Optimization of immunoturbidimetric assay system enhanced by β2-microglobulin latex

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Abstract. In this study, three-factor and three-level orthogonal experiment was used to optimize the detection system of clinical renal function marker β2-microglobulin latex enhanced immune turbidity, so as to prepare a detection system with a wider linear range, solve the false negative problem in clinical detection of high-concentration samples, and improve the detection accuracy. Based on latex enhanced immunoturbidimetry, automatic biochemical analyzer was used to establish the linear relationship between the β2-microglobulin standard with different concentrations and the absorbance change value of 546 nm, and optimize the antibody source, latex microsphere particle size, activator concentration, sealer concentration and buffer system in the β2-microglobulin detection system. The linear range and accuracy of the optimized detection system were evaluated. After optimization, the optimal detection system was obtained, and its detection linear range was 0.2-30 mg/L, which reached the upper limit of the reference range of β2-microglobulin in normal population by 10 times, and the accuracy was in line with clinical standards, which solved the false negative problem of high-concentration samples in clinical testing, eliminated the repeated dilution process of high-concentration samples, simplified the detection steps, and improved the detection accuracy.

1. Introduction

Beta2-Microglobulin (B2M) is a 12 kda protein made up of 119 amino acids that is encoded by a gene located on chromosome 15 [1]. It is an essential component of MHC class I molecules and provides structural integrity and stability to the MHC class I-peptide complex [2]. B2M mutations are known to occur with varying frequencies in various tumor types and some tumors like bladder cancer are known to downregulate B2M in the absence of hard mutations within the gene [3]. Downregulation of B2M is generally believed to facilitate escape of tumors from immunosurveillance and immune-mediated destruction Downregulation of B2M is generally believed to facilitate escape of tumors from immunosurveillance and immune-mediated destruction [4] our results underscore that loss of B2M and HLA-A expression is specific to tumor cells but in the majority of cases it is not permanent and can be upregulated through immunotherapy treatment [5-6].

The detection reagent of β2-microglobulin prepared by latex enhanced immunoturbidimetric method is the most effective method for clinical detection of β2-microglobulin at present. However, at present, the reagents used for automatic biochemical analyzer β2-microglobulin latex turbidimetry are mostly imported and packaged, which is expensive, and the linear range of detection is 6 times that of the upper limit of the reference range of the normal population, that is, 18 mg/L. False negative problems caused by high concentration of β2-microglobulin in blood samples (hook effect) often occur in clinical testing, so it is necessary to dilute high-value samples frequently, increasing the time and cost of manual testing.

In this study, three-factor and three-level orthogonal experiment was used to optimize the bet2-microglobulin latex enhanced immune turbidimetric detection system, so as to prepare a detection system with a wider linear range. The detection system was compared with commercial reagents to detect the linear range, and the repeatability and accuracy of the system were evaluated, providing a theoretical basis for the accurate detection of clinical high-value samples.

2. Experimental material

2.1. Raw materials and reagents

β2-microglobulin Standard (Guangdong Fipeng, China), β2-microglobulin Quality Control Product (UK RANDOX), latex microspheres (Japan JSR), 1-ethyl -(3-Dimethylaminopropyl) carbodiimide Hydrochloride (EDC) (Shanghai Aladdin, China), N-hydroxysuccinimide (NHS) (Shanghai Aladdin, China) Polyclonal sheep resistance (19.8 mg/mL, Boyan, Beijing, China), polyclonal rabbit resistance (10 mg/mL, reached in Beijing, China), polyclonal sheep resistance (24.4 mg/mL, Nanjing Ollikon, China), bovine Serum albumin (BSA) (Roche, Germany), phosphate buffer

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(PBS) (Sinopharm Group), Trimethylol aminomethane buffer (TRIS) (Suzhou, China), 2-morpholine ethanesulfonic acid buffer (MES) (Suzhou, China).

2.2. Instrument and equipment

BSA224S electronic balance (Sartorius, Germany), PB-10 acidity meter (Sartorius, Germany), HHS-24 electric thermostatic water bath (Yiheng, Shanghai, China), GL-21M low-temperature high-speed centrifuge (Xiangyi, Hunan, China), O8-3G magnetic Stirrers (Meiyingpu, Shanghai, China), Scientz-1500F ultrasonic cell Crusher (Xinzhi, Ningbo, China), BS2000 automatic biochemical analyzer (Mindrays, Shenzhen, China).

3. Experimental method

3.1. Optimization of β2-microglobulin detection system

3.1.1 Antibody source, latex microsphere size and activator concentration sieve

According to the previous study, three kinds of β2-microglobulin polyclonal sheep antibody (19.8 mg/mL), polyclonal sheep antibody (24.4 mg/mL) and polyclonal rabbit antibody (10 mg/mL) were selected, and their concentration in the system was 300 mg/L. Latex microspheres with particle sizes of 88 nm, 107 nm and 147 nm were selected, and their concentration in the system was 2.5 g/L. The concentration ratio of activator EDC and NHS is 1:1, and its concentration in the system is 0.5 mM+0.5 mM, 1 mM+1 mM, 1.5mM+1.5mM, respectively. According to the previous study, three kinds of β2-microglobulin polyclonal sheep antibody (19.8 mg/mL), polyclonal sheep antibody (24.4 mg/mL) and polyclonal rabbit antibody (10 mg/mL) were selected, and their concentration in the system was 300 mg/L. Latex microspheres with particle sizes of 88 nm, 107 nm and 147 nm were selected, and their concentration in the system was 2.5 g/L. The concentration ratio of activator EDC and NHS is 1:1, and its concentration in the system is 0.5 mM+0.5 mM, 1 mM+1 mM, 1.5mM+1.5mM, respectively.

A total of 9 groups of experiments with three factors and three levels were designed according to the orthogonal experiment method, as shown in Table 1. The detection concentrations of the 9 groups of experiments were 0.2mg /L, 1.88mg /L, 3.75mg /L, 7.5mg /L, 15mg /L, and 30mg /L β2-microglobulin antigen standards by automatic biochemical analyzer, respectively. The absorbance A1 before antigen antibody binding was determined at 546 nm wavelength, and the absorbance A2 after antigen antibody binding was determined 3 min later. The absorbance change value △A, △A=A2-A1 before and after the immunoturbidimetric reaction was calculated. The linear relationship between β2-microglobulin with different concentrations and △A was established by calculating the average value. The linear correlation coefficient R2 should be greater than 0.95, and the experimental condition with the maximum R2 is the best condition.

Table 1. Orthogonal test of antibody source, microsphere size and activator concentration

Experimental Group	Antibody sources	Microsphere size	EDC+NHS concentration
1	Polyclonal Sheep antibody (19.8 mg/mL)	88 nm	0.5mM + 0.5mM
2	Polyclonal Sheep antibody (19.8 mg/mL)	107 nm	1.0mM + 1.0mM
3	Polyclonal Sheep antibody (19.8 mg/mL)	147 nm	1.5mM + 1.5mM
4	Polyclonal Sheep antibody (24.4 mg/mL)	88 nm	1.5mM + 1.5mM
5	Polyclonal Sheep antibody(24.4 mg/mL)	107 nm	0.5mM + 0.5mM
6	Polyclonal Sheep antibody(24.4 mg/mL)	147 nm	1.0mM + 1.0mM
7	Polyclonal rabbit antibody(10 mg/mL)	88 nm	1.0mM + 1.0mM
8	Polyclonal rabbit antibody(10 mg/mL)	107 nm	1.5 mM+ 1.5 mM
9	Polyclonal rabbit antibody(10 mg/mL)	147 nm	0.5mM + 0.5mM

3.1.2 Buffer with pH determined

Three buffer systems of PBS, MES and TRIS were selected, and three gradients were set for each buffer concentration, which were 20 mM, 30 mM and 50 mM respectively. The pH value of each buffer is set up three gradients, respectively 7.0, 7.5, 8.0. A total of 9 groups of experiments with three factors and three levels were designed according to the orthogonal experiment method, as shown in Table 2. The 9 groups of experiments were respectively detected by automatic biochemical analyzer with different concentrations of β2-microglobulin antigen standard, and the detection method was the same as 3.1.1.

Table 2. Orthogonal test of buffer liquid system, concentration and pH

Experimental Group	Buffer liquid system	Buffer concentration	pH
1	PBS	20 mM	7.0
2	PBS	35 mM	7.5
3	PBS	50 mM	8.0
4	MES	20 mM	7.5
5	MES	35 mM	8.0
6	MES	50 mM	7.0
7	TRIS	20 mM	8.0
8	TRIS	35 mM	7.0
9	TRIS	50 mM	7.5

3.1.3 Determination of the concentration of antibody, microsphere and sealer

The antibody concentration in the system was set to three gradients, which were 300 mg/L, 350 mg/L and 400 mg/L respectively. Three gradients were set for the concentration of latex microspheres, which were 2.50 g/L, 2.75 g/L and 3.00 g/L, respectively. Three gradients were set for the concentration of sealer BSA, which were 3.00 g/L, 5.00 g/L and 8.00 g/L, respectively. A total of 9 groups of experiments with three factors and three levels were designed according to the orthogonal experiment method, as shown in Table 3. The 9 groups of experiments were respectively detected by automatic biochemical analyzer with different concentrations of β2-microglobulin antigen standard, and the detection method was the same as 3.1.1.

Table 3. Orthogonal test of concentrations of antibody, microspheres and sealer BSA

Experimental Group	Antibody concentration	Microsphere concentration	BSA concentration
1	300 mg/L	2.50 g/L	3.00 g/L
2	300 mg/L	2.75 g/L	5.00 g/L
3	300 mg/L	3.00 g/L	8.00 g/L
4	350 mg/L	2.50 g/L	5.00 g/L
5	350 mg/L	2.75 g/L	8.00 g/L
6	350 mg/L	3.00 g/L	3.00 g/L
7	400 mg/L	2.50 g/L	8.00 g/L
8	400 mg/L	2.75 g/L	5.00 g/L
9	400 mg/L	3.00 g/L	3.00 g/L

3.2. Performance evaluation of β2-microglobulin detection system

3.2.1 Evaluation of linear range

The detection system optimized in this study and commercial reagents (Ningbo Meikang, China) were used to detect β2-microglobulin antigen standards at different concentrations by automatic biochemical analyzer, and the detection method was the same as 3.1.1.

3.2.2 Accuracy evaluation

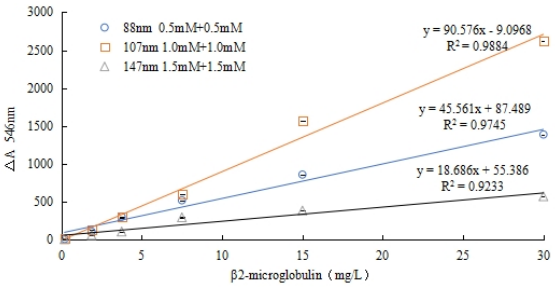
Automatic biochemical instrument was used to detect 2 concentration β2-microglobulin quality control products, each quality control product was repeated for 3 times, and the average value (M) was calculated. The calibrated concentration of the quality control product was denoted as (T), and the relative deviation (Bi) was calculated according to the formula Bi=(M-T)/T×100%. The relative deviation required by clinical testing standards should be ≤±10%.

4. Results and analysis

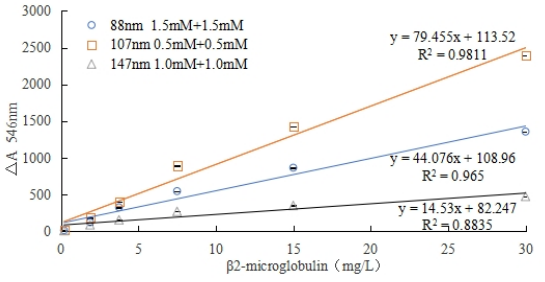
4.1. Optimization of β2-microglobulin detection system

4.1.1 Antibody source, latex microsphere particle size and activator concentration screening results

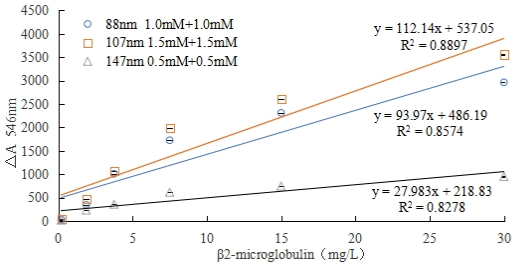
By screening 3 kinds of antibodies from 3 sources, 3 kinds of latex microspheres and 3 kinds of concentration activators, the influence of raw material properties on the binding of antibodies to latex microspheres was studied. Automatic biochemical analyzer was used to detect 0.2mg /L-30 mg/ Lβ2-microglobulin antigen standard, and the linear relationship between the concentration of β2-microglobulin standard and △A was established. And the correlation coefficient R2 was calculated. As can be seen from Figure 1, the linear equation of polyclonal sheep resistance (19.8 mg/mL) combined with 107 nm latex microspheres under the activation of 1 mM EDC+1 mM NHS is y = 90.576x -- 9.0968, and the correlation coefficient R is the maximum value 0.92884. The best antibody to polyclonal sheep antibody (19.8 mg/mL) was determined, the best latex microsphere particle size was 107 nm, and the best EDC+NHS concentration was 1 mM+1 mM.



A. Polyclonal sheep antibody (19.8 mg/mL) group.



B. Polyclonal sheep resistance (24.4 mg/mL) group.

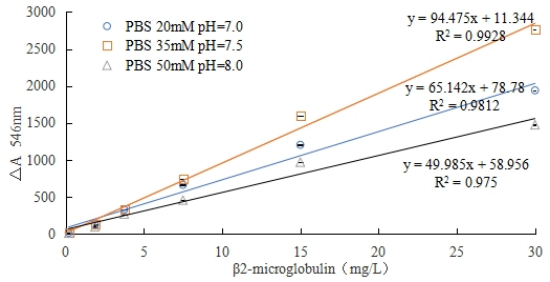


C. Polyclonal rabbit resistance (10 mg/mL) group.

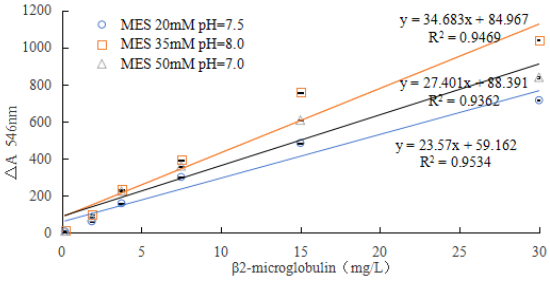
Fig. 1. Results of the orthogonal test of antibody source, microsphere size and activator concentration.

4.1.2 Buffer and pH range screening results

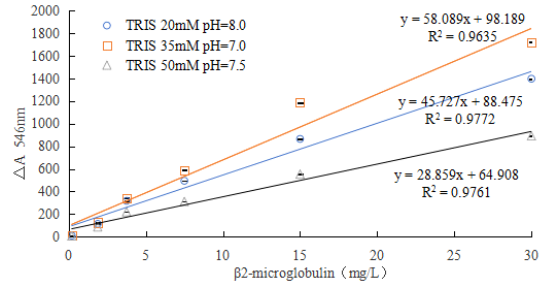
The influence of buffer capacity on the coupling of antibody and latex microspheres was studied by screening different systems, concentrations and pH buffers. Automatic biochemical analyzer was used to detect 0.2mg /L-30 mg/ Lβ2-microglobulin antigen standard, the linear relationship between β2-microglobulin standard concentration and △A was established, and the linear correlation coefficient R2 was calculated. As can be seen from Figure 2, when the buffer is 35 mM PBS buffer (pH 7.5), the linear equation is y = 94.475x +11.344, and the correlation coefficient R2 reaches the maximum value of 0.9928. The optimal concentration of 35 mM PBS buffer (pH 7.5) is used for the coupling of antigen and antibody.



A. PBS buffer group.



B. MES buffer group.

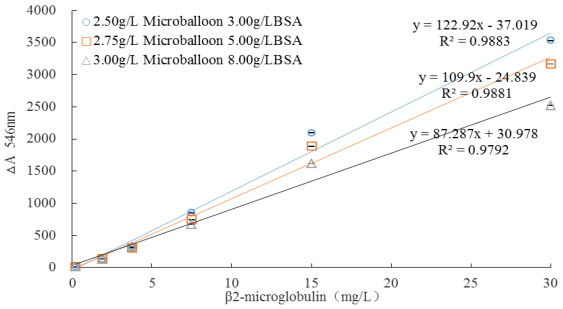


C. TRIS buffer group.

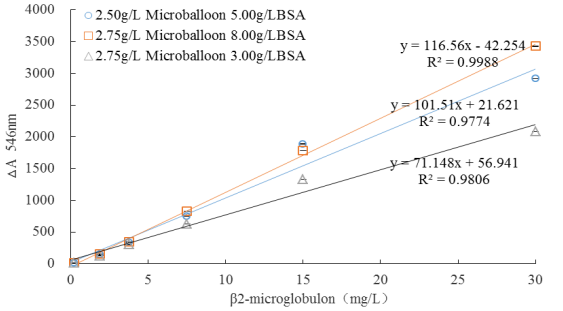
Fig. 2. Buffer, buffer concentration and pH orthogonal test results.

4.1.3 Concentration screening results of antibody, microspheres and sealer BSA

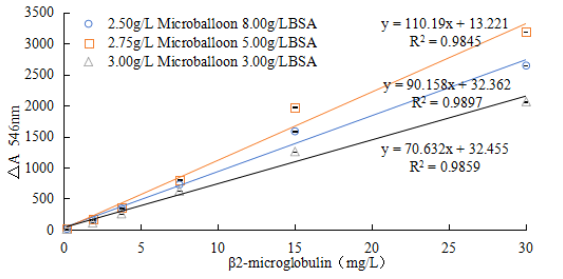
By screening different concentrations of antibodies, latex microspheres and BSA sealer, the influence of raw material concentration on the coupling of antibodies and latex microspheres was studied. Automatic biochemical analyzer was used to detect 0.2 mg/L-30 mg/ Lβ2-microglobulin antigen standard, and the absorbance change △A before and after antigen-antibody reaction was determined at 546 nm wavelength. The linear relationship between β2-microglobulin standard concentration and △A was established, and the linear correlation coefficient R2 was calculated. As can be seen from FIG. 3, the linear equation y = 116.56x -- 42.254 under the blocking action of 8 g/L BSA with 350 mg/L antibody binding 2.75 g/L microsphere, the correlation coefficient R^2 reaches the maximum value of 0.9988, and the optimal antibody concentration is determined to be 350 mg/L. The optimal concentration of latex microspheres was 2.75 g/L, and the optimal concentration of BSA was 8 g/L.



A. 300 mg/L antibody group.



B. 350 mg/L antibody group.



C. 400 mg/L antibody group.

Fig. 3. Results of orthogonal test of antibody, microsphere and BSA concentration.

According to the optimization results of the above β2-microglobulin detection system, the best reaction detection system was 35 mM PBS buffer with pH 7.5. 350 mg/L polyclonal sheep antibody; The size of the latex microspheres was 107 nm and the concentration was 2.75 g/L; The concentration of activator was 1 mM EDC+1 mM NHS; And the concentration of the blocking agent BSA is 8 g/L.

4.2. Performance evaluation of the best detection system for β2-microglobulin

4.2.1 Results of linear range evaluation between the detection system and commercial reagents in this study

The detection system in this study and the commercial reagent (Ningbo Meikang) were respectively detected on the automatic biochemical instrument with the concentration of 0.2mg /L-30 mg/ Lβ2-microglobulin antigen standard. The results are shown in Figure 4. The detection system in this study was within the range of 0.2-30 mg/L. The linear equation is y = 119.52x -- 47.013, R2=0.9984, and the linear equation of the commercial reagent is y = 84.614x + 704.38, R2= 0.7275. The results show that the linear relationship of the detection system in this study is better than that of the commercial reagent, indicating that the linear range of the detection system in this study is wider. The linear range covered the reference value range of β2-microglobulin in normal population 0-3 mg/L, and reached 30 mg/L, which was 10 times of the upper limit of the reference value.

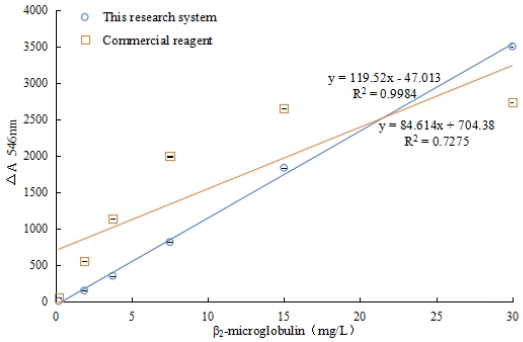


Fig. 4. Linear range test results of the detection system and commercial reagents in this study.

4.2.2 Accuracy results

Two concentration β2-microglobulin quality control products were tested in the automatic biochemical instrument, and each quality control product was repeated for 3 times. The results were shown in Table 4. The relative deviation of quality control product 1 was 0.49%, and the relative deviation of quality control product 2 was 1.36%, and the accuracy was in line with clinical standards.

Table4. Test results of β2-microglobulin quality control products,

Samples	Quality Control 1			Quality Control 2		
Number of measurements	1	2	3	1	2	3
Determination value	2.73	2.75	2.69	4.22	4.19	4.24
Average (M)	2.72			4.22		
Calibration Concentration (T)	2.71			4.16		
Relative Deviation (Bi)	0.49%			1.36%		

5. Conclusion

In this study, three factor and three level orthogonal experiment was used to optimize the clinical renal function marker β2-microglobulin latex enhanced immunoturbidimetric detection system, and the best β2-microglobulin detection system was 35 mM PBS buffer with pH 7.5. 350 mg/L polyclonal sheep antibody; The size of the latex microspheres was 107 nm and the concentration was 2.75 g/L; The concentration of activator was 1 mM EDC+1 mM NHS; The concentration of the blocking agent BSA was 8 g/L; The detection linear range of the system reached 0.2-30 mg/L, covering the reference range of 0-3 mg/L for the normal population, reaching the upper limit of the reference value of 30 mg/L, which was superior to the commercial reagents used in clinical testing, and the accuracy of the system met the clinical standards, and solved the false negative problem of high concentration samples in clinical testing. It eliminates the repeated dilution process of high-concentration samples, simplifies the detection steps, and improves the detection accuracy.

6. Outlook

At present, the clinical application of β2-microglobulin is still under continuous research. With the deepening of research, there are still the following problems to be solved in the optimization and development of β2-microglobulin detection kit: (1) The stability of β2-microglobulin detection reagents is required to be high in clinical practice, and molecular chaperone is prepared to be used as a stabilizer to improve the stability of the kit. (2) Clinical blood samples contain different interfering substances, including chylous blood, hemolysis, jaundice blood and ascorbic acid interference, it is necessary to verify the influence of different concentrations of interfering substances on the measured value.

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