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ORIGINAL RESEARCH ARTICLE

Screening of anti-human albumin monoclonal antibody by chemiluminescence immunoassay

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ABSTRACT

In this study, chemiluminescence immunoassay was used to screen clones of mouse anti-human albumin monoclonal antibody (anti-ALB McAb). The method is simplified as a one-step operation and the experimental parameters are optimized by orthogonal experiment [L9 (34)]. The parameters of the positive clone / blank control luminescence value (SNR) are the largest and the method is evaluated and statistical analysis. The results showed that the coated antibody was 3mg / L, the ratio of the enzyme protein A was 1: 2000, the incubation time was 304 times and the signal - to - noise ratio was 1284, which was several times higher than other combinations. The linear range was 20-20000ng / L, the precision was good, the average CV was 5.32% and the average inter-assay CV was 8.82%. The results showed that the method can be used for cloning and screening of anti-ALB McAb and the method is obviously superior to ELISA and other traditional methods, especially the simple and rapid operation of one step is also suitable for high antibody screening of other antibodies.

Keywords*: chemiluminescence immunoassay, anti-human albumin monoclonal antibody, orthogonal test, clone screening*

Introduction

Theoretical basis

Chemiluminescence is a special phenomenon that occurs in the chemical reaction process. The reaction product is excited in the reaction in the excited state, when the transition from the excited state back to the ground state, in the form of optical radiation to release a certain amount of energy. The combination of highly sensitive chemiluminescence techniques with highly specific immune responses has established a technique for chemiluminescence immunoassay (CLIA) which is a class of immunoluminescent reagents, enzymes that directly label antibodies or antigens. The method has the advantages of high sensitivity, strong specificity, no radiation and long validity period of the label and full automaticization. Therefore, chemiluminescence immunoassay has been widely used in clinical diagnosis, drug analysis, food testing and environmental analysis and other fields[1].

Protein A is a constituent component of the bacterial cell wall, which has a high affinity for the Fc region of the antibody IgG[2] and the Fab region of the antibody IgG binds specifically to the antigen. In this study, protein A and horseradish peroxidase (HRP) were used as secondary antibody. The anti-human albumin monoclonal antibody was screened by indirect enzyme-linked immunosorbent assay (ELISA). Since the human albumin antigen and the protein A enzyme conjugate can be combined with different regions of the antibody to be screened on the luminescent reaction microplate, there is no interference with each other so that the hair is formed in one step. Protein-antibody IgG-protein A enzyme conjugate complex and the free component was removed by washing. Finally, the presence or absence of anti-human albumin monoclonal antibody was determined by enzyme-linked photolysis.

Purpose Meaning

With the increasing use of chemicals, the incidence of drug-induced renal injury increased significantly. Detection of microalbuminuria in urine is one of the sensitive indicators of diagnosis of nephropathy in order to achieve rapid detection of microalbumin which has great significance[3]. In this study, we used chemiluminescence immunoassay to screen anti-ALB McA to prepare high specific monoclonal antibody and to develop clinical diagnostic reagents. Cloning screening is a very critical step in the preparation of monoclonal antibodies. Methods commonly used are ELISA, immunoprecipitation, binding inhibition experiments, flow cytometry, immunoblotting, immunohistochemistry and functional neutralization experiments[4] In recent years, CLIA research has progressed rapidly has become a highly sensitive, highly specific and highly automated rapid detection method which is gradually replacing the traditional immunoassay method of immunoassay immunoassay for monoclonal antibody cloning screening literature. The aim of this study was to establish a more sensitive, rapid and simple method for screening high-throughput CLIA clones by using CLIA for cloning and screening of anti-human albumin monoclonal antibodies. The aims are to overcome the traditional detection methods for a long time, low sensitivity, complex operation and other defects and to improve the efficiency of cloning screening[5].

Methodology establishment

As the experimental results of more experimental factors, the main human serum albumin coating concentration, incubation time and protein A enzyme conjugate dilution concentration. Therefore, the use of orthogonal test method reduces the amount of experiment and improves the experimental efficiency. In this experiment, the three-factor three-level orthogonal experiment design experiment was used to optimize the experimental parameters by orthogonal experiment L9 (34) table to determine the positive clonal luminescence value / Blank control Luminous value (SNR) is optimal when combined with experimental conditions. According to the size of the SNR, the monoclonal antibody was selected as the basis for selecting the optimal experimental conditions. After the optimal experimental conditions were determined, the parameters such as sensitivity, precision and linear range were evaluated by statistical analysis under the experimental conditions to confirm the accuracy and reliability of the method[6].

Overview of research at home and abroad

Chemical luminous sensitivity is high but of poor selectivity. In 1977, Halmann, based on the principle of radioimmunoassay, established a technique - chemiluminescent immunoassay (CLIA)[7], which is chemiluminescence, by combining high-sensitivity chemiluminescence techniques with highly specific immune responses Reagents directly labeled antibody or antigen of a class of immunoassay method with high sensitivity, specificity, no radiation, the validity of the label and can achieve full automation. Luminol and its derivatives, acridine ester derivatives, horseradish peroxidase and alkaline phosphatase are the most commonly used markers in chemiluminescence immunoassays. Chemiluminescence immunoassay has been widely used in clinical drug analysis, food detection and environmental analysis[8]. With the development of novel agents, labeling, labeling technology, the application of new solid carriers and the continuous use of technology.

CLIA analysis method is diverse, suitable for a wide range and has been applied to antigen, hapten and antibody immunoassay which is fast and effective. Its linear range is relatively wide and in line with clinical testing needs. CLIA has become an advanced trace or ultra-trace material detection technology. Its medicine, chemistry, biology and food testing has a broad application prospects. At present, CLIA has made some achievements in immunoassay, but it needs to be explored. The focus of attention is on improving the sensitivity and specificity of immunodiagnosis and developing new analytical techniques. CLIA technology development trend is to optimize the preparation of antibodies, synthesize new luminescent markers, improve labeling technology and the establishment of new immunoassay methods[9].

Materials and methods

Test material

Test reagents

Anti-human albumin monoclonal antibody (McAb3D4, McAb8H2) cell culture supernatant, anti-human albumin monoclonal antibody cell culture supernatant (negative control), mouse anti-human albumin antiserum (positive control), ELISA substrate (ALB) were purchased from Sigma, USA. Protein A and horseradish peroxidase (HRP) were purchased from Shanghai Xibao Company. Chemiluminescent end (A, B) by Tailun

Biotechnology Co., Ltd. Bovine serum albumin (BSA) was purchased from Hangzhou Jiangbin Biotechnology Co., Ltd. Chemiluminescent microplates were purchased from Xiamen Yunteng Biotechnology Co., Ltd.

Preparation of test reagents

(1) 0.02 mol / l pH7.2 Phosphate buffer (PBS) preparation: refer to the literature[10] preparation.

(2) Preparation of protein A enzyme conjugate (HRP-PA): 5 mg of horseradish peroxidase and protein A were weighed separately and prepared by simple sodium periodate method[11]. Add the same amount of neutral glycerol, set low temperature refrigerator frozen reserve.

(3) Anti-human albumin monoclonal antibody (clone 3B8) Preparation of IgG: Mouse ascites with anti-albumin monoclonal antibody (clone number 3B8, manufactured by our laboratory) 10 ml Reference method and DEAE52 column chromatography[11], confirmed by polyacrylamide gel electrophoresis> 95%, Coomassie brilliant blue method to determine the protein content with 0.02M pH7.2 PBS diluted to 200μg / ml, sub-installed, 4 ℃ refrigerator Standby[12].

Test equipment

KJ-20180-type chemiluminescence analyzer was purchased from Shijiazhuang Kangpu Biotechnology Co., Ltd. KJ-201A-type oscillator Jiangyan City Health Medical Equipment Co., Ltd. DNM-9602 ELISA analyzer Beijing Pulang New Technology Co., Ltd. (1μl / 10μl, 20-200μl) was purchased from Tai Long Medical Equipment Co., Ltd. Electronic analytical balance (1/000) was purchased from Shanghai Jingke Company.

Test methods

According to the literature and pre-test data, the proposed test parameters were grouped into 3 factors: A factor: ALB coating concentration (mg / L) was 0.3, 1.0, 3.0; B factor: HRP-PA dilution ratio was 1: 1000: 1: 2000, 1: 5000; C factor: incubation time (min) is 30,45,60. According to L9 (34) table for orthogonal design, random block arrangement. Nine sets of test parameters were obtained (see Table 1).

Table 1. L9 (34) table for orthogonal design, random block arrangement

Group	ALB Concentration (mg/L)	HRP-PA Dilution Ratio	Incubation Time (min)
1	0.3	1:1000	30
2	1.0	1;2000	30
3	3.0	1:2000	30
4	0.3	1:5000	45
5	1.0	1:5000	45
6	3.0	1:1000	45
7	0.3	1:2000	60
8	1.0	1:1000	60
9	3.0	1:5000	60

Luminescence analysis of microtiter plate coating

Take a removable 96-well microplate (12 x 8 holes). 410mg / L human serum albumin solution with 0.05mol / L pH 9.6 carbonate buffer were diluted to 0.3mg / L, 1.0mg / L, 3.0mg / L three concentrations, each concentration of the three Plate, add 100μl per well, 4 ℃ refrigerator overnight. Remove from the refrigerator with 0.01 mol / L pH 7.2PBST and washed for 3 times, each 2-3min, pat dry. Each well was added 1% BSA solution, 200μl / well per well, 37 ℃ water bath closed 2h. Remove from the water bath with 0.01 mol / L pH 7.2PBST and wash for 3 times, each 2-3min, pat dry. Held into the 37 ℃ oven dry, in the frame and slat blank paste logo. It will be coated with a good board and desiccant into the plastic bag, sealed and keep frozen.

Optimization of test parameters

(1) Open the quasi-automatic chemical luminous analyzer, according to Table 1 prepared test procedures. 1, 2, 3 group for the program 1 (incubation 30 minutes), 4,5,6 group for the program 2 (incubation 45 minutes), 7,8,9 group for the program 3 (incubation 60 minutes).

(2) Each group of slats is in accordance with Table 2 in different holes were added samples.

Table 2. Orthogonal test sample table (unit: μl)

Sample	Microplate number							
	A	B	C	D	E	F	G	H
Saline	50	50	-	-	-	-	-	-
Negative control	-	-	50	50	-	-	-	-
Positive control	-	-	-	-	50	50	-	-
Monoclonal antibody(3D4)	-	-	-	-	-	-	50	50

(3) Take the program 1 respectively, each slate, each first by Table 2, then add the same table 1 by adding different dilution ratio of HRP-PA to the slats, each hole are 50μl. Set the micro-oscillator mixed evenly into the luminous analyzer, the incubation, washing, plus substrate, read the hole luminescence value.

(4) Procedure 2 and Procedure 3 are the same as Program 1 operations.

(8H3) signal-to-noise ratio, signal-to-noise ratio = positive hole luminescence value / blank hole (physiological saline) luminescence value were calculated by the same method of each group.

(6) Establishment of test parameters: By comparing the luminous value and the SNR (monoclonal antibody / blank) of each group, the test parameters of the corresponding group of SNR were selected as the optimal test conditions of CLIA screening method and further methodological confirmation.

Evaluation of test methods

Establishment of standard operating procedures

The parameters were optimized by the orthogonal test, and the HRP-PA was programmed as a test specification step.

Sensitivity and Linear Range Analysis

The purified 200 mg / L anti-albumin monoclonal antibody (3B8) IgG was diluted to 200 ng / L with 0.02 mol / L pH 7.2 PBS and then diluted 4 times in 10-fold ratio at 200.00, 20.00, 0.20,0.0 ng / L and at the same time to do five negative control respectively. The standard operating procedures were measured the luminous value of each tube. The average luminous value of 2.1 is the CUT-OFF value and the sensitivity of the CUT-OFF value is higher than the CUT-OFF value. At the same time, the anti-albumin monoclonal antibody IgG concentration is Abscissa draws the standard curve. After logarithmic fitting, the two points corresponding to the straight line are the linear range of the test and the correlation coefficient is calculated.

Precision analysis

(1) Anti-human albumin monomer (McAb8H2) cell culture supernatant was used as a high concentration of 0.02 mol / L pH 7.2 PBS diluted 1:10 as a high concentration sample and then diluted 1: 1000 times as a low concentration specimen.

(2) Standard deviation (S) and coefficient of variation (CV) of the high and low concentration samples were calculated for 10 times, and the above high and low concentration samples were continuously measured 10 times in the same batch.).

(3) Inter-assay variation: the high and low concentrations of specimens were divided into five batches, each batch of 3 tubes, calculate the number of X per batch and then the batch between the X, S and CV.

Statistical analysis

The statistical analysis and mapping of this experiment are SPSS 17.0 statistical software.

Test results and discussion

Orthogonal test results

Orthogonal test: The average value of each group of test results in Table 1, the signal to noise ratio of each group into a histogram (Figure 2) easier to analyze. From the table 3, the maximum value of the monochromatic (3D4) luminescence value and the signal-to-noise ratio is the third group, and the maximum value of the positive control luminescence value is in the 8th group, but the maximum SNR is consistent with the monoclonal antibody. This may be due to the fact that the positive control is antiserum, which differs from monoclonal antibody as a single IgG subclass, but contains different subclasses of IgG, because the protein A is different from the different subclasses, resulting in inconsistencies.

Table 3. Analysis of orthogonal test results

Group	Blank Luminescent value	Luminescent monoclonal antibody (3D4)			Positive control	
		Luminous value	Signal to noise ratio		Luminous value	Signal to noise ratio
1	2124	60902	28.7		99569	46.9
2	302	187818	621.9		54818	181.59
3	296	379646*	1284.0*		66192	223.9*
4	257	5566	21.7		34732	135.1
5	332	38123	114.8		31690	95.5
6	531	143969	271.1		48940	92.1
7	687	20339	29.6		113992	165.8
8	1261	289474	229.6		163364*	129.6
9	287	85068	574.6		65013	29.9

Combined with the experimental parameters in Table 1 and Table 3, the results of comprehensive analysis of the test results with the HRP-PA dilution ratio decreased, the incubation time is increased without increasing the detection of the sample luminescence value, thus reducing the signal to noise ratio. This also reflects the general law of immunological reactions which is only in the antigen and antibodies in the appropriate ratio in order to make the most complete reaction. It also shows the necessity and importance of the optimization of the experimental parameters.

The purpose of cloning screening is to find out the positive clones in the near future, so the signal-to-noise ratio is a key indicator to determine the merits of the screening method. The maximum value of the signal-to-noise ratio of this test, whether the multi-resistance or monoclonal antibody is the third group especially the monoclonal antibody up to 1284, several times higher than the other groups (Figure 3), so the third group of parameters for the optimization combination. The corresponding test parameters of 3.0 mg / L human serum albumin package, HRP-PA dilution ratio of 1: 2000 and incubation time of 30min. Establish a standard operating procedure for cloning screening.

Methodological evaluation results

Sensitivity analysis

Sensitivity analysis results in Table 4, negative control does not contain anti-human albumin specific IgG, calculate the average luminous value of 865.4, CUT-OFF value = 865.4 × 2.1 = 1817.3. From Table 4, we can see that the corresponding luminescence value (1903) is slightly larger than the CUT-OFF value (1817.3) when the anti-human albumin monoclonal antibody IgG concentration is 0.02 ng / L. Therefore, the sensitivity of the test method is 0.02 ng / L. Indicating that CLIA sensitivity than the traditional ELISA method 2-3 orders of magnitude. It can be used for positive clonal screening, can be detected in the early cell culture, shorten the monoclonal antibody preparation cycle.

Precision analysis

(1) The results of the variation in the batch are shown in Table 5. The intra-assay variation of the high and low concentration samples is about 5%, the precision is better at high concentration, and the average CV is 5.32%.

(2) The results of the inter-assay variation are shown in Table 6. The results showed that the

Table 4. Sensitivity and linear analysis results

Sample number	Anti-human albumin monoclonal antibody IgG		Negative control	
	Concentration (ng / L)	Luminous value	Concentration (ng / L)	Luminous value
1	0.02	1903	0	865
2	0.20	3954	0	902
3	2.00	9286	0	884
4	20.00	22878	0	795
5	200.00	426394	0	881

Table 5. Precision analysis within the batch

Sample type	Sample Luminous Value										X	S	CV
	1	2	3	4	5	6	7	8	9	10			
Low concentration	2041	2244	2207	2436	2148	2461	2209	2341	2098	2289	2247.4	137.4	6.10%
High concentration	14026	12336	12461	13254	13331	13203	13312	12711	12025	13002	12966.	588.7	4.53%

Table 6. Inter-batch precision analysis

Sample type	Test Batch Average Luminous Value					X	S	CV
	1	2	3	4	5			
Low concentration	2200	2356	1948	2320	2469	2258.6	198.4	8.78%
High concentration	11902	13756	13208	11790	14485	13028.2	1171.1	8.86%

variation between the high and low concentrations was similar, with an average CV of 8.8%.

Comprehensive analysis of the above results, CLIA technology through the use of protein A enzyme conjugate as a secondary antibody, one step to complete the test can be used with anti-albumin monoclonal antibody cloning screening which is simple, fast, sensitive and reliable. The optimum screening parameters were 3.0 mg / L human serum albumin package, HRP-PA dilution ratio was 1: 2000, incubation time was 30 min.

This test is based on the orthogonal test to optimize the parameters established by the standard operating procedures, sensitive, accurate and reliable. Regardless the detection of monoclonal antibody culture supernatant, polyclonal antiserum, or purified antibody IgG showed a lot of advantages of CLIA technology, this test can be used for anti-albumin monoclonal antibody cloning screening, while other monoclonal antibodies Cloning screening also has reference. (1) The test uses the automatic instrument to detect and improve the accuracy of the test while also excluding the manual operation may introduce the error. (2) The test is characterized by the use of HRP-PA (1). The use of HRP-PA As a secondary antibody and it is possible to complete the test in one step, simplify the operation procedure, shorten the reaction time and reduce the error introduced by the complicated process. However, protein A in the case of low salt with different IgG subclass affinity difference. The impact of weak affinity antibody screening remains to be further studied.

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