# Immuno-Reproduction Assays in Semen Biology

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**Abstract**

The practical advantage of an immunological methodology to the study of carbohydrates, proteins, and other cellular structures is that when carefully carried out, results are highly specific, sensitive, and repeatable. All immunological assays in semen biology involve measurement of the interaction of targeted antibody/antigen (Ab-Ag) with that of Ag-Ab. Since most of the investigations in the semen biology involve either quantitative or qualitative assay of interaction of Ab with Ag, we have divided such assays in two parts. Part one, the qualitative assays, includes the enzyme-linked immunosorbent assay (ELISA) with its four common variants, namely, indirect ELISA, direct competitive ELISA, antibody-sandwich ELISA, and radioimmunoassay (RIA). Part two, the quantitative assays, includes double immunodiffusion (DID), Western blot, and dot blot assays. Additionally, tests for screening of antisperm antibody (ASA) on the sperm cells as well as biological fluids have been explained. This chapter also includes isolation of Ab (specifically IgG) by chromatography method. The comparison of various assays has been provided in the relevant sections.

**Keywords**

Antisperm antibodies • Elisa • Western blot • Immunodiffusion • Dot blot • Immunobead

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N. Srivastava, M. Pande (eds.), *Protocols in Semen Biology (Comparing Assays)*,
DOI 10.1007/978-981-10-5200-2_19

## 19.1 Introduction

Any experiment involving production of antibodies (Ab) invariably involves assessment of interaction of antibody against specific antigen (Ag). This interaction (binding) can be measured in the solution by several means such as by measuring the insoluble Ab-Ag complexes in the gel and by determining the adsorption of soluble Ag to solid-phase Ab (or its converse) or fluorescent quenching or by equilibrium dialysis. In the chapter, we have outlined the procedures that are quick, easy to carry out, sensitive, repeatable, inexpensive, and specific involving semen biology. Thus, the chapter describes enzyme-linked immunosorbent assay (ELISA) and its three variants as qualitative assays and double immunodiffusion (DID), Western blot, and dot blot assays as quantitative assays of binding of targeted Ab with Ag. Researcher may bear in mind that precise assessment of binding of Ab with Ag requires a clear and proper selection of assay, careful consideration of animal species from which Ab or Ag is to be procured, and appropriate attention to each and every detail throughout the many steps of the selected protocols.

After following repeatable, accurate, as well as simple assays for evaluating antibody activity, the investigators can then proceed to production of antibodies. Though there are several assays dealing with the production of antibodies, we have outlined a chromatography assay for isolation of most commonly found Ab, viz., IgG in semen. In semen biology involving animals particularly bovines, detection of antisperm antibody (ASA) holds great importance for their role in the repeat breeding syndrome [1]. Therefore, the chapter also outlines tests to determine ASA on sperm cells as well as in biological fluids.

## 19.2 Comparative Merits of Assays Employed in Immuno-Reproduction

Since the first description of ELISA techniques in 1971 by Engvall and Perlman [2], these have become the assays of choice for evaluating soluble antigens and antibodies. The ELISA techniques are inexpensive, highly versatile, quantitative, and sensitive requiring a few equipment and chemicals. The ELISA is similar in principle to radioimmunoassay, excepting that an enzyme conjugate replaces the radioactive label. The reagents used in ELISA are quite stable; e.g., *p*-nitrophenyl phosphate (NPP) (at 4 °C) and 4-methylumbelliferyl phosphate (MUP) can be stored for several months at RT.

The indirect ELISA is employed to detect specific Ab whereas a direct competitive ELISA or an antibody-sandwich ELISA is used to study soluble antigens. Electrophoresis and immune-fixation are relatively recent techniques that combine the merits of agarose gel electrophoresis and accuracy of the AB-Ag interaction. In comparison to the above techniques, double immunodiffusion (DID) is the simplest and most often employed technique to study Ab-Ag reaction. Though all three assays are useful for investigating Ab activity of polyclonal antisera, DID is not a very effective assay to investigate monoclonal Ab binding to monoclonal antisera (Table 19.1).

## 19.3 Assays in Immuno-Reproduction

**General Principle**

In all the protocols mentioned above, Ag and Ab are adsorbed onto plastic microtiter plates to prepare solid-phase reactants. This is followed by incubation of these reagents with secondary or tertiary reactants, which are covalently coupled, to an enzyme. Thereafter, unbound conjugates are flushed out with the addition of a fluorogenic or chromogenic substrate. Following the hydrolysis of the substrate by the bound enzyme conjugate, either a colored or a fluorescent product is generated. Finally, this product is detected visually or with a microtiter plate reader. Results are interpreted based on the proportion of amount of product generated in relation to the amount of analysate in the test mixture. Of the different protocols described hereafter, the first support protocol is carried out to optimize the various ELISAs,

**Table 19.1** ELISA techniques: a comparison

| Technique | Application | Requirements | Remarks |
|---|---|---|---|
| Indirect | Ab screening, epitope mapping | Ag: pure or semi-pure | Requires large amount of Ag, does not require specific Ab [3] |
| | | Test solution with Ab | |
| | | Enzyme conjugates that bind Ig of immunized species | |
| Direct competitive | Ag screening to detect soluble Ag | Ag: pure or semi-pure | Rapid assay for measuring cross-reactivity |
| | | Test solution with Ag | |
| | | Enzyme-Ab conjugates specific for Ag | |
| Antibody-sandwich | Ag screening to detect soluble Ag | Ab: pure or semi-pure | Most sensitive; however requires relatively large quantity of Ab |
| | | Test solution with Ag | |
| | | Enzyme-Ab conjugates specific for Ag | |
| RIA | Detection of specific Ab or Ag | Requires specific equipment and permission from concerned regulatory agencies | Very sensitive and specific; large samples can be processed and cost-effective, but requires specific equipment and is hazardous due to the use of radioactive isotopes |
| Double immunodiffusion | Binding of Ab with Ag | Very simple, quick, suitable for polyclonal Ab binding | Not suitable for monoclonal Ab and monovalent Ag [3] |

*Ab* antibody, *Ag* antigen, *RIA* radioimmunoassay

whereas the second support protocol presents a procedure for preparing alkaline phosphatase conjugates [3].

### 19.3.1 Quantitative Assays: Enzyme-Linked Immunosorbent Assay (ELISA)

These are quantitative assays, which can be employed to determine the concentration of antibodies and/or antigen in a given sample. The following assays can be used for quantitative estimation of antigen or antibody:

- An indirect ELISA
- A direct competitive ELISA
- An antibody-sandwich ELISA

Other assays such as double immunodiffusion, Western blot, and dot blot are quantitative assays.

#### 19.3.1.1 Indirect ELISA to Detect Specific Antibody

**Principle**

In indirect ELISA for detection of Ab, the following steps are involved:

(a) Coating of wells of microtiter plates with Ag
(b) Incubation with test solutions containing specific Abs against Ag
(c) Washing away of unbound Abs
(d) Addition of a solution containing a developing reagent (e.g., alkaline phosphatase conjugated to protein-A, protein-G, or antibodies against the test solution antibodies)
(e) After incubation, washing of unbound conjugate
(f) Adding the substrate solution and incubating
(g) Assessing the amount of substrate hydrolyzed with a spectrophotometer or spectrofluorometer

Results are interpreted based on the principle that the measured amount is proportional to the amount of specific antibody in the test solution. Visual observation can also be employed to detect hydrolysis.

**Application**

- For detecting specific antibodies in antisera or hybridoma supernatants.
- This assay can be employed when purified or semi-purified Ag is available in small quantities (in milligrams). One mg of purified Ag will allow screening of 80–100 microtiter plates.

**Materials**

Developing reagent: protein-A, alkaline phosphatase conjugate; protein-G, alkaline phosphatase conjugate; or Anti-Ig, alkaline phosphatase conjugate (Support Protocol-II); blocking buffer, antigen solution, DW, 4-methylumbelliferyl phosphate (MUP) or *p*-nitrophenyl phosphate (NPP), test antibody samples, 0.5 M NaOH, substrate solution, PBS containing 0.05% $NaN_2$ (PBSN), multichannel pipette, disposable pipette tips, microtiter plate (or Immulon 2/Immulon 4), plastic squirt bottles, microtiter plate reader (spectrophotometer (405 nm filter) or spectrofluorometer with 365/450 nm ex/em filter)

**Basic Protocol**

(a) Carry out crisscross serial dilution analysis (Support Protocol-I) to determine the optimal concentration of the developing reagent (conjugate).
(b) Carry out crisscross serial dilution analysis (Support Protocol-I) to determine the final concentration of the antigen-coating reagent.
(c) This reading is utilized to prepare an Ag solution in PBSN (the final concentration of Ag is usually 0.2–10.0 μg/mL). Accordingly, 6 mL of Ag solution is prepared for each plate.

**Coating plate with Ag**

(d) Dispense 50 μL of Ag solution into each well of an Immulon microtiter plate using a multichannel pipette.
(e) Tap or shake the plate to ensure even distribution of Ag solution over the bottom of each well.
(f) Incubate overnight at 37 °C for 2 h after wrapping coated plates in plastic wrap seal.
(g) Carry out rinsing of coated plate by filling wells with deionized or DW dispensed either from a plastic squirt bottle or from the tap over a sink. After rinsing, flick the water into the sink and rinse with water twice.

**Blocking residual binding capacity of plate**

(h) Take a squirt bottle filled with blocking buffer and fill each well with it dispensed as a stream followed by incubation for 30 min at RT.
(i) Rinse plate three times as described in step "g."
(j) Keep ready several paper towels on the bench top. Following the last rinse, wrap each plate in a large tissue paper and gently flick it face down onto prepared paper towels to remove residual.

**Adding antibodies to the plate**

(k) To each of the coated wells, pour 50 μL of antibody samples diluted in blocking buffer.
(l) Wrap plate in plastic wrap and incubate ≥2 h at RT.

**Washing of the plate**

(m) Rinse the plate three times as in step "g" above.
(n) After filling each well with blocking buffer, vortex and incubate 10 min at RT.
(o) Rinse the plate three times as in step "g" above.
(p) Following the last rinse, remove remaining liquid as in step "j."

**Adding developing reagent to plate**

(q) In each well of the microplate reader, add 50 μL of developing reagent in blocking buffer (at optimal concentration determined in step 1).
(r) Wrap each plate in plastic wrap and incubate for 2 h at RT.
(s) Wash plates as in steps "m" to "p" above.

**Adding substrate and measuring hydrolysis**

(t) To each well add 75 μL of MUP or NPP substrate solution and incubate for 1 h at RT.
(u) Monitor hydrolysis: qualitatively (visual inspection) or quantitatively (microtiter plate reader). Add 25 μL of 0.5 M NaOH to stop hydrolysis.

**Observations**

(a) Appearance of a yellow color indicates hydrolysis of NPP (visual observation). Microtiter plate (405 nm filter) can also be used to measure NPP hydrolysis.
(b) Use illumination with a long wavelength UV lamp in a darkened room to visually detect hydrolysis of MUP. Use microtiter plate (365/450 nm ex/em filter) to detect MUP hydrolysis.

**Points to Ponder**

(a) Good conjugates of much specificity are commercially available. Use conjugates prepared with antibodies specific for Ig κ and γ light chains to detect all antibodies that bind to Ag. Otherwise, screening of monoclonal antibodies may be carried out preferably using protein-A or protein-G enzyme conjugates. It is easy to purify and characterize monoclonal antibodies that bind specifically to protein-A or protein-G.
(b) Concentration of pure Ag is usually $\leq$2 μg/mL. Though pure Ag preparations are not essential, in general, Ag concentration in the solution would be $>$3% of the protein. For semi-purified Ag preparations, this concentration of protein in the Ag solution should be increased. The total protein concentration of about $>$10 μ/mL usually saturates $>$85% of the available sites on Immulon microtiter plates.
(c) For some Ag, efficiency of coating of plates may vary for each Ag at different pH.
(d) For wrapping the plates, plastic wrap is easier to use and works as well; however, individual adhesive plate sealer is available commercially. Plates once sealed can be stored at 4 °C with Ag solution for several months.
(e) Rinsing the plates with DW is cheaper than with buffer and is as effective.
(f) Allowing more time for binding of Ab may permit increase in specific signal.
(g) Following final rinsing, wrap the plates in plastic wrap. This can be stored for months at 4 °C before the substrate is added.
(h) Compared to NPP, the fluorogenic system using MUP is up to 100 times. Moreover, the spontaneous hydrolysis rate of MUP is much less as compared to NPP.
(i) Carry out all steps after coating the microtiter plates in solutions containing 0.05% Tween 20 and a carrier protein (0.25% BSA or gelatin).
(j) During quantitative determination of Ag or Ab concentrations using ELISA, keep all experimental conditions constant between experiments (up to the final wash after incubation with conjugate – including incubation times, wash times, reagent concentrations, and temperature). This is especially important while using polyclonal Ab and complex mixtures of Ag.
(k) Draw a standard curve on each plate since the efficiency of binding and other microenvironmental conditions vary from plate to plate.
(l) Analyze all samples at least in duplicate.
(m) Researcher must note that the concentration of the quantitated reagent must lie within the dynamic range of the standard curve.

(n) ELISA is generally designed to take up to 6 h, but time course may vary.
(o) Fluorogenic ELISA is generally 10–100 times faster than assays using chromogenic substrate.

## Support Protocol-I

### Determining Optimal Reagent Concentrations (Crisscross Serial Dilution Analysis/Checker Board Assay)

**Principle**

To determine optimal concentration of reagents to be used in ELISA, perform crisscross serial dilution analysis. In the ELISA support protocol described below, all three reactants – a solid-phase coating reagent, a secondary reagent (binds to the primary reagent), and an enzyme-conjugated tertiary developing reagent (binds to the secondary reagent) – are serially diluted and analyzed by a crisscross matrix analysis. After determining the optimal concentration of reagents to be used under particular assay, keep these variables constant from experiments to experiments. Depending upon the need to optimize the previous protocols, these three reagents will vary.

**Materials**

Coating reagent, secondary reagent, developing reagent, and 17 × 100 mm and 12 × 74 mm test tubes.

**Procedure**

**Preparing coating-reagent dilutions**

(a) Place four 17 × 100 mm test tubes in a rack and add 6 mL PBSN to the last three test tubes.
(b) In test tube 1, prepare a 12 mL solution of coating reagent at 10 μL/mL in PBSN. Transfer 6 mL of tube 1 solution to the tube 2; mix by pipetting up and down six times.
(c) Repeat this transfer and mix for tubes 3 and 4; the tubes now contain the coating reagent in 10, 5, 2.5, and 1.25 μL/mL.
(d) Dispense 10 μL of the coating-reagent solutions using multichannel pipette into wells of the four Immulon microtiter plates (i.e., each plate is filled with one of the four dilutions).
(e) Incubate overnight at room temperature or at 37 °C for 2 h.
(f) Place and block plates with blocking buffer as in steps “g” to “i” of basic protocol.

**Preparing secondary-reagent dilutions**

(a) Take five 12 × 74 mm test tubes in a rack and add 3 mL blocking buffer to the last four tubes.
(b) In tube 1, prepare a 4 mL solution of secondary reagent at 200 ng/mL in PBSN. Transfer 1 mL of tube 1 solution to the tube 2; mix by pipetting up and down six times.
(c) Repeat this transfer and mix for tubes 3 and 4; the tubes now contain the coating reactant at 200, 50, 12.5, 3.125, and 0.78 ng/mL.
(d) Dispense 50 μL of the secondary-reagent solutions into the first five columns of all four coated plates. The most dilute solution is dispensed into the column 5, while solutions of increasing concentrations are added successively into column 4, 3, 2, and 1. In this way the fifth column contains 0.78 ng/mL, whereas the first column 200 ng/mL. Incubate for 2 h at room temperature.
(e) Wash plates as in steps “m” to “p’ of basic protocol.

**Preparing developing-reagent dilutions**

(a) Place five 17 × 100 mm test tubes in a rack and add 3 mL blocking buffer to the last four tubes.
(b) In tube 1, prepare a 6 mL solution of developing reagent at 500 ng/mL in blocking buffer.
(c) Transfer 3 mL of the tube 1 solution into tube 2 and mix.
(d) Repeat this transfer and mix for tube 3 and 4.
(e) The tubes now contain the developing reagent at 500, 250, 125, 62.6, and 31.25 ng/mL.

(f) Dispense 50 μL of the developing-reagent solutions into the wells of rows 2–6 of each plate, dispensing the most dilute solution into row 6 and solutions of increasing concentrations successively into rows 5, 4, 3, and 2.
(g) Incubate for 2 h at RT.

**Measuring hydrolysis**

(a) Add 75 μL of MUP or NPP substrate solution to each well.
(b) Incubate for 1 h at RT and measure the degree of hydrolysis visually or with a microtiter plate reader.

**Observations**

An appropriate assay configuration using NPP as a substrate results in 0.50 absorbance units/h at 405 nm or 1000 to 1500 fluorescence units/h when using MUP as a substrate. In the basic and alternate protocols, these results are used to adjust optimal concentrations.

Depending upon the individual assay variables, reagent concentrations are predetermined by the investigator. Use 500 ng/mL of enzyme-antibody conjugate (the relative fluorescence is at −1000 relative fluorescence units, the time of hydrolysis is 1 h, and the sensitivity is at 780 pg/mL of homologous Ag) in the ELISA. On the other hand, if the probable detection of the assay is only 3.12 ng/mL of homologous Ag, then reduce the concentration of conjugate to 125 ng/mL.

**Points to Ponder**

In the case of especially sensitive assay, sometimes it is necessary to increase the secondary reactant concentrations so the tube 1 solution is 1000 ng/mL.

## Support Protocol-II

### Preparation of Antibody-Alkaline Phosphatase Conjugates

In this support protocol, Abs are mixed with alkaline phosphatase followed by cross-linking by incubation with glutaraldehyde for 2 h. Stop the reaction by adding lysine and ethanolamine contained in PBS-LE. Desalt the mixture on a small Sephadex G-25 sizing column and analyze the fractions to detect those containing conjugate.

Though a number of different enzymes have been successfully used in ELISA, viz., horseradish peroxidase, gluco-amylase, urease, and alkaline phosphatase, the last one is the most used and a highly recommended enzyme. This is so because of excellent intrinsic stability, rapid catalytic rate, ease of conjugation availability, and resistance to inactivation by common laboratory reagent.

**Materials**

˃0.2 mg/mL antibody in PBS, alkaline phosphatase in NaCl solution (Sigma), 25% glutaraldehyde (EM grade, Sigma), 10 mL Sephadex G-25 column, and 0.2 μm filter

**PBS-LE solution**

1.83 g lysine (100 mM).
0.611 g ethanolamine (100 mM).
Dilute to PBS 100 mL.

**Borate-buffered saline (BBS) pH 8.5**

66.5 g $Na_2B_4O_7.10H_20$ (0.17 M).
7.0 g NaCl (0.12 M).
Dilute to 1000 mL DW.

**Blocking buffer (store at 4 °C)**

0.05 g Tween 20 (0.05%).
0.0372 g EDTA (1 mM).
0.25 g BSA (0.25%).
0.05 g $NaN_2$ (0.05%).
Dilute to 100 mL BBS.

**Blocking buffer with $MgCl_2$**

0.024 g $MgCl_2$ (2.5 mM).
Dilute to blocking buffer 100 mL.

**MUP substrate solution (store at RT)**

6 mg 4-methylumbelliferyl phosphate (0.2 mM).
4.15 g $NaCO_3$ (0.05 M).
4.7 mg $MgCl_2$ (0.5 mM).
Dilute to 100 mL DW.

**NPP substrate solution (store at 4 °C)**
111.33 mg *p*-nitrophenyl phosphate (3 mM).
4.15 g $NaCO_3$ (0.05 M).
4.7 mg $MgCl_2$ (0.5 mM).
Dilute to 100 mL DW.

**Test antibody solution**
Dilute hybridoma supernatant (1:50 and ascetic fluid and antisera (1:500) in blocking buffer.
Take diluted nonimmune sera as a negative control. Prepare Ab dilutions in cone- or round-bottom microtiter plates before adding them to Ag-coated plates.

**Test antigen solution (store at 4 °C)**
0.2–10 μg/mL Ag in PBSN

**Wash buffer**
1 mL fetal calf serum (FCS), heat-inactivated 60 min, 56 °C.
0.05 g $NaN_2$.
Dilute to Hank's balanced salt solution (HBSS) 100 mL.

**Procedure**

(a) Prepare a 1:3 mixture of Ab-alkaline phosphatase in PBS (˃0.2 mg/mL total protein concentration).
(b) Add 2.5% glutaraldehyde to 0.2% solution while vortexing.
(c) Incubate for 2 h at room temperature; stop reaction by adding an equal volume of PBS-LE.
(d) Desalt the sample by chromatography on a 10 mL Sephadex G-25 column in PBSN: bed volume of the column should be five to ten times larger than the reaction volume.
(e) Collect fractions that are one-half of the reaction volume.
(f) Assay fractions by transferring 2 μL into tubes containing 0.5 mL NPP substrate solution.
(g) Pool the first five fractions that strongly hydrolyze NPP.
(h) Mix the pool 1:2 in blocking buffer containing 2.5% $MgCl_2$.
(i) Filter through a 0.2 μm filter and store at 4 °C.

**Points to Ponder**

(a) Gelatin can be used as a substitute for BSA. Five percent instant skim milk has been used successfully in many experiments, but sometimes nonspecific binding with Ab may interfere in results.
(b) To analyze 200–800 microtiter plates, an initial preparation of 0.5 mg Ab and 1.5 mg alkaline phosphatase (because of long shelf life and the high specific activity of most antibody-alkaline phosphatase conjugates) will usually produce enough conjugate.
(c) Source of appropriate Ab and conjugates can be found in Linscott's Directory of Immunological and Biological Reagents.

### 19.3.1.2 Direct Competitive ELISA to Detect Soluble Antigen

**Principle**
In this assay, binding of specific antibody-enzyme conjugates to antigen-coated plates is inhibited by test solutions containing soluble antigens. This is incubated with a mixture of conjugates and inhibitor followed by washing of unbound conjugate and addition of substrate. Keep the amount of Ag in the test solution in proportion to the inhibition of substrate hydrolysis. This can be quantitated by interpolation onto an inhibition curve, which is generated with serial dilutions of a standard Ag solution.

**Applications**

(a) Useful to detect or quantitate soluble Ag.
(b) When both specific Ab and milligram quantities of purified or semi-purified Ag are available, this assay proves most useful.
(c) By substituting specific antibody-enzyme conjugate, direct competitive ELISA can be adapted as an indirect assay. In this protocol, detect the amount of specific Ab using a species-specific or isotype-specific conjugate as a tertiary reactant.

**Materials**

Standard Ag solution, test Ag solution, specific antibody-alkaline phosphatase conjugate (refer to Support Protocol-II), round- or cone-bottom micropipette plates.

**Procedure**

(a) Apply crisscrossing serial dilution analysis to determine the optimal concentration of coating reagent and antibody-alkaline phosphatase conjugate. At this stage, the concentrations of both Ag (coating reagent) and the conjugate (developing reagent) (Support Protocol-I) vary.
(b) 2x conjugate solution is prepared by diluting (to twice the optimal concentration) the specific Ab-alkaline phosphatase conjugate in blocking buffer.
(c) Use 50 μL of Ag solution to coat and block wells of Immulon microtiter plate as in steps "b" to "j" of basic protocol.
(d) Prepare a standard inhibition curve using Ag concentration. This is done by preparing six 1:3 serial dilutions of standard Ag solution in blocking buffer (Support Protocol-I for serial dilutions).
(e) Mix and incubate conjugate and inhibitor by adding 75 μL of 2x conjugate solution (from step "a") and 75 μL inhibitor – either test solution or standard Ag solution (from step "d") to each well of a round- or cone-bottom microtiter plate.
(f) Draw the conjugate and inhibitor solution by pipetting up and down in the pipette tip, and mix three times followed by incubation for ≥30 min at RT.
(g) Uninhibited control samples are prepared by mixing equal volumes of 2x blocking buffer and conjugate solution.
(h) To an Ag-coated plate (from step "c"), transfer 50 μL of the mixture of conjugate plus blocking buffer (from step "g") or conjugate plus inhibitor (from steps "e" to "f") and incubate for 2 h at a RT.
(i) Wash plate as in steps "m" to "p" of the basic protocol.
(j) After adding 75 μL of NPP or MUP substrate solution to each well, incubate for 1 h at RT.

**Observations**

(a) Read plates on the microtiter plate reader after ≥1 h. During this period, precise measurement of the inhibition is possible because sufficient substrate is hydrolyzed in the uninhibited reactions.
(b) Standard antigen-inhibitor curve is prepared from the inhibition produced by the dilutions of the standard Ag solutions as in step "d."
(c) Plot: x axis = Ag concentration (a log scale) and y axis = fluorescence or absorbance (a linear scale).
(d) Interpret the results by interpolating the concentration of Ag in the test solutions from the standard curve prepared above.

**Points to Ponder**

(a) Prepare 3 mL ab-alkaline phosphatase conjugate for each plate. In step "a" the final concentration of Ab is usually 25–500 ng/mL.
(b) In step "d" for each plate to be assayed, prepare ≥75 μL of each dilution.
(c) For precise quantitation of the amount of Ag in the test solutions, test Ag solutions should inhibit conjugate binding between 15 and 85%. Sometimes to produce inhibitions within this range, assaying two or three different dilutions of the test solutions is required (step "f").
(d) For duplicate assay of samples, place each sample should be in adjacent columns on the same plate.
(e) When specific heterogenous Ab possess significantly different affinities or if in the case of heterogenous forms of the Ag, the dynamic range of the inhibition curve may deviate from linearity. As long as the above does not occur, the test Ag concentration can be accurately interpolated from the inhibition curve.

### 19.3.1.3 An Antibody-Sandwich ELISA to Detect Soluble Antigens

**Principle**

In this protocol to detect Ag, the wells of the microtiter plates are coated with specific (capture) Ab, unlike previous experiments where it

was coated with Ag, and incubated with test solutions containing Ag. This is followed by washing of unbound Ag and addition of a different Ag-specific Ab conjugated to enzyme (i.e., developing reagent) and plate is incubated. Thereafter, unbound conjugate is washed out and substrate is added. After further incubation, measurement on the degree of substrate hydrolyzed is done. This is proportional to the amount of Ag in the test solution.

**Application**

Most useful of all ELISA techniques for detection of Ag (five times more sensitive)

**Materials**

Specific antibody or immunoglobulin fraction from antiserum

**Procedure**

(a) Capture Ab (mono- or polyclonal) is prepared by diluting specific Ab or immunoglobulin fraction in PBSN to a final concentration of 0.2–10 μg/mL.
(b) Crisscross serial dilution analysis (Support Protocol-I) is carried out to determine the concentration of conjugate and capture Ab required to measure the desired Ag concentration. Capture Ab solution is prepared in PBSN at this concentration.
(c) Coat wells of a Immulon plate with capture antibody solution as in steps "d" to "g" of basic protocol.
(d) Block wells as in steps "h" to "j" of basic protocol.
(e) Prepare a standard antigen-dilution series by successive 1:3 dilutions of the homologous antigen stock in blocking buffer (Support Protocol-I).
(f) Prepare dilutions of test antigen solutions in blocking buffer.
(g) Add 50 μL aliquots of the Ag test solutions and the standard Ag dilutions (from step "e") to the antibody-coated wells, and incubate ≥2 h at a RT.
(h) Wash plate as in steps "m" to "p" of the basic protocol.
(i) Add 50 μL of specific antibody-alkaline phosphatase conjugate and incubate 2 h at RT.
(j) Wash plate as in steps "m" to "p" of the basic protocol.
(k) Add 75 μL of MUP or NPP substrate solution to each well and incubate 1 h at RT.

**Observations**

(a) Read the plates on a microtiter plate reader.
(b) To quantitate low-level reactions, the plate can be read again after several hours of hydrolysis.
(c) Prepare a standard curve constructed from the data produced by serial dilutions of the standard antigen (step "e").
(d) Plot Ag concentration on the *x*-axis (a log scale) and fluorescence or absorbance on the *y* axis (a linear scale).
(e) Interpolate the concentration of Ag in the test solutions from the standard antigen-inhibition curve.

**Points to Ponder**

(a) Though standard curves are not required for qualitative assay, these can be used to precisely determine the amount of Ag in test samples.
(b) To ensure that at least one of the dilutions can be accurately measured, carry out 1–2 two serial dilutions of the initial Ag test solutions. However, in most protocol systems, test solutions with 1–100 ng/mL of Ag can be accurately measured (step "f").
(c) Run samples in duplicate or triplicate for accurate quantitation. Include the standard Ag-dilution series on each plate.
(d) Perform pipetting rapidly to minimize differences in time of incubation between samples.
(e) The conjugate concentration (specific Ab) is typically 25–400 ng/mL (step "h").
(f) In protocols involving the capture Ab specific for a single determinant, the prepare conjugate from Ab which recognize different determinants remaining available after the Ag is bound to the plate by the capture Ab.

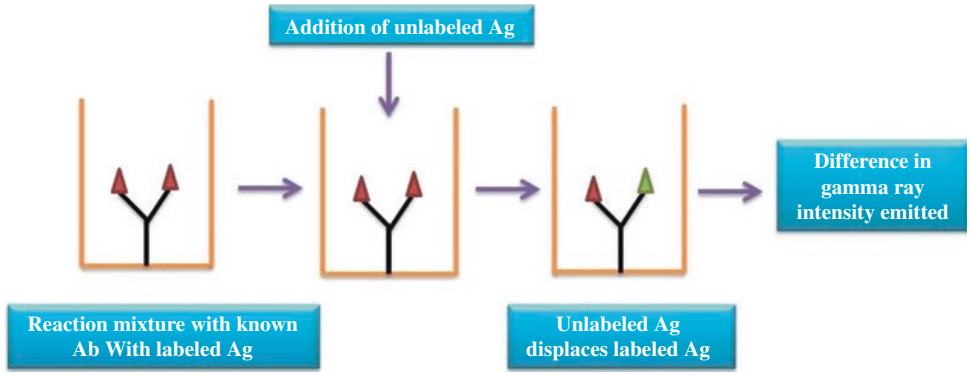


**Fig. 19.1** Radioimmunoassay reaction. In this assay, reaction mixture comprising of known antibodies (Ab) with labeled antigen (Ag) is mixed with unlabeled antigen. The unlabeled antigen then displaces the labeled antigen, and the difference in emitted gamma ray intensity is read

**Radioimmunoassay (RIA)**

RIA is an immunological technique to analyze any antigen (Ag) or antibody (Ab) in a given sample. The assay involves competitive binding of radiolabeled antigen and unlabeled antigen to a high-affinity antibody. The technique is very versatile, quick (usually 2 days or less), sensitive (< ng/mL), and specific (antibody dependent), and thousands of samples can be processed a day. However, the technique has a disadvantage of being hazardous due to the use of radioactive substances and requires expensive equipment (gamma or beta counter).

RIA involves three principles which make it most specific and sensitive (0.0006–0.006 μg antibody/mL) than other immune assays:

(a) An immune reaction, i.e., Ag-Ab binding.
(b) A competitive binding or competitive displacement reaction. (It gives specificity.)
(c) Measurement of radio emission. (It gives sensitivity.) (Fig. 19.1)

In classical RIA, a known quantity of an Ag is made radioactive (by labeling it with gamma-radioactive isotopes of iodine, for example, 125-I, attached to tyrosine, or beta-emitting isotopes such as tritium (3H)). This is followed by mixing of radiolabeled (hot) Ag with a known amount of Ab for that Ag, resulting in their specific binding. Thereafter, cold Ag (a sample of serum containing an unknown quantity of that same Ag, unlabeled) is added. This results in cold Ag to compete with the tagged (hot) Ag for Ab binding sites. Following increase in the concentration of cold Ag, more of it will bind to the Ab. This displaces the hot Ag reducing the ratio of Ab-bound radiolabeled Ag to free radiolabeled Ag. The radioactivity of the bound Ag remaining in the supernatant is measured using a gamma counter after separating the bound Ag from the unbound Ag. The common used steps in RIA technique is similar to sandwich ELISA described before.

### 19.3.2 Quantitative Assays to Detect Antigen-Antibody Reaction

In experiments where mere detection of the presence of antibody or antigen in a given sample is required quantitative assays can be employed. These assays are:

- Double immunodiffusion
- Western blot
- Dot blot

#### 19.3.2.1 Double Immunodiffusion Assay

**Principle**

Gel-diffusion techniques are one of the earliest and frequently used methods to detect specific antibodies and antigenicity of proteins. The unique nature of Ag-Ab interactions determines

the principle of this procedure. In this protocol, when polyvalent Ab with moderate-to-high intrinsic affinities are added to Ag at the proper ratio – called the zone of equivalence – lattice of Ag-Ab complexes form and precipitate out of the solution. At the equivalence zone, a line of insoluble precipitation forms when gradients of Ag and Ab are established by diffusion from adjacent wells in a bed of agar. This can be stained and viewed through naked eyes confirming the presence of specific antibodies against a particular antigen.

**Materials**

Noble agar, 1 mg/mL antigen, antisera, 2 × 3 inch microslides (thoroughly cleaned), boiling and 56 °C water baths, 50 °C oven, template, 15 G stainless steel needle (blunt-ended and beveled) or immunodiffusion punch set (EC apparatus), 10 μL Hamilton syringe, humidified chamber (enclosed plastic container with moistened tissue paper or cotton), staining rack and dish, Whatman 3MM filter paper

**PBSN solution**

100 mL PBS
0.05 g sodium azide ($NaN_2$)

**PEG 6000 (JT Baker)**

4 g PEG 6000
100 mL PBSN

**Noble agar solution (0.5, 1, and 2%)**

0.125/0.25/0.5 g Noble agar
25 mL PBSN

**Staining solution**

0.5% (wt/v) Coomassie Brilliant Blue R-250.
40% (v/v) ethanol.
10% (v/v) glacial acetic acid.
50% (v/v) DW.
Store at RT.

**Destaining solution**

15% (v/v) ethanol
05% (v/v) glacial acetic acid
80% (v/v) DW.
Store at RT.

**Procedure**

This procedure is applied in test solutions that are a mixture of antigen and antibodies.

**Pre-coating Microslides with Agar**

(a) Take two microslides, and clean them thoroughly using absolute alcohol.
(b) Place them on a perfectly horizontal surface.
(c) Dissolve 0.5% Noble agar by boiling it in a conical flask; take care to avoid overspilling.
(d) Place about 1–1.5 mL of melted agar over each microslide forming a very thin layer.
(e) Take care so that agar does not flow out of slide, and allow it to solidify.
(f) Allow gel to dry out at 50 °C for 4 h or overnight at RT.

**Preparing Analytical Gel**

(a) Dissolve 2% Noble agar by boiling in a conical flask; take care to avoid overspilling.
(b) Place coated microslides on a perfectly horizontal surface.
(c) Cool the 2% melted agar to 56 °C and mix with PEG solution (at 56 °C) at 1:1 ratio.
(d) Pipet 4.5 mL evenly over each slide, not disturbing until gel sets completely.
(e) Place the slides over a template, and either using needle or immunodiffusion punch set, carefully punch wells as per the need, i.e., one for antigen and at least four for antibodies.
(f) Remove agar plugs using a Pasteur pipette attached to a vacuum line.

**Loading the Gel**

(a) Prepare three antigen solutions (250 μg, 500 μg, and 1 mg/mL in PBSN).
(b) Using Hamilton syringe, fill the central wells with antigen solutions separately.
(c) Fill surrounding wells with undiluted hyperimmune sera (wells holds 5–10 μL).
(d) Allow samples to diffuse through wells.
(e) Incubate the microslides in humid boxes at 37 °C for 24–72 h.
(f) Observe for development of precipitin lines at 24 h interval.

**Washing and Staining the Gels**

(a) Place gels in a staining rack that in turn is placed in a staining dish filled with PBSN.
(b) Incubate for 24 h at room temperature with gentle stirring.
(c) Replace PBSN with fresh solution and repeat the procedure.
(d) Remove salt by replacing PBSN with DW for 4 h at RT.
(e) Remove gels from staining rack and place face up on a flat surface.
(f) Dry the gels by covering with 3MM filter paper and leaving over night at RT.
(g) Place dry gels in the staining rack and immerse in staining solution for 10 min at RT.
(h) Destain the gel by immersing in the destaining solution for 4 min.
(i) Repeat the destaining process until precipitin lines are maximally visible and background stain is negligible.

**Modified Procedure**

In test samples where antigens and/or antibodies are purified, a simple and quick method as given below can be followed:

(a) Take two microslides, and clean them thoroughly using absolute alcohol.
(b) Place them on a perfectly horizontal surface.
(c) Dissolve 2% Noble agar by boiling in a conical flask; take care to avoid overspilling.
(d) Place about 4.5 mL of melted agar over each microslide (forming a thickness of 1.5 mm).
(e) Take care so that agar does not flow out of the slide; allow it to solidify.
(f) Once agar solidifies, punch holes in a concentric manner using hub of a needle.
(g) Remove excess agar from the wells using needle.
(h) Now fill central well protein solution (100 μg in 1 mL of PBSN).
(i) Fill surrounding wells with serially diluted hyper immune sera (1:2–1:16 in PBSN).
(j) Repeat this procedure for each test antigen and antibody separately.
(k) Incubate the microslides in humid boxes at 37 °C for 48–72 h.
(l) Observe for development of precipitin lines at varying time interval.
(m) Presence of lines (Fig. 19.2) indicates hyperimmune sera (IgG) against antigen.
(n) Immerse the gels in staining solution; destain, dry, and store as permanent record.

**Observations**

DID assay in which immunoprecipitates are stained with Coomassie Brilliant Blue can be sensitive to low concentration (25 μg/mL) of antibodies. In unstained gels concentrations of 100 μg/mL of antibodies as immunoprecipitation lines can be viewed. Observe details of precipitation lines for the presence of spurs, lines of identity, and double precipitin lines which can reveal information about the antigenic specifications of various antisera and information about structure of the antigen.

**Points to Ponder**

(a) This assay requires high concentrations of antigen and antibodies in test solutions.
(b) Gel-diffusion assay is relatively insensitive to antibodies with low affinity.
(c) The initial Ag and Ab concentration must be able to support the equivalence zone. Thus, usually three different Ag concentrations are recommended. Consider more sensitive techniques like ELISA when there is failure to observe no lines of precipitation even after staining with silver stain.
(d) A dried agar pre-coat provides an adhesive base that prevents the analytical agar from separating from the slides during staining and destaining treatments.
(e) PEG stabilizes immunoprecipitates and increases their visibility.
(f) To remove agar plugs, always use a weak vacuum, taking care not to disturb surrounding agar field.
(g) To increase the amount of reagent loaded, wells can be filled two or three times. After the liquid is absorbed into the gel (5–10 min), wells may be refilled again.

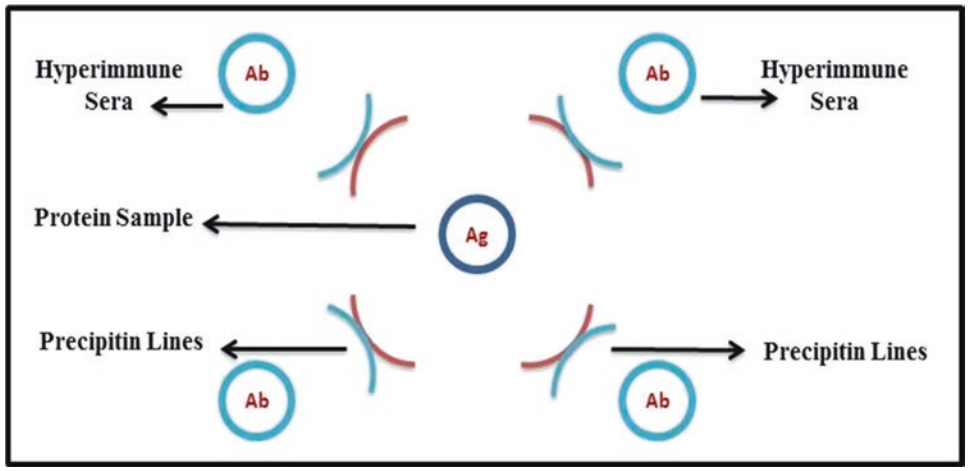


**Fig. 19.2** Double immunodiffusion (DID) assay. In this assay, five wells (central well surrounded by four wells) are punched in the solidified agar over a clean glass slide. The central well contains protein sample (antigen, Ag), whereas four surrounding wells contain hyperimmune sera (antibodies, Ab). After incubation in the humid chamber, the migration and subsequent meeting of the Ag and Ab result in band formation (stained with Coomassie Blue) known as "precipitin" lines in the immunodiffusion assay

(h) Gels should not be in direct contact with moistened tissue in dehumidifier chamber.
(i) Washing is done to remove proteins that are not precipitated.

#### 19.3.2.2 Western Blot or Enzyme-Linked Immune-Transfer Blot

**Principle**

Enzyme-linked immune-transfer blot is done to detect the specificity of the raised antisera. In this procedure, the proteins resolved on SDS-PAGE are transferred electrophoretically onto the nitrocellulose membrane (e.g., NCM, Sigma-Aldrich, USA) using blotting apparatus (e.g., ATTO, Japan). Checkerboard ELISA is performed before Western blot to determine the appropriate concentration of antibody against specific antigen (protein). We have reproduced the procedure of Towbin et al. 1979 [4] here.

**Materials**

Nitrocellulose membrane, Whatman filter paper No. 3, transfer buffer, PBS, Tween 20, skim milk, diluted antibodies against protein (hyperimmune sera if this has been raised in laboratory animals otherwise procure commercially), goat anti-rabbit HRPO conjugate, substrate solution, and blotting apparatus

**Washing buffer (PBST)**

0.5 g Tween 20.
Dilute to 1000 mL PBS (pH 7.4).

**PBST with SM powder (5%) pH 7.4**

5 g skim milk
100 mL PBST

**Blocking solution**

3.0 g bovine serum albumin.
Dilute to 100 mL PBST.

**Transfer buffer**

18.2 g Tris base.
86.5 g glycine.
1200 mL methanol.
Dilute to 6000 mL DW.

**Coating buffer (0.05 M carbonate buffer, pH 9.5)**

1.5 g sodium carbonate.
2.93 g sodium bicarbonate.
0.02 g sodium azide.
Dilute to 1000 mL DW.

**Substrate solution**

25 mg ortho-phenylenediamine.
25 mL citric acid phosphate buffer (pH 5.0).
Mix well and add.

25 µL hydrogen peroxide ($H_2O_2$).
Protect from light.

**Stopping solution ($H_2SO_4$, 1 M)**
5.4 mL sulfuric acid (concentrated)
94.6 mL DW

**Procedure**

(a) Run the protein in SDS-PAGE to get a desired band.
(b) After electrophoresis, take the gel off from the plates and keep in transfer buffer to remove excess SDS from the gels.
(c) Take 8–10 Whatman filter paper No. 3 of a size little larger than the gel and soak in transfer buffer.
(d) Stack filter paper one by one on the anode plate.
(e) Take care to avoid air bubbles in between stacks.
(f) Take NCM pre-wet in transfer buffer and place it over the filter paper stacks.
(g) Now place gel containing protein band carefully to avoid air bubbles.
(h) Mark the orientation of the membrane with respect to gel.
(i) Take 8–10 Whatman FP No. 3 of a size little larger than the gel and soak in transfer buffer.
(j) Stack filter paper one by one on the gel.
(k) Saturate the complete stack with ice-cold transfer buffer.
(l) Now place the cathode plate in position over the stack.
(m) Apply a current of 3–5 mA/cm$^2$ for 3 h.
(n) Stain the gel afterward to check the efficiency of transfer (Fig. 19.3).

**Development of Blot**

(a) Incubate the NCM after transfer overnight in 5% (w/v) skim milk in PBST for blocking the nonspecific binding sites.
(b) After blocking, wash the membrane thrice with PBST (5 min each).
(c) Incubate with 1:400 diluted rabbit anti-protein serum at 37 °C for 2 h.

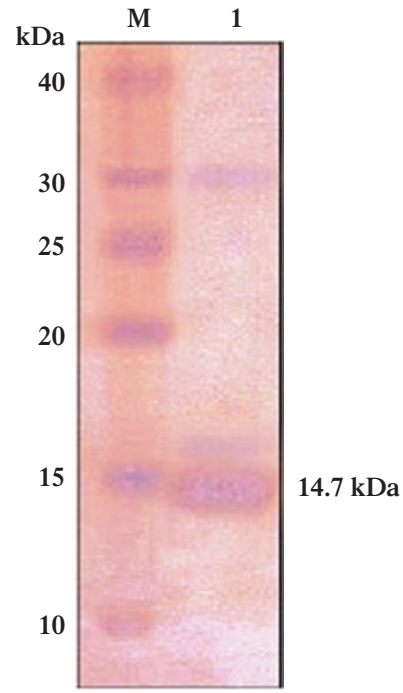


**Fig. 19.3** Western blot or enzyme-linked immune-transfer blot. Microphotograph shows Western blot of a protein using antisera raised against it. M indicates the lane with bands of marker, whereas "l" shows the lane where antisera have reacted with the target protein forming a thick band

(d) Wash the membrane thrice with PBST (5 min each) again.
(e) Incubate for 1 h at 37 °C with 1:5000 diluted goat anti-rabbit HRPO conjugate.
(f) After washing, detect the protein antibody reaction by incubating the membrane with substrate solution.
(g) Terminate the color reaction by washing the membrane with DW to prevent background coloration.
(h) Stain and observe band (Fig. 19.3; Table 19.2).

### 19.3.2.3 Dot Blot ELISA

**Principle**

A dot blot ELISA is a simple and quick assay that may be employed to determine if selected antibodies and detection system are effective and to determine appropriate starting concentration of primary antibody for Western blot.

**Table 19.2** Troubleshooting

| Observation | Reason | Solution |
|---|---|---|
| No bands/faint bands observed | Insufficient antibody | Low affinity Ab, increase Ab concentration (two to fourfold higher) |
| | | Lost activity Ab, perform dot blot |
| | Insufficient protein | Increase the amount of total protein loaded on gel |
| | | Use a positive control (recombinant protein) |
| | Poor transfer | Wet NCM in methanol |
| | | Ensure good contact between NCM and gel |
| | Incomplete transfer | High MW protein may require more time for transfer |
| | | To ensure transfer is complete, stain the membrane with Ponceau S, Amido Black, or India Ink |
| | Sodium azide contamination | Sodium azide can quench HRP signal |
| | Washing frequency | Reduce the number of washes to minimum |
| | Inactive conjugate | Mix enzyme and substrate in a tube; if weak color our develops, make fresh or purchase new reagents. |
| Extra bands | Nonspecific binding of primary antibody | Reduce primary Ab concentration/amount of total protein loaded on gel concentration |
| | Nonspecific binding of secondary antibody | Run a control with the secondary Ab alone (omit primary Ab); if bands develop, choose an alternative secondary Ab |
| Diffuse bands | Excessive protein on gel | Reduce amount of protein loaded |

**Materials**

Nitrocellulose membrane (NCM), primary antibody, recombinant protein, Western Glo Chemiluminescent detection reagents, Tween 20 blocking solution (TTBS).

**Tween 20 blocking solution (TTBS, pH 7.4)**

Tris (50 mM)
Sodium chloride (0.5 M)
Tween 20 (0.05%)

**Basic Protocol**

(a) Take a strip of nitrocellulose membrane.
(b) Blot 10 μL of different concentrations of recombinant protein onto the membrane.
(c) Blot 10 μL of different concentrations of cell lysates onto the membrane.
(d) Blot 10 μL of 100 μg/mL of primary antibody onto the membrane.
(e) Incubate the membrane for 1 h at room temperature, ensuring that the blots are dry before going to the next step.
(f) Block the membrane with 5% dry milk in TTBS for 1 h at RT.
(g) Pour off the block buffer, but keep membrane wet at all times for the remainder of the procedure.
(h) Incubate the membrane with primary antibody for 1 h at RT in TTBS.
(i) Wash the membrane three times (10 min each) in TTBS on rocker.
(j) Incubate the membrane with secondary antibody for 1 h at RT in TTBS.
(k) Wash the membrane three times (10 min each) in TTBS on rocker.
(l) Detect with Western Glo Chemiluminescent detection reagents.
(m) Expose to film.

**Observations**

Investigator may find arrange blots in the following manner (Fig. 19.4):

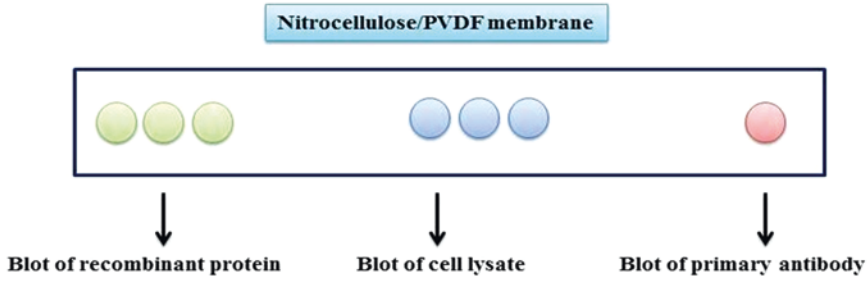


**Fig. 19.4** Dot blot ELISA. A quick and simple assay employed to evaluate if selected Abs and detection system are efficient and to determine appropriate initial concentration of primary Ab for Western blot

## 19.4 Isolation of Immunoglobulin (IgG)

**Principle**

Gamma globulin is a very powerful immunogen and is usually prepared as an ammonium sulfate precipitate of the whole serum.

**Materials**

Ammonium sulfate, dilute ammonia solution, hyperimmune sera, DDW, NaCl, centrifuge, glass rod, dialysis tubing, $NaHCO_3$, EDTA, KCl, $Na_2HPO_4$, $k_2HPO_4$, and $BaCl_2$

**Ammonium sulfate solution (saturated)**

100 g ammonium sulfate.
Dilute to 100 mL DW.
Dissolve at 50 °C, allow to stand at RT, and adjust the pH to 7.2 using dilute $NH_3$.

**PBS (0.15 M, pH 7.2)**

0.8 g sodium chloride.
0.02 g potassium chloride.
0.115 g disodium hydrogen phosphate.
0.02 g potassium dihydrogen phosphate.
Dilute to 100 mL DW.

**Sodium bicarbonate + EDTA (dialysis) solution**

2 g sodium bicarbonate.
0.05 g EDTA.
Dilute to 100 mL DW.

**Desalting solution (Barium chloride, 1%)**

1 g Barium chloride.
Dilute to 100 mL DW.

**Procedure**

(a) Take hyperimmune sera in a test tube and allow clotting by keeping it in slating position at RT.
(b) Free the clot from sides of the walls to aid retraction of serum.
(c) Collect the serum by the pipette and centrifuge at 3000 g for 15 min.
(d) Dilute serum with normal saline (1:2), and add saturated ammonium sulfate solution to the final concentration of 45% (v/v).
(e) Stir at 4 °C for 30 min.
(f) Centrifuge the precipitate at 3000 g 4 °C for 30 min.
(g) Wash the precipitate with saturated ammonium sulfate solution (45%) and centrifuge.
(h) Redissolve the precipitate in the same volume of PBS as the original serum.
(i) Centrifuge to remove any insoluble material.
(j) Re-centrifuge the globulin using a final concentration of 40% saturated ammonium sulfate solution.
(k) Centrifuge and wash the pellet with 40% saturated ammonium sulfate solution.
(l) Redissolve the precipitate in the minimum volume of PBS.
(m) Dialyze the globulin against PBS at 4 °C (four changes).

**Activation of Dialysis Tubing**

(a) Select dialysis tubing of suitable diameter and length as per sample volume.
(b) Submerge it in the dialysis solution and boil for 10 min.

(c) Discard the solution and again boil it in the dialysis solution for 10 min.
(d) Wash in DW water 4–5 times.
(e) Boil again in DW for 5 min.
(f) Wash in DW twice.
(g) Activated dialysis tubing can be stored at 4 °C up to 3 months.

**Desalting of Sample by Dialysis**

(a) Seal the dialysis tubing from one end by a thread.
(b) Pour the sample in it from the other end and seal this end also.
(c) Hang it in beaker containing 100 volume of PBS.
(d) Keep the beaker on the magnetic stirrer and leave it at 4 °C for 2 h.
(e) Similar procedure is followed for four changes.
(f) To check completion of dialysis (desalting), add desalting solution.
(g) Appearance of precipitate indicates incomplete dialysis.
(h) Repeat dialysis if required.

**Protein Estimation**

(a) Determine the absorbance of the sample at 280 nm in a UV spectrometer.
(b) An OD of 1.0 (1 cm curve) is equivalent to an r-globulin concentration of 0.74 mg/mL.

**Alternate Protocol for IgG Isolation**

The ion exchange chromatography procedure as described by [3] was employed to purify the antibody which is described here. Always ascertain the presence of antibody in the given sample using the quantitative assays described before.

**Materials**

Ammonium sulfate solution (saturated, 34%), phosphate buffer (pH 8.0), and DEAE-Sephadex media

**Procedure**

(a) Take 10 mL of hyperimmune sera in a 50 mL tube, and add 20 mL of saturated ammonium sulfate solution (SAS) slowly by continuously stirring at 4 ° C for 2 h.
(b) Centrifuge the solution at 9000 g for 10 min and collect precipitate.
(c) Dialyze the precipitate overnight against 0.01 M phosphate buffer (pH 8.0) at 4 °C.
(d) Take pre-cleaned glass columns and mount on the stand after marking at 20 cm height.
(e) Pour the DEAE-Sephadex media till the mark.
(f) Equilibrate the packed column with 0.01 M phosphate buffer (pH 8.0) at RT.
(g) Apply the previously dialyzed sample onto the top of equilibrated column.
(h) Stir the top of the column bed when flow rate of 2 mL/5 min decreases.
(i) Wash the column bed in two steps.
(j) In the first step, pass three bed volumes (35 ml, first washing) of 0.01 M phosphate buffer (pH 8.0).
(k) Observe absorbance of the fraction (5 mL) at 280 nm to ascertain the presence of proteins.
(l) Repeat first washing till the OD shows no trace of proteins in the elute.
(m) Pass 0.03 M phosphate buffer containing 100 mM NaCl; collect each 5 mL elute (two washes).
(n) Observe the OD of the eluted 5 mL fractions in spectrophotometer at 280 nm.
(o) Select the samples with peak OD and analyze in 15% SDS-PAGE.
(p) Estimate the protein content as described before.

## 19.5 Detecting Antisperm Antibody (ASA) in Samples

Spermatozoon is a foreign protein in the female and thus may lead to the generation of antibodies against it resulting in failure of conception [1].

Though this condition is not so common, it anyway warrants a screening of the animals. At the outset, spermatozoa demonstrating agglutination (sticking of motile spermatozoa to each other tail to tail, head to head, or in a mixed way) (see Chap. 3), the presence of sperm antibodies may be the reason.

The mere presence of ASA is an insufficient reason for the diagnosis of sperm autoimmunity. A researcher must demonstrate that the sperm function is severely hampered by Abs; this is usually done by a sperm-mucus penetration test (refer to the chapter on functional assay). Moreover, zona binding and the AR functions are also interfered by ASA.

Two immunoglobulin classes of ASA in semen almost exclusively are IgA and IgG. Of the two, IgM antibodies, being larger, are filtered out by blood-testis barrier and hence rarely found in semen. Clinical importance of IgA is greater than IgG antibodies [5]. IgA and IgG are found on sperm cells or in biological fluids in related screening assays.

Assays for ASA are grouped into two types: assays for ASA on spermatozoa (direct assays) and assays for ASA in sperm-free fluids (indirect assays; seminal plasma, blood, and secretions from female genital tract) [6]. In IB-indirect test, the diluted, heat-inactivated fluid suspected of containing ASAs is incubated with Ab-free washed donor sperm. Donor spermatozoa will show attachment of any ASAs in the suspect fluid. These bound spermatozoa are then assessed in the IB-direct test.

A comparison of merits and demerits of various assays employed for detecting ASA has been provided in Table 19.3.

MAR, Mixed antiglobulin reaction; IB, Immunobead, IVF, In vitro fertilization.

**Table 19.3** Comparative merits of assays employed for ASA detection

| Assay | Merits | Demerits |
|---|---|---|
| MAR test | On fresh semen sample, inexpensive, quick, and sensitive (Rajah et al. 1992) [7] | Provides less information than IB test (Bronson et al., 1984) [8]. Results from MAR test do not always agree with IB tests (MacMillan & Baker, 1987) [9] |
| IB (direct) test | On washed spermatozoa, results are correlated with IVF assay; provides information about masked Ab on the sperm surface | More time-consuming than the MAR test |
| IB (indirect) test | Cervical and body fluids can be tested for ASA | Depends on motile sperm number; hence any decrease in motility will affect result |

## 19.5.1 Assays for Antisperm Antibodies (Direct Assays)

For detection of ASA on spermatozoa, the following two assays are mostly followed:

(i) Mixed antiglobulin reaction (MAR) test
(ii) Immunobead (IB-direct) test

### 19.5.1.1 The Mixed Antiglobulin Reaction (MAR) Test

**Principle**

In this test, a "bridging" antibody (anti-IgG or anti-IgA) is used. The "bridging" Abs bring the Ab-coated beads into contact with fresh spermatozoa (with superficial IgG or IgA). Mix untreated fresh semen separately with treated red blood cells coated with IgG or IgA or latex particles (beads) to perform the direct IgG and IgA MAR tests. This is followed by adding a mono-specific anti-IgG or anti-IgA. The presence of IgG or IgA is indicated by the formation of mixed agglutinates between beads and motile spermatozoa.

**Materials**

ASA-positive semen, ASA-negative semen, IgG−/IgA-coated latex beads, and anti-IgG/IgA

**Procedure**

(a) Gently mix the semen sample.
(b) Take two microscopic slides and place aliquots of 3.5 μL of semen separately (replicates).

(c) For each test, include one slide with 3.5 μL of ASA-positive semen and one with 3.5 μL of ASA-negative semen as controls.
(d) Add 3.5 μL of IgG-coated latex beads to each droplet of test and control semen, and mix by stirring with the pipette tip.
(e) Add 3.5 μL of antiserum against IgG to each semen-bead mixture, and mix by stirring with the pipette tip.
(f) Cover the suspension with a coverslip.
(g) Place the slides horizontally in a humid chamber for 3 min at RT.
(h) Examine the wet preparation with phase-contrast optics at ×200 or ×400 magnification after 3 min and again after 10 min.
(i) Repeat the procedure using IgA instead of IgG-coated beads and anti-IgA instead of anti-IgG antibodies.

### Observations

(a) The beads will adhere to the spermatozoa having Ab on their surface. At initial examination, the motile spermatozoa will be seen moving around with a few or even a group of beads attached. At later stages, with sticking together of many spermatozoa, movement will be greatly restricted. In contrast, sperm devoid of surface Ab will be seen swimming freely between the particles.
(b) For calculating the number of spermatozoa (%) adhering to beads, the following points may be observed:
    (i) Score only motile spermatozoa and determine the percentage of motile spermatozoa that have two or more beads attached.
    (ii) Ignore tail-tip binding and evaluate at least 200 motile spermatozoa in each replicate.
    (iii) Record the class (IgG or IgA) and the site of binding of the latex particles to the spermatozoa (head, mid-piece, principal piece) (Table 19.4).

**Table 19.4** Observation on bead-bound spermatozoa

| No. of bead-bound sperm | Remarks |
|---|---|
| 100% at 3 min | Take this as test results; do not observe after 10 min |
| <100% at 3 min | Observe the slide after 10 min |
| Immotile at 10 min | Take the value at 3 min as the result |

### Inference

Since the reference value is not available for animal species, investigators are advised to follow the average value as threshold value of the group of animals they are examining.

Abshagen et al. [10] reported that when more than 50% of the sperm population shows binding with Ab, penetration into the cervical mucus and in vivo fertilization tend to be significantly impaired. However, binding of beads to the tail tip only is not associated with impaired fertility [11].

### Points to Ponder

(a) Antisperm antibodies can be present without causing sperm agglutination.
(b) Agglutination can be caused by factors other than sperm antibodies.
(c) These assays are not suitable for detecting cytotoxic antibodies that kill all spermatozoa or inhibit sperm motility.
(d) In MAR tests agglutination between beads serves as a positive control for Ab-Ag recognition.
(e) Positive control spermatozoa in MAR test can be produced by incubation in a serum known to contain antibodies.
(f) Use anti-IgG or IgA specific to the species of the animal being tested.
(g) The objective of the MAR test is to measure the number (%) of agglutinated motile spermatozoa. Confusion may occur with respect to unattached normal sperm that are close to beads. Attachment of such spermatozoa with beads can frequently be ascertained by lightly tapping the coverslip using a small pipette tip. Positive binding is indicated by the concerted movement of beads with active spermatozoa.

#### 19.5.1.2 The Immunobead-Direct Test

**Principle**

In this test, washed spermatozoa are directly mixed with beads coated with covalently bound rabbit antihuman (or the particular test species) immunoglobulins against IgG or IgA. The presence of IgG or IgA on the surface of the spermatozoa is evaluated by visual observation indicating binding of beads with anti-IgG or anti-IgA to motile cells. The procedure described by Bronson et al. 1982 and Clarke et al. [12, 13] is described here.

**Materials**

Dulbecco's glucose-phosphate-buffered saline (PBS)/bovine serum albumin (BSA) or Tyrode's-BSA solution

**Buffer I**

0.3 g of Cohn Fraction V BSA.

Dilute to 100 mL of Dulbecco's PBS/Tyrode's medium.

**Buffer II**

0.5 g of Cohn Fraction V BSA.

Dilute to 100 mL of Dulbecco's PBS/Tyrode's medium.

Filter all solutions through 0.45 μm filters and thaw at 25–35 °C before use.

**Preparing the Immunobeads**

(a) Add 0.2 mL of stock bead suspension to 10 mL of buffer I in separate centrifuge tubes.
(b) Centrifuge at 500 g for 5–10 min.
(c) Decant and discard the supernatant from the washed immunobeads.
(d) Gently re-suspend the beads in 0.2 mL of buffer II.

**Preparing the Spermatozoa**

(a) The amount of semen required for the assay is determined from the sperm concentration and motility, as shown in Table 19.5.
(b) Gently mix the semen sample well.

**Table 19.5** Determination of quantity of semen to use for an immunobead test

| Sperm concentration (million/mL) | Sperm motility (progressive, %) | Volume of semen required (mL) |
|---|---|---|
| >50 | – | 0.2 |
| 21–50 | >40 | 0.4 |
| 21–50 | <40>10 | 0.8 |
| 10–20 | >40 | 1.0 |
| 10–20 | <40>10 | 2.0 |
| <10>5 | >10 | >2.0 |

Note: Above values are for human semen samples and hence need recalibration for different animal species.

(c) Transfer the required amount of semen to a centrifuge tube.
(d) Make up the volume to 10 mL with buffer I.
(e) Centrifuge at 500 g for 10 min.
(f) Decant and discard the supernatant from the washed spermatozoa.
(g) Gently re-suspend the sperm pellet in 10 mL of fresh buffer I.
(h) Centrifuge again at 500 g for 10 min.
(i) Decant and discard the supernatant.
(j) Gently re-suspend the sperm pellet in 0.2 mL of buffer II.

**Procedure**

(a) For each test, include one slide with 5 μL of ASA-positive semen and one with 5 μL of ASA-negative semen as controls.
(b) Place 5 μL of the washed sperm suspension (test) on a microscope slide.
(c) Add 5 μL of anti-IgG immunobead suspension beside each sperm droplet.
(d) Mix above two droplets by stirring with the pipette tip.
(e) Cover the suspension with a coverslip.
(f) Place the slides horizontally in a humid chamber for 3 min at RT.
(g) Examine the wet preparation with phase-contrast optics at ×200 or ×400 magnification after 10 min.
(h) Repeat the procedure using IgA instead of IgG-coated beads and anti-IgA instead of anti-IgG antibodies.
(i) Repeat the procedure using the anti-IgA immunobead suspension.

**Observation**
As for MAR test

**Interpretation**
As for MAR test

**Points to Ponder**

(a) If the aliquot is more than 1.0 mL, wash thrice.
(b) For samples with low sperm motility (<10%), carry out IB-indirect test.
(c) To ensure that all binding is assessed within 10 min, it is best to stagger the preparation of the slides.

### 19.5.2 Tests for Antisperm Antibodies in Sperm-Free Fluids (Indirect Assays)

The immunobead (IB-indirect) test is employed to evaluate the presence of ASA in body fluids.

#### 19.5.2.1 The Immunobead-Indirect Test

The test described here is employed to determine ASA in sperm-free heat-inactivated fluids (seminal plasma, testicular fluid, serum, or bromelain-solubilized cervical mucus). Ab-free donor's spermatozoa take up ASA present in the tested fluid which are then evaluated as in the direct immunobead test.

**Materials**
Bromelain (10 IU/mL, a broad specificity proteolytic enzyme for cervical mucous testing) and other chemicals as described for IB-direct test

**Preparing the Immunobeads**
As described for IB-direct test

**Preparing the Spermatozoa**
As described for IB-direct test

**Preparing the Sample Fluid to be Tested**
Inactivate any complement in the solubilized cervical mucus, serum, seminal plasma, or testicular fluid by heating at 56 °C for 30–45 min.

**Cervical Mucous**
Dilute cervical mucus (1:2) with 10 IU/mL bromelain.
Stir with a pipette tip and incubate at 37 °C for 10 min.
After liquefaction, centrifuge at 2000 g for 10 min.
Use the supernatant immediately for testing, or freeze at −80 °C.

**Body Fluids**
Dilute the heat-inactivated sample (1:4) with buffer II.

**Procedure**

(a) Include known-positive and known-negative samples, as described before.
(b) Mix 50 μL of washed test sperm suspension with 50 μL of 1 + 4 diluted fluid to be tested.
(c) Incubate at 37 °C for 1 h.
(d) Centrifuge at 500 g for10 min.
(e) Decant and discard the supernatant.
(f) Gently re-suspend the sperm pellet in 10 mL of fresh buffer I.
(g) Centrifuge again at 500 g for 10 min.
(h) Decant and discard the supernatant.
(i) Repeat the washing steps f, g, and h above.
(j) Gently re-suspend the sperm pellet in 0.2 mL of buffer II.
(k) Perform the IB test, as described earlier with the fluid-incubated test spermatozoa.

**Observation**
As for MAR test

**Interpretation**
As for MAR test

**Points to Ponder**

For repeatability of the results, permit sufficient time for the sperm-Ab interaction, since up to 10 min might be required for the mixed agglutination to become visible. However, investigator must bear in mind that time lag lowers sperm motility; this is important since the test depends on the presence of motile spermatozoa.

## 19.6 Background Information

Recent advancements in the assays involving immuno-reproduction have opened very exciting avenues of science for the investigators of semen biology. Yalow and Berson [14], at the Veterans Administration Hospital in Bronx, New York, to study iodine metabolism and blood volume, developed the protocol of the first radioisotopic technique. This technique was later adapted by them to study the mechanism of the body to utilize insulin. These researchers showed that the inefficient use of insulin causes type II (adult onset) diabetes. The earlier misconception was that lack of insulin is the reason for diabetes to occur. This precise measurement technique was perfected by Yalow and Berson in 1959 [15] naming it as radioimmunoassay (RIA). RIA is extremely sensitive (measures one trillionth of a gram of material per mL of blood). RIA quickly became a standard laboratory tool for its advantage of requirement of the small samples for measurement. Dr. Yalow earned the Nobel Prize for Medicine in 1977 (the second woman ever to win it) for this revolutionary development [16]. In her acceptance speech, Dr. Yalow said, "The world cannot afford the loss of the talents of half its people if we are to solve the many problems which beset us."

It was much later that the general method for assaying Ab and Ag binding was developed. At first enzyme-linked immunosorbent assay (ELISA), a highly sensitive and quantitative technique, was developed [2]. This was followed by development of electrophoresis and immunofixation, a technique which involves advantages of agarose gel electrophoresis and the specificity of Ab-Ag reaction, and then the double immunodiffusion assay, a simple but not useful for evaluating monoclonal Ab to monovalent Ag.

Of all the ELISA techniques, Ab-sandwich assays are the most sensitive (detects concentration of protein Ag of 100 pg −1 ng/mL). Generally, sandwich ELISAs are an order of magnitude more sensitive than ELISA in which Ag is directly bound to plates. The ELISA techniques are designed to take 6 h, but an investigator can abbreviate or expand the incubation time as needed. Stronger specific signals can usually be obtained by prolonged incubation because equilibration binding between the soluble and solid phases frequently takes 5–10 h. Fluorogenic ELISAs are generally 10–100 times quicker than assays using chromogenic substrates.

## Literature Cited

1. Srivastava SK, Shinde S, Singh SK, Mehrotra S, Verma MR, Singh AK, Nandi S, Srivastava N, Singh SK, Kumar H, Ghosh SK (2016) Anti-sperm antibodies in repeaters: frequency, detection and validation of threshold levels employing sperm-immobilization, − agglutination and immunoperoxidase assay in Cattle. Reprod Domest Anim 52:195–202. doi:10.1111/rda.12877
2. Engvall E, Perlman P (1971) The enzyme-linked immunosorbent assay (ELISA): quantitative assay of immunoglobulin G. Immunochemistry 8:871–879
3. Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W (1992) Currant protocols in immunology. In: Coico R (ed) Current protocols. Wiley, Greene Publishing Associates, Inc./Willey, New York
4. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci U S a 76:4350–4354
5. Kremer J, Jager S (1980) Characteristics of antispermatozoal antibodies responsible for the shaking phenomenon, with special regard to immunoglobulin class and antigen reactive sites. Int J Androl 3:143–152
6. Cooper TG (ed) (2010) WHO laboratory manual for the examination and processing of human semen. 5th edn. World Health Organization, Geneva, Switzerland.
7. Rajah SV, Parslow JM, Howell RJ, Hendry WF (1992) Comparison of mixed antiglobulin reaction and direct immunobead test for detection of sperm-bound antibodies in subfertile males. Fertil Steril 57:1300–1303
8. Bronson RA, Cooper GW, Rosenfeld D (1984) Sperm antibodies: their role in infertility. Fertil Steril 42:171–183

9. MacMillan RA, Baker HW (1987) Comparison of latex and polyacrylamide beads for detecting sperm antibodies. Clin Reprod Fertil 5:203–209
10. Abshagen K, Behre HM, Cooper TG, Nieschlag E (1998) Influence of sperm surface antibodies on spontaneous pregnancy rates. Fertil Steril 70:355–356
11. Chiu WW, Chamley LW (2004) Clinical associations and mechanisms of action of antisperm antibodies. Fertil Steril 82:529–535
12. Bronson RA, Cooper GW, Rosenfeld D (1982) Detection of sperm specific antibodies on the spermatozoa surface by immunobead binding. Archives Androl 9:61
13. Clarke GN, Clarke GW, Elliott PJ, Smaila C (1985) Detection of sperm antibodies in semen using the Immunobead test: a survey of 813 consecutive patients. Am J Reprod Immunol Microbiol 7:118–123
14. Berson SA, Yalow RS (1959) Recent studies on insulin-binding antibodies. Ann N Y Acad Sci 25(82):338–344
15. Yalow RS, Berson SA (1960) Plasma insulin concentrations in nondiabetic and early diabetic subjects. Determinations by a new sensitive immuno-assay technic. Diabetes 9:254–260
16. Vare EA, Ptacek G (2002) Patently female: from AZT to TV dinners: stories of women inventors and their breakthrough ideas. Wiley, New York, p 99

## Key References

17. Coligan and co-workers (1992) See above [3] Provides protocols and comparative merits/demerits of various ELISA and other techniques employed in assessment of interaction of binding of Ab with Ag.
18. WHO laboratory manual (2010) See above [6] Provides excellent information on some of the immunological assays mentioned in this chapter.