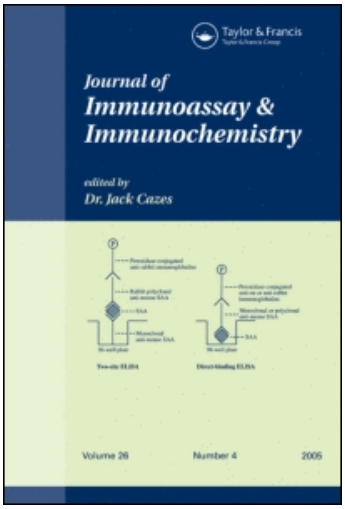

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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To cite this Article Gill, Pooria , Forouzandeh, Mehdi , Rahbarizadeh, Fatemeh , Ramezani, Reihaneh and Rasaee, Mohammad Javad(2006) 'Production of Anti-Digoxigenin Antibody HRP Conjugate for PCR-ELISA DIG Detection System', Journal of Immunoassay and Immunochemistry, 27: 4, 303 — 318
To link to this Article: DOI: 10.1080/15321810600861910
URL: http://dx.doi.org/10.1080/15321810600861910

Journal of Immunoassay & Immunochemistry, 27: 303–318, 2006

ISSN 1532-1819 print/1532-4230 online
DOI: 10.1080/15321810600861910

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Abstract: There are several methods used to visualize the end product of polymerase chain reactions. One of these methods is an ELISA-based detection system (PCR-ELISA) which is very sensitive and can be used to measure the PCR products quantitatively by a colorimetric method. According to this technique, copies of DNA segments from genomic DNA are amplified by PCR with incorporation of digoxigenin-11-dUTP. Samples are analyzed in a microtiter plate format by alkaline denaturation and are hybridized to biotinylated allele-specific capture probes bound to streptavidin coated plates. Use of the produced anti-digoxigenin antibody horseradish peroxidase conjugate and the substrate 2,2′-azino-di-3-ethylbenzthiazolinsulfonate (ABTS) detected the hybridized DNA. One of the key components in this procedure is the anti-digoxigenin antibody HRP conjugate. Described here is the preparation, purification, and characterization of anti-digoxigenin antibody HRP conjugate for use in the PCR-ELISA DIG detection system. Several biochemical protocols and modifications were applied to increase the sensitivity and specificity of this conjugate for an efficient and cost-effective product.

Keywords: Digoxigenin-BSA conjugate, Anti-digoxigenin antibody HRP conjugate, PCR-ELISA

INTRODUCTION

The polymerase chain reaction (PCR) is one of the most used molecular biology techniques; it is employed for the amplification of nucleic acid

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sequences.[1] There are several procedures with different sensitivities for the detection of the PCR products. A combination of PCR amplification with allele-specific hybridization in a microtiter plate format, using a colorimetric ELISA-based detection system, PCR-ELISA, was developed which provides highly sensitive and quantitative detection of PCR products.[2,3] By this method, specific detection of microorganisms and mutant gene alleles is possible.[4–6] But, there are several reports that the test is expensive.[4,5] To overcome this problem, we planned to produce the main components of the kit in our laboratory, so as to reduce the cost of the test to a reasonable amount for its routine use. In this study, we prepared, purified, and characterized a key component of the PCR-ELISA DIG detection kit, i.e., the anti-DIG antibody HRP conjugate.

EXPERIMENTAL

Conjugation of Digoxigenin-3-O-methylcarbonyl-ε-aminocaproic Acid N-Hydroxysuccinamide Ester to Bovine Serum Albumin

Bovine serum albumin (BSA) was selected as a carrier protein for immunogen preparation and the digoxigenin-BSA conjugate was thus prepared.[7] The molar reaction mixture of BSA to digoxigenin-3-O-methylcarbonyl-ε-aminocaproic acid N-hydroxysuccinamide ester was 1:70. 4.62 mg of BSA powder (Sigma) was dissolved in 15 mL of phosphate buffered saline (PBS), pH 8.5, and cooled in an ice bath for 30 min with gentle stirring. 3.27 mg of digoxigenin-3-O-methylcarbonyl-ε-aminocaproic acid N-hydroxysuccinamide ester (Roche) was dissolved in 8.18 mL of dimethylsulfoxide (DMSO, Sigma) and incubated for 30 min in a dark room, with stirring. The reaction mixture was added, dropwise, to a stirred bovine serum albumin solution in an ice bath during 4 h. The mixture was incubated at room temperature for 24 h in a dark room with gentle stirring. To remove the unreacted digoxigenin-3-O-methylcarbonyl-ε-aminocaproic acid N-hydroxysuccinamide ester and the solvent, the digoxigenin-BSA conjugate solution was dialyzed against PBS buffer (pH 7.0–7.2) for 24 h at 4°C with stirring. 200 μL of this conjugate was mixed with 800 μL of glacial sulfuric acid and the optical absorption spectrum was recorded at wavelengths from 340 nm to 450 nm.[8] The final concentration of digoxigenin-BSA conjugate was measured.[9]

Qualitative Analysis of the Prepared Digoxigenin-BSA Conjugate by Uncompetitive ELISA

Wells of microtiter plates were coated with 5 μg/100 μL and 10 μg/100 μL of digoxigenin-BSA conjugate (in PBS, pH 7.0–7.2, overnight at 37°C) and

blocked with 5% skimmed milk (in PBS, pH 7.0–7.2, for 1 h at 37°C).[10] Similarly, microtiter plates were coated with 5 μg/100 μL and 10 μg/100 μL of BSA and blocked as a non-specific binding index, simultaneusely. Two dilutions (1:1,500 and 1:3,000) of anti-digoxigenin antibody HRP conjugate (Roche) were prepared in an universal conjugate buffer (Roche) and 100 μL from each of the dilutions was added to each well. The wells were washed 5 times with phosphate buffer, pH 7–7.2. The color was developed with 100 μL/well of ABTS substrate solution (Roche) after 30 min at 37°C and measured at 405 nm with multi channel ELISA reader (Tecan).[4,5,10]

Dosage of Immunogen and Timing of Injections

150 μg/mL of the immunogen (digoxigenin-BSA conjugate) was combined with an equal volume of complete Freund's adjuvant and injected intradermaly into a male-deutsche rabbit of 1 kg weight. The booster immunizations were first administered between 90 days, at 2 week intervals, with incomplete Freund's adjuvant and, then, during 90 days at 20 day intervals, intramuscularly, with 1/2 initial dosage of immunogen. Antisera titeration was started 5 days after the third immunization and continued 5–6 days after every antigen injection. The blood samples were centrifuged at 4°C and the sera were collected and stored at −70°C.[11]

Titration of Rabbit Anti-Digoxigenin Antibodies of Whole Serum

Titration assay was performed by ELISA.[12] For this purpose, 16 wells of a microtiter plate were coated with 0.5 μg/100 μL of digoxigenin-BSA (in PBS, pH 7.0–7.2, overnight at 37°C) and blocked with 5% skimmed milk in PBS, pH 7.0–7.2, for 1 h at 37°C. In another 16 wells of the microtiter plate, as a non-specific binding index, 0.5 μg/100 μL of BSA was coated. Serial dilutions of whole serum of the rabbit were prepared in the universal conjugate buffer (Roche) and 100 μL of each dilution was added to each well. The wells were washed 5 times with phosphate buffer (pH 7.0–7.2) and 100 μL of diluted (1:2,000) mouse anti-rabbit antibody HRP conjugate (Roche) was added to each well and incubated for 30 min at 37°C. At the end of the incubation time, the wells were washed and 100 μL of substrate (ABTS) was added to each well and incubated for 30 min at 37°C. The color was measured at 405 nm.[4,5,10]

Purification of Anti-Digoxigenin Antibodies

An immunized animal was bled and blood was collected in a clean, dry glass bottle and allowed to clot at 4°C overnight until the clot was retracted. It

helped to ring the clot with a glass rod to promote separation. The sample was then centrifuged and the serum was separated immediately. For the preparation of immunoglobulins from the whole serum, an equal volume of saturated ammonium sulfate solution (pH 8) was added to the sample, dropwise, and stirred gently on a cold magnetic stirrer.[12] After all of the ammonium sulfate solution was added, the mixture was placed at 4°C for at least 1 h to allow the immunoglobulin to precipitate, then centrifuged at 10,000 × g for 30 min. The precipitate was dissolved in a volume of PBS approximately equal to half the volume of the original antiserum and dialyzed against PBS for 2 days at 4°C. In order to prevent non-specific binding with streptavidin and biotin-conjugated BSA, further purification of antibodies was performed by passing the product through the affinity columns of streptavidin and biotin-conjugated BSA. The immunoglobin fraction collected from the above columns was further purufied by protein A affinity chromatography.[13] The eluted antibodies from the protein A column were collected and dialyzed against PBS at 4°C overnight.

Preparation of Rabbit Anti-Digoxigenin Immunoglobin Conjugate to Horseradish Peroxidase

The purified anti-digoxigenin antibodies were conjugated to horseradish peroxidase.[14] Brifely, 7.5 mL of anti-digoxigenin antibody solution (0.5 mg/mL, measured by Bradford assay) was dialyzed against 1 L of 0.1 M carbonate buffer, pH 9.2, overnight, at 4°C with gentle stirring. 6.6 mg of horseradish peroxidase (Sigma) was dissolved in 500 μL of 0.1 M carbonate buffer, pH 9.2. 500 μL of freshly prepared sodium periodate solution (1.71 mg/mL) was added dropwise to the enzyme solution with gentle stirring, capped tightly, and incubated for 2 h at room temperature in the dark. The dialyzed antibody solution was added, dropwise, to the prepared enzyme solution during 4 h, under gentle stirring, in the dark. 8 mL of the antibody-enzyme mixture was added to a 20 cm × 3 cm^2 column fitted with glass wool and blocked with parafilm. Then, 2 g of Sephadex G-25 was added to the column and incubated for 3 h at room temperature, in the dark. The column was eluted with 0.1 M carbonate buffer until the eluent became clear. A 1:20 volume of freshly prepared $NaBH_4$ (5 mg/mL in 0.1 mM NaOH) was added to the eluted solution and 30 min later another volume (1:10) of freshly prepared $NaBH_4$ solution was added to the latter and incubated for 1 h at 4°C. An equal volume of saturated ammonium sulfate solution (pH 8) was added to the above mixture and stirred gently for 30 min at 4°C.[12] The mixture was centrifuged for 15 min (10,000 × g at 4°C) and the supernatant was discarded. The pellet was resuspended in 2.5 mL of TEN buffer (Tris/EDTA/NaCl buffer, pH 7.2) and dialyzed against 3 L of TEN buffer for 24 h at 4°C. After 24 h, the TEN solution

was changed and the dialysis was continued for another 4 h. Subsequently, 20 mg/mL of BSA and 20% glycerol were added to it and stored at 4°C.[14]

Characterization of Anti-Digoxigenin-HRP Conjugate

Preparation of Checkerboard of Anti-Digoxigenin-HRP Conjugate

A checkerboard assay was performed by ELISA, with minor modifications.[10] Briefly, different concentrations of digoxigenin-conjugated BSA were coated onto the wells of a microtiter plate and blocked with 5% skimmed milk. Several concentrations of BSA were also coated and blocked with skimmed milk in a microtiter plate, to serve as a non-specific binding. Serial dilutions of anti-digoxigenin-HRP (our prepared conjugate and Roche Co. conjugate) were prepared in the universal conjugate buffer and 100 μL of each dilution was added to each well. The wells were washed 5 times with phosphate buffer. The color was developed with 100 μL/well ABTS substrate solution after 30 min at 37°C and measured at 405 nm.

Average Affinity Measurement of Anti-Digoxigenin-HRP

A competitive ELISA assay was performed for measurement of average affinity of two anti-digoxigenin-HRP conjugates.[15,16] Different cocentrations of free digoxigenin ligand molecules (0, 0.1, 1, 10, 100, 1,000 ng/well) were added to the semisaturated concentrations of anti-digoxigenin antibodies and coated DIG-BSA conjugates in competitive ELISA assays. After 1 h at 37°C, the following steps were performed according to the previously described protocols.

Bacterial Strain

Mycobacterium tuberculosis H37Rv (RIVM) was cultured on Löwenstein-Jensen medium at 37°C for 21 days.

Isolation of DNA

Mycobacterial DNA was isolated by a boiling procedure.[17] A single colony of bacteria was diluted in 150 μL Tris-EDTA buffer (10 mM Tris-base, 1 mM EDTA, pH 8) and vortexed vigorously, then incubated in a 95°C water bath for 15 min; the lysate was centrifuged at 10,000 × g for 10 min. The supernatant, which contained the DNA, was seperated and used for the polymerase chain reaction.

Oligonucleotides

The following oligomers were synthesized (M. W. G. Biotech GmbH, Germany) and used for amplification, labeling, and colorimetric detection of 200 bp of TUF gene:[18]

TUF15 (5′CCTGGTGGTCGATGGGCGA 3′)
TUF18 (5′CCTCTGTCGAGGAACTGATGA 3′)
5′biotin-modified TUF26 (5′ACGAGGAAGTTGAGATCG 3′)

DIG-Labeling PCR

Each reaction contained 3 μL of the lysate DNA. The reaction mixture consisted of 25 μM TUF15 and TUF18 primers, 1.25 U Taq DNA polymerase (Roche), 1X reaction buffer (Roche), 1.5 mM $MgCl_2$, 2 mM dATP, dCTP, dGTP, 1.9 mM dTTP and 0.1 mM DIG-dUTP (Roche). The cycling reaction was performed as follows: 1 cycle of 5 min 95°C, 30 cycles of 30 sec 94°C, 30 sec 66°C, 1 min 72°C, and 1 cycle of 10 min 72°C using an Eppendorf gradient master cycler.[19]

DIG-Detection ELISA Checkerboard

The PCR-ELISA checkerboard was performed according to the protocols received from Roche Applied Sciences. The produced anti-digoxigenin-HRP was used and compared with the Roche anti-digoxigenin-HRP (as a standard control).

2.5 μL of DIG-labeled PCR product was added to 10 μL of denaturing solution (Roche) and the mixture was incubated at room temperature for 10 min. 112.5 μL of hybridization solution (Roche), containing 5 μM biotin-modified TUF26 probe, was mixed with the denatured DIG-labeled PCR product and 100 μL of the mixture was transferred to a microtiter streptawell (Roche) immediately. In order to increase the stringency of the probe attachment to the single stranded DNAs, the incubation was performed at 53°C in a hybridyzer oven (Techne), for 75 min, with gentle shaking. Non-specific binding was measured by adding an equal volume of distilled water instead of denatured PCR product to the hybridization solution containing the probe. The wells were washed 5 times with washing solution (Roche). 100 μL of different dilutions (1:100, 1:200, 1:400, 1:800, 1:1,000 and 1 : 2,000) of anti-digoxigenin HRP conjugate in conjugate buffer (Roche) was added to each well and incubated at 37°C for 30 min. The wells were washed 5 times with washing solution. Then, 100 μL of ABTS substrate solution (Roche) was added to each well and treated as explained previously.[19,20]

RESULTS AND DISCUSSION

Conjugation of Digoxigenin-3-O-methylcarbonyl-ε-aminocaproic Acid N-Hydroxysuccinamide Ester to Bovine Serum Albumin

Absorbance at 380 nm was used to calculate the amount of digoxigenin derivative coupled with bovine serum albumin, assuming that the millimolar extinction coefficient at 380 nm for the digoxigenin derivative is 3.2. The hapten:protein molar ratio was 8.5 (Figure 1) and the concentration of the conjugate was 100 μg/mL.

Qualitative Analysis of the Prepared Conjugate by Uncompetitive ELISA

The results obtained from a noncompetitive ELISA test showed that the wells that were coated with digoxigenin-BSA conjugate have higher ODs than BSA coated wells (Table 1).

Immunization and Anti-Digoxigenin Antibody Titration

Eight weeks after the initial immunization, antisera with suitable titers were obtained. The titers of anti-digoxigenin antibody in rabbit sera were 9,600,

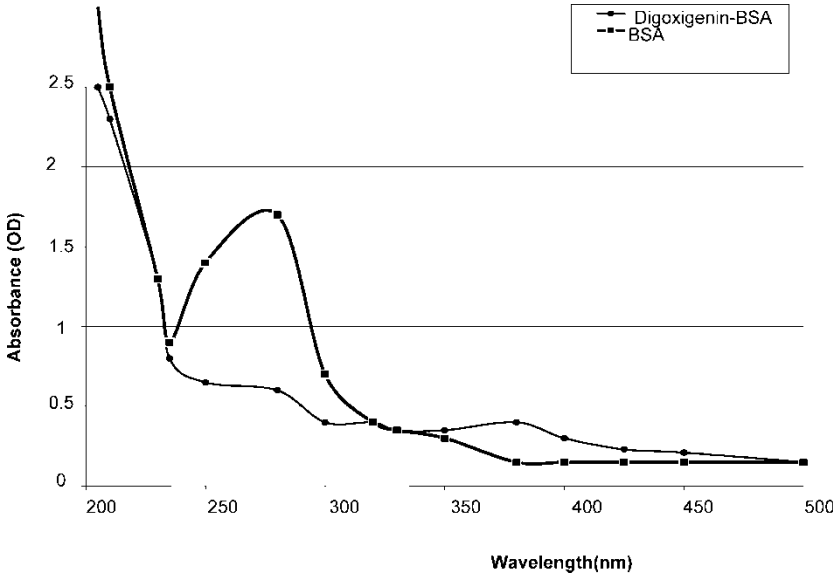

Figure 1. Optical absorption spectrum of digoxigenin-BSA conjugate. Molar ratio of hapten:carrier protein = OD_{380} of conjugate/OD_{380} of carrier protein × 3.2.

Table 1. Uncompetitive ELISA test for qualitative analysis of digoxigenin-BSA conjugation

Anti-DIG-HRP conjugate dilutions	DIG-BSA conjugate (5 μg/100 μL)	BSA (5 μg/100 μL)	DIG-BSA conjugate (10 μg/100 μL)	BSA (10 μg/100 μL)
1 : 1500	2.07 ± 0.2	0.22 ± 0.1	2.20 ± 0.2	0.23 ± 0.1
1 : 3000	3.42 ± 0.2	0.22 ± 0.1	3.67 ± 0.2	0.24 ± 0.1

The results are expressed in term of optical density.
All assays were performed in duplicate.

64,000, 128,000, 128,000, 192,000, 192,000, 192,000, and 192,000 at 50, 65, 80, 95, 105, 125, 145, and 165 days after primary immunization, respectively (Figure 2).

Characterization of Anti-Digoxigenin-HRP Conjugate

Checkerboard of Anti-Digoxigenin-HRP Conjugate

The result obtained from checkerboard for the prepared anti-digoxigenin antibody HRP conjugate was 1 : 6,400 dilution for 50% binding to 13.8 ng/well of digoxigenin-conjugated BSA (Figure 3). The result for Roche HRP-conjugated anti-digoxigenin showed 1 : 200 dilution for 50% binding to 13.8 ng/well of digoxigenin-conjugated BSA (Figure 4).

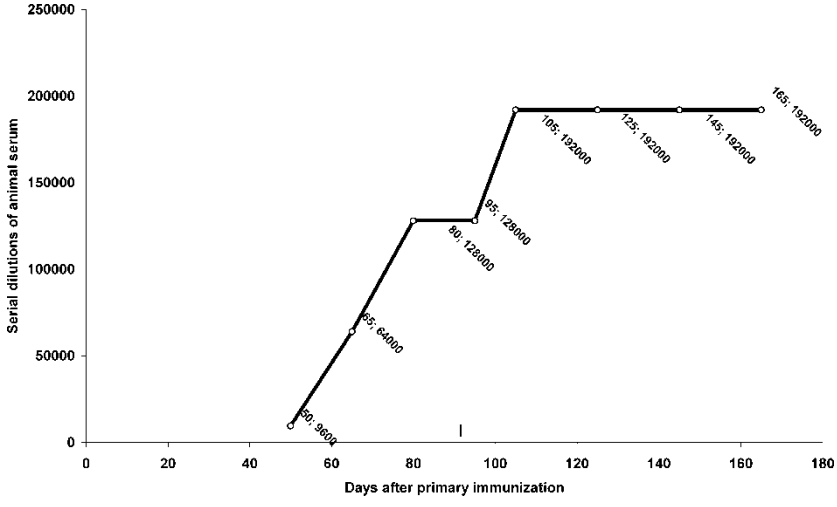

Figure 2. Titers of antibody in immunized-rabbit sera.

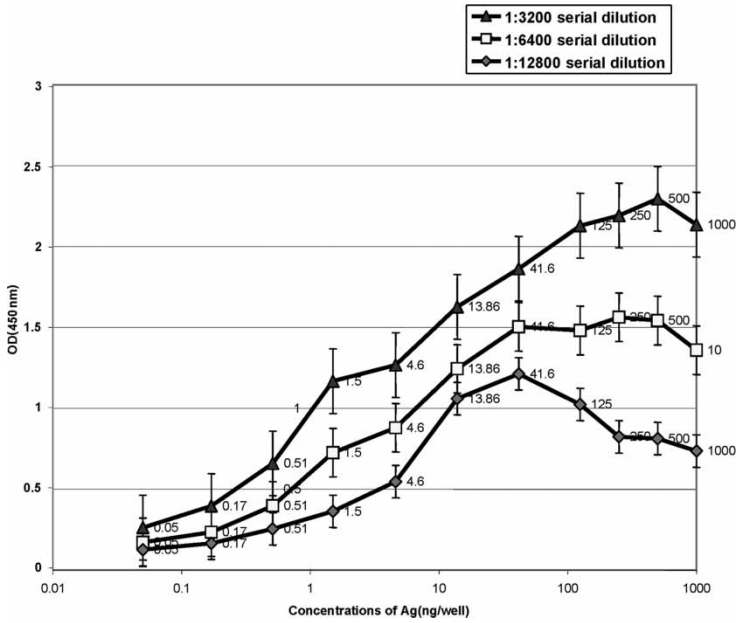

Figure 3. Checkerboard of anti-digoxigenin HRP conjugate prepared in this research work.

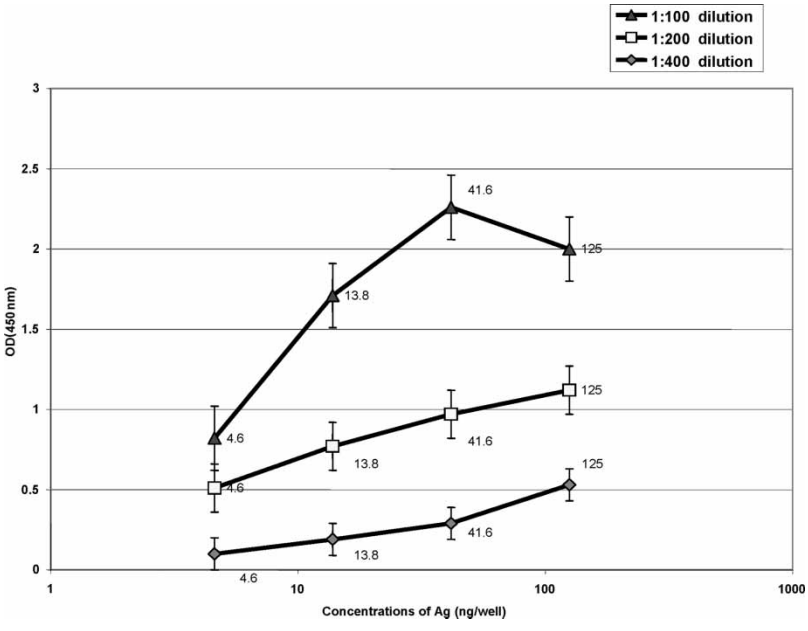

Figure 4. Checkerboard of anti-digoxigenin HRP conjugate(Roche).

Standard Competitive Curves and Average Affinity Measurement

According to the optical density measurements of competitive standard ELISA assays, the standard curves (Figure 5) and logit-log transformation plots (Figures 6 and 7) of anti-digoxigenin antibody HRP conjugates were plotted. The average affinities for the anti-DIG HRP conjugate obtained from the Roche Company and our conjugate were found to be $1.8 \times 10^{10}\ M^{-1}$ and $1.5 \times 10^{10}\ M^{-1}$, respectively (Figures 8 and 9).

DIG-Detection PCR- ELISA Checkerboard

The optical densities belonging to serial dilutions of both anti-digoxigenin antibody HRP conjugate are shown in Table 2. These values reveal that the highest dilution of our prepared antibody HRP conjugate has an optimum OD in the DIG-detection PCR- ELISA system; however, the 1 : 200 dilution of the Roche conjugate is the final optimum OD of the test; it may have been made accordingly by the Roche Co.

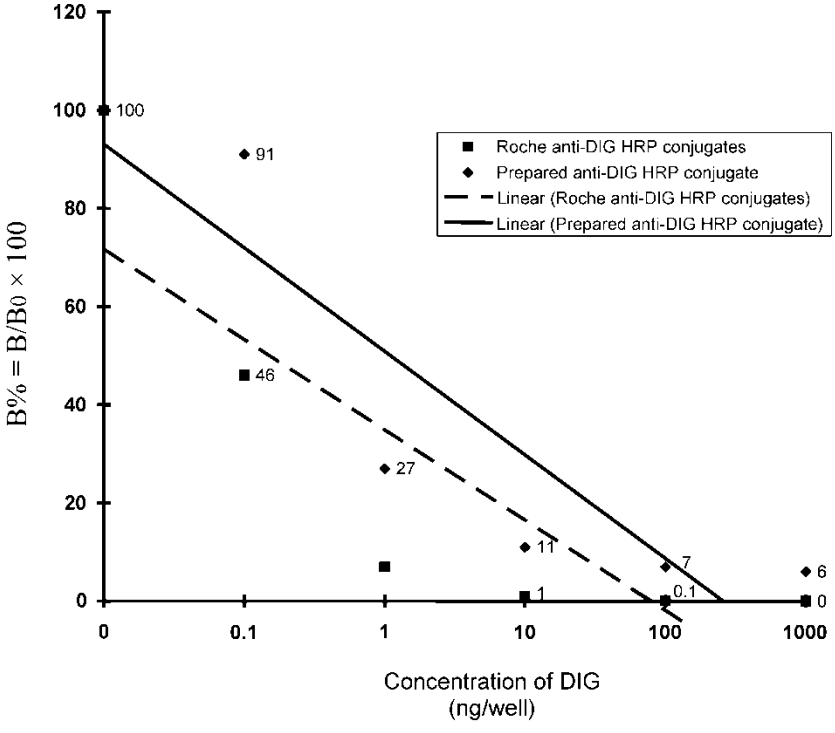

Figure 5. Competitive standard curves of anti-DIG HRP conjugates.

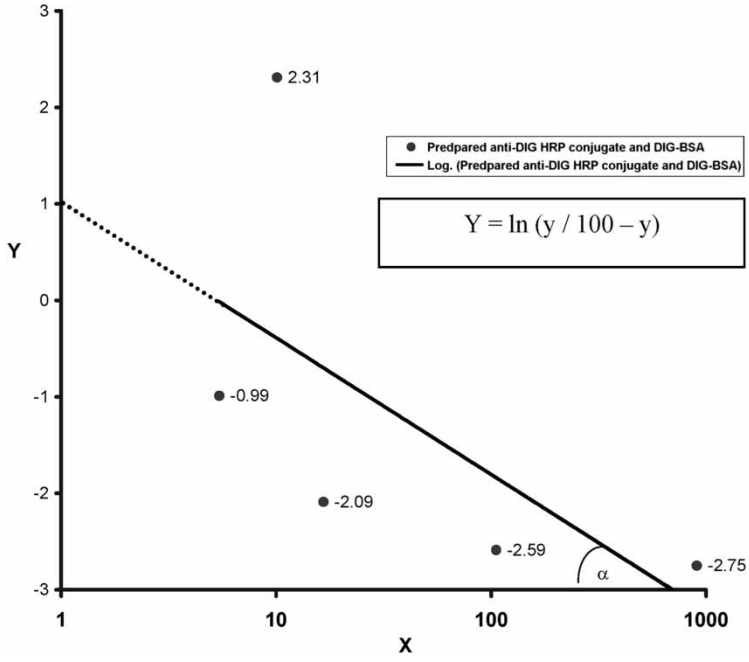

Figure 6. Logit-log transformation plot of our prepared anti-DIG HRP conjugate and DIG-BSA. y: B%; X: B (nM).

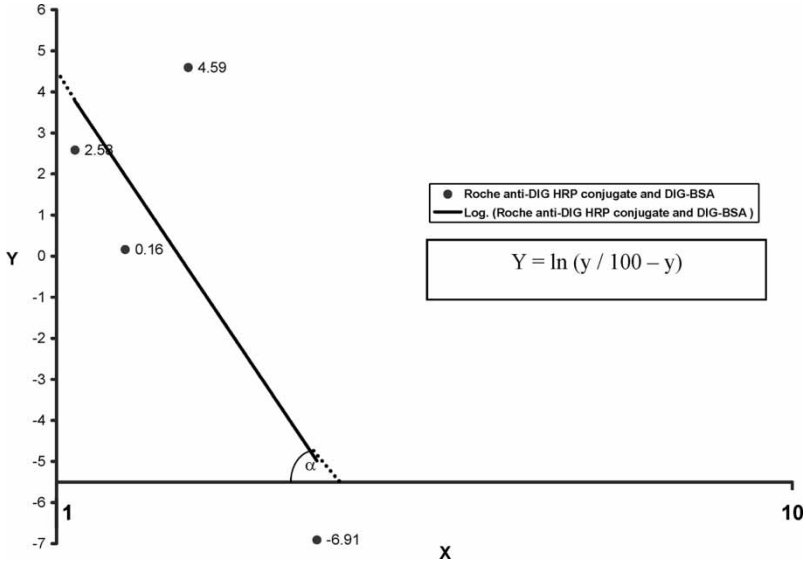

Figure 7. Logit-log transformation plot of Roche anti-DIG HRP conjugate and DIG-BSA. y: B%; X: B (nM).

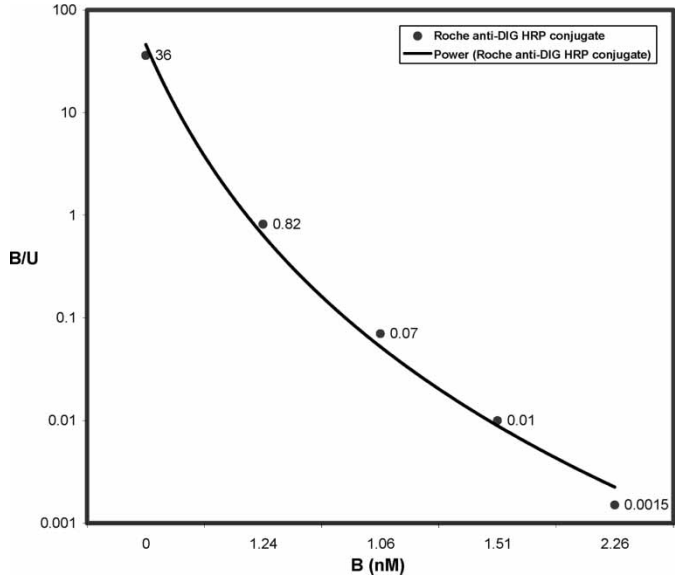

Figure 8. Skatchard plot of Roche anti-DIG HRP conjugate and DIG-BSA. B/U = (B−NSB)/T−(B−NSB). B (nM) = B/U × Molar concentration of Digoxigenin (nM). Ka = Y-intercept/X-intercept.

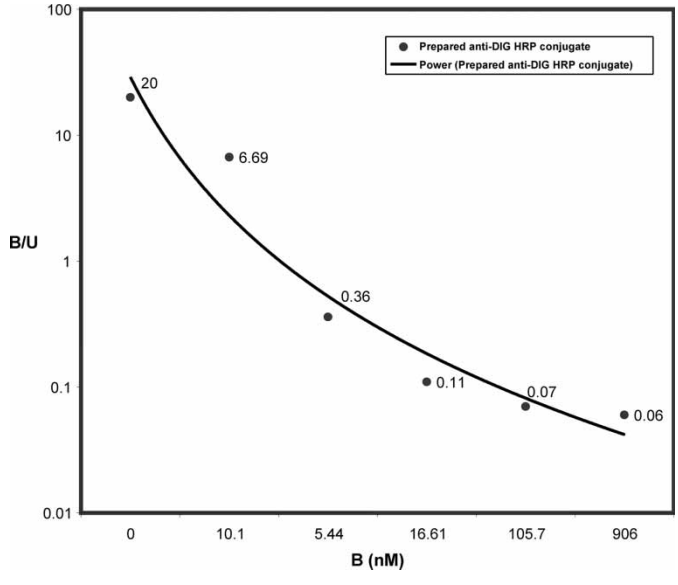

Figure 9. Skatchard plot of prepared anti-DIG HRP conjugate and DIG-BSA. B/U = (B-NSB)/T-(B-NSB). B (nM) = B/U × Molar concentration of Digoxigenin (nM). Ka = Y-intercept/X-intercept

Table 2. DIG-detection PCR-ELISA checker board

Anti-digoxigenin antibody HRP conjugate	Serial dilutions of anti-digoxigenin-HRP conjugate											
	1 : 100		1 : 200		1 : 400		1 : 800		1 : 1,000		1 : 2,000	
	PCR product	NSB	PCR product	NSB	PCR product	NSB	PCR product	NSB	PCR product	NSB	PCR product	NSB
Roche anti-digoxigenin-HRP conjugate	2 ± 0.2	0.2 ± 0.1	1.1 ± 0.2	0.1 ± 0.1	0.6 ± 0.2	0.0 ± 0.1	0.4 ± 0.2	0.0 ± 0.1	0.2 ± 0.2	0.0 ± 0.1	0.1 ± 0.2	0.0 ± 0.1
Our prepared anti-digoxigenin-HRP conjugate	2.9 ± 0.2	0.4 ± 0.1	2.7 ± 0.2	0.3 ± 0.1	2.5 ± 0.2	0.3 ± 0.1	2.3 ± 0.2	0.2 ± 0.1	2.0 ± 0.1	0.2 ± 0.1	1.8 ± 0.2	0.1 ± 0.1

The results are expressed in term of optical density.

All assays were performed in duplicate.

CONCLUSION

Enzyme-linked immunosorbent assay (ELISA) is a widespread laboratory test that can be used for the qualitative and quantitative detection of specific regions of target DNA, so named PCR-ELISA.[2] It could have many applications in molecular diagnosis of pathogenic microorganisms and mutation detection of genetic disorders, including SNPs, provided the materials used for the test become cost effective. In many publications, it has been reported that the PCR-ELISA is an expensive method[2,4,5] because all of the reagents used in PCR-ELISA must obtained commercially. There are simplified protocols that can be used, with some modifications, for preparing most of the reagents involved in this test. One of the key components of this system is the anti-digoxigenin antibody HRP conjugate, which has been prepared from a rabbit source in this study. Procedures for preparing the other components (such as denaturation solution, hybridization buffer, washing buffer, and conjugate buffer) have been reported previously.[6,21]

According to the results obtained, the rabbit immunized against DIG-BSA conjugate raised a high titer antiserum. So, the dosage of the prepared immunogen and the timing of injections have been performed efficiently. The results of ELISA checkerboards for two enzymatic conjugates revealed that the two dilutions (1 : 6,400 of prepared anti-DIG-HRP and 1 : 200 of Roche material) of both anti-DIG-HRP conjugates gave equivalent results and were suitable for average affinity comparisions. Subsequently the standard curve and affinity measurements showed that the average affinity of the produced antibodies was suitable for preparing an effective enzymatic conjugate for use in PCR-ELISA tests. The PCR-ELISA checkerboard results showed that the prepared anti-digoxigenin-HRP conjugate could give equivalent results at higher dilutions, when compared with a commercially available conjugate.

In this study, from 5 mL of antiserum, we could produce an amount of anti-DIG HRP conjugate which is sufficient to perform 4,000 PCR-ELISA tests, with a much lower budget than could be spent for an equal number of commercially available kits. Therefore, the production of high quality anti-digoxigenin antibody HRP conjugate with specifications that could match the requirement of a PCR-ELISA test is possible to be prepared in any laboratory with basic facilities.

ABBREVIATIONS

DIG, digoxigenin; OD, optical density; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; DNA, deoxyribonucleic acid; DMSO, dimethyl sulfoxide; BSA, bovine serum albumin; HRP, horseradish peroxidase; ABTS, 2,2′-azino-di-3-ethylbenzthiazolinsulfonate; PBS, phosphate buffer saline; TEN, Tris/EDTA/NaCl; EDTA, ethylen diamine

tetraacetic acid; RIVM, the national institute for public health and the environment, the Netherlands; dATP, deoxyadenosine triphosphate; dGTP, deoxyguanosine triphosphate; dCTP, deoxycytidine triphosphate; dTTP, deoxytymidine triphosphate; dUTP; deoxyuridine triphosphate; NSB, non-specific binding index.

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Received January 12, 2006
Accepted February 14, 2006
Manuscript 3193