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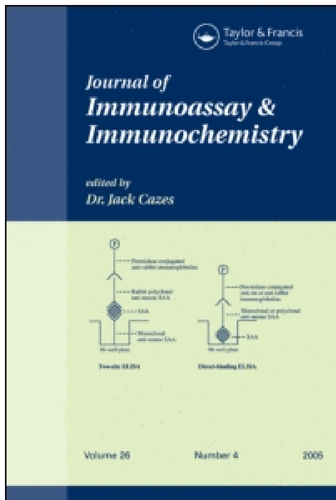

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Substitution of Carbonate Buffer by Water for IgG Immobilization in Enzyme Linked Immunosorbent Assay

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ABSTRACT

The first step of enzyme linked immunosorbent assay (ELISA), namely, adsorption of antigen or antibody to the plastic microtiter well plate, was studied as a function of insolubility of IgG in water. Immobilization efficiency was assessed in terms of number of wells

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coated per milliliter of primary antiserum. We have compared different coating/immobilization protocols, i.e., direct and indirect immobilization of primary antibody to the plastic microtiter well plate using carbonate buffer and phosphate buffer with glutaraldehyde. We have observed efficient coating when the immobilization of primary antibody through an immunobridge technique was performed, where water was used as a coating medium. It gave a higher number of wells coated per milliliter of anti-serum (primary or secondary) than other compared coating protocols and it allowed the use of serum (non-immune) and anti-serum (primary and secondary antibody) dilutions, avoiding the need for γ-globulin purification from normal and immunized serum.

Key Words: Immobilization; Coating; Immunoassay; Cortisol; IgG; ELISA.

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INTRODUCTION

The acronym, ELISA, coined by Engvall and Perlmann,[1] has become a standard abbreviation in many scientific journals. ELISAs are immunoassays in which one immuno-reactant is immobilized on a solid phase and the "signal generator" or "reporter" is an enzyme; it is one of the most widely used techniques for the study of low level analytes in biological fluids, which is often preferred to radioimmunoassay. Although many variations have been introduced into the original method with respect to the number of stages or nature of analyte under study (antigen or antibody), these assays always include a preliminary step corresponding to immobilization of antigen or antibody.

The simple passive adsorption that is the recognition that the antibodies could be directly coated onto plastic tubes was a major milestone in immunoassay development.[2] The proteins form the majority of molecules that immobilized to solid phase, either by passive adosrption or by covalent coupling. While devising ELISA for steroid hormones, we noticed significant variations in the amounts of primary antibody bound to microtiter wells with respect to the solubility behavior of IgG when carbonate buffer, phosphate buffer with glutaraldehyde, and water were used as the coating medium. Optimization of the parameter led to a significant increase in microtiter wells coated per milliliter of primary antibody through the immunobridge technique.

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EXPERIMENTAL

Cortisol, cortisol-3-*O*-carboxymethyl-oxime (cortisol-3-O-CMO), cortisol-21-hemisuccinate (cortisol-21-HS), horseradish peroxidase (HRP) type VI (EC 1.11.1.7), bovine serum albumin (BSA), gelatin, thimerosal, dextran T-70, 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide-HCl (EDAC), *N*-hydroxysuccinimide (NHS), adipic acid dihydrazide (ADH), glutaraldehyde (GA), and freund's complete adjuvant (FCA) were all purchased from Sigma Chemical Company, St. Louis, MO, USA. Tetra methyl benzidine with hydrogen peroxide solution (TMB/H_2O_2) and microtiter plates, were procured from Bangalore Genei, Bangalore, India and Greiner, Germany, respectively. All other chemicals and buffers were of analytical grade.

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Buffer

1. The most frequently used buffer was 10 mM phosphate containing 0.9% NaC1 (10 mM PBS), pH 7.0, ($Na_2HPO_42H_2O$: 0.895 gm/L and NaH_2PO_4 $2H_2O$: 0.39 gm/L), and 0.1% NaN_3.
2. Carbonate buffer 0.05 M, pH 9.5 (Na_2CO_3: 3.66 gm/L and NaH_2CO_3: 1.87 gm/L).
3. HRP conjugate dilution buffer was 10 mM acetate buffer (10 mM AB), pH 5.6 (CH_3COONa: 0.84 gm/L and 1 N CH_3COOH 1.5 mL/L), containing 0.1% thimerosal, sodium salicylate, dextran T-70, and 0.3% BSA.
4. Microtiter well blocking and stabilizing buffer was 10 mM PBS containing 0.2% BSA, 0.1% gelatin, thimerosal, dextran T-70, ethylene diamine tetra acetic acid di-potassium salt (EDTA K-salt), and 0.01% gentamicin sulfate.

Biological and Immunological Materials

The study was approved by the Institute's ethics review committee. Normal rabbit serum (NRS), collected from non-immunized New Zealand white rabbits.

Primary antibody: Cortisol antiserum was generated against cortisol-3-*O*-CMO–BSA as an immunogen in New Zealand white rabbit.[3]

Secondary antibody: Antispecies antiserum as secondary antibody generated in goats using purified normal γ-globulin of rabbit as an immunogen.[4]

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Preparation and Dilution of Cortisol-21-HS–ADH–HRP Conjugate

Horseradish peroxidase was activated and coupled to ADH by using the periodate method of Wilson and Nakane,[5] with some modification. The cortisol-21-HS was conjugated to ADH coupled HRP by an activated ester method with modification.[3] The complete procedure for coupling ADH to HRP and then carboxyl-derivative of steroid to ADH coupled HRP was described in the preceding article.[6]

Immunobridge Technique for Primary Antibody Immobilization

Step I: Hydrophobic Immobilization of Normal γ-Globulin

Normal rabbit serum was diluted in distilled water 1:1000, and stirred for 30 min at room temperature, 250 μL was dispensed in each well of the microtiter plate and incubated overnight at 4°C. After incubation, the contents of the plate were decanted and the plate was washed under tap water five to six times by filling, decanting, and tapping.

Step II: Immunochemical Binding of Secondary Antibody to the Immobilized Normal γ-Globulin

The anti rabbit goat serum (secondary antibody) was diluted (1:4000) in 10 mM PBS containing 0.1% NaN_3 and 0.3% gelatin 250 μL was added to all the wells and incubated for 2 h at 37°C. Decanting and washing were performed as before.

Step III: Immunochemical Immobilization of Antigen Specific Antibody on Immobilized Secondary Antibody

The cortisol antiserum was serially diluted in phosphate buffer containing 0.1% NaN_3 and 0.3% gelatin, 1:500, 1:1000, 1:2000, 1:4000, and 1:8000, and 200 μL was added into the wells (one dilution per eight well strip); for non-specific binding (NSB), 200 μL of phosphate buffer was added in a separate eight-well strip and incubated for 1 h at 37°C. Decanting and washing were performed as before.

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Step IV: Blocking of Unoccupied Sites of Microwell Surface and Drying

Microwell blocking and stabilizing buffer, 250 µL, was added to all the wells, incubated for 1 h at 37°C, contents decanted, and the plate was dried at room temperature and kept at 4°C in a polythene bag containing silica gel. This was stable for more than one year.

Existing Procedures of Immobilization

Direct Immobilization Using Carbonate Buffer

The IgG was purified from cortisol antiserum,[3] and serially diluted, 100, 50, 25, 10, and 5 µg/mL, in 0.05 M carbonate buffer, 200 µL, was added in the wells (one dilution per eight well strip) whereas, for NSB, 200 µL of carbonate buffer was added into a separate eight-well strip and incubated overnight at 4°C. Decanting, washing, blocking, and drying were performed as before.

Direct Immobilization Using PBS Containing Glutaraldehyde

The purified γ-globulin of cortisol antiserum was serially diluted, 100, 50, 25, 10, and 5 µg/mL in 10 mM PBS containing 0.1% glutaraldehyde, 200 µL was dispensed into the wells (one dilution per eight-well strip) whereas, for NSB, 200 µL of 10 mM PBS containing 0.1% gluraraldehyde was added into a separate eight-well strip and incubation, decanting, washing, blocking, and drying were performed as before.

Indirect Immobilization Through Secondary Antibody Using Carbonate Buffer

The anti rabbit gamma-globulin (ARGG), was purified from goat serum,[3] diluted in 0.05 M carbonate buffer, pH 9.5, (10 µg/mL), 250 µL was dispensed in all the wells, and incubation and washing were performed as mentioned before. To the ARGG immobilized microtiter wells, cortisol antiserum serially diluted in 10 mM PBS, 1:500, 1:1000, 1:2000, 1:4000, and 1:8000, 200 µL was added into the wells (one dilution per eight-well strip) whereas µ for NSB, 200 µL of PBS was added into a

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separate eight-well strip and incubated for overnight at 4°C. Decanting, washing, blocking, and drying were performed as before.

Indirect Immobilization Through Secondary Antibody Using PBS Containing Glutaraldehyde

Two hundred and fifty microtiters of purifed ARGG (7.5 μg/mL), in 10 mM PBS containing 0.1% glutaraldehyde, was added to all the wells and incubated for overnight at 4°C. Decanting and washing were performed as before. To the ARGG coated microtiter wells, cortisol antiserum diluted in 10 mM PBS, 1:500, 1:1000, 1:2000, 1:4000, and 1:8000, 200 μL, was added to the wells (one dilution per eight-well strip) whereas, for NSB 200 μL of PBS was added in separate eight-well strip and incubation, decanting, washing, blocking, and drying were performed as before.

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Preparation of Substrate Solution

Substrate solution was prepared from TMB/H_2O_2 (Bangalore Genei, India). According to the manufacturer protocol, 100 μL of TMB/H_2O_2 solution was diluted to 2 mL (1:20 dilution) in water. This solution was freshly prepared just before its use.

Determination of Optimal Loading of Primary Antibody Using Cortisol-21-HS–ADH–HRP Conjugate

In order to estimate the amount of primary antibody immobilized on microtiter wells by different immobilization procedures, as described previously, was determined by adding 100 μL of cortisol-21-HS–ADH–HRP conjugate diluted serially in conjugate dilution buffer, 1:500, 1:1000, 1:2000, and 1:4000 in wells (one dilution per two wells in vertical fashion) and incubated for 1 h at 37°C.

After incubation, the contents of the wells were decanted and washed in running tap water five to six times by filling, decanting, and tapping. Finally, for measuring bound enzyme activity (which is a direct function of primary antibody), 100 μL of substrate solution was added to all the wells and incubated for 15 min at 37 °C. The reaction was stopped by adding 100 μL of 0.5 M H_2SO_4 and the color intensity was measured at 450 nm in a Tecan-Spectra ELISA plate reader.

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RESULTS

We compared the immunobridge procedure with existing two procedures of primary antibody immobilization, namely, (i) direct and (ii) indirect. Tables 1 and 2 indicate the number of wells coated by 1 mL of primary or secondary antibody by different coating procedures, respectively. The optimal loading of primary and secondary antibody achieved in different types of immobilization protocols were given below.

Immunobridge Immobilization Technique

The optimal bound enzymatic signal in the immunobridge immobilization technique was achieved when 250 μL of 1:4000 diluted secondary antibody and 200 μL of 1:8000 diluted cortisol primary antiserum were used per well. This means that, from 1 mL of secondary antibody, antiserum 16,000 wells and from 1 mL of cortisol primary antiserum 40,000 wells were able to be coated.

Direct Coating

The optimal bound enzymatic signal in direct coating of cortisol primary antibody IgG by carbonate buffer and PBS containing 0.1% glutaraldehyde was obtained when 200 μL of 10 μg/mL and 8 μg/mL IgG per well in respective buffer were utilized. When IgG was purified using ammonium sulfate precipitation and ion-exchange chromatography from cortisol primary antiserum, it yielded 12 mg of IgG/mL. This means that, from 1 mL of cortisol primary antiserum (IgG), 6,000 wells by carbonate buffer and 7,500 wells by PBS (containing 0.1% glutaraldehyde), respectively, were able to be coated.

Indirect Coating

The optimal bound enzymatic signal in indirect coating of cortisol primary antibody through secondary antibody IgG by carbonate buffer and PBS containing 0.1% glutaraldehyde was obtained when 250 μL of 10 μg/mL and 7.5 μg/mL IgG per well in respective buffer were utilized. When IgG from secondary antibody was purified using ammonium sulfate and ion-exchange chromatography, it yielded 15 mg of IgG/mL. This means that, from 1 mL of second antibody IgG, 6,000 wells by

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Table 1. Number of microtiter wells coated from 1 mL of primary antibody by different immobilization procedures.

Immobilization procedure	Immunoreagent	Dilution	Amount/well	Number of wells coated
Immunobridge	Primary antibody	1:8000	200 µL	40,000
Indirect carbonate buffer	Primary antibody	1:4000	200 µL	20,000
PBS with GA	Primary antibody	1:4000	200 µL	20,000
Direct carbonate buffer	Primary antibody (12 mg IgG/mL)	10 µg/mL	200 µL	6,000
PBS with GA	Primary antibody (12 mg IgG/mL)	8 µg/mL	200 µL	7,500

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Table 2. Number of microtiter wells coated from 1 mL second antibody by different immobilization procedures.

Immobilization procedure	Immunoreagent	Dilution	Amount/well	Number of wells coated
Immunobridge	Second antibody	1:4000	250 μL	16,000
Indirect carbonate buffer	Second antibody IgG (15 mg IgG/mL)	10 μg/mL	250 μL	6,000
PBS with GA	Second antibody IgG (15 mg IgG/mL	7.5 μg/mL	250 μL	8,000

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carbonate buffer and 8,000 wells by PBS containing 0.1% GA were able to be coated. To the above coated secondary antibody IgG wells 200 μL of 1:4000 diluted cortisol primary antibody was utilized. This means 20,000 wells were able to be coated by these methods.

DISCUSSION

Hydrophobicity is the basis of passive adsorption of protein to solid surface.[7] Increase in hydrophobicity, either in immunoreagent[8–10] or solid surface,[11] increases the immobilization of immunoreagent. To increase the hydrophobicity of immunoreagent for immobilization onto solid support, various synthetic cross linkers have been coupled to immunoreagent.[8–10] In the immunobridge immobilization procedure, the increase in hydrophobicity of normal γ-globulin was achieved very simply by using water as a diluting medium. In this strategy, the physicochemical property, that is, insolubility of γ-globulin in water, was exploited for hydrophobic immobilization. In the first step of immunobridge immobilization procedure, normal rabbit serum was diluted with water, the γ-globulins present in the serum become hydrophobic in nature (tend to precipitate out) and become immobilized onto the solid surface. In the second step, buffer diluted secondary antiserum was added, the anti-species antibody present in antiserum immobilized immunochemically onto solid surface, immobilized normal γ-globulin. Finally, buffer diluted primary antiserum was added, antigen specific antibody present in antiserum was immunochemically immobilized onto the anti-species secondary antibody. The anti-species specific secondary antibody forms the immunochemical bridge between non-immunized γ-globulin and antigen specific primary antibody because both of them were from same species.

The number of wells coated with per milliliter of primary antiserum is more with the immunobridge procedure than by direct and indirect procedures. This may be because passive adsorption onto the hydrophobic surface can produce substantial conformational and functional changes in proteins.[12] If antigen specific antibody is immobilized onto the solid surface directly, this restricts the movement of antibody and can considerably decrease the rate of reaction with antigen,[13] and also antibody loses its affinity towards antigen.[14] Alternatively, the immobilization process, whether covalent or passive, will result in partial denaturation of antibody because antibody is being held at the surface of the solid phase and will result in antibody affinity constant reduction.[15] This loss of affinity can result in increased usage of primary

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antibody and, thus, limits the direct coating of primary antibody onto the solid surface.[16]

The indirect immobilization of primary antibody through secondary antibody,[17] proteins A, G, and L[18] and avidin–biotin linkages[19,20] onto solid surface has advantages such as unalteration of the kinetics of the primary antibody–antigen reaction. This may be because of orientation of immobilized primary antibody with binding sites towards the solution. But, for obtaining optimal loading of primary antibody to solid surface, a large quantity of these reagents are required for immobilization in purified forms, whereas, avidin–biotin linkages require extra coupling steps; all of these make solid phase immunoassay costly.

The immunobridge immobilization technique, where a physicochemical property of immunoglobulin, i.e., insolubility in water, is exploited, has some advantageous over indirect and direct coating procedures; these are (a) economic utilization of primary and secondary antibody; (b) nonrequirement of purified normal γ-globulin and immunoglobulin, thereby eliminating loss in primary or second antibody by the process of purification, orientation of immobilized primary antibody with binding sites towards solution and, hence, increase in numbers of wells coated per milliliter of primary or secondary antibody as compared to the direct or indirect immobilization procedure; (c) applicability to a wide range of assays; and (d) may be suitable for simultaneous multi-analyte assays.

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