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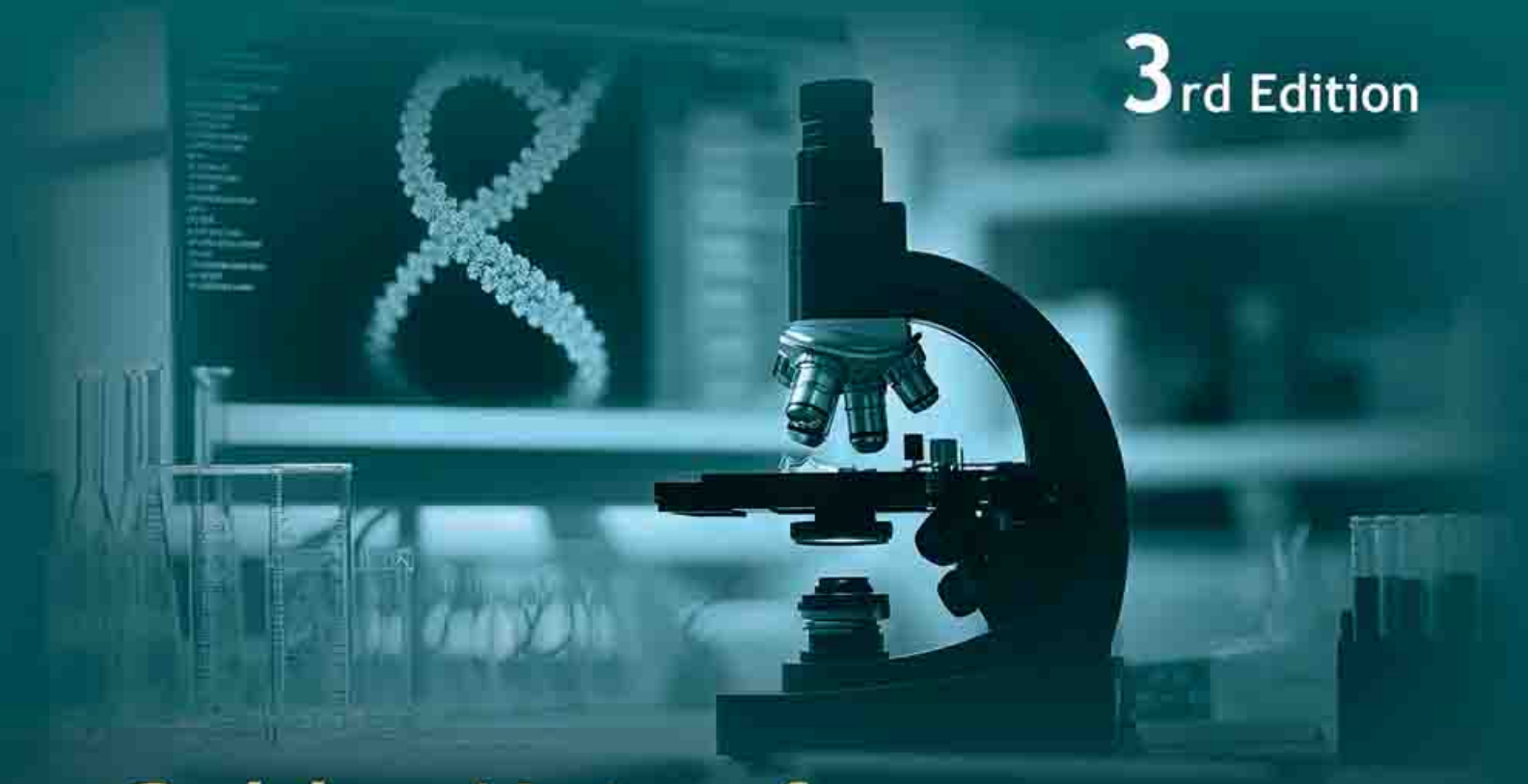

3rd Edition

Golden Notes for MEDICAL LABORATORY SCIENCE

Previously Known as
Concise Book of Medical Laboratory Technology—Methods and Interpretations

Ramnik Sood
Madhu Choudhary



Dr. Wise
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CHAPTER 23

Diagnostic Immunology

Nonenzymatic, Quantitative Techniques (Immunodiffusion, Electrophoresis and Turbidimetry)

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QUALITATIVE DETERMINATION OF PLASMA PROTEINS BY IMMUNOPRECIPITATION

The principle of immunoprecipitation was first described by Kraus. Originally, immunoprecipitation reactions carried out in test tubes were detected by the fact that turbidity can be observed following the mixing of antigen (Ag) and antibody (Ab) solutions. Centrifugation of such samples results in an insoluble sediment. Layering the antigen solution on top of the antibody solution in a narrow test tube, without mixing, results in a ring-like turbidity at the contact area within a short time. This so-called "ring test" reveals the presence of Ag-Ab precipitation in the solution studied. A distinction of characteristics between the various antigens and antibodies cannot be made.

Q. What is immunodiffusion method of oudin?

With this method, the Ag-Ab precipitation takes place in a gel medium. Due to the different diffusion rates of heterogeneous antigens (e.g., the proteins of blood serum) the differentiation of the various antigens is rendered possible. The linear (one dimensional) double diffusion technique, i.e., diffusion of antigens and antibodies from opposite directions, results in the formation of multilayered precipitations if heterologous Ag solutions and multivalent antisera are used.

Agar is most frequently employed for the gel because of its transparency and solidification characteristics.

Q. What is the double diffusion method of ouchterlony?

Agar gel is poured uniformly onto glass plates or into Petri dishes and allowed to solidify. Holes (wells) are cut into the agar gel which are filled with Ag and Ab solutions, respectively. Both the antigens and antibodies diffuse radially, and form, upon confluence, immunoprecipitates. These immunoprecipitates often take the form of a curved line. Because of its numerous possible modifications, the versatile arrangement of Ag-Ab containing wells, etc., this technique is widely applied in immunological, biochemical and medical laboratories.

Methodology

Macrotechnique

Reagents

- Phosphate buffer solution 0.15 mol/L, pH 7.1. This solution is obtained by mixing 67 mL of solution I with 33 mL of solution II.
  - *Solution I:* Distilled water is added to 26.70 g of $Na_2HPO_4 . 2H_2O$ to total 1,000 mL.
  - *Solution II:* Distilled water is added to 20.41 g of KH_2PO_4 to total 1,000 mL.
- **Agar:** Behringwerke agar purum or agar of equal quality which possesses satisfactory transparency.
- **Antisera:** Antisera to plasma proteins.

Preparation of agar gel slides

Four grams of purified agar is mixed with 100 mL of phosphate buffer solution 0.15 mol/L, pH 7.1, and 300 mL of distilled water. Approximately 40 mg of thimerosal are added as a preservative. This mixture is heated at 100°C until the solution is clear. Undissolved particles can be removed by centrifugation of the hot solution at 3,000 rpm for 2–3 minutes.

Twenty-two milliliters of the hot agar solution is then pipetted into level Petri dishes of 8 cm diameter. Upon standing for a short time, the agar solution will solidify, forming a gel. Holes (wells) are cut into the agar gel by means of a glass or metal punch. For routine examinations, it is best to cut holes of 0.6 to 0.7 cm in diameter approximately 2 cm apart (as measured from the center of the holes). Punch sets of various patterns are available commercially. After cutting the holes, a small amount of agar gel is heated with several drops of water in a test tube. A few drops of this diluted agar solution are added to each hole to seal the gaps between the agar gel and the bottom of the Petri dish.

Gel diffusion procedure

Petri dishes prepared in this manner can be used for many types of immunoprecipitation reactions. Multivalent antisera (e.g., antiserum to human serum), heterogeneous antigen solution (e.g., human serum), purified proteins (human IgG, human IgA, or human albumin), or specific antisera (e.g., antisera to plasma proteins) can be analyzed by this method.

The wells in the agar plate are filled completely with approximately 0.1 mL of antigen solution or antiserum. Highly precipitating antisera react optimally with antigen solutions of concentrations between 1 and 10 mg/mL. The Petri dish is covered to prevent drying of the agar, then allowed to stand at room temperature for 2–3 days.

During this period, immunoprecipitates form. Each individual antigen produces a single precipitin line, the location of which depends upon the rate of diffusion and the concentration of the antigen. The diffusion rate of protein depends primarily upon the molecular weight. High molecular globulins (e.g., macroglobulins) are precipitated in the proximity of the point of application, in contrast to antigens of lower molecular weight (e.g., albumin) which are precipitated nearer to antibody well. Immunologically, identical proteins in adjacent wells form precipitin lines which merge completely ("reaction of identity"). Partial immunological identity (partially shared antigenic determinants) is reflected by formation of precipitin arcs, which partially fuse and partially intersect forming so-called "spurs" (e.g., γ G-globulin) and γ A-globulin). Immunological nonidentity is reflected by intersection of the precipitin lines without fusion (e.g., albumin and γ G-globulin).

Microtechnique

A microtechnique, using small agar gel plates, with smaller Ag wells and shorter distances between them, is applicable, under certain circumstances. Examples include the need to examine small amounts of test material, or if test results are needed in a short time period. Plates for this microtechnique may be prepared with a 2% agar solution as used for immunoelectrophoresis. Three mL quantities of the hot agar solution are pipetted onto precleaned glass slides (2''× 2'') and the agar is then allowed to solidify. The agar wells are cut either individually or by means of a template.

Recording of Results

Photographic recording of results of gel diffusion test is the most satisfactory method of documentation. Precipitin lines can be copied directly onto photographic paper. For this purpose, the surface of the agar gel is carefully rinsed with tap water to remove dust or other undissolved particles in the antisera or antigen solutions. To obtain an optically uniform surface, the agar is covered by tap water or physiological saline solution. The Petri dish is then placed on photographic paper (extra hard) in a dark room and illuminated from above with a lamp of approximately 200 watts. When optimally exposed and developed, the photographic paper will exhibit even fine precipitin lines with great sharpness.

Staining of the precipitin patterns of gel diffusion preparations can be achieved by the methods described under "immunoelectrophoresis" if this should prove necessary.

Possible Sources of Error

- Specific antisera to plasma proteins are obtained by immunizing rabbits or goats with highly purified plasma proteins. Frequently, traces of contaminating antibodies in such antisera must be removed by absorption. Absorbed antisera may contain minute amounts of antigens (human plasma proteins) used for this purpose. This possibility must be considered when precipitin lines occur between the antisera wells, where two or more types of antisera are used.
- Occasionally, circular precipitates develop around the antigen wells. These "halos" occur particularly when markedly lipemic or aged samples of sera or plasma are used. These nonspecific precipitations are easily distinguished from specific immunoprecipitates by their circular arrangements. Strongly hemolytic sera may mimic nonspecific precipitates on photographic records.

Q. Describe immunoelectrophoresis in detail.

Reagents and Equipment

Agar

Agar used for IEP should have a low calcium concentration and should yield a transparent gel with a low solidification

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point. Agar of good quality can be prepared by washing commercially available agar. It is important to wash agar thoroughly with distilled water since electrophoretic separation of serum proteins depends partly upon the purity of agar. For example, in unwashed agar the protein migration to the anode in the electrical field is limited; the majority of proteins migrate to the cathode due to the marked electro-osmotic potential. Agar specifically prepared for IEP is available from Behring diagnostics.

Particularly suitable as a carrier medium for IEP is the low-ion agarose, which is obtained by the removal of the agaropectin moiety of agar.

Buffer

Michaelis diethylbarbiturate acetate buffer solution pH = 8.2, μ = 0.1.

A total of 13.38 g of sodium 5, 5-diethylbarbiturate and 8.83 g of sodium acetate trihydrate are dissolved in distilled water to yield 1.5 liters. The pH is adjusted to 8.2 by adding approximately 180 mL 0.1 N hydrochloric acid. This solution possesses an ionic strength of μ = 0.1.

Antisera

Depending upon the specific problem to which the technique is being applied (e.g., IEP analysis of human serum) either the multivalent antisera (i.e., antisera containing numerous antibodies, such as those directed against the various proteins of human serum) or the specific antisera (i.e., antisera directed against specific antigens, such as albumin, IgG, etc.) can be employed. Such antisera can be prepared in horses, rabbits, goats chickens, or other suitable animals. The higher the immunoprecipitation titer of the antiserum (as shown by the ring test), the stronger are the precipitin lines on IEP.

With rabbit antiserum to human serum, approximately 30 different human serum proteins can be demonstrated by IEP. A somewhat different immunoelectrophoretic pattern is obtained with horse antihuman antiserum. This difference is due to an altered Ag-Ab ratio which is dependent upon the species specific differences of antibody structures. Rabbit antisera form strong precipitin lines in a wide range of Ag-Ab ratios, whereas horse antisera produce finer and sharper precipitin lines in a rather narrow range of Ag-Ab ratios. For this reason, it may be advisable to use various dilutions when using horse antisera.

Agar Gel Slides

Two grams of pure agar is dissolved in 50 mL of diethylbarbiturate acetate buffer, pH 8.2 (μ = 0.1) (can check by pH meter), and 50 mL of distilled water. This solution is heated for 15 minutes in a water bath of 100°C. Any undissolved particles can be removed from the heated agar by centrifugation at 3,000 rpm for 2–3 minutes. Ten mg of Thimerosal is added as a preservative. The hot agar solution is applied by pipette to alcohol cleansed, level glass slides. 3 mL of agar are placed onto each glass slide. After a few minutes the agar solidifies and the glass slides thus prepared are put into moisture chamber. A simple plastic container with Petri dish of water could serve as a moisture chamber.

It is advisable to prepare just sufficient agar solution to cover the glass slides since repeated heating of the gel leads to alterations which interfere with electrophoretic process. Holes and troughs are punched through the agar layer with a die or suitable modification. On both sides of the trough, a hole is made. Agar remaining in the punch is aspirated by a syringe connected to the barrel of the punch by rubber tubing.

Application of Antigens

The antigen mixture, e.g., serum, to be electrophoresed is introduced into the wells by a 26-gauge needle attached to a tuberculin syringe or by means of an appropriate micropipette. Complete fillings of the wells require about 0.002 mL of serum or protein solution. Each slide provides two Ag wells, permitting antigen analysis in duplicates or comparison of two different antigens. For the analysis of enzymatically cleaved agents or substances which diffuse readily, it is recommended that only one antigen be electrophoresed, since migration to the other side of the Ab trough is a possibility.

Electrophoresis

In principle, the apparatus used for IEP is similar to that used for paper electrophoresis. The ends of the antigen-containing agar covered microscope slides are placed on two parallel bars. Filter paper strips establish the contact between the agar on the slides and the buffer in the troughs. The slides must be horizontal in position. The voltage between the ends of the slides is 45 V (6 volts/cm). Adequate electrophoretic separation is achieved within 45 minutes. Under these conditions the agar slides warm to approximately 25–28°C. Therefore, the application of a lid is necessary to prevent desiccation.

Application of Antiserum

Subsequent to electrophoretic separation, the agar strip from the precut center trough is removed with a 19-gauge injection needle attached to tuberculin syringe. The amount of antiserum applied is approximately 0.04 to 0.06 mL. It is advantageous to vary the antigen and

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antiserum concentrations. A shorter antibody trough can be used to examine only one serum fraction (e.g., IgG) in order to economize on antisera. After application of antiserum, the slides are placed in a moisture chamber for diffusion. The electrophoretic separated proteins (i.e., antigens) and the antiserum diffuse toward one another and the homologous agents undergo an Ag-Ab reaction by forming precipitin lines at the points of confluence. Depending upon the concentration of antigens and antisera, optimal immunoprecipitates are formed between 20 hours and longer.

Possible Sources of Errors

- Slides must be cleaned with chromic sulfuric acid.
- The electrophoretic separation of the serum protein must be within an optimal range. Too short or too long a separation distance may be caused by faulty buffer composition (pH, μ), by incomplete contact between agar slides and filter paper strips, or by improper voltage. It is important to maintain uniform distribution of voltage (not of current) through the agar.
- Wells of proper diameters and the correct distance between the Ag well and Ab trough are decisive for satisfactory IEP. Deviation from these specifications may result in marked dislocations and/or distortion of precipitin patterns.
- The agar gel slides should be used within 2 or 3 days after preparation and must be stored in moisture containing sealed containers, preferably at 4°C.
- The sera to be examined should be fairly fresh or stored by freezing at –20°C. Bacterial contamination or autocatalytic processes may result in marked alterations of serum samples, leading to changes in electrophoretic mobility, solubility, etc.
- Normal control sera should be examined simultaneously with abnormal sera.

Fixation of Precipitin Patterns

Immunoelectrophoretic patterns can be recorded by photographing the unstained or stained precipitin lines. For most purposes, direct photography of the slide is sufficient.

Staining of the precipitin patterns is occasionally of value for chemical characterization of the precipitin lines. The agar slides are washed with physiological saline for 1 or 2 days, in order to remove the nonprecipitated protein from the agar. They are then covered by the filter paper and dried completely either in the incubator at 37°C (or in air at room temperature). After removal of the filter paper, the agar slides are placed in 2% acetic acid solution for approximately 5 minutes. The various staining techniques performed are as follows.

Protein Stains

- **Amidoschwarz (amido black):** 0.5% amidoschwarz 10B in methanol-glacial acetic acid (9:1). Stain for 5–10 minutes, then wash with methanol-glacial acetic acid (9:1), for approximately 15 minutes.
- **Azocarmine:** 0.5% azocarmine B in methanol-glacial acetic acid (9:1). Stain for 15 minutes; wash with methanol-glacial acetic acid solution (9:1).
- **Light green:** 0.5% green SF in 5% trichloroacetic acid. Stain for 1 hour; wash with 5% trichloroacetic acid solution.
- **Bromophenol blue:** 0.1% bromophenol blue in $HgCl_2^-$ saturated methanol. Wash with methanol. Green stain results from acid solution, blue stain from alkaline solution.

Lipoprotein Stains

- **Oil red:** 0.5% oil red O in 50% ethanol (filtered). Stain for approximately 2 hours. Wash with 50% ethanol.
- **Sudan black:** Sudan black (0.1%) is dissolved in 60% ethanol at 37°C, with occasional stirring, during a 24 hours time period. This solution is then filtered at 25°C and stored in dark containers. Before use, 0.1 mL of a 30% sodium hydroxide solution is added to 160 mL of the Sudan black solution. After staining for 2 hours, the slides are then washed using 50% ethanol.

Q. Discuss Grabar and Williams' method of immunoelectrophoresis.

This represents a combination of physicochemical and immunochemical techniques. The material to be examined (e.g., tissue exudates, serum, etc.) is separated electrophoretically on agar gel and, subsequently, subjected to the effect of a precipitating antiserum. This is done by placing antiserum in a trough in the agar parallel to the electrophoretically separated proteins. Both the antibodies and antigens diffuse toward one another, forming, upon confluence, well-defined precipitin lines.

To date, approximately 30 different precipitin lines in human serum can be detected by immunoelectrophoresis (IEP), indicating an equal number of individuals serum proteins. Of late, many of these serum proteins have been characterized.

The immunoelectrophoretic technique is a valuable addition to available methods for characterization of proteins. This technique is usefully employed in all areas of protein chemistry and in connection with the investigation of a large number of clinical problems connected with protein metabolism. In particular, the microtechnique of IEP has found widespread acceptance.

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Q. Describe the peroxidase reaction (for haptoglobin and hemopexin).

- **Benzidine:** Dissolve 0.2 g benzidine in 100 mL of distilled water, then add 0.5 mL glacial acetic acid and 0.2 mL hydrogen peroxide 30%. When stained for 10–20 minutes, the specific precipitates become dark greenish-blue in color.
- **Fixation of the color:** Wash briefly with distilled water, stain for 10 minutes in a 0.1% solution of Ni $(NH_4)_2$ $(SO_4)_2$, thereafter for 12 hours in 0.2% Ni $(NH_4)_2(SO_4)_2$. The specific color is bluish black.

Ceruloplasmin Stain

p-Phenylenediamine: Solution must be prepared immediately before use. Dissolve 21.6 mg of p-phenylenediamine in 100 mL of sodium acetate buffer solution (pH 5.7, μ = 0.1); add 10 mL of a solution consisting of 0.65 g sodium azide in 1,000 mL of distilled water, and warm to 37°C. Stain for 2 hours at 37°C. Wash twice for 2 hours in sodium acetate buffer (pH 5.7). This technique is suitable for fresh samples only.

Cholinesterase Stain

Indoxyl acetate: 5 mg indoxyl acetate is dissolved in 0.5 mL acetone, to which are added immediately thereafter 22 mL of a diethylbarbiturate acetate buffer solution pH 8.2, μ = 0.05 (1 volume of buffer μ = 0.1 and one volume of distilled water) and 2.5 mL of copper acetate solution 0.1 μmol/L (0.018) g copper acetate in 100 mL distilled water). This solution must be used within 8 hours after preparation. After staining for 2 hours, and subsequent washing in tap water for 1 hour, cholinesterase is indicated by blue color.

Lipid-protein Double Staining

A volume of 0.5% oil red solution in 50% ethanol is suitable as a lipid stain. Place the slide in the dye mixture for approximately 2 hours, then wash 50% ethyl alcohol. A volume of 0.5% light green solution in 5% trichloroacetic acid is then immediately applied to achieve the specific protein stain. The staining time for proteins is approximately 1 hour. Wash with trichloroacetic acid.

The slides must be photographed after to keep a permanent record.

Identification and Interpretation of Precipitin Lines

The analysis of immunoelectrophoretic patterns requires a certain degree of experience and training. It is imperative to be able to recognize the various specific precipitin lines. Various techniques are available to achieve identification of given precipitin line by experimental means:

- Employment of specific antisera
- Specific stains
- Selective absorption
- Employment of purified proteins.

Q. Discuss the practical applications of Immuno-electrophoresis.

Increased immunoglobulin concentrations are indicated by elongated and thickened precipitin lines of IgG, IgA and IgM immunoglobulins and antigen excess result in positions of these precipitin lines closer to the antibody trough in comparison with those obtained with normal sera. Although IEP does not lend itself to precise quantitation of serum proteins, marked deviations from normal concentrations may be recognized.

Increased IgG, IgA and IgM are frequently associated with acute and chronic liver diseases. This is the case in acute hepatitis, in which a particularly impressive increase in IgM occurs. In infectious mononucleosis, chronic hepatitis and Laennec's cirrhosis all three major immunoglobulins are frequently, but not invariably, increased. In lipoid hepatitis the IgG-globulin precipitin line may be markedly accentuated. Collagen diseases, including Sjögren's syndrome and systemic lupus erythematosus (SLE), as well as certain infections, may be associated with hyperimmunoglobulinemia as indicated by IEP. In trypanosomiasis, the IgM may be excessively increased. Certain virus diseases, such as Coxsackie infections, are often associated with increased immunoglobulins.

In most cases of sarcoidosis IEP has not revealed abnormal serum protein changes, though definite IgA increases have been observed by some. IEP revealed increased immunoglobulin contents in a variety of additional diseases such as dermatitis herpetiformis and dermatitis gestationis and pernicious anemia in which IgA, and mongolism in which the IgA and IgG were increased. Besides all the causes mentioned above, the disorders mentioned under hypoalbuminemia, monoclonal and polyclonal gammopathies discussed under serum protein in clinical chemistry chapter can also be assessed by using IEP techniques.

Quantitative Determination of Plasma Proteins by Immunoprecipitation

The reagents used for quantitative determination are specific antisera which react stoichiometrically with the proteins to be determined and form precipitates with them. These immune reactions may occur either in solutions or in

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gels which contain the antiserum in even distribution. In the first case, the quantity of immune precipitate, measurable at appropriate dilutions as turbidity or by other means, gives a quantitative measure of the antigen concentration. By using, as the reaction medium, a gel containing antiserum, the reagent is arranged in the form of a stationary phase into which the antigen can penetrate either by diffusion or under the influence of an electrical field. The resulting precipitates, which assume different configurations depending on the method used, can be quantitatively evaluated.

In the four most widely used methods to be described, reaction conditions have been selected which ensure that the quantity of specific antiserum is constant and exceeds the quantity of antigen, while the concentrations of the antigens to be determined can vary within wide ranges.

FUNDAMENTAL QUANTITATIVE CONSIDERATIONS

Q. Describe the principle of photometric method.

Principle: This method is a modification based on the determination of the nitrogen content of isolated immunoprecipitates as described by Heidelberger. In contrast to the tedious technique, the antigen and antibody are allowed to react together at high dilution. Under these conditions, the reaction product of the immune reaction does not result in the formation of the precipitate which sediments, but merely in the appearance of turbidity. The extinction (e.g., at 450 nm) can be used as a measure of the turbidity. If the quantity of antiserum is kept constant and the antigen concentration is varied, a curve can be obtained. When the antigen is present in excess, soluble reaction products are formed. For this reason, only the ascending limb of the curve can be used for quantitative determinations. By reference to this part of the curve, the concentration of antigen in an unknown solution can be determined.

Q. What is single linear immunodiffusion of Oudin?

Principle: Gel containing the antiserum is allowed to solidify in a test tube, and the antigen solution is poured on top of it. The precipitate formed at the boundary layer migrates into gel zone. The distance of the front of the precipitate from the boundary surface is proportional to the square root of the diffusion time, t.

$$h = k \times \sqrt{t}$$

As the migration velocity of the antigen h

$$k = \frac{h}{\sqrt{t}}$$

is proportional to the logarithm of its concentration CAg and the distance h through which the precipitate migrates may be expressed by the following equation:

$$\log CAg = a \times k + b = a \times \frac{h}{\sqrt{t}} + b$$

Accordingly, if CAg as a function of $\frac{h}{\sqrt{t}}$, is plotted in a semilogarithmic system, the result is a straight line. Its slope is represented by *a* and it intersects the ordinate at *b*. The slopes *a* and *b* are thus constants determined by the Ag-Ab system under consideration. The length of the cylinder of precipitate is proportional to the negative logarithm of antibody concentration.

The fundamental difference between the techniques of single linear immunodiffusion and single radial immunodiffusion and electroimmunodiffusion (EID) described below is simply that in the former the antigen concentration is determined from the speed of migration of an immunoprecipitate; while in the latter methods, it is determined by measuring the precipitin area on the length of a precipitate which remains constant after a certain time period.

Since the diffusion velocity of any molecule is dependent on temperature, it is essential in using Oudin's technique that the temperature be kept strictly constant during the reaction period.

The results of EID by Laurell's method and single radial immunodiffusion as described by Mancini, Carbonara and Heremans are not significantly affected by temperature fluctuations. One advantage of Oudin's method is that the solutions require less exact volume measurements, and pipetting errors which may affect other methods are of minor importance.

Q. Describe the principle of single radial immunodiffusion.

Principle: Dissolved antigen molecules diffuse radially from a cylindrical well into an agar gel layer of uniform thickness containing the corresponding antiserum. The resulting precipitate assumes the form of a cylinder or ring. When diffusion ceases, the surface area of the base of the cylinder is directly proportional to the quantity of antigen, at a predetermined concentration of antiserum in the reaction gel.

If the circular area πr^2 (i.e., area of central well + area of precipitate) is plotted on a graph as a function of the quantity of antigen QAg (i.e., antigen concentration × volume of antigen solution), the result is a straight-line relationship expressed by the equation $\pi r^2 = k \times QAg + S$.

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Point S at which the straight line intercepts the ordinate is a function of the size of the central well. And the slope *k* is inversely proportional to the antiserum concentration in the gel and the gel thickness. By standardizing the technical conditions, it is possible to keep constant the variables contained in *k* and *S* so that the measured radius or diameter of the precipitate ring is a function solely of the quantity of antigen introduced. With the aid of standardized protein preparations, it is possible to construct a reference curve which can be used to determine the antigen concentrations of unknown solutions. It is essential, however, to deliver identical volumes of the antigen solutions, standard and test solutions, into central wells.

Differences in temperature do not affect the results of this reaction. The only effect of a rise in temperature is to accelerate diffusion and thereby the appearance of a measurable precipitin ring. However, the temperature should not be allowed to rise above 37°C because the gel may melt and because of the risk of irreversible damage to the thermolabile proteins.

Q. What is electroimmunodiffusion?

Principle: In the course of the electrophoretic migration of an antigen through agarose gel containing the corresponding antiserum, it produces an extended trail of an immunoprecipitate. The length 'I' of the precipitate is a measure of the antigen concentration provided that the latter is contained in a fixed volume of solvent.

Depending on the electrophoretic migration rate of an antigen, the precipitin peaks may appear more or less rapidly. Fast moving proteins usually produce long narrow precipitin bands ending in a point, while proteins of lower electrophoretic mobility produce broader precipitates with rounded ends. Because of their slow migration rates, electrophoresis takes longer; diffusion of the antigen at right angles to the direction of electrophoresis may be responsible for the broadening of the peaks. The migration velocity of the antigens is affected by the field strength and the pH of the buffer solution employed. Careful standardization of these factors is essential for reproducible results. This makes the method somewhat more elaborate than comparable methods based on diffusion alone. On the other hand, the results are usually available in 2 or 3 hours, while the methods of Oudin and Mancini require at least 20–30 hours.

Q. Discuss in detail single radial immunodiffusion.

Equipment

Antiserum-agar gel plates of single radial immunodiffusion by the method of Mancini, Carbonara and Heremans as modified by Augener can be prepared in polystyrene Petri dishes (8 cm diameter) having perfectly flat bottoms.

Application of exact amounts of antigen samples is best achieved by the use of a microliter syringe with which exact amounts of 1 µL can be delivered.

The immunoprecipitin rings can be measured with a measuring microscope or a magnifying lens with 0.1 mm scale. A simple way of measuring is by using a measuring template (with Tripartigen platesfrom Behring Diagnostics, these are given as part of the kit for immunoglobulin, etc. quantitations).

Procedure

A microliter syringe is used to fill holes in the agar gel layer with 2 µL of antigen solution. At least three different dilutions of each standard solution are required to plot a curve. The remaining holes are used for the solutions being analyzed. Each is filled with 2 µL of the test specimen in suitable dilution; the diluent is physiological saline. After introducing the reagents, the plates are left in a moisture chamber at room temperature. The results are preferably read after 2 days, although an approximate reading can be obtained in 1 day. After 2 days, the circular immunoprecipitates in the gel layer are distinct and can easily be measured. For *Tripartigen* (from Behring Diagnostics) a Tripartigen ruler (scale) is provided to read the ring diameter and the quantitation is done by noting the corresponding value given in appropriate units from the reference chart provided. With every kit, a standard serum/specific protein solution can also be had for comparing results.

Q. What are immunoassays?

Immunoassays are assays that detect the presence of an antigen in the human body with the help of an antibody or detect the presence of an antibody with the help of an antigen. In this text for simplicity, all further information provided is based on considering antibody as a reagent to detect antigen in the human body fluids.

The first reported immunoassays were homogeneous. They are attributed to Kraus (1897), who coined the term '**precipitin**' for the precipitate formed upon mixing an antigen and an antibody.

Meyer in 1922, employed sheep erythrocytes to serve as a label and conjugated human immunoglobulin to them. Antiimmunoglobulin antibodies appearing in rheumatoid arthritis patients were shown to cause visible clumping of these erythrocytes. This method was known as **hemagglutination**.

Singer and Plotz replaced the erythrocytes with latex particles, which were easier to standardize, and these assays

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are popularly known as **latex agglutination tests**. High degree of sensitivity for a wide variety of antigens/antibodies, which can be detected by these latex agglutination assays, has promoted their usage worldwide for screening since, 1956 in clinical laboratories. The simplicity of performance and obviating the need for equipment, have made these assays extremely popular.

The need for quantitative estimation, and higher sensitivity led to the development of **radioimmunoassays** (RIA) first in 1959 by Berson and Rosalyn Yalow. The first RIA developed was used to detect and quantify insulin.

Since then immunoassays have been used to detect and quantify a variety of molecules native to humans such as proteins, hormones as well as foreign molecules such as bacteria, viruses and parasites.

Qualitative Immunoassays

Qualitative immunoassay techniques provide test results, which only help to identify or indicate the presence of analytes. Various techniques for qualitative detection of antigens have been in use, which include latex agglutination, passive gel diffusion, IEP and Western blotting. These techniques at the best can give a semiquantitative or comparative information about analytes under assay.

Single immunodiffusion technique uses the diffusion of an antigen into agar impregnated with antibody. **Double immunodiffusion technique** allows the direct comparison of two or more test materials providing a simple and direct method for determining whether the antigens in the test specimens are identical, cross-reactive, or nonidentical. **Immunoelectrophoresis** has been used over the years for detection of several different antigens present in a common solution. The latex agglutination assays though simple to use are subject to variations in results as the interpretation pattern between negative and weakly reactive samples may vary between laboratory to laboratory and person to person.

Lower sensitivity for many analytes and the need for correct quantification of analytes for:

- ❖ Effective monitoring of disease
- ❖ For differential diagnosis to aid correct therapy, have created the need for more sensitive and precise quantitative immunoassays.

Quantitative Immunoassays

Quantitative results of immunoassays are extremely useful in:

- ❖ Establishing the extent of severity of a disease
- ❖ Assessing the course and stage of clinical condition
- ❖ Differential diagnosis of many diseases
- ❖ Monitoring response to therapy
- ❖ Accurate prognosis of disease.

Various techniques have been used to develop quantitative methods that include radial immunodiffusion (RID) and electroimmunoassays, turbidimetric and nephelometric assays and labeled immunochemical assays.

The RID and electroimmunoassay (rocket electrophoresis) though reliable, are slow, relatively involvement intensive, and expensive. This limits their usage in routine laboratories. In many laboratories, the gel-based techniques are restricted to qualitative studies or are used as reference methods.

During the last decade, the gel techniques are increasingly being replaced by optical detection methods.

The various techniques by which quantitative immunoassays are performed can be broadly grouped as follows:

Heterogeneous Immunoassays

These assay systems employ an antibody immobilized on a solid phase, which captures the corresponding antigen from the sample. A second labeled antibody specific to a different epitope of the antigen is used as a basis for signal generation. After the immunochemical reaction has taken place, the bound and unbound-labeled antibodies are separated. The concentration of antigen is then estimated by measuring bound or unbound-labeled antibodies through an appropriate signal generation and measurement system.

Heterogeneous immunoassays can be performed by various techniques such as:

- ❖ Radioimmunoassays (RIA)
- ❖ Enzyme immunoassays (EIA)
- ❖ Fluorescent enzyme immunoassay
- ❖ Chemiluminescent enzyme immunoassay.

The difficulties associated with separation of bound and unbound-labeled antibodies, the need for dedicated instrumentation and labor-intensive procedures has prompted the usage of heterogeneous assays in specialty laboratories mainly through use of expensive automation.

The need for simpler, affordable, user-friendly assay techniques for detection of routinely encountered clinical analytes still remained to be explored. With the tremendous progress made in instrumentation technology, optics, and software, the face of quantitative estimation for routine parameters has changed dramatically in recent years. Simultaneous development in purification techniques for polyclonal antibodies, emergence of monoclonal antibodies with high specificity and avidity have been instrumental in the development of homogeneous assay techniques which

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are simple to perform and easily adaptable for routine laboratory analysis.

Homogeneous Immunoassays

These assays require only the mixing of a sample (antigen) and the immunochemical reagents (antibody) followed by detection of signal. These assays do not require separation of free or bound-labeled materials in the test system for the detection or measurement of the antigen. In homogeneous immunoassays the immunochemical binding produces a detectable signal (agglutination, absorbance, fluorescence, etc.). The simplicity and flexibility associated with the performance of homogeneous assays has made their usage popular with laboratorians worldwide.

The homogeneous assays can be performed by different techniques such as:

- Turbidimetry
- Nephelometry
- Homogeneous enzyme immunoassays
- Enzyme-multiplied immunoassay technique (EMIT)
- Enzyme inhibitor immunoassay
- Enzyme complementation immunoassay
- Substrate linked fluorescence immunoassay (SLFIA)
- Scintillation proximity assay (SPA)
- Electrochemiluminescence (ECL)
- Luminescent oxygen channeling immunoassay (LOCI).

The clinical chemistry analyzers (photometers) were originally developed for colorimetric estimation of chemical or enzymatic reactions. Subsequently, it was shown that the visible scattered light in Kraus's precipitin reaction could be measured by turbidimetry and nephelometry on photometers, to quantitate the immune complex formation. These systems utilize the fast reaction between an antigen with their corresponding antibodies in a liquid phase.

The technique of quantitation by turbidimetry and nephelometry is apparently similar to the popular absorption spectrophotometry used in routine clinical laboratories and hence, adaptable by high throughput as well as small and medium laboratories easily.

Q. What is the principle of spectrophotometry, and how is it used in clinical and research laboratories?

Spectrophotometers work on the basis of the Beer's and Lambert's law.

Beer's Law

When a colored solution is illuminated with a monochromatic light (light of a single wavelength), its absorbance is proportional to the concentration of the colored solution when the light path is constant, i.e., A α C where A is the absorbance of light, C is the concentration of solution.

Lambert's Law

When a colored solution is illuminated with a monochromatic light, its absorption is proportional to the length of the light path, when the concentration of the solution is constant, i.e., A α L, Where A is the absorbance of light, L is the length of the light path.

Beer-Lambert Law

Combining the two laws together, we have the Beer-Lambert law, which states that when a colored solution is illuminated by a monochromatic light, its absorbance is proportional to the concentration of the solution and the length of the light path, i.e., A α C α L.

$$A = K \times C \times L \qquad \text{Equation (1)}$$

where K is a constant

In all photometric estimations a reference standard whose concentration is known is used and its color intensity is compared with the color intensity of the test sample, i.e.,

$$A_t = K \times C_t \times L$$
$$A_s = K \times C_s \times L$$

where A_t—Absorbance of test, C_t—Concentration of test, A_s—Absorbance of standard, C_s—*Concentration of the standard*.

Since the pathlength is constant (1 cm) in the spectrophotometer, L is constant, concentration of the standard C_s is known, therefore

$$C_t = \frac{A_t}{A_s} \times C_s \qquad \text{Equation (2)}$$

It has been observed with most biochemistry analytes that as the concentration of analyte increases linearly, the absorbance also increases linearly within the pathophysiological concentration. When a graph of concentration vs absorbance is plotted, a straight-line graph is obtained **(Fig. 23.1A)**. A single standard method using a standard of known concentration or a factor method can be employed for calculating the concentration of the unknown.

Certain reactions, however, may not follow the Beer-Lambert law within the pathophysiological concentration for an analyte and, hence, do not provide a straight-line graph. For such analytes, the unknown cannot be determined using a single standard. A graph using different concentrations of standards vs absorbance has to be plotted on a graph paper. The plotted curve is known as the standard curve **(Fig. 23.1B)**.

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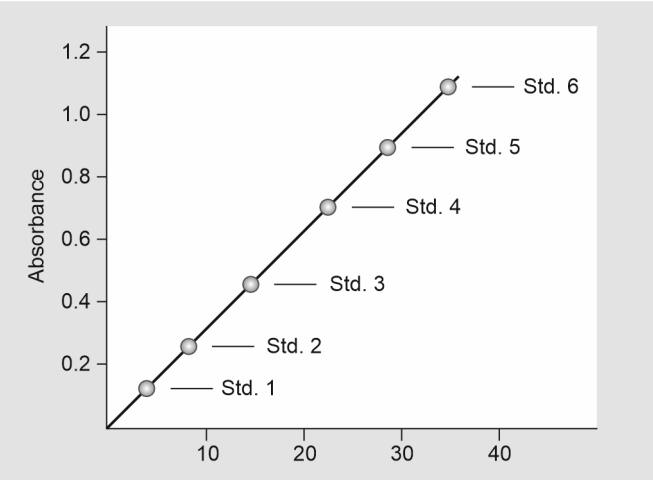

Fig. 23.1A: Illustration of a straight-line graph obtained by plotting absorbance vs concentration of analyte for reactions which obey Beer-Lambert law.

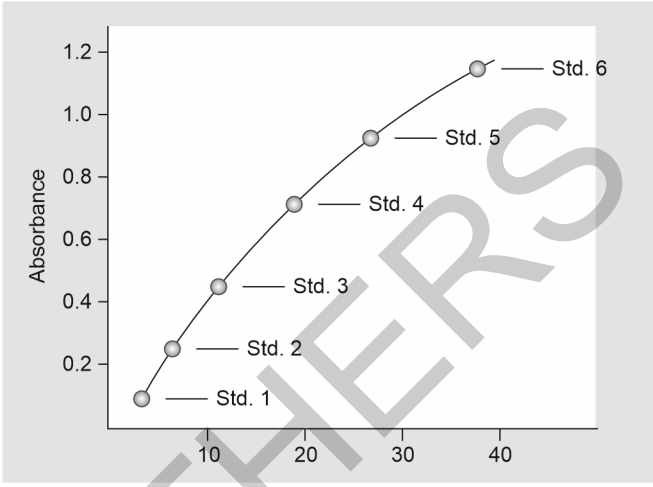

Fig. 23.1B: Illustration of a standard curve obtained by plotting absorbance vs concentration of analyte for reactions which do not obey Beer-Lambert law.

The concentration of the unknown can be interpolated from this standard curve.

Measuring Principles in Biochemistry

Criteria for Wavelength Selection

It has been established that when the wavelength of light used is complementary to the color of the chemical complex to be measured, peak absorbance is obtained. Thus, selection of the wavelength depends on the color of the complex to be measured.

Complementary Filters for Measuring Color Complexes

Color of the complex	*Wavelength/color*
Yellow	405/Violet
Red	505/Green
Blue-violet	546/Green
Green	630/Red

For example, for the estimation of hemoglobin using cyanmethemoglobin method, a red colored complex, which is formed during the reaction, is measured using a green filter.

Reading Methods

The measurements of biochemical reactions using enzymes, substrates or specific chemicals are read by methods mentioned below.

Equilibrium Methods

Also known as end point methods. Here, the absorbance of end product is measured when the reaction between the reagent and sample has virtually come to equilibrium (end) and the substrate has been converted into a stable end product. The reaction ceases when the equilibrium is reached. The concentration of the test specimen can be calculated by using the equation 2 as described earlier.

Kinetic Methods

Also known as rate methods where the rate of change of absorbance (ΔA) produced in a fixed time interval is measured.

The kinetic measurements are of two types:

1. **Fixed time analysis:** Where the ΔA produced by the reaction between the reagent and the substrate is measured by stopping the reaction at a fixed time interval.
2. **Continuous monitoring:** Where the ΔA produced is monitored continuously as the reaction proceeds.

   Results of the unknown are derived using a factor (K) in the kinetic methods, which is usually provided by the manufacturer or can be calculated as:

$$K = \frac{V_{total} \times 1{,}000}{V_{sample} \times t \times \in \times d}$$

V_{total} = total volume of the reaction mixture, V_{sample} = volume of sample, t = time, $\in$ = molar extinction coefficient of the chromogen, d = length of the light path.

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STANDARDIZATION OF TIME INTERVAL FOR RATE REACTIONS

Determination of the reaction rate involves the measurement of the amount of change in absorbance (ΔA) produced in a defined time interval. Depending on the reaction, kinetics between a specific reagent and the substrate, the time interval for reading the ΔA can be selected to measure the reaction rate. The different types of reaction curves, which can be obtained as the reaction progresses, typically follow the following patterns **(Figs. 23.2A to C)**:

- **Curve A:** If a graph similar to 'curve A' is obtained then any time interval can be selected for reading reactions, as the rate of change is constant during the entire reaction run.
- **Curve B:** Correct results can be obtained only if the rate is measured along segment II. Incorrect results are obtained if the ΔA is measured during the lag phase (I) or during the phase III.
- **Curve C:** Deviates from linearity over its entire course and ΔA fall off with time. At no time does it give rate of constant changes. Such reaction curves are not suitable for measurements and the reagent systems have to be optimized to obtain correct reaction curves.

Measurement of Immune Complexes by Spectrophotometry

Unlike in classical biochemistry, where the reactants are clear and endpoints are expressed as absorbances, the behavior of light differs for solutions containing suspensions or particulates. Such particles (insoluble immune complexes) are formed as the reaction between antigens and antibodies takes place.

When light of suitable wavelength is allowed to pass through a reaction solution containing antigens (analytes) and the initial absorbance is measured, the absorbance is minimum at this point **(Fig. 23.3)**.

Subsequently, the reagent containing corresponding antibody solution is then added to the antigen in the

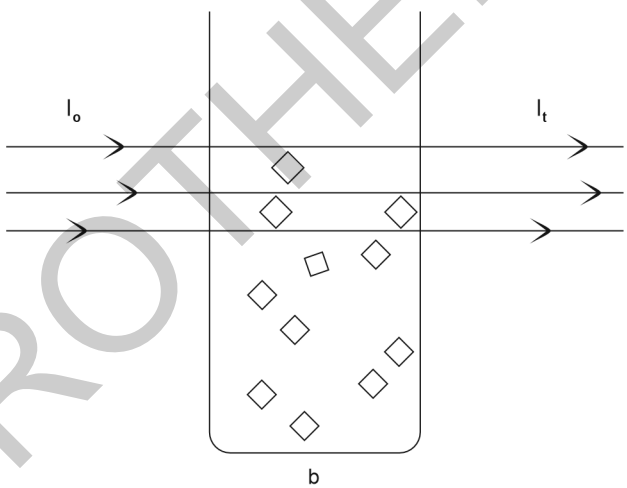
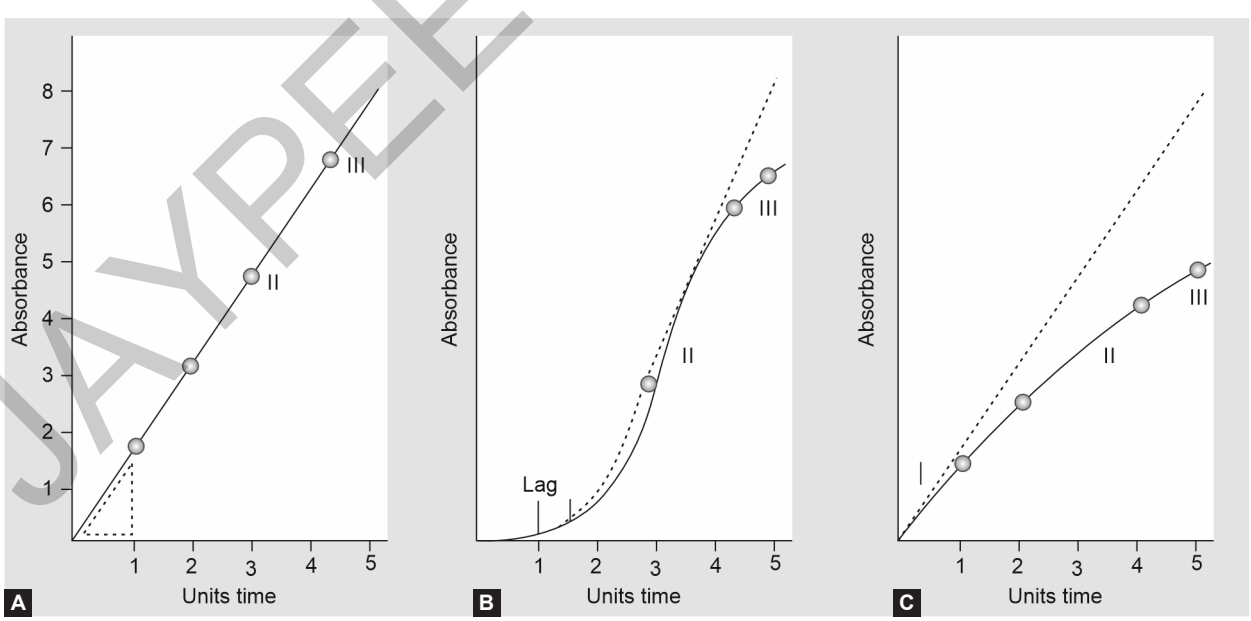


Fig. 23.3: Behavior of light in solution containing antigens where I_o is the intensity of incident light, it is the intensity of transmitted light and 'b' is the cuvette containing antigens in the reaction solution.



Figs. 23.2A to C: Forms of graphs showing change in reaction rate as a function of time: (A) the rate is constant in all the segments I, II and III; (B) A measurement at segment II will be representative of constant rate, but at segment I (lag phase) and segment III it will be less; (C) The rate falls of continuously.

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cuvette and allowed to react. An agglutination reaction begins when a single molecule of antibody binds to at least two corresponding binding sites on different antigen particles. As the reaction proceeds, the agglutinating particles aggregate and form immune complexes. Immune complexes increase in size, become larger, resulting in an increase in turbidity and the scattering of the incident light. Thus, a decreasing part of the incident light (I_o) is transmitted as the reaction proceeds. Spectrophotometers read this decrease in the intensity of the transmitted light as absorbance **(Fig. 23.4)**.

This measurement of reduction in the intensity of the transmitted light at 180° is defined as *turbidimetry*. The turbidity is proportional to the analyte concentration, which in turn is proportional to the amount of agglutination. Based on this proportional relationship, the amount of analyte in the sample causing the turbidity can be easily determined. It should be noted that the nature of immunochemical reaction is exactly the same in turbidimetry and nephelometry. However, it is the detection principle applied for measurement, which differentiates turbidimetry from nephelometry **(Fig. 23.5)**.

Nephelometry measures light scattered or reflected towards the detector, which is away from direct path of the transmitted light. Routine spectrophotometers cannot be used for nephelometry and hence, nephelometers are required. Most nephelometers measure light scattering at a 90° angle. However, in order to measure the forward scatter intensity caused by light scattering from large particles, some nephelometers are designed to measure scattered light at an angle other than 90°.

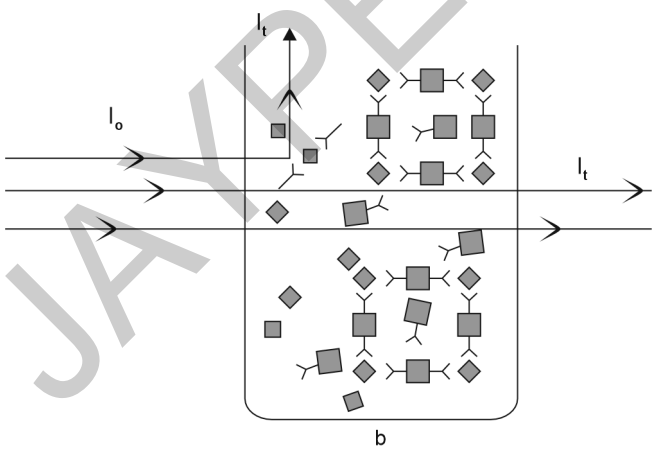

Fig. 23.4: Behavior of light in solution containing immune-complexes (Ag-Ab), where I_o is the intensity of incident light, I_t is the intensity of transmitted light, I_s is the intensity of scattered light and 'b' is the cuvette containing immune complexes in the reaction solution.

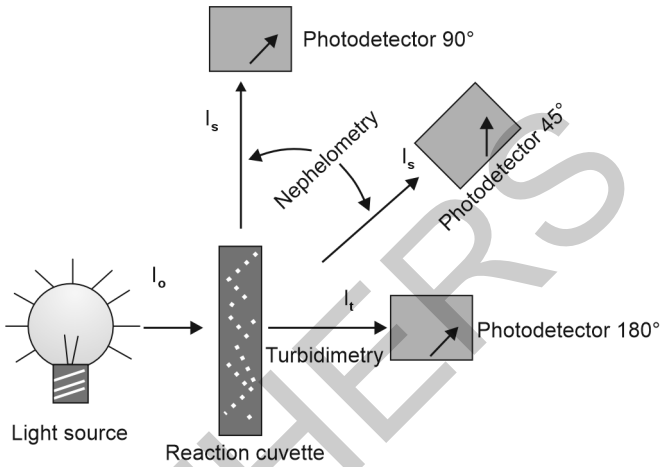

Fig. 23.5: Detection principles in turbidimetry and nephelometry where I_o is the intensity of incident light, 'b' is the reaction cuvette, I_t is the intensity of transmitted light measured by detector at 180°, I_s the intensity of scattered light measured by detectors placed at 90° and 45°.

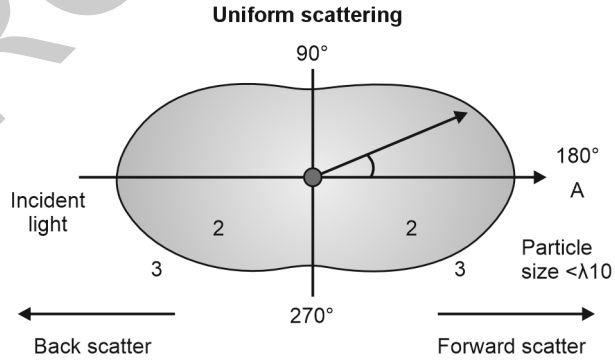

Fig. 23.6: Illustrating Rayleigh scattering for an immune complex with particle size <λ10 of the wavelength of incident light.

Selection of Wavelength for Measuring Immune Complexes

The optimum wavelength for optical measurement of immune complexes increases with the size of immune complex to be measured. In general, if the size of the immune complex formed is less than 1/10th the size of the wavelength of incident light, then the light scattering is relatively symmetrical **(Fig. 23.6)**. This uniform scattering of light is known as **Rayleigh scattering**.

On the other hand, when the size of the immune complex to be measured is more than 1/10th the size the wavelength of the incident light of there is concentration of scattered light in forward direction at an angle of 45° or less, away from the axis of the incident light beam **(Fig. 23.7)**.

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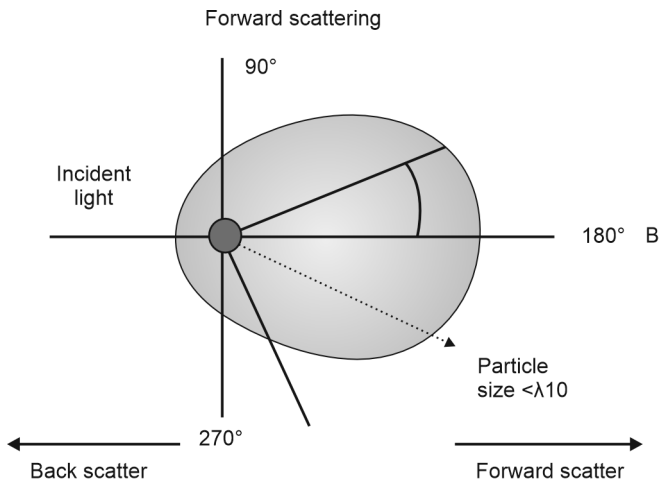

Fig. 23.7: Illustrating Rayleigh-Debye scattering for a immune complex with particle size >λ10 of the wavelength of incident light.

This type of scattering is referred to as **Rayleigh-Debye scattering**.

Careful examination of both the figures **(Figs. 23.6 and 23.7)** show that the intensity of scattered light for forward and back scatter (0° and 180°) from small particles is equal but less at 90° (Rayleigh scattering). As the size of the particle becomes larger, the angular dependence of light scattering becomes dissymmetrical, increasing in forward scattering and decreasing in backward scattering.

The Rayleigh and Rayleigh-Debye expressions provide useful information about scattering of light by small and intermediate size particles and are important for the optimization of analytical instrumentation for measuring light using turbidimetric and nephelometric assays.

The upper limit on size of immune complexes exhibiting Rayleigh scattering is about 40 nm when a visible light at 400 nm is used. Many of the plasma proteins such as immunoglobulins, albumin, etc., fall below this limit. As the immune complexes, become larger in size from 40 to 400 nm, the angular dependence of scattered light at 400 nm loses the symmetry around the 90° axis, and shows an increase in forward scattering. Some plasma proteins of the IgM class, aggregating immunoglobulin/antigen complexes fall into this size category. For measuring such complexes a bigger wavelength of light depending on the size of the immune complexes formed, should be used. For latex based assays using a latex particle of approximately 200–300 nm, light of wavelength between 500–600 nm would be ideal for measuring the immune complexes formed.

Biochemical measurements	***Nephelometric measurements***	***Turbidimetric measurements***
Measures absorbance scattering at an by colored complexes formed as a result of biochemical reactions	Measures light reduction in angle away from the incident light due to formation of immune complexes	Measures light at 180°, intensity of light transmitted (as absorbance) at 180°due to the formation of immune complexes
Selection of wavelength of light is complementary to the color of the chemical complex to be measured	Selection of wavelength of light depends on the size of the immune complex formed	Selection of wavelength of light depends on the size of the immune complex formed

The choice between turbidimetry and nephelometry will depend on application and the available instrumentation. Until recently, it was assumed that for relatively clear solutions in which the transmission of light in the forward direction is greater than 95% small changes in absorption due to turbidity were difficult to measure with precision. The stability and resolution of modern microprocessor driven spectrophotometers and automated clinical chemistry analyzers have greatly improved their ability to measure turbidity with dependable accuracy and precision. Turbidimetric methods have today become competitive in sensitivity with nephelometric methods for immunological quantitation of such solutions.

For some analytes, the signal amplification and assay sensitivity requires the usage of conjugation chemistry to attach antibodies to inert and uniform latex particles. Such reagent systems are referred to as particle enhanced turbidimetry (PET).

PET reagents usually use latex of approximately 200–300 nm for conjugating antibodies to facilitate formation of larger immune complexes and thereby generate detectable signals. This leads to decrease in scattering of light at 90° and increase in forward scattering. Turbidimetric assays, therefore, have better precision for measuring larger immune complexes.

In the context of contemporary technology, turbidimetric assays are gaining popularity over nephelometric determination due to their simplicity and overall consistency.

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Q. Differentiate between turbidimetry vs nephelometry.

Turbidimetry	*Nephelometry*
Measures reduction in intensity of transmitted light at 180° due to the formation of immune complexes	Measures scattering of light at an angle (usually 90°) away from the incident light, due to the formation of immune complexes
• Can be performed on most spectrophotometers sensitivity competitive with nephelometric for small immune complexes such as serum proteins • More precise for measuring large immune complexes	• Requires dedicated nephelometers sensitive for measuring small immune complexes such as serum proteins • Less precise for measuring large immune complexes due to forward scattering of light
• Blanking and reading reaction can be performed in the same measuring cuvette • Provides better precision due to slower reaction kinetics as blanking of immunochemical reaction can be monitored in a single cuvette	• Blanking has to be performed in separate measuring cuvette • Because of the fast reaction kinetics it is difficult to obtain a sample and a reagent, sample and reagent blank in case of nephelometry

Q. What are the considerations for measurements of turbidimetric immunoassays (TIA)?

As far back in the year 1929, Heidelberger and Kendall have quantitatively described the formation of a precipitate when reacting an antigen with an antibody. They demonstrated that when an increasing amount of an antigen is added to a constant amount of corresponding antibody, the resulting degree of precipitate formed follows a bell-shaped curve as shown in **Figure 23.8**. To obtain the Heidelberger curve, the antigen concentration is plotted against the absorbances obtained from measuring the Ag-Ab reaction.

The Heidelberger-Kendall curve can be divided in three zones as follows:

1. **The antibody excess zone:** In the first stage of the reaction, there is a large excess of binding sites in the reaction mixture available for the antigen to bind. First the antigen binding sites are quickly saturated by antibody before cross-linking begins to occur. This results in formation of small Ag-Ab complexes. In this zone, the absorbances increase proportionally to the analyte concentration.
2. **Zone of equivalence:** In the second stage of reaction binding sites available for the antigen are proportionate to the antigen concentration. Here, the probability of cross-linking is more likely resulting in formation of large immune complexes. As the saturation point is reached, there is neither free antigen nor free antibody in the reaction mixture. To this zone, the absorbances increase with the increasing analyte concentration, but does not increase proportionally.
3. **The antigen excess zone:** In the third stage of the reaction, the relative concentration of the antigen is so high that most of the binding sites are overcrowded, hindering the formation of real precipitate and favoring the formation of small immune complexes. This is called the prozone effect or the hook effect. The term "prozone" is inappropriately used to describe "postzone" or "antigen excess" in day-to-day parlance. The existence of prozone effect causes very high concentrations of antigen to produce signals, which are similar to the signals generated, by moderate concentrations of the same antigen. It is imperative to know for the assay design as to what concentration of analyte will cause a prozone effect in a turbidimetric immunoassay for a given antibody reagent system.

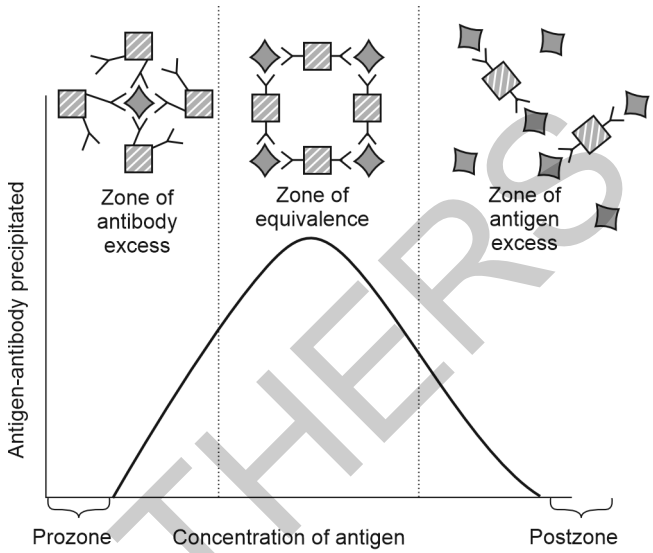

Fig. 23.8: The quantitative immunoprecipitin curve (Heidelberger-Kendall curve).

When the Ag-Ab reaction takes place and the formation of the immune complex is measured optically by turbidimetry, then the absorbance and reaction kinetics in the three zones will follow the following pattern:

Heidelberger-Kendall	*Absorbance curve*
Antibody excess zone	Increases towards maximum
Equilibrium	Reaches maximum
Antigen excess zone	Decreases below maximum

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The Heidelberger-Kendall immunoprecipitin curve forms the fundamental basis for all homogeneous Ag-Ab assays including turbidimetry and is usually referred to as the dose response curve.

For many analytes of diagnostic importance, the Ag-Ab reactions neither follow the Beer-Lambert law nor provide a linear relationship between concentration and turbidity. Estimating the concentrations of analytes using a single standard, as in biochemical analysis therefore, results in inaccurate results near the zone of equivalence. The ΔA is directly proportional to the concentration of analyte only in the initial region of the antibody excess zone. Use of single standard for calculating concentration of analyte may be acceptable only for lower analyte concentrations. As the analyte concentrations increase, the error in measurement will start to magnify. Therefore, for having a larger measuring range, the turbidimetric assays use that part of the dose response curve, which covers the maximum portion of the antibody excess region and demonstrates a linear reaction as the standard curve.

The standard curve is plotted using a number of standards containing different concentrations of analyte being measured (usually 5–6). The highest concentration of the standard is chosen in such a way that the analyte absorbance at that concentration will lie on the linear extreme of the standard curve. The lowest concentration of the analyte is usually selected below the reference values of the analyte of interest.

The linear range between the highest and lowest standards used for the preparation of standard curve is referred to as the measuring range of the assay **(Fig. 23.9)**.

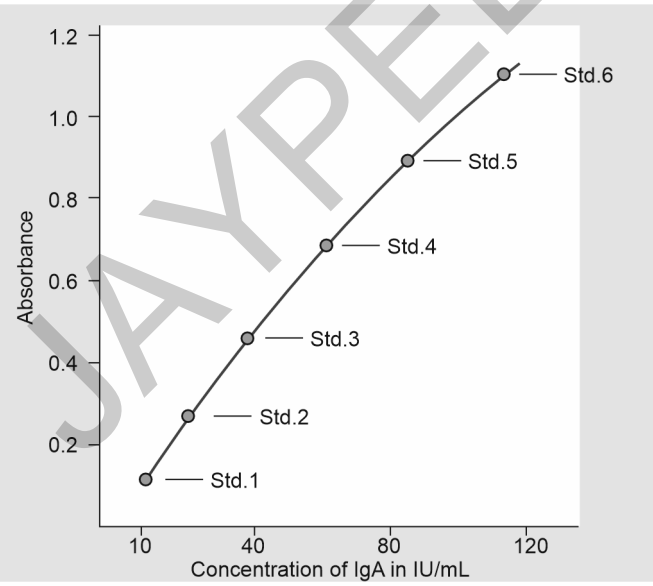

Fig. 23.9: Illustrating standard curve for IgA using lowest standard as 7 IU/mL and highest standard as 120 IU/mL.

Q. Discuss the optimization, standardization and quality control of turbidimetric assays.

To measure the Ag-Ab reactions, reliably all the factors that affect the reaction rate, other than the concentration of the antibody, must be optimized and controlled. As the reaction velocity is at its maximum under optimal conditions, a larger analytical signal is obtained that can be more accurately and precisely measured as compared to a smaller signal obtained under suboptimal assay conditions.

Investigations of the factors affecting Ag-Ab reactions were extensively studied by Heidelberger and Kendall. In addition to the relative proportions of immune reactants, other conditions such as temperature, ionic strength of the medium, characteristics of the antibody, such as avidity and affinity are important for formation of Ag-Ab immune complex. These principles need to be applied to the reagent system optimization for immunoturbidimetry.

Ionic Strength

It has been observed that the Ag-Ab reactions are strongly influenced by the nature of ionic medium in which the reaction is carried out. The ionic strength of the reaction environment has a profound effect on the quantum and the rate of the Ag-Ab reaction. As the ionic strength increases, the depth of the electrical double layer that forms around the charged molecules is compressed, reducing the distance over which repulsive forces that keep the molecules apart can act. This in effect leads to the promotion of aggregation. The reduction in charge on the other hand influences the electrostatic attraction between oppositely charged species, thereby reducing the specific binding between antigen and antibody.

pH

The reaction pH also influences the rate of aggregate formation. The rate of reaction is found to be fairly consistent at a pH of 6.0–8.0. Reduction in pH leads to some proteins having net positive charge (those with a pH above the reaction pH) leading to nonspecific agglutination with negatively charged proteins or particles.

Temperature

As is well known, temperature influences the rate of formation of immune complexes and it should be optimized to obtain accurate results. Assays are usually designed with an incubation temperature of 37°C because it is the most common temperature used in the routine of clinical laboratories for clinical assays.

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Enhancers

For enhancing the Ag-Ab reactions, polymeric compounds, such as PEG and BSA, may be included in the buffer system. These compounds facilitate the formation of immune complex and help in amplification of signals and improve the assay system sensitivity.

Interference

Many interfering factors, such as bilirubin and lipids, are normally present in the samples apart from the analyte of interest. High concentrations of some of these interfering factors are frequently encountered in clinical samples. They may influence signal generation and, therefore, can interfere in the assay result. Minimizing the influence of these factors help provide accurate and precise assay results.

Turbidimetric assays usually employ a suitable buffer in the assay design to optimize assay conditions and the desired ionic strength, pH and enhancement required for the reaction medium. In addition, the buffer is also useful in reducing the influence of interfering factors present in the sample. The buffer used in turbidimetric assays is generally referred to as *activation buffer*.

Characteristics of Antibody Used as a Reagent

Intrinsic characteristics of antibody employed as reagents have a profound effect on the Ag-Ab reactions. The specificity and affinity of the antibody to the antigenic sites affect the sensitivity of the assay and signal generation time. Usually, when high specificity and affinity antibodies are used, a strong agglutination reaction will readily result. In contrast, antibodies with low affinity, even if highly specific, tend to react slowly and form a weak immune complex, thereby lowering the detectable signal. The avidity of antibody is also an important consideration in the formation of immune complexes. This characteristic of the antibody determines the degree of stability of the Ag-Ab complexes at the antigen-binding sites. The tendency of the complexes to dissociate and disperse decreases substantially as the avidity of the antibody increases.

An antibody without cross-reactivity and with a good titer is a prerequisite for a reliable turbidimetric assay utilizing antibody as a principal reagent. In addition, the antibody must be formulated as a clear solution to give a low reagent blank and should be free from particulate matter.

Standardization and Calibration

During the course of treatment, individual patients are likely to have tests carried out for the same analyte by different methods, and to have results checked against reference intervals that were set elsewhere. To achieve agreement between different methods, a single recognized source of reference preparation is needed.

The reference preparation should:

- Have value assignment in meaningful units
- Be stable and identical to the analyte in the test samples
- Be free of interference from the test sample matrix
- Be standardized by a reference method
- Demonstrate intermethod agreements.

Most International Reference Preparations (IRPs) and Certified Reference Materials (CRMs) such as CRM 470 for immunoassay analytes, can be obtained from the main custodians of International Biological Standards, such as National Institute for Biological Standards and Controls (NIBSC) or WHO and Community Bureau of Reference of the Commission of European Communities (BCR) or the IFCC.

As the availability of International Standards is limited, it is a practice to prepare sets of secondary standards, from which future lots of calibrators can be assigned values. The secondary standards act as an intermediate between IRP primary standard and future lots of calibrators for assay runs. The calibrator sets are made in bulk and values are assigned with reference to the secondary standards.

As discussed, the immunoturbidimetric assays require a set of 5 or 6 calibrators to obtain a standard curve. The quantitative values of unknown analyte obtained from the standard curve will be highly dependent on correct assignment of values to the calibrator used for preparing the standard curve.

Quality Control

The tendency of most immunological reagents to produce changes in reactivity over time requires the application of quality control procedures to ensure the satisfactory analytical performance of immunometric assays on a day-to-day basis. Similarly, in the case of turbidimetric immunoassays reagent stability within a defined usable time span is a prime requirement of the reagent systems, so is the need for accurate and stable controls to validate reagent functioning, precision and accuracy.

Reading Principles in Turbidimetry

For turbidimetric measurements, both end point and rate measurements are applicable. However, the factor method for calculating the concentration of the unknown is not preferred in the kinetic methods by turbidimetry due to the nonlinear nature of relationship between absorbance and analyte concentrations.

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Once the assay system has been designed, the analyzers used for reading must be able to operate according to the principles mentioned below with respect to the addition of reagents and reading of signals (absorbance).

Real Sample Blanking

In this system first the activation buffer (R1) is added to the sample cuvette (S). Then the sample is added, mixed and allowed to stabilize (preincubation period). The first reading (A1) is then taken at the end of preincubation period.

The antibody reagent (R2) is subsequently added to the above mixture and mixed gently. Turbidity develops due to the reaction between the antigen and the antibody over a short period of time. A second reading is taken at the defined time interval (usually 2–10 minutes).

The difference ΔA_S **(Table 23.1)** between the two readings represents the absorbance generated as a result of Ag-Ab reaction.

If required, the absorbance due to the reagent ΔA_B can be measured by running in parallel a reagent blank in a separate cuvette (R) using saline in place of sample **(Table 23.2)**.

ΔA_R thus obtained of the reagent blank can be subtracted from ΔA_S of the sample to calculate the absorbance generated due to the Ag-Ab reaction in the sample.

Table 23.1: Real sample blanking system: Using sample cuvette.

Signals			
	First reading (A1)	Second reading (A2)	ΔA_s (A2–A1)
	Absorbance due to:	Absorbance due to:	Absorbance due to:
Sample cuvette (S)	• Sample • Buffer	• Sample • Buffer • Reagent • Immune complexes	• Reagent • Immune complexes

Table 23.2: Real sample blanking system: Using reagent cuvette.

Signals			
	First reading (A1)	Second reading (A2)	ΔAR (A2–A1)
	Absorbance due to:	Absorbance due to:	Absorbance due to:
Reagent cuvette (R)	• Buffer • Blank sample (saline)	• Buffer • Blank • Sample • Reagent	Reagent

The reagent blank facility may not be available in many semiautomated analyzers. However, the reagent assay system can be optimized to provide a very low reagent blank in order to obviate the need for correcting the reagent blank signals which can contribute to the complete reaction absorbance.

The principle of taking a reading just before the addition of antibody solution (R2) is referred to as *'true sample blanking'* or *'real sample blanking.'*

Immediate Mixed Blanking

In this system initially the activation buffer, sample and the antibody reagent solution are all mixed simultaneously. Then as fast as possible usually 10 to 20 seconds after mixing, the first reading A1 is taken. This 10 to 20 seconds delay time in taking a reading is referred to as lag phase. The reaction is allowed to proceed further and the second reading A2 is measured at the preselected time interval. The increase in absorbance ΔA (A2–A1) represents the signal generated due to the Ag-Ab reaction **(Table 23.3)**.

This method eliminates the need for determination of reagent blank as it measures the increase in absorbance after equilibration of all the reagents and sample. Hence, absorbance generated both due to interfering substances in the sample and the reagent would be blanked during the first reading.

In all Ag-Ab reactions in the initial contact phase the reaction kinetics do not follow a systematic pattern. As this initial chaotic phase settles, the reaction pattern and the absorbances move proportionately. This pattern depends upon the intrinsic nature of the antibody, such as affinity, avidity, etc., and also the concentration of the analyte being measured.

Depending on the assay system requirements, it is desirable that the initial chaotic phase is not included in the measurement of absorbance. Typically, a lag phase would vary from ten to thirty seconds from analyte to analyte. It is, therefore, imperative to follow diligently the recommended

Table 23.3: Immediate mixed blanking system.

Signals			
	First reading (A1)	Second reading (A2)	ΔA_s (A2–A1)
	Absorbance due to:	Absorbance due to:	Absorbance due to:
Sample cuvette	• Reagent • Sample • Buffer	• Reagent • Buffer • Sample • Ag-Ab reaction	Ag-Ab reaction

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time assigned for the lag phase for precise blanking in the "*immediate mixed blanking*" method.

Reaction Kinetics and its Effect on Blanking

The reaction kinetics of an antigen-antibody also guides as to the appropriateness of the blanking system. As the reaction kinetics is not the same for all Ag-Ab systems, for a system with slow reaction kinetics, e.g., IgA, a first reading 10–20 seconds after mixing with the antibody is not very critical **(Fig. 23.10)**.

However, for a system with fast reaction kinetics, e.g., IgG **(Fig. 23.11)**, half of the reaction would have taken place within 10 to 20 seconds when the first reading is taken. Here, a poorly defined point for the first reading would be obtained.

The implications of '*immediate mixed blanking*' can be demonstrated by comparing the standard curves obtained for the six calibrators of latex enhanced reagent system for measurement of IgA **(Fig. 23.12A)** and IgG **(Fig. 23.12B)** at zero seconds and ten seconds, respectively.

The standard curve obtained for IgA **(Fig. 23.12A)** is practically not affected by the difference between the two ways of blanking indicating that a delay of ten seconds is not very significant.

Whereas for a nonenhanced system with fast reaction kinetics for measurement of analytes such as IgG **(Fig. 23.12B)**, a delay of 10 seconds becomes very critical.

There is considerable signal development during the first ten seconds. This results in decreased difference between A1 and A2 **(Fig. 23.11)**. The loss of signal increases with increasing concentration of IgG in the calibrators.

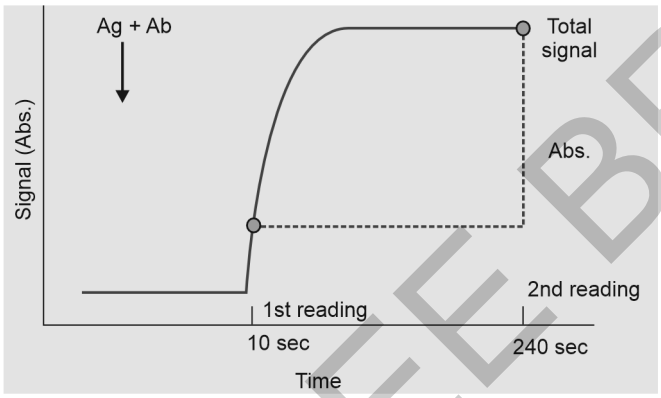

Fig. 23.10: Signal development as a function of time. Figure illustrating 'immediate mixed blanking' using IgA as an example.

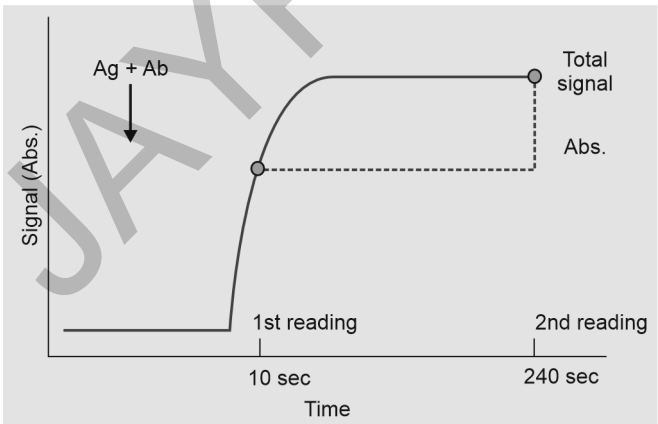

Fig. 23.11: Signal development as a function of time. Figure illustrating 'immediate mixed blanking' using IgG as an example.

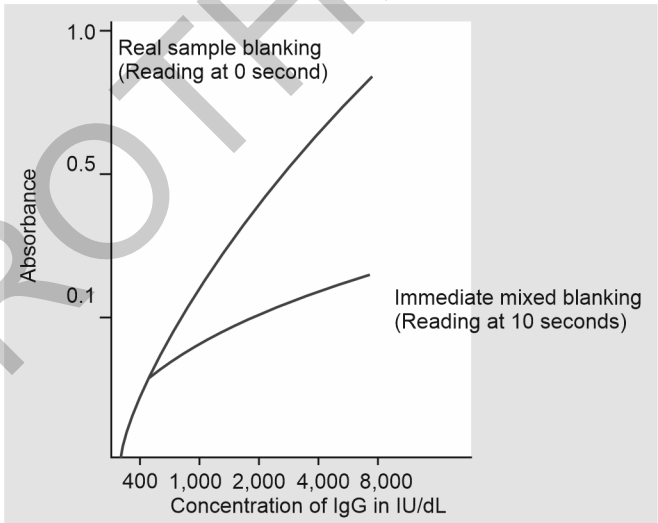

Fig. 23.12A: Standard curves for IgA obtained with 'real sample blanking' and 'immediate mixed blanking'.

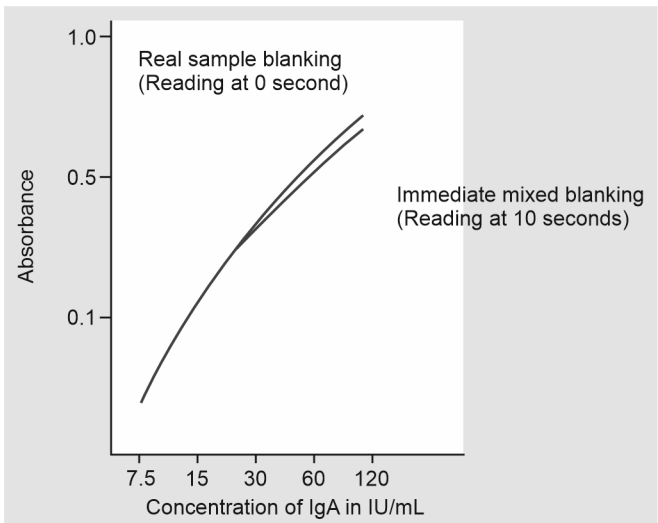

Fig. 23.12B: Standard curves for IgG obtained with 'real sample blanking' and 'immediate mixed blanking'.

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It can be observed from **Fig. 23.12B** that the curve for "*immediate mixed blanking*" tends to get flatter with the increasing concentration of IgG, resulting in a decrease in the precision of the analysis.

It would be desirable to optimize both slow reacting systems and assay systems based on particle enhanced turbidimetry (latex-based assays) where the reagent absorbance is very high, based on "*immediate mixed blanking*".

Whereas for systems with fast reaction kinetics such as IgG, the assays should be optimized using the "*real sample blanking*" principle as the sample blanking and the immunochemical reaction can be optimized separately.

Q. What are the concepts of assay optimization?

While optimizing reagent system for immunoturbidimetric assays, it is important to optimize the dose-response curve by titrating the amount of sample (antigen) and the antibody concentration in the reagent until a dose-response curve as shown in **Figure 23.13**, is obtained.

A portion of the antibody excess zone of dose-response curve is then selected as the "*range for the standard curve*".

Detection Limit

The lowest concentration of an antigen, which gives a detectable signal compared to the background noise, is defined as the detection limit or analytical sensitivity of the analysis. It is defined as the minimum concentration of analyte that is statistically unlikely to form part of the range of signals seen in absence of analyte. Usually, the detection limit is set as the lowest signal where the standard deviation around that signal is less than one third of the signal itself. The lowest concentration selected for the calibration of the assay is usually above the detection limit.

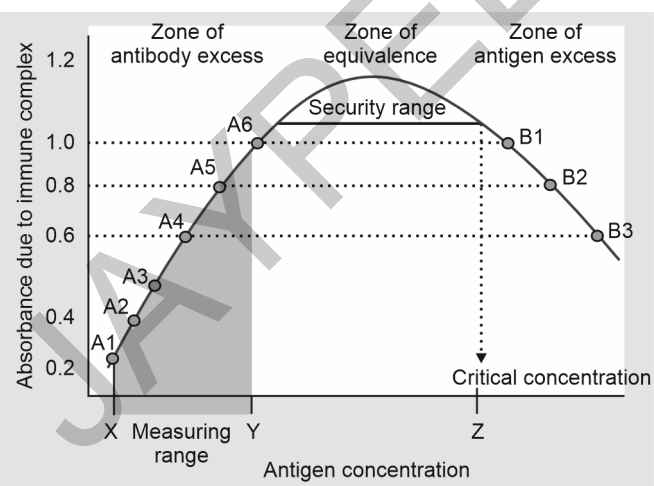

Fig. 23.13: Dose-response curve Figure illustrating measuring range (X–Y), critical concentration (Z), standard curve (A1–A6), erroneous interpolation (where Abs. val.1.0 corresponds to A6 and B1, Abs. val. 0.8 corresponds to A5 and B2 and Abs val. 0.6 corresponds to A4 and B3), security range (Y–Z).

Measuring Range

As long as the analyte signal is higher than the signal of the lowest calibrator and lower than the signal of the highest calibrator, the assay system will operate accurately for the said analyte and a concentration value of the sample can be interpolated.

The interval between the signal generated by the lowest calibrator to the signal generated by the highest calibrator, which gives proportionate and measurable and a linear signal, is referred to as the measuring range of the assay system.

Security Range

The critical point **(Cx in Fig. 23.13)**, in the antigen excess zone of the dose-response curve corresponds to the maximum concentration value of analyte, which gives a signal value higher than the signal value of the calibrator of highest concentration, and just before the value **(B_1 in Fig. 23.13)** at which erroneous interpolation begins. The interval between the signal of highest standard and the signal of the critical concentration can be referred to as the security range of the assay system for the analyte.

Reagent Optimization

The risk of obtaining signals in the antigen excess zone are more relevant in analytes like C-reactive protein or immunoglobulins such as IgG where the concentration between normal and pathological sample can differ by manifolds. It is necessary to make sure that the concentration values lying in the antigen excess zone (critical point) are beyond the concentrations which can be expected to occur in clinical samples during routine analysis.

The relation between the security range and measuring range is very important in optimizing assay systems. As mentioned earlier the shape of the dose-response curve depends on the ratio between the antigen and the antibody. At a constant antibody concentration, an increase in the measuring range will result in a narrower security range, leading to antigen excess at a lower antigen concentration **(Fig. 23.14)**.

The implications of increasing the antibody reagent concentrations can be practically demonstrated using IgG as an analyte and antihuman IgG as a reagent. The measuring range and the security range can be expanded

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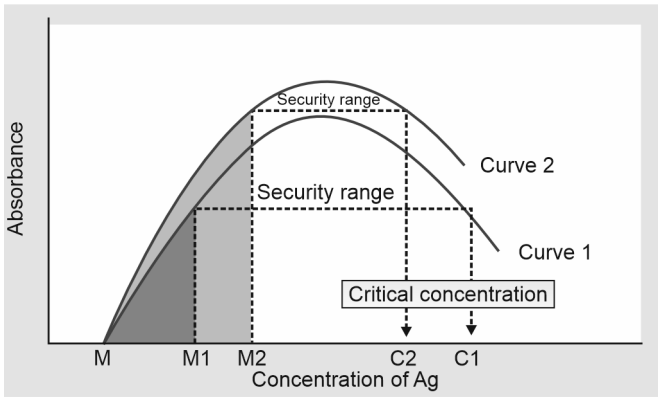

Fig. 23.14: Effect of increasing measuring range on security range. M–M1 = measuring range for curve 1, C1 = critical concentration for curve 1 M–M2 = Measuring range of curve 2, C2 = critical concentration for curve 2.

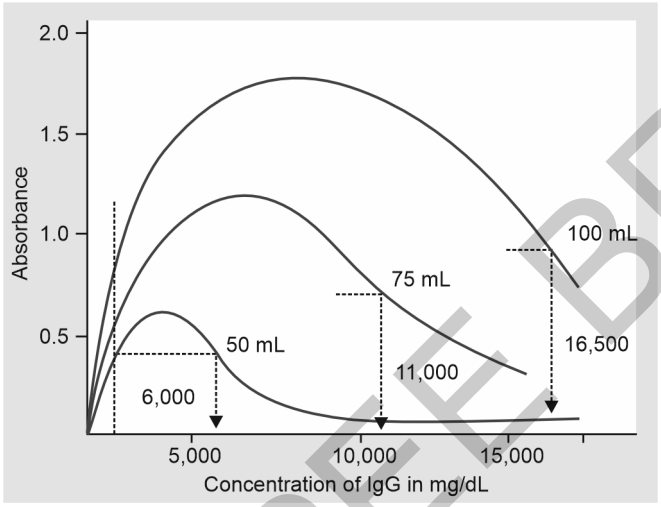

FIG. 23.15: Effect of increasing antibody concentration on security range. Where 50, 75 and 100 µL are volumes of antibody and 6,000, 11,000 and 16,500 are their respective critical concentrations.

by increasing the antibody concentration. However, this expansion can only be done to a point where it is still possible to have the desired sensitivity for lower analyte concentration.

Figure 23.15 shows the effect of increasing the antibody concentration on the security ranges while keeping constant, the measuring range of the dose-response curve.

When the volume of an antibody solution of concentration 'X' used is 50 µL, the security range obtained is around 5,000 mg/dL. As the volume of the same antibody concentration is increased to 75 µL, the security range increases to >10,000 mg/dL. With a further increase in volume of the same antibody concentration to 100 µL, the security range shifts to >15,000 mg/dL. But this increase in measuring range is possible till a certain limit of increasing volume of antibody solution. If the volume of antibody solution is increased further, there will be a decrease in the absorbance at the lowest concentration of the measuring range due to antibody excess, resulting in compromising with assay sensitivity.

By adjusting the sample dose and the antibody concentration, a measuring range 20 to 25 times the lowest calibrator value can be possibly optimized, with a security range still giving a warning up to the pathophysiological concentration. A wide measuring range combined with a wide security range offer the advantage of a few reruns and maximum security against antigen excess problems.

Standard Curve

Once the dose-response curve for a reagent has been optimized, a standard curve can be obtained by using a number of dilutions of the calibrator (preferably 5–6) covering the optimal measuring range. The lowest calibrator should be chosen to give a signal significantly higher than the background noise. The highest calibrator should be selected to allow measurements for a reasonably wide range of analyte concentrations and still leaving space for a fair security range **(Fig. 23.16)**.

A curve is fitted to the signals obtained for calibrator dilutions and can be stored in the memory of the instrument. Different curve fitting programs can be made available in instrument software.

Many of the instruments are equipped with a facility to give a 'warning' that indicate reruns of the test with dilution of the sample with high concentration values of analyte. This warning is given as long as the sample signal is higher than the signal of the highest calibrator.

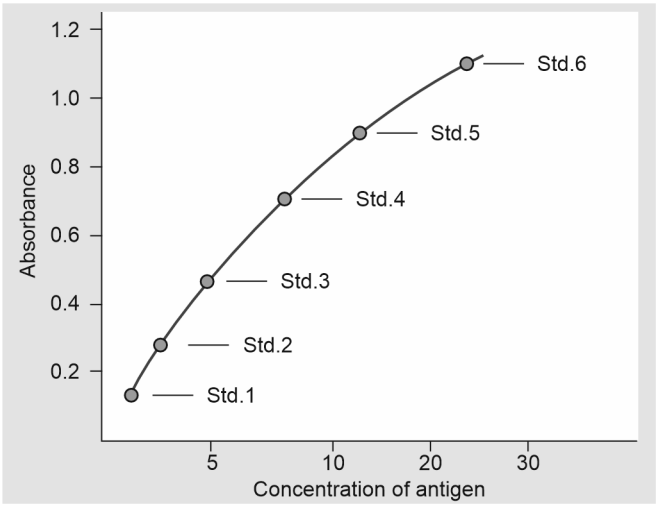

Fig. 23.16: Illustrating standard curve.

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The validity of the stored standard curve should be checked with known controls at regular time intervals.

Q. What instruments are needed for turbidimetry?

The development of automated instruments for the clinical laboratory began in the 1950s at the same time as the demand for test such as IgA, CRP, HBsAg escalated dramatically. One of the benefits of automation is a reduction in the variability of results and errors of analysis by eliminating tasks that are repetitive and monotonous for a human and that can lead to boredom or inattention. The significant improvement in quality of laboratory tests in recent years owes much to the combination of well-designed instrumentation with good analytical methods.

The photometric requirements of turbidimetric analysis are no different from those of photometric biochemistry analysis. However, the photometer used must be provided with means to maintain the contents of the cuvette at a constant temperature during the reaction along with compatible software to run the various steps of the reagent and reagent kinetics appropriately.

The chemistry analyzers (spectrophotometers) available utilize either or both of the two modes of measuring absorbance.

Aspiration Mode

In this mode, the chemical reaction is carried out in a test tube/cuvette. The instrument aspirates the reaction mixture from the test tube/cuvette, which enters into the flow cell (internally built reading chamber), where the absorbance is measured. After the absorbance is measured, the reaction mixture is passed through a different outlet and is collected in a waste collecting bottle. As all the measurements are done in a single flow cell, the flow cell has to be washed after each test. Improper washing may affect the test results of the subsequent tests. Especially, if latex enhanced tests are used, the latex has a tendency to stick and form a permanent coating on the internal walls of the flow cell resulting in variation in the wavelength of the incident light, and hence, lead to erroneous results. Even nonenhanced antibody reagents are proteinaceous and cleaning of the flow cell would remain a critical issue.

When Ag-Ab complexes are aspirated, because of the force of aspiration, the formed immune complexes would be structurally disturbed and may break down into smaller complexes, which would result in lower absorbance values. Moreover, the immune precipitates formed may block the aspiration tube or the flow cell itself.

For turbidimetric assays designed with real sample blanking reading principle, it would be inconvenient to run the test in the aspiration mode. Initially, the sample mixed with the activation buffer would have to be aspirated first and absorbance A1 measured. Then again the activation buffer and the sample will have to be taken in another test tube and the principle reagent added and the reaction read after a fixed time interval. Hence, for running one assay twice, double the amount of activation buffer and sample will have to be used.

Cuvette Mode

In this mode, the reaction of the reagent and sample takes place in a measuring cuvette, and absorbance is read in the same measuring cuvette itself. Hence, assays using any of the reading principles can be conveniently read in cuvette mode without wastage of reagents.

Instruments applying this mode of measuring have an advantage. As the reactions are run in external cuvettes, the instruments are safe from the effects of reagents. Moreover, availability of standardized optically clean disposable cuvettes eliminates the carryover effects of the previous tests.

Classification of Analyzers

There are several types of analyzers available in the market. They may be grouped in two categories:

1. Semi-automated analyzers
2. Automated analyzers

Semiautomated Analyzers

Instruments with an absorbance linearity of 2.0 are suitable for turbidimetric estimations of both particle enhanced and nonparticle enhanced reagent systems. Most of the instruments with the above specification can be used for turbidimetric measurements using the absorbance mode. In the absorbance mode as the calibration curve cannot be stored, it has to be drawn manually.

Among the instruments working on cuvette mode for measuring absorbance, very few instruments have software interface programed with a facility to store the calibration curve utilizing both the principles of reading, i.e., 'real sample blanking' and 'immediate mixed blanking' in the multistandard mode. Many instruments with cuvette modes are known to have programs in multistandard mode to store calibration curve for assay systems using the real sample blanking techniques only.

Automated Analyzers

The automated analyzers can be grouped in two categories:

1. Centrifugal analyzers
2. Static instruments (noncentrifugal analyzers).

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Centrifugal analyzers

In these analyzers, the cuvettes are arranged in circle (rotor) that can be rotated at a velocity of about 1,000 rpm. The shape of each cuvette allows application of sample and reagent (reaction buffer, antibody) in separate compartments. When the rotor starts to spin, the contents of these compartments are mixed simultaneously and held in place in the cuvette by centrifugal force. Readings of all the cuvettes are performed at essentially the same time (i.e., when the rotating cuvettes are passing the optical measuring device). Two reading systems are used: either parallel to the length of the cuvette where the volume in the cuvette is proportional to the light path or perpendicular to the length of the cuvette where the width of the cuvette equals the light path.

Static instruments

In these instruments, the cuvettes are mostly arranged in a circle (rotor), and this is slowly rotated in step at a fixed time interval (cycle time). Access to the cuvette is possible only at these intervals for sample or reagent application and reading. Mixing is in most cases performed with a mechanical stirring device. Modern instruments seem more and more to be based on these principles.

All instruments operate under software control. A part of this software is the user interface that makes it possible to program the instrument to perform analysis and calculation according to an optimized protocol. The analytical parameters available for user control vary from instrument to instrument.

Some instruments, however, are "closed instruments" which implies that all parameter settings are read into the instruments by bar coded reagents. In this case, the user cannot control the assay and will have to rely entirely on the manufacturer and their instructions.

The applications and reference values of important clinical analytes are shown in **Table 23.4**.

Given below are serum proteins with clinical conditions where they are raised and diminished **(Table 23.5)**.

Table 23.4: Applications, reference values of important clinical analytes.

Analyte	*Description*	*Reference values*	*Applications*
RF	Quantitation of rheumatoid factors	<10 IU/mL	Detection of RA, differential diagnosis of RA from rheumatic fever and other rheumatic disorders
CRP	Quantitation of C-reactive protein	Adults and children <0.6 mg/dL	Detection of inflammatory conditions, measuring the severity of conditions, differential diagnosis of bacterial and viral infections Monitoring the response to therapy
CRP US	Quantitation of ultrasensitive levels of C-reactive protein	<0.05 mg/dL	Prognostic cardiac marker
ASO	Quantitation of antistreptolysin 'O'	Children: <150 IU/mL Adults: <200 IU/mL	Detection of Group A streptococcal infections such as sore throat, rheumatic fever, rheumatic heart disease
MA	Quantitation of urinary albumin	<20 mg/L	Detection of microalbuminuria. Monitoring the effect of ACE inhibitor or intervention strategies for reducing UAE*
IgA	Quantitation of immunoglobulin IgA	70–400 mg/dL	Chronic infections of GI and respiratory tract, anaphylactic transfusion reactions, monitoring progress of IgA myeloma
IgG	Quantitation of immunoglobulin IgG	700–1,600 mg/dL	IgG myeloma, IgG deficiency, assessment of the progression and response to treatment of IgG myeloma
IgM	Quantitation of immunoglobulin IgM	40–230 mg/dL	Monitoring patients with Waldenström's macroglobulinemia Estimating frequent, chronic and acute infections
IgD	Quantitation of immunoglobulin IgD	3–14 mg/dL	Screening for congenital infections. Monitoring IgD myeloma
IgE	Quantitation of immunoglobulin IgE	Adult 3–423 IU/mL	Assessment of atopic diseases, dermatologic and parasitic infections
C3	Quantitation of complement component C3	90–180 mg/dL	C3 deficiency, recurrent infections detection and monitoring of immune complex disorders such as SLE, vasculitis, glomerulonephritis, autoimmune hemolytic anemia
C4	Quantitation of complement component C4	10–40 mg/dL	Congenital deficiency in lupus erythematosus Hereditary angioneurotic edema recurrent infections

Contd...

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Contd...

Analyte	*Description*	*Reference values*	*Applications*
AT III	Quantitation of antithrombin II	17–30 mg/dL	Evaluating patient at risk of developing thrombotic-embolic disease. In surgical patients receiving heparin, assessment of thrombotic risk of contraceptive or estrogen therapy
Apo A-1	Quantitation of apolipoprotein A-1	Males: 105–175 mg/dL Females: 105–205 mg/dL	Independent risk factor for coronary artery disease
Apo B	Quantitation of apolipoprotein B	Males: 60–140 mg/dL Females: 55–130 mg/dL	Elevated Apolipoprotein B levels are associated with atherosclerosis
Lp (a)	Quantitation of lipoprotein(a)	<300 mg/L	Risk factor for coronary heart disease that is independent of all other lipid parameters
β2-M	Quantitation of β2-Microglobulin	<60 years: 0.8–2.4 mg/L >60 years: <3.0 mg/L	• Prognosis of multiple myeloma • Early detection of renal transplant rejection, differentiation of glomerular and tubular nephropathies • Monitoring therapeutic response of patients with nonsecretory myeloma or light chain disease
Cp	Quantitation of ceruloplasmin	20–60 mg/dL	• Diagnosis of Wilson's disease • Menkes disease, nutritional copper deficiency
Hp	Quantitation of haptoglobin	30–200 mg/dL	Diagnosis and monitoring of hemolytic diseases

*UAE = Urinary albumin excretion

Note: The above reference values are for guidance only. As the reference values are related to age, geographical and methodological differences and vary widely. Each laboratory should define its own reference range for the relevant population.

Table 23.5: Serum proteins in different clinical conditions

Protein	*Increased in*	*Decreased in*
Serum albumin	Rare, usually associated with hemoconcentration	Acute phase response, severe liver disease, nephrotic syndrome, other renal diseases, malnutrition, pregnancy, premature infants
Complement		
Complement C3	APR (infection, inflammation etc.), biliary obstruction, obstructive jaundice, diabetes mellitus, gout, some connective diseases (excluding SLE)	Autoimmune diseases, immune complex diseases, mixed cryoglobulinemia, serum sickness, chronic renal failure, thrombotic thrombocytopenic purpura, neonatal respiratory distress syndrome, genetic deficiency
Complement C4	APR, RA, SLE, rheumatic fever, ankylosing spondylitis, temporal arteritis, acute viral hepatitis, MI, DM, malignancies, thyroiditis, irritable bowel syndrome, pneumonia, pregnancy	Acquired deficiencies resulting from: • *Hypercatabolism:* Disease in which circulating immune complexes are likely to lead to acquired hypocomplementemia, subacute bacterial endocarditis, mixed cryoglobulinemia, hereditary angioneurotic edema, progressive glomerulonephritis Hyposynthesis: Protein calorie malnutrition, liver disease, Sjögren's syndrome • *Congenital deficiencies:* Associated with increased frequency of scleroderma, chronic hepatitis, autoimmune hepatitis, Henoch-Schönlein purpura
Lipoproteins		
Apolipoprotein A-1	Familial hyperalphalipoproteinemia, modest alcohol intake, estrogen use, exercise, thyroid hormones androgen use	Chronic renal failure, tangier disease, diabetes, hypertriglyceridemia, liver diseases, familial and nonfamilial hypoalphalipoproteinemia, certain drugs,

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Protein	***Increased in***	***Decreased in***
Apolipoprotein B	Premature atherosclerosis, familial defective Apo B, hyperapobetalipoproteinemia, tendon xanthomata, hepatosplenomegaly, diabetes mellitus, chronic renal disease, tangier disease, hypothyroidism, nephrotic syndrome, familial hypercholesterolemia	Familial hypobetalipoproteinemia, abetalipoproteinemia, neuromuscular degeneration, chronic anemia, exercise, liver disease, acute inflammation, certain drugs, neurologic disease
Lipoprotein (a)	Coronary artery disease, cerebrovascular disease, peripheral vascular disease, nephrotic syndrome, DM (variable), cancer, gout, APR, familial hypercholesterolemia	Cirrhosis (particularly primary biliary cirrhosis), certain drugs (nicotinic acid, neomycin, oral estrogen), some steroids such as stanozolol
Immunoglobulins		
IgA class	• *Polyclonal:* Chronic liver disease, alcoholic and nonalcoholic cirrhosis, chronic respiratory infections, GI diseases, some immunodeficiency states, RA, ankylosing spondylitis, nephropathy oligoclonal: May be observed in electrophoresis of IgA myeloma • *Monoclonal:* Multiple myeloma (IgA type)	Infancy and early childhood, selective IgA deficiency, protein-losing syndromes, congenital rubella, macroglobulinemia or non-IgA multiple myeloma
IgG class	• *Polyclonal:* Autoimmune diseases, chronic liver disease, chronic or recurrent infections, sarcoidosis, some parasitic infections, intrauterine contraceptive devices • *Oligoclonal:* Lymphoid or nonlymphoid malignancies, various autoimmune disorders, infections • *Monoclonal:* IgG myeloma, lymphoma, • Monogammopathies of unknown significance	Agammaglobulinemia, hypogammaglobulinemia, omenn's syndrome, X-linked hyper-IgM syndrome, nephrotic syndrome, non-IgG myelomas, infancy, pregnancy
IgM class	• Polyclonal: Viral infections (hepatitis A, CMV), parasitic infections (filariasis, malaria), chronic liver disease, hyper-IgM dysgammaglobulinemia, collagen vascular disease, primary biliary cirrhosis, primary sclerosing cholangitis • Monoclonal: Waldenström's macroglobulinemia, malignant lymphoma, reticulosis, cold agglutinin/hemolysin disease	Immunodeficiency states (Wiskott-Aldrich syndrome), non-IgM or IgM myeloma, infancy and early childhood, lymphoma
IgD class	IgD myeloma, chronic infections (pyelonephritis), connective tissue disease, Hodgkin's disease, some forms of liver disease	Various hereditary and acquired deficiency syndromes
IgE class	IgE myeloma, allergic rhinitis, atopic dermatitis, bronchial asthma, hay fever, thymic dysplasia, selective IgA immunodeficiency, eosinophilic gastroenteritis, Wiskott-Aldrich syndrome, Loeffer's syndrome, hyper-IgE syndrome, active SLE nephritis, certain drugs (particularly gold compounds)	Some progressive neoplastic diseases, ataxia-telangiectasia, hypogammaglobulinemia, hypersensitivity
CRP	APR, bacterial infections, viral infections, rheumatic fever, active RA, vascular disorders, MI, Crohn's disease, ulcerative colitis, renal transplant failure, early pregnancy, intrauterine devices, malignancies with widespread metastases	None described
Fibrinogen	APR, nephrotic syndrome, hemodialysis patients, pregnancy, estrogen therapy, contraceptives, acromegaly	Consumption coagulopathies, recurrent pulmonary embolism, recurrent stroke, DIC, incompatible blood transfusion reactions, obstetrical complications, inherited deficiency, prostatic carcinoma, liver disease, certain drugs (e.g., tamoxifen, anabolic steroids, nicotinic acid)

Contd...

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Protein	*Increased in*	*Decreased in*
Haptoglobin (Hp)	• APR, RA, biliary obstruction, nephritis, ulcerative colitis, aplastic anemia. • Major depression, corticosteroid therapy, androgen use	Ineffective erythropoiesis (sickle cell anemia, folic acid deficiency), intravascular hemolysis, progressive tumors of liver and marrow, severe liver disease, pregnancy, estrogen therapy, newborns
Proteinase Inhibitor		
AT III	APR, vitamin K deficiency	Inherited deficiency, acute thrombosis, DIC, consumptive coagulopathies, certain chemotherapeutic drugs, severe liver disease, estrogen therapy, nephrotic syndrome, heparin therapy, some contraceptive medications
Other Markers		
Serum β2-microglobulin	Decreased glomerular filtration, lymphoproliferative disorders, myeloma, RA, viral infections, anticancer drugs, newborns, ESRD, Crohn's disease, Sjögren's syndrome, dialysis related amyloidosis, certain anti-inflammatory drugs	None reported
APR = acute phase reactant, RA = rheumatoid arthritis, SLE = systemic lupus erythematosus, MI = myocardial infarction, DM = diabetes mellitus, ESRD = end-stage renal disease		

EXAMPLES OF TURBIDIMETRIC IMMUNOASSAYS

Q. Describe the turbidimetric immunoassay for determination of rheumatoid factors Quantia RF (multi-standard).

Principle

Quantia-RF® is a turbidimetric immunoassay for the determination of rheumatoid factors and is based on the principle of agglutination reaction. The test specimen is mixed with Quantia-RF® latex reagent (R2) and activation buffer (R1) and allowed to react. The presence of RF in the test specimen results in formation of an insoluble complex resulting in an increase in turbidity, which is measured at wavelength 578–630 nm. The increase in turbidity corresponds to the concentration of RF in the test specimen.

Notes

- In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
- The reagents that are derived from human source have been tested for HBsAg and HIV antibodies and are found to be nonreactive. However handle the material as if infectious.
- Reagents contain 0.1% sodium azide as preservative. Avoid contact with skin and mucosa. On disposal flush with large quantities of water.
- The reagents can be damaged due to microbial contamination or exposure to extreme temperatures. It is recommended that the performance of the reagents be verified using known controls periodically.
- Gently mix the Quantia-RF® latex reagent well before use to disperse the latex particles uniformly to improve test performance.
- The working reagent should be mixed gently.
- Do not use vortex mixers for mixing. Gently mix the reagents and samples during test procedures.
- As the reagents within lots have been matched, reagents from different lots must not be interchanged.
- Calibrators of different manufacturers must not be used with Quantia-RF® reagents.
- The calibration curve must be validated periodically with known controls.
- The Quantia-RF® assay is recommended only for analyzers with cuvette mode. Though any semi-automated analyzer with appropriate programing facility can be used, for best results it is recommended to use Quantiamate analyzer. Fully automated analyzers may be used, provided the reagent has been standardized on the system.
- The procedures mentioned in this pack insert are based on a minimum reading volume of 500 µL (0.5 mL). In case of instruments where minimum volume required for reading absorbance is 1.0 mL, use double the quantity of reagents and samples mentioned in the test procedure.

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Specimen Collection and Preparation

- No special preparation of the patient is required prior to specimen collection by approved techniques.
- Only serum should be used for testing. Should a delay in testing occur, store the samples at 2–8°C. Samples can be stored for up to 3 days at 2–8°C, provided they are not contaminated. Do not use hemolyzed, icteric, or highly turbid sera. Turbid or particulate serum samples must be clarified by centrifugation at 2,000 rpm for 15 minutes. Use the clear supernatant for testing.

Additional Material Required

Spectrophotometer with 578–630 nm wavelength filters and cuvette mode, stopwatch, well calibrated micropipettes, disposable tips, isotonic saline, particulate free distilled water, test tube rack, incubator/waterbath set at 37°C, optically clean disposable cuvettes such as Quantiamate semi micro cuvettes/glass cuvettes.

Note: Though any filter between the wavelengths 578–630 can be used, optimum results are obtained with 630 nm wavelength filter.

Test Procedure

Bring reagents and specimen to room temperature before use.

Assay conditions:

Test tube No.	1	2	3	4

- Wavelength : 630 nm
- Reaction temperature : 37°C
- Cuvette : 1 cm path length

Method for Preparation of RF Calibration Curve

The Quantia-RF® calibrator must be reconstituted exactly with 1.0 mL of distilled water, wait for 10 minutes, gently swirl the vial till the solution attains homogeneity. Once reconstituted it is ready to use for preparation of RF calibration curve. The Concentration (S) of RF in the reconstituted calibrator is as mentioned at the end of this package insert.

Dilute the calibrator serially as mentioned below for preparation of calibration curve.

Test tube No.	1	2	3	4
Calibrator dilution No.	D1	D2	D3	D4
Isotonic saline	-	100 µL	100 µL	100 µL
Calibrator	100 µL	100 µL	100 µL	100 µL
Concentration of RF in IU/mL	160	80	40	20

The above four dilutions of the calibrator including the highest 120 IU/mL (D1) and lowest 15 IU/mL (D4) concentrations of measuring range must be used for the preparation of the calibration curve.

Note: Increasing concentration of calibrators must be used for preparing the calibration curve.

Test Procedure for Preparation of Calibration Curve

- Zero the instrument with distilled water.
- Pipette 450 µL of Quantia-RF® activation buffer (R1) and 50 µL of Quantia-RF® latex reagent (R2) in the measuring cuvette. Mix well and incubate for 5 minutes at 37°C.

OR

- Pipette 500 µL of the Quantia-RF® working reagent in the measuring cuvette. Mix well and incubate for 5 minutes at 37°C.
- Add 10 µL of calibrator (D1), mix gently and start the stopwatch simultaneously.
- Read absorbance (A1), exactly at 10 seconds, and absorbance (A2) again at the end of exactly 2 minutes.
- Repeat steps No. 2–4 for each diluted calibrator (D2 to D4) for preparing calibration curve.
- Calculate ΔA (A2–A1) for each calibrator (D1 to D4). Plot a graph of ΔA versus concentration of RF on the graph paper provided with the kit.

"The calibration curve" so obtained is valid only for the same lot of Quantia-RF® reagents.

Test Procedure for Specimen

For determination of RF concentration in the test specimen:

- Follow steps 2–4 as mentioned in the above procedure for calibration curve using the test specimen in place of the calibrator.
- Calculate ΔA (A2–A1) for the test specimen.

Validation Criteria

If ΔA of the test specimen is less than ΔA obtained for the standard of highest concentration (D1) then the concentration of RF in the test specimen can be determined directly by interpolating ΔA of the test specimen from the calibration curve.

If the ΔA of the diluted test specimen is higher than ΔA of standard with highest concentration (D1) then the test has to be rerun by carrying out further dilution of test specimen such as 1:10, 1:20 etc., till the ΔA of the diluted test specimen is less than ΔA of D1. Then proceed for calculations.

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Calculations

- Interpolate ΔA of the diluted test specimen on the calibration curve and obtain the RF concentration 'C' of the diluted test specimen.
- Multiply the RF concentration 'C' with the dilution factor (F) of the test specimen for obtaining the concentration of RF in the neat test specimen.
  Concentration of RF in the neat test specimen in IU/mL = C × F
  (Where 'F' is the dilution factor of the test specimen, i.e., 10 for 1:10 dilution of test specimen and so on).

Reference Values

- The reference value for RF was determined to be <20 IU/mL with Quantia-RF® on Quantiamate.
- It is recommended that each laboratory must define its own reference range for relevant population taking into account all affecting factors.

Remarks

- Usage of well-calibrated equipment and accessories and procedures is critical for achieving correct results.
- When ΔA obtained for the test specimen is greater than the ΔA of the standard with highest concentration then, it indicates that the concentration of RF in the test specimen is beyond the measuring range of the Quantia-RF® assay. Such specimens should be rerun with further dilutions.
- Markedly lipemic, hemolyzed, and contaminated serum samples could produce erroneous RF values.
- Use of plasma rather than serum can result in erroneous RF values.
- Do not read results beyond 2 minutes.
- Rheumatoid factors are not exclusively found in rheumatoid arthritis but sometimes in syphilis, systemic lupus erythematosus, hepatitis, and hypergammaglobulinemia also.
- It is recommended that results of the tests should be correlated with clinical findings to arrive at final diagnosis.
- Quantia-RF® assay is sensitive to the presence of IgM RF with heterogeneous specificity.

C-REACTIVE PROTEIN

C-reactive protein (CRP) is an abnormal serum glycoprotein produced by the liver during acute inflammation or infections. CRP is synthesized by the liver under regulatory control of cytokines. Interleukins 1b and 6 and tumor necrosis factors are the most important regulators of CRP synthesis. The intact CRP molecule is a pentameric protein with identical subunit arranged in a doughnut shaped polymer.

Q. Describe the clinical significance of CRP.

The function of CRP is felt to be related to its role in the innate immune system. Similar to IgG it activates complement, binds to Fc receptor and acts as an opsonin for various pathogens. Interaction of CRP with Fc receptors leads to the generation of proinflammatory cytokines that enhance inflammatory response. Unlike IgG, which specifically recognize distinct antigenic epitopes, CRP recognizes altered self and foreign molecules based on pattern of recognition. This recognition provides an early defense and leads to a proinflammatory signal and activation of the humoral immune response. CRP binds to apoptotic cells, protects the cells from assembly of terminal complement components and sustains an anti-inflammatory innate immune response.

All acute inflammatory process (infectious and noninfectious and certain malignant conditions result in rise in serum CRP, as a nonspecific phenomenon. CRP production is a nonspecific response to disease and it can never on its own be used as a diagnostic test. However, if the CRP results are interpreted in the light of clinical information on the patient it can provide exceptionally useful information.

Levels of CRP increase very rapidly in response to trauma, inflammation and infection and decrease very rapidly with the resolution of the condition. An activated CRP is always associated with pathological changes. Hence, determination of CRP is of great value in diagnosis, treatment and monitoring of inflammatory condition. Measurement of CRP may be helpful to know whether the patient is getting better, or if there are any complications arising.

CRP Measurements Help in Diagnosis and Management

Rheumatology	The rheumatic diseases exhibit joint or soft tissue symptoms, such as back pain, and myalgia. However, such symptoms may also be due to psychogenic factors. The elevation of acute phase proteins confirms the presence of organic disease but a value within the reference range does not exclude a mild local disease. In conditions like ankylosing spondylitis, serum CRP may be elevated before the onset of clinical symptoms

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