 50ng/mL folate and vitamin B_{12} standard solution, FBP concentration 7.5μg/L, IF concentration 0.4μg/L; G1-G3, coating time 0.5h, incubating time 1h, 0ng/mL folate and vitamin B_{12} standard solution, FBP concentration 25μg/L, IF concentration 1.33μg/L; H1-H3, coating time 0.5h, incubating time 1h, 0ng/mL folate and vitamin B_{12} standard solution, FBP concentration 7.5μg/L, IF concentration

0.4μg/L; I1-I3, coating time 1h, incubating time 0.5h, 50ng/mL folate and vitamin B_{12} standard solution, FBP concentration 25μg/L, IF concentration 1.33μg/L; J1-J3, coating time 1h, incubating time 0.5h, 50ng/mL folate and vitamin B_{12} standard solution, FBP concentration 7.5μg/L, IF concentration 0.4μg/L; K1-K3, coating time 1h, incubating time 0.5h, 0ng/mL folate and vitamin B_{12} standard solution, FBP concentration 25μg/L, IF concentration 1.33μg/L; L1-L3, coating time 1h, incubating time 0.5h, 0ng/mL folate and vitamin B_{12} standard solution, FBP concentration 7.5μg/L, IF concentration 0.4μg/L; M1-M3, coating time 1h, incubating time 1h, 50ng/mL folate and vitamin B_{12} standard solution, FBP concentration 25μg/L, IF concentration 1.33μg/L; N1-N3, coating time 1h, incubating time 1h, 50ng/mL folate and vitamin B_{12} standard solution, FBP concentration 7.5μg/L, IF concentration 0.4μg/L; O1-O3, coating time 1h, incubating time 1h, 0ng/mL folate and vitamin B_{12} standard solution, FBP concentration 25μg/L, IF concentration 1.33μg/L; P1-P3, coating time 1h, incubating time 1h, 0ng/mL folate and vitamin B_{12} standard solution, FBP concentration 7.5μg/L, IF concentration 0.4μg/L.

Table 1. Coating time, incubating time and concentration assay for simultaneous detection of vitamin B_{12} and folate

	Coating time	Concentration (ng/mL)	Incubation time 0.5h		Incubation time 1h	
			FBP 25μg/L, IF 1.33μg/L	FBP 7.5μg/L, IF 0.4μg/L	FBP 25μg/L, IF 1.33μg/L	FBP 7.5μg/L, IF 0.4μg/L
Folate	0.5h	50	173±29.1	213.7±10.5	187±28.8	272.7±16.9
		0	6817±60	2227.3±42.1	5501.3±48.5	1820±147.3
	1h	50	168.3±13.4	218.7±12.4	184±36.3	239±21.7
		0	7422±183	2212.3±48.8	6589±162.5	2079.7±92.6
vitamin B_{12}	0.5h	50	160.7±9.3	136.3±9	168.7±30.2	138.7±9.5
		0	6239.7±219.3	1395.3±30.7	5806.3±204	1215.3±21.6
	1h	50	159.3±6.4	138.3±7.1	170.3±15.5	137±22.1
		0	5834.7±140.7	1441±41.3	4962.3±119.5	1138.7±130.4

Serum pretreatment, standard curve and dilution recovery: Vitamin B_{12} was incubated with linker1 for 30min, while folate was incubated with linker10 for 30min. Then stop solution was added into the mixture above and incubated for 30min. Mix folate/linker10 and vitamin B_{12}/linker1 together, coat the assay plate, and then shake the plate for 30min. After that, folate/linker10 was binded to site10 and vitamin B_{12}/linker1 was binded to site1. Wash the plate.

Standard curves for folate and vitamin B_{12} were made as follows: folate and vitamin B_{12} were together dissolved in 1%BSA solution by different concentration: 50ng/mL, 10ng/mL, 2ng/mL, 0.4ng/mL, 0.08ng/mL and 0ng/mL. 24uL folate and vitamin B_{12} standard solution was mixed with 48uL ruthenium-labeled FBP and IF, and 50uL of the mixture solution was added into the wells of plate as the standard concentration. The plate was incubated for 30min on oscillator. The plate was washed and read buffer was added into the wells.

As was shown in figure 4, ECL signals were increasing with vitamin B_{12} and folate concentration decreasing, in line with competitive assay principle. The R-square of standard curve was respectively 0.998 for vitamin B_{12} and 0.999 for folate.

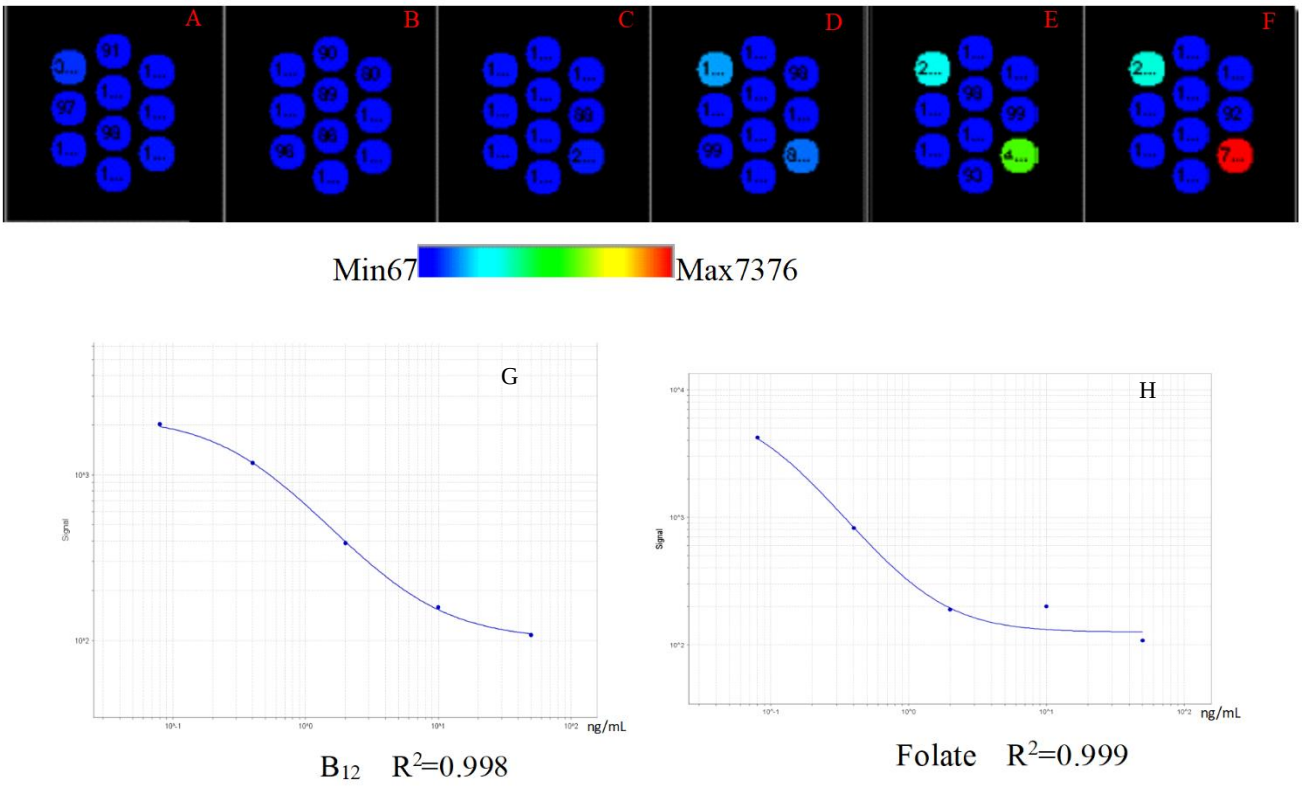

Figure 4. Standard curves of vitamin B_{12} and folate simultaneously detected without pretreatment. A, folate and vitamin B_{12} concentration 50ng/mL; B, folate and vitamin B_{12} concentration 10ng/mL; C, folate and vitamin B_{12} concentration 2ng/mL; D, folate and vitamin B_{12} concentration 0.4ng/mL; E, folate and vitamin B_{12} concentration 0.08ng/mL; F, folate and vitamin B_{12} concentration 0ng/mL; G, the standard curve of vitamin B_{12}; H, the standard curve of folate.

Pretreatment using the first method was done as follows: Biotin-labeled folate was incubated with linker 10 for 30min, and stop solution was added and incubated for 30min. Biotin-labeled vitamin B_{12} was incubated with linker 1 for 30min, and then stop solution was added and incubated for 30min. Folate/linker10/stop solution, vitamin B_{12}/linker1/stop solution were mixed as the ratio of 1:1. 50uL mixture solution were coated on those wells of the U-PLEX plate for 0.5h. Wash the plate three times. Folate and vitamin B_{12} were together dissolved in 1%BSA solution by different concentration: 100ng/mL, 33.3ng/mL, 11.1ng/mL, 3.7ng/mL, 1.23ng/mL, 0.41ng/mL and 0ng/mL. 12uL SRM1950 serum were mixed with 1.33uL NaOH (pH14), and then pH was about adjusted into 13, at that time vitamin B_{12} and folate were released from binding proteins[17]; folate and vitamin B_{12} standard solution was processed as the same steps of SRM1950 above. 1.33uL pH1 HCl solution was added to the solution to adjust the pH of solution, because folate and vitamin B_{12} could be respectively binded to FBP and IF at pH9.3[17]. Then 9.34uL solution of Dithiothreitol and Sodium 2-Mercaptoethanesulfonate was added into the solution. 48uL Ruthenium-labeled FBP and IF were added into the solution above, and 50uL mixture solution were added into the wells of plate. The plate was incubated for 30min on oscillator. The plate was washed and read buffer was added into the wells.

As was shown in figure 5, ECL signals of vitamin B_{12} and folate were increasing with the concentration decreasing, in line with competitive assay principle. The R-square of standard curve was

respectively 0.991 for vitamin B_{12} and 0.993 for folate. As was shown in Table 2, the folate recovery rate was 74.8%, and the dilution recovery rate was 77.8% according to the SRM1950. 12uL serum volume was enough for the first pretreatment method.

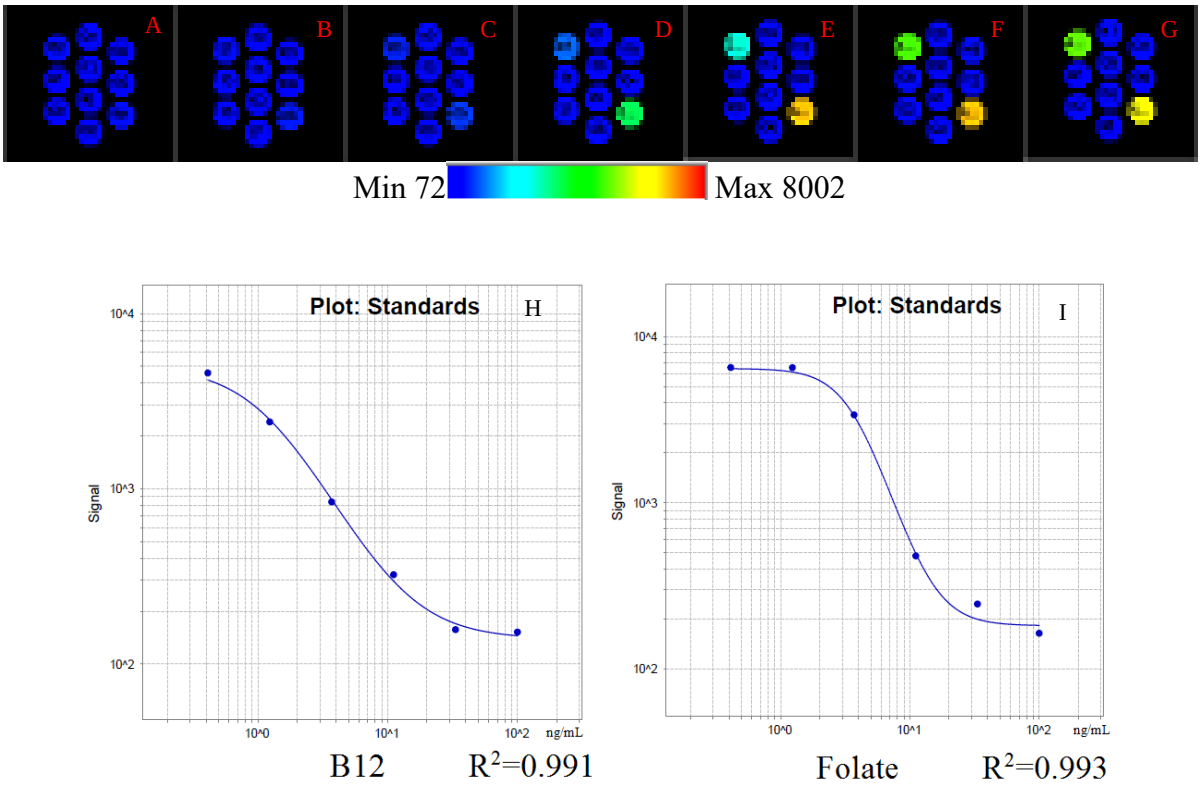

Figure 5. Simultaneous detection results of vitamin B_{12} and folate using the first pretreatment method. A, folate and vitamin vitamin B_{12} standard solution concentration 100ng/mL; B, folate and vitamin B_{12} standard solution concentration 33.3ng/mL; C, folate and vitamin B_{12} standard solution concentration 11.1ng/mL; D, folate and vitamin B_{12} standard solution concentration 3.7ng/mL; E, folate and vitamin B_{12} standard solution concentration 1.23 ng/mL; F, folate and vitamin B_{12} standard solution concentration 0.41ng/mL; G, folate and vitamin B_{12} standard solution concentration 0ng/mL; H, the standard curve of vitamin B_{12} detecting; I, the standard curve of folate detecting. 12uL Folate and vitamin B_{12} standards of different concentration were mixed with 1.33uL NaOH (pH14), and then pH was adjusted into 13. Then 1.33uL pH1 HCl solution was added to the solution to adjust the pH of solution. 9.34uL Dithiothreitol and Sodium 2-Mercaptoethanesulfonate were added into the solution. 48uL Ruthenium-labeledd FBP and IF were added into the solution above. 50uL of the mixture solution were added into the wells of plate (the plate was coated as the steps above: biotin-labeled folate was incubated with linker 10 for 30min, and stop solution was added and incubated for 30min. Biotin-labeled vitamin B_{12} was incubated with linker 1 for 30min, and then stop solution was added and incubated for 30min. Folate/linker10/stop solution, vitamin B_{12}/linker10/stop solution were mixed as the ratio of 1:1. 50uL mixture solution were coated on those wells of the U-PLEX plate for 0.5h. Wash the plate three times.) The plate was incubated and shaked for 30min on oscillator. Wash the plate, add read buffer, and read the ECL signals of the plate.

Table 2. Dilution recovery rate of folate in SRM1950 using the first pretreatment method

	Total folate(microbiological analysis)	Total folate Recovery
Standard reference material 1950	30.6 nmol/L, equal to 13.50ng/mL	/
Detecting value	10.1ng/mL	74.8%
Detecting value of dilution 1:2	3.93ng/mL	77.8%

Pretreatment using the second method was done as follows: Folate and vitamin B_{12} standards were together dissolved in 1%BSA solution by different concentrations: 100ng/mL, 33.3ng/mL, 11.1ng/mL, 3.7ng/mL, 1.23ng/mL, 0.41ng/mL and 0ng/mL. 12uL Folate and vitamin B_{12} standards of different concentration and 12uL SRM1950 were respectively mixed with 0.21uL NaOH (pH14), and then pH was adjusted into 12, and at that time vitamin B_{12} and folate were released from binding proteins[17]. Then 1.33uL pH1 HCl solution was added to the solution to adjust the pH of solution. Folate and vitamin B_{12} could be respectively binded to FBP and IF at pH9.3[17]. 10.46uL Dithiothreitol and Sodium 2-Mercaptoethanesulfonate were added into the solution. 48uL Ruthenium-labled FBP and IF were added into the solution, and 50uL mixture solution were added into the wells of plate. The plate was incubated for 30min on oscillator. The plate was washed and read buffer was added into the wells.

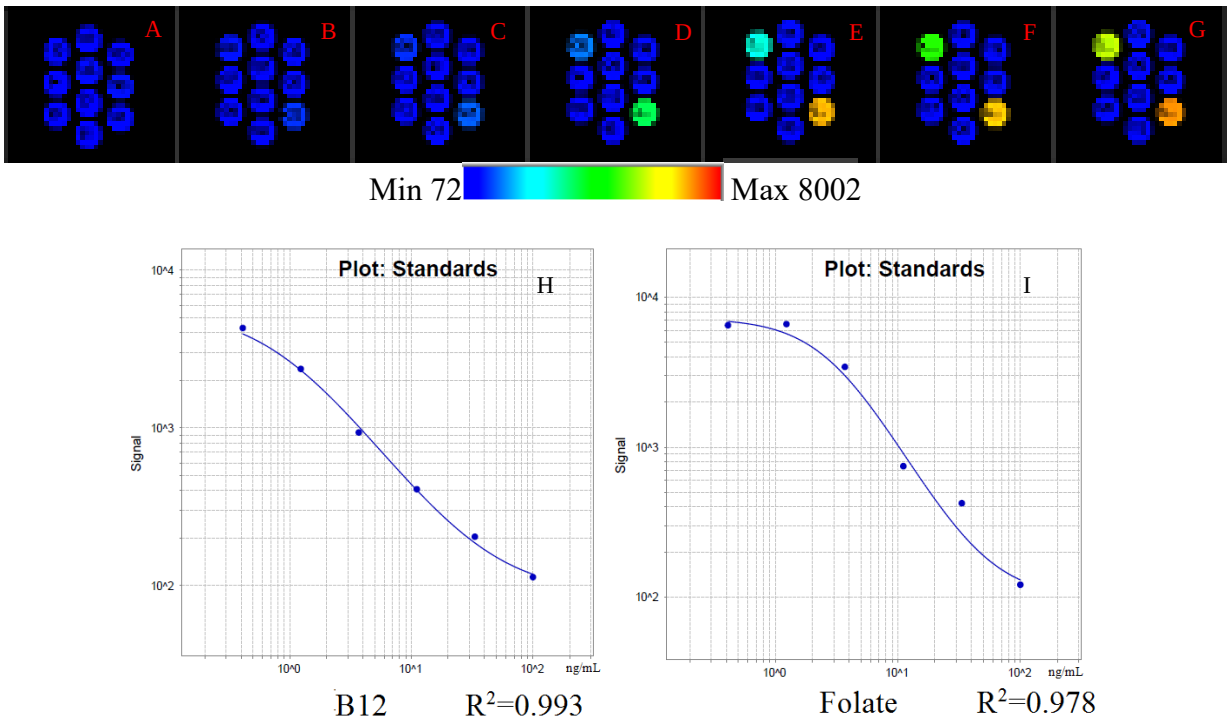

Figure 6. Simultaneous detection results of vitamin B_{12} and folate using the second pretreatment method. A, folate and vitamin B_{12} standard solution concentration 100ng/mL; B, folate and vitamin B_{12} standard solution concentration 33.3ng/mL; C, folate and vitamin B_{12} standard solution

concentration 11.1ng/mL; D, folate and vitamin B_{12} standard solution concentration 3.7ng/mL; E, folate and vitamin B_{12} standard solution concentration 1.23 ng/mL; F, folate and vitamin B_{12} standard solution concentration 0.41ng/mL; G, folate and vitamin B_{12} standard solution concentration 0ng/mL. H, the standard curve of vitamin B_{12} detecting; I, the standard curve of Folate detecting.12uL Folate and vitamin B_{12} standards of different concentration were mixed with 0.21uL NaOH (pH14), and then pH was adjusted into 12. Then 1.33uL pH1 HCl solution was added to the solution to adjust the pH of solution. 10.46uL Dithiothreitol and Sodium 2-Mercaptoethanesulfonate were added into the solution. 48uL Ruthenium-labeledd FBP and IF were added into the solution above. 50uL of the mixture solution were added into the wells of plate, (the plate was coated as the steps above: biotin-labeled folate was incubated with linker 10 for 30min, and stop solution was added and incubated for 30min. Biotin-labeled vitamin B_{12} was incubated with linker 1 for 30min, and then stop solution was added and incubated for 30min. Folate/linker10/stop solution, vitamin B_{12}/linker10/stop solution were mixed as the ratio of 1:1. 50uL mixture solution were coated on those wells of the U-PLEX plate for 0.5h. Wash the plate three times.) The plate was incubated and shaked for 30min on oscillator. Wash the plate, add read buffer, and read the ECL signals of the plate.

As was shown in Figure 6, ECL signals of vitamin B_{12} and folate were increasing with the concentration decreasing, in line with competitive assay principle. The R-square of standard curve was respectively 0.993 for vitamin B_{12} and 0.978 for folate. According to the reference value of SRM1950, the recovery rate was only 35.2% and the dilution recovery was 208% shown in Table 3.

Table 3. The recovery rate of folate in SRM1950 using the second pretreatment method

	Total folate(microbiological analysis)	Total folate Recovery
Standard reference material 1950	30.6 nmol/L, equal to 13.50ng/mL	/
Detecting value	4.75ng/mL	35.2%
Detecting value of dilution 1:2	4.95ng/mL	208%

To sum up, compared to the two pretreatment methods, pretreatment using the first method was chosen as the suitable method for further experiment. The volume of detection serum was only 12uL, while the volume of plasm was 200uL for detection vitamins in biological samples by LC-MS[17]. Compare to pretreatment of LC/MS method, the pretreatment of this method was easy and time-saving[17].a

Whlgham's study[18] showed that the standard curve and linear response were acceptable in the simultaneous assay for vitamin B_{12} and folate using a kit ("SimulTRAC"; Schwarz/Mann, Orangeburg) containing both standards, both binders, and label solution containing ^{125}I-labeled folate and [^{57}Co]B_{12}. Compared with the radioligand results of Whlgham's study, the standard curve is accepted and there may be a wider detecting range in this assay of ruthenium-labeled binders. Raniolo's study[15] showed

that the commercial assay kit method for simultaneous estimation of vitamin B_{12} and folate concentration was time and labour saving compared with the non-commercial methods, but the method had still radiation hazard. In conclusion, it's valuable for the further assay of the ruthenium-labeled binder method for folate and vitamin B_{12}.

Abano's study[19] showed that simultaneous detection of water-soluble vitamins should be rapid and suitable for routine analysis as well as inexpensive to set up. Ciulu's study [20] showed that water-soluble vitamins (B2, B3, B5,folic acid, and C) was simultaneously determined by LC with UV-vis detection within 17min only in honey, but not in other food materials. Vidovic's study [21] showed simple simultaneous determination of some water-soluble vitamins (vitamin C, B1, B2, B3, B5 and B6) by high performance liquid chromatography (HPLC), but the method didn't cover folate and vitamin B_{12}. Recent literature pays attention to those vitamins including folate and vitamin B_{12}, important to the treatment of anaemia, especially in pregnancy[19]. Much literature[19] reported detection of water-soluble vitamins by Liquid Chromatography with electrochemical detection for blood plasm. Electrochemical detection has low detection limits with high sensitivity, but don't detect the oxidation product of some water-soluble vitamins. HPLC could determine all the water-soluble vitamins by coupling with electrospray ionization mass spectroscopy (ESI-MS), but the method was expensive [19].

The reported methods for simultaneously detecting folate and vitamin B_{12} are shown in the following Table 4. There're radioimmunoassay, HPLC/LC, HPLC-MS/MS for simultaneously detecting multiple vitamins including folate and vitamin B_{12}. Every simultaneous method's merits and defects are listed in the Table 4. The electrochemiluminescence method might be a new method for simultaneous folate and vitamin B_{12} detection in serum, with its merit of simple operation and time-saving.

Table 4. The reported methods for simultaneously detecting folate and vitamin B_{12}

Simultaneous assay methods	Merits	Defects	Simultaneous detecting vitamins
Radioimmunoassay[15,18，22-23]	Simple operation, time-saving	Radiation hazards, cross reaction	Folate and vitamin B_{12} in serum
Liquid Chromatography (LC) [14]; high-performance liquid chromatography tandem mass spectrometry (HPLC) - Solid phase extraction with polypyrrole nanofibers [24]	Good linearity, acceptable accuracy; Low detection limits	Not cost-effective, a skilled analyst needing	nicotinamide, pyridoxine, folic acid, cyanocobalamin (vitamin B_{12}) in pharmaceutical preparations [14]; Folic acid, vitamin B12, B2 in urine [24]
high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) [25]	high sensitivity and accuracy	Expensive to set up, a skilled analyst needing	Caffeine, taurine, vitamin B1, B2, B3, B9 (folate), B12 in tobacco products [25]

4. CONCLUSIONS

ECL signals of 21℃were higher than those of 37℃. At 21℃, ECL signals of pH5.5 were higher than those of pH7.3 for linker1 detecting vitamin B_{12} and linker10 detecting folate. Coating 0.5h was chosen as suitable coating time. Higher protein incubation concentration (FBP 25μg/L, IF 1.33μg/L) had significantly higher signals than other incubation concentration (FBP 7.5μg/L, IF 0.4μg/L). Incubating 0.5h in competitive combining assay had higher signals than incubating 1h.

The first serum pretreatment method was chosen for the further analysis, the method was processed as follows: the solution was combined with NaOH solution (pH=1) as the ratio of 9:1, then 1/9 volume HCl of the mixture solution was added, dithiothreitol and sodium 2-Mercaptoethanesulfonate were added into the solution, the R-square of standard curve was respectively 0.991 for vitamin B_{12} and 0.993 for folate.

Simultaneous detection of vitamin B_{12} and folate was initially established using ECL method. 12uL was enough for the volumn of detection serum, detection time was as quickly as 2-3 hours, and the method was convenient and high-throughout. Further methods evaluation and optimation were still needed for the application.

There are some problems about vitamin detection, such as long detection time, large detection volume of biological samples and expensive equipment platform. As the development of accurate nutrition, it's important to make the vitamin detection routine and universal. This research focuses on simultaneous and high-throughout detection technology for multiple vitamins, especially folate and vitamin B_{12} easily deficient in human body. Specific binding proteins of these vitamins were used to establish a high-throughout simultaneous detection electrochemiluminescence method. The method was established by linker selection, experiment temperature selection, sample dilution solution pH selection, coating time selection, serum pretreatment method selection, standard curve evaluation, incubation time and concentration evaluation. This study could provide some technical support for solving the bottleneck problems in human nutrition detection, and might contribute to the development of accurate nutrition for both individuals and populations.

THE AUTHORS DECLARE NO COMPETING INTERESTS.

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ABBREVIATIONS
FBP: folate binding protein; IF: intrinsic factor; ECL: electrochemiluminescence; ELISA: enzyme-linked immunology sorbent assay; MSD: Meso Scale Discovery Company

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