Turbidimetry

TECHNICAL SERIES

TULIP DIAGNOSTICS (P) LTD.

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Foreword

TULIP Diagnostics (P) Ltd. is a part of the innovative **TULIP** Group of companies based at Goa, India.

The group's commitment in building products of international standards, through indigenous R&D has accorded the company virtual leadership in most product segments in the Indian marketplace. Its state-of-art manufacturing facility conforms to the strictest FDA (India) and GMP regulations. In its efforts to build world-class Quality products, the group has recently received the ISO 9001(2000) certification from TUV.

It is this commitment to Quality, which has given the group international acclaim. The products are now exported to over 45 countries globally with an ever-increasing user base.

With decades of experience in *in-vitro* diagnostics (IVD), **TULIP** has created a strong knowledge base. **TULIP** believes that in the knowledge-based society of the 21st century, regular upgradation of knowledge is essential not only for better diagnosis and patient care, but also to improve the overall quality of life.

Publishing of **Technical Series** is one such initiative to make available to the Laboratory professionals and clinicians updated knowledge that is vital for them to set trends in their day-to-day practice.

Gerneral Back ground

Diagnosis is a decision point. The decision is the intention to treat. It is the point at which sufficient evidence has been accumulated to state, beyond reasonable doubt, that the patient is or is not suffering from a particular disease.

Laboratory tests remain one of the mainstays on which the clinicians rely for diagnosis and management of the patient. Laboratory tests are indicated for;
1. **Detection:** The presence or absence of a particular substance e.g. testing for infectious diseases like VDRL, HBsAg.
2. **Quantification:** accurately determining the concentration of a particular substance as an aid to diagnosis or differential diagnosis (e.g. Concentration of CRP in differential diagnosis of Viral and bacterial infections) and for establishing the extent of the Clinical condition (e.g. RF in measuring disease severity).
3. **Monitoring:** The course of clinical condition or response to therapy.
4. **Prognosis:** For predicting the probability of occurrence of a disease/disorder (e.g. Microalbuminuria for predicting Diabetic nephropathy) or predicting the outcome of a disease/disorder.

Microscopy, Biochemical assays, Microbiology procedures, and Immunoassays are various techniques that fulfil the requirements of routine laboratory tests to meet the needs of the clinicians.

Certain clinical analytes can be measured by specific techniques only, whereas for the measurement of certain analytes options exist for selecting the techniques of measurement. For example urinary albumin can be measured by biochemical methods such as pyrogallol red or coumassie blue. But for the diagnosis of Microalbuminuria, a condition where urinary excretion of albumin is in the range of 30-300 mg/L the accuracy of the measurements by biochemical methods is questionable because these methods also react with other proteins in addition to albumin which are frequently found to be present in urine. Immunochemical methods (Immunoassays) which are more sensitive and specific have a distinct advantage and hence are preferred.

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. Anti-immunoglobulin antibodies appearing in rheumatoid arthritis patients were shown to cause visible clumping of these erythrocytes. This method was known as **haemagglutination.**

Singer and Plotz replaced the erythrocytes with latex particles, which were easier to standardise, and these assays are popularly known as **latex agglutination tests**. High degree of sensitivity for 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 equipments, 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 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, immunoelectrophoresis (IEP) and western blotting. These techniques at the best can give a semiquantitative or comparative information about analyte 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 non-identical. **Immunoelectrophoresis** has been used over the years for detection of several 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 & 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 and electroimmunoassays, turbidimetric and nephelometric assays, and labelled immunochemical assays.

Radial immunodiffusion (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;
Heterogeneous Immunoassays: These assay systems employ an antibody immobilized on a solid phase, which captures the corresponding antigen from the sample. A second labelled 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 labelled antibody are separated. The concentration of antigen is then estimated by measuring bound or unbound-labelled antibodies through an appropriate signal generation and measurement system.

Heterogeneous immunoassays can be performed by various techniques such as;
- Radio immunoassays (RIA)
- Enzyme Immunoassays (EIA)
- Fluorescent Enzyme Immunoassay
- Chemiluminescent Enzyme Immunoassay

The difficulties associated with separation of bound and unbound-labelled 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 the 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 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 labelled materials in the test system for the detection or measurement of the antigen. The immunochemical binding produces a detectable signal (agglutination, Absorbance, fluorescence etc.). The simplicity & 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)
- Elecrtrochemiluminiscence (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 & nephelometry on photometers, to quantitate the immunecomplex formation. These systems utilize the fast reaction between an antigen with their corresponding antibodies in a liquid phase.

The technique of quantitation by turbidimetry & 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.

SPECTROPHOTOMETRY

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.

$$\text{i.e. } A \propto C \text{ when L is constant}$$

Where A is the absorbance of light, C is the concentration of solution, L is the length of the light path.

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.

$$\text{i.e. } A \propto L \text{ when C is constant}$$

Beer-Lambert's law
Combining the two laws together we have the Beer-Lambert's 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.

$$\text{i.e. } A \propto C \propto L$$
$$A = K \times C \times L \qquad \text{Equation (1)}$$
$$\text{Where K is a constant}$$

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.

$$\text{i.e } A_t = K \times C_t \times L$$
$$A_s = K \times C_s \times L$$

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 in the spectrophotometer(1cm), 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 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.

Fig 1A

Fig 1 B
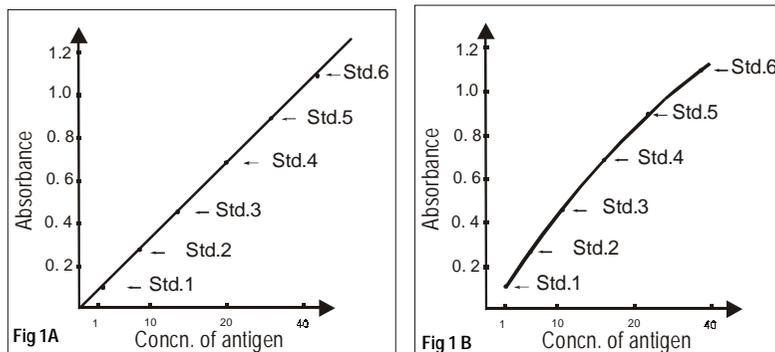
Certain reactions however, may not follow the Beer-Lambert's law within the pathophysiological concentration for an analyte and hence do not provide a straight-line graph. For such analytes the unknown cannot be determined using a single standard. A graph using different concentration of standards Vs absorbance has to be plotted on a graph paper. The plotted curve is known as the **standard curve** (fig. 1B). 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	Color of the filter
Yellow	405 Violet filter
Red colored complex	505 Green filter
Red colored complex	505 Green filter
Blue-violet colored complex	546 Green filter
Green colored complex	630 Red filter

For the estimation of Haemoglobin using cynamethaemoglobin method, a red colored complex, which is formed during the reaction, is measured using a green filter.

● Reading Principles
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 all the substrate has been converted into a stable endproduct. The reaction ceases when equilibrium is reached. The concentration of the test specimen can be calculated by using the equation;

$$C_t = \frac{A_t}{A_s} \times C_s$$

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

The kinetic measurements are of two types;
- **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.

- **Continuous monitoring** - Where the ΔA produced is monitored continuously.

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;

$$K = \frac{V_{total} \times 1000}{V_{sample} \times t \times e \times s}$$

V_{total}- total volume of the reaction mixture, V_{sample}- Volume of sample, t -time, e- molar extinction coefficient of the chromogen, s - Cuvette thickness

Standarisation 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 type of reaction curves, which can be obtained as the reaction progresses, typically follow the following patterns;
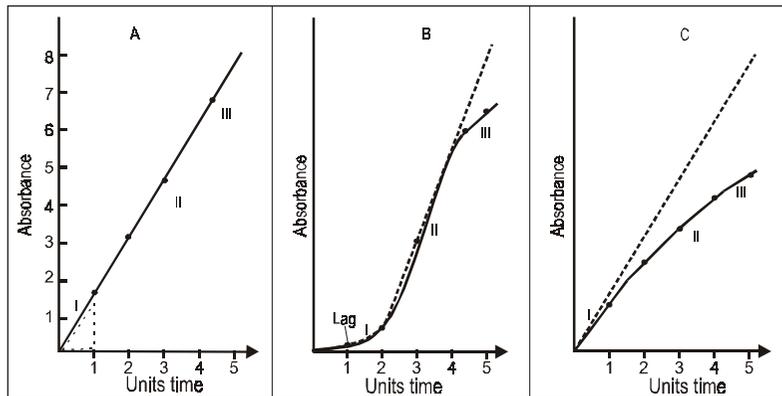
Fig. 2 . Forms of graph showing change in reaction rate as a function of time.
In A, the rate is constant in all the segments I, II, III. In B, a measurement at segment II will be representative of constant rate, but at segment I (lag period) and segment III it will be less. In C, the rate falls off continously.

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 reagents have to be optimised to obtain better reaction curves.

Measurement of Immunecomplexes 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 insoluble immunecomplexes are formed as a 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.
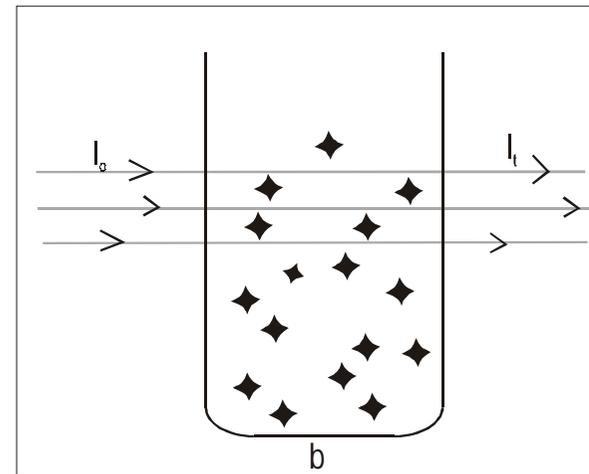
Fig.3 Behaviour of light in solution containing antigens
where I_o is the intensity of incident light, I_t is the intensity of transmitted light and 'b' is the cuvette containing antigens in the reaction solution.

Subsequently the reagent containing antibody solution is then added to the antigen and allowed to react. An agglutination reaction takes place 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 is transmitted as the reaction proceeds. Spectrophotometers read this decrease in the intensity of the transmitted light as absorbance.
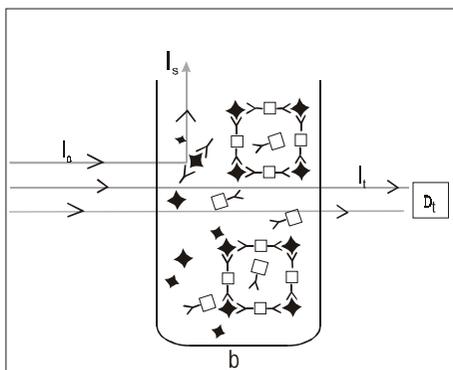
Fig.4 Behaviour of light in solution containing immunecomplexes(Ag-Ab)
where I_o is the intensity of incident light, I_t is the intensity of transmitted light, Is is the intensity of scattered light and 'b' is the cuvette containing immunecomplexes in the reaction solution.

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 inturn 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.
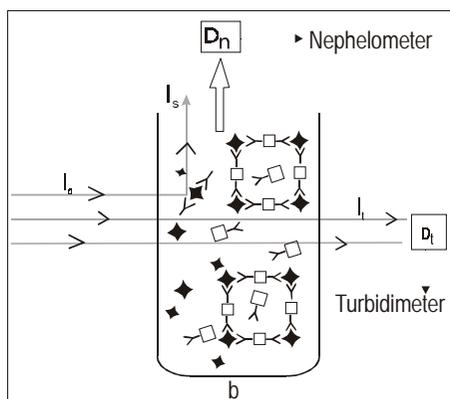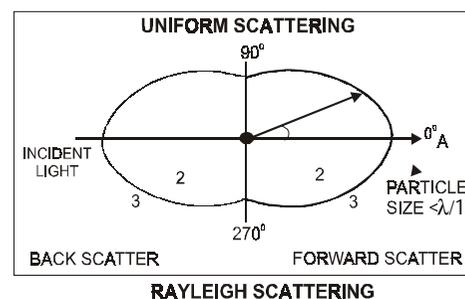

Fig.5 Detection principles in turbidimetry and nephelometry
Where I_o is the intensity of incident light, 'b' is the reaction cuvette, I_t is the intensity of transmitted light measured as absorbance by detecter Dn at 180^0, I_s is the intensity of scattered light measured by detector Dn placed at 90^0.

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°.

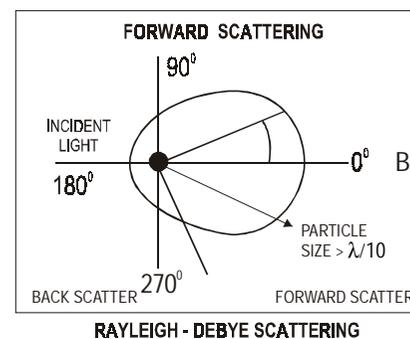**Selection of wavelength for measuring Immunecomplexes:**
The optimum wavelength for optical measurement of immune complexes increases with the size of immune complex to be measured.
In general, if the size of the immune complex formed is less than I/10th the size of the wavelength of incident light, then the light scattering is relatively symmetrical (Fig.6) . This **uniform scattering** of light is known as **Rayleigh scattering.**

Fig.6 illustrating scattering of light for a immune complex with particle size < λ/10 of the wavelength of incidence light

On the otherhand when the size of the immunecomplex to be measured is more than 1/10th the size the wavelength of the incident light 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. 7). This type of scattering is referred as **Rayleigh-Debye scattering.**

Fig.7 illustrating scattering of light for a immune complex with particle size >λ/10 of the wavelength of incident light

Careful examination of both the figures (fig. 6 & 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 dissymetrical, 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 in 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 becomes larger in size from 40-400 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 300 nm, light of wavelength between 500- 600 nm would be ideal for measuring the immune complexes formed

The choice between turbidimetry & nephelometry will depend on application & the available

Biochemical Measurements	Nephelometric measurements	Turbidimetric measurements
Measure absorbance of light at 180°,by colored complexes formed as a result of biochemical reactions	Measures light scattering at an angle away from the incident light due to formation of immune complexes	Measures reduction in 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 immunecomplex formed.	Selection of wavelength of light depends on the size of the immunecomplex formed.

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 analysers 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. In the context of contemporary technology turbidimetric assays are gaining popularity over nephelometric determination.

TURBIDIMETRY VS NEPHELOMETRY

TURBIDIMETERY	NEPHELOMETRY
Measures reduction in intensity of transmitted light at 180°C due to the formation of immune complexes	Measures scattering of light at an angle (usually 90°C) away from the incident light, due to the formation of immune
Can be performed on most spectrophotometers	Requires dedicated Nephelometer
Sensitivity Competitive with nephelometeric measurements for small immune complexes such as serum proteins	Sensitive for measuring small immune complexes such as serum proteins
More precise for measuring large immune complexes	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.	Blanking has to be performed in separate measuring cuvette.
Provides better precision due to slower reaction kinetics as blanking of reagent , sample & Immunochemical reaction can be monitored in a single cuvetteve	Because of the fast reaction kinetics it is difficult to obtain a sample and a reagent blank in case of Nephelometry,

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 fig. 8. To obtain the Heidelberger curve the antigen concentration is plotted against the absorbances obtained from measuring the Ag-Ab reaction.
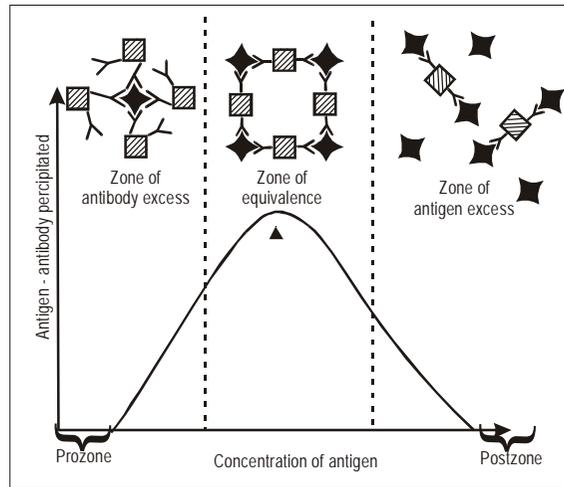

Fig.8 The Quantitative immunoprecipitin curve (Heidelberger-Kendall curve)

The Heidelberger-Kendall curve may be divided in three Zones;
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 antigen-antibody complexes. **Here the absorbance increases 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. **Here also the absorbance increases with the analyte concentration, but does not increase proportionally.**

3. The antigen excess Zone
In the third stage of the reaction, the 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 as the prozone effect or the hook effect. Prozone is inappropriately used to describe "postzone" or "antigen excess" in day to day parlance. The existence of prozone effect will cause very high concentrations of antigen to produce signals, which are similar to the signals generated, by moderate concentrations of antigen. It is imperative to know, what concentration of analyte will cause a prozone effect in a turbidimetric immunoassay for a given antibody reagent system.

When an antigen-antibody reaction takes place and the formation of the immune complex is measured optically by turbidimetry than the absorbance and reaction kinetics in the three zones will be as follows:

Heidelberger- Kendall Curve	Absorbance
Antibody excess zone	Increases towards maximum
Equilibrium	Reaches Maximum
Antigen excess zone	Decreases below maximum

This quantitative immunoprecipitin curve forms the fundamental basis for all homogeneous antigen-antibody assays including turbidimetry and is usually referred to as the **dose response curve.**

For many analytes of diagnostic importance, the antigen-antibody reactions neither follow the Beer-Lambert's 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. As 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 concentration. As the analyte concentration increases 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 different concentrations of standards (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.
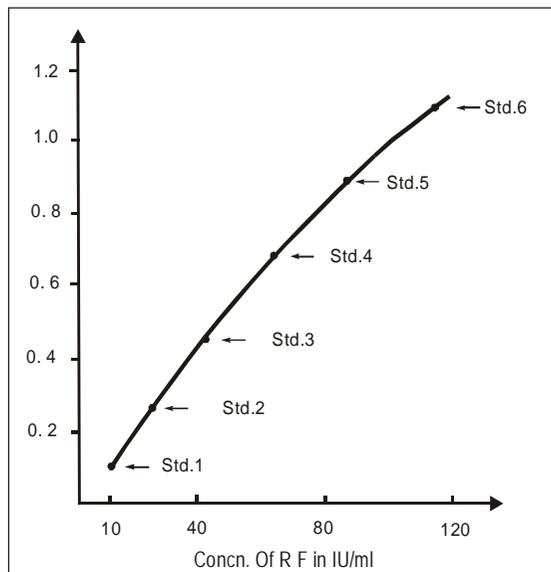

Fig . 9 illustrating standard curve for RF

For e.g. for a RF assay where the reference interval is 10 IU/ml, the lowest concentration used is 7 IU/ml (<10 IU/ml). The highest concentration of the standard selected is 120 IU/ml above which there is no linearity in reaction. Thus the measuring range for such a RF assay would be 7.5-120IU/ml.

The lowest concentration of the assay is selected in such a way that it is more than the **detection limit** (analytical sensitivity) of the assay. For e.g. the aforesaid RF assay should have a detection limit of around 5 IU/ml.

Optimization, Standardization, and Quality Control of turbidimetric assays

To measure the antigen- antibody 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 antigen-antibody 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 optimisation for Immunoturbidimetry.

Ionic Strength: It has been observed that the antigen-antibody 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 otherhand influences the electrostatic attraction between oppositely charged species, thereby reducing the specific binding.

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 non specific agglutination with negatively charged proteins or particles.

Temperature: Temperature as is well known influences the rate of formation of immune complexes and it should be optimised to obtain accurate results. Assays are usually designed with an incubation temperature of 37°C because it is the most common temperature in the routine of clinical laboratories.

Enhancers: For enhancing the Ag-Ab reactions, polymeric compounds such as PEG, 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. Minimising the influence of these factors help provide accurate and precise assay results.

Turbidimetric assays usually employ a suitable buffer in the assay design to optimise assay conditions and the desired ionic strength, pH and enhancement for the reaction. 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 antigen-antibody 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 immunecomplex, thereby lowering the detectable signal. The avidity of antibody is also an important consideration in the formation of immunecomplexes. 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 utilising 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.

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 reagents are referred to as **Particle Enhanced Turbidimetry (PET).**

Standarisation 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 standardised by a reference method
- Demonstrate inter-method agreements.

Most International Reference Preparations (IRP's) & Certified Reference Materials 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 W. H. O.

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 & 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 five or six 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.

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).

1.   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).

Table 1.

	Signals		
	First reading (A1)	Second reading (A2)	ΔA_s (A2-A1)
Sample cuvette (S)	Absorbance due to: ● Sample ● Buffer	Absorbance due to: ● Sample ● Buffer ● Reagent ● Immunecomplexes	Absorbance due to: ● Reagent ● Immunecomplexes

The difference ΔA_s (refer table 1) between the two readings represents the absorbance generated as a result of antigen-antibody 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 2.

	Signals		
	First reading (A1)	Second reading (A2)	ΔA_B (A2-A1)
Reagent cuvette (R)	Absorbance due to: ● Buffer ● Blank sample (saline)	Absorbance due to: ● Buffer ● Blank sample ● Reagent	Absorbance due to: ● Reagent

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

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'**.

2.   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 –20 seconds after mixing, the first reading A1 is taken. This 10-20 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 (refer table 3).

Table 3.

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

This method eliminates the need for determination of reagent blank as it measures the increase in absorbance after equilibration of the reagent 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 & 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 time assigned for the lag phase for precise blanking in the immediate mixed blanking method.

Reaction kinetics & 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 for e.g. RF, a first reading 10-20 seconds after mixing with the antibody is not very critical (fig 10).
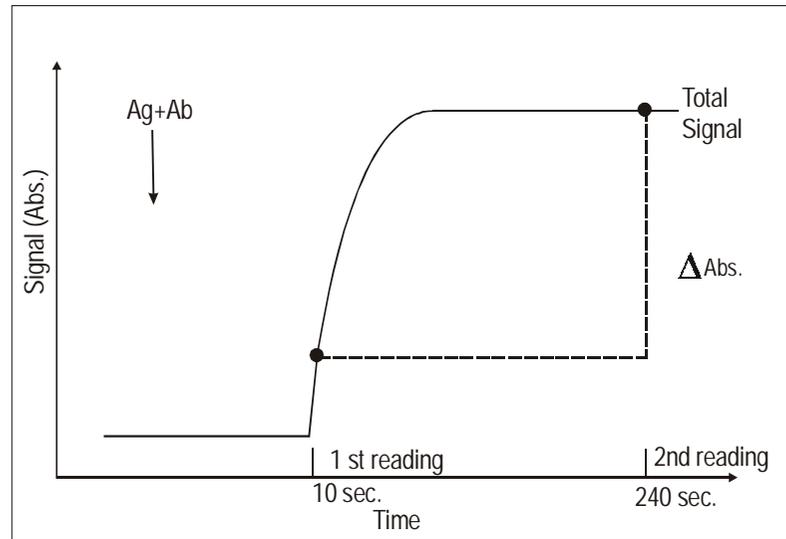

Fig.10 Signal development as a function of time.
Fig. illustrating 'Immediate mixed blanking' using RF as example.

However for a system with fast reaction kinetics for e.g. IgG (fig. 11), half of the reaction would have taken place within 10-20 seconds when the first reading is taken. Here a poorly defined point for the first reading would be obtained.
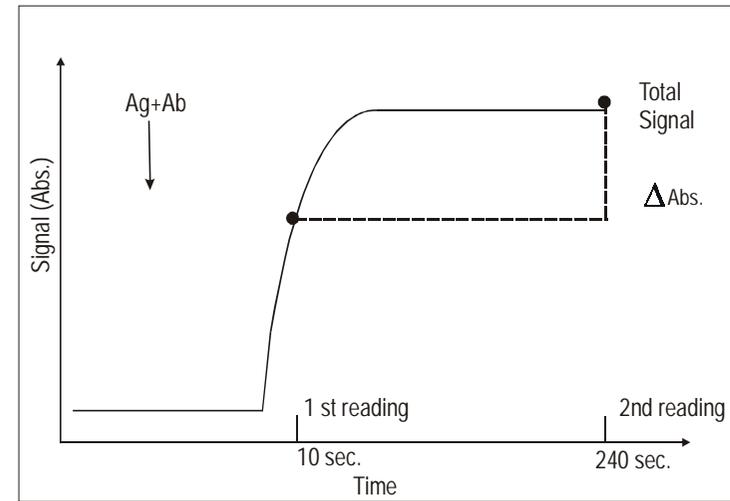

Fig.11 Signal development as a function of time.
Fig. illustrating 'Immediate mixed blanking' using IgG as example.

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 RF (fig. 12A) and IgG (fig 12B) at Zero seconds and 10 seconds respectively.
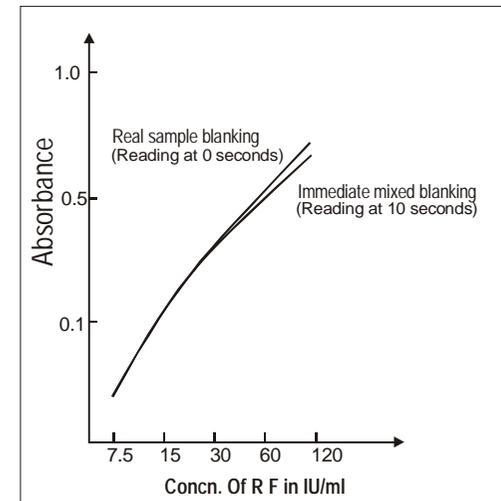

Fig.12 A. Standard curves for RF obtained with 'real sample blanking' & 'immediate mixed blanking'.

The standard curve obtained for RF (Fig.12A) is practically not affected by the difference between the two ways of blanking indicating that a delay of 10 seconds is not very significant.

Whereas for a non-enhanced system with fast reaction kinetics for measurement of analytes such as IgG (Fig. 12 B), a delay of ten seconds becomes very critical.

There is considerable signal development during the first ten seconds. This results in decreased difference between A1 & A2 (fig. 11). The loss of signal increases with increasing concentration of IgG in the calibrators.
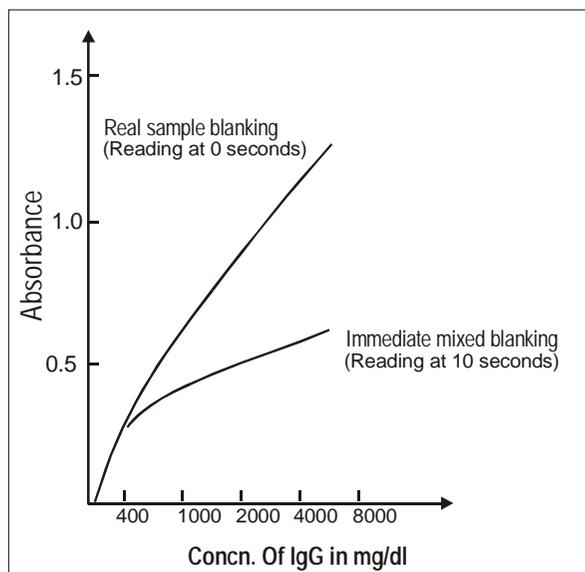

Fig.12 B. Standard curves for IgG obtained with 'real sample blanking' & 'immediate mixed blanking'.

It can be observed from fig.12 B 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 optimised using the **"real sample blanking"** principle as the sample blanking and the immonochemical reaction can be optimised separately.

CONCEPTS OF ASSAY OPTIMISATION

While optimizing reagent for Immunoturbidimetric assay system, it is important to optimize the dose-response curve **by iterating the amount of sample (antigen) and the antibody concentration** in the reagent until a dose response curve as shown in figure 13 is obtained**.**
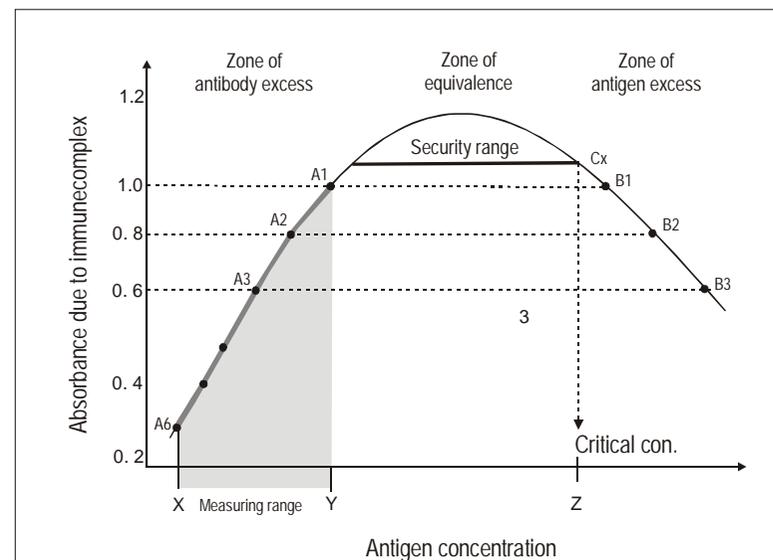

Fig.13 Dose response curve
Fig. illustrating measuring range (X-Y), Critical concentration (Z), standard curve (A1-A6), erroneous interpolation (where Abs. val.1.0 corresponds to A1 and B1, Abs. val. 0.8 corresponds to A2 and B2 and Abs val. 0.6 corresponds to A3 and B3), security range (Y-Z).

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.

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 signal , is referred to as **the measuring range** of the assay system.
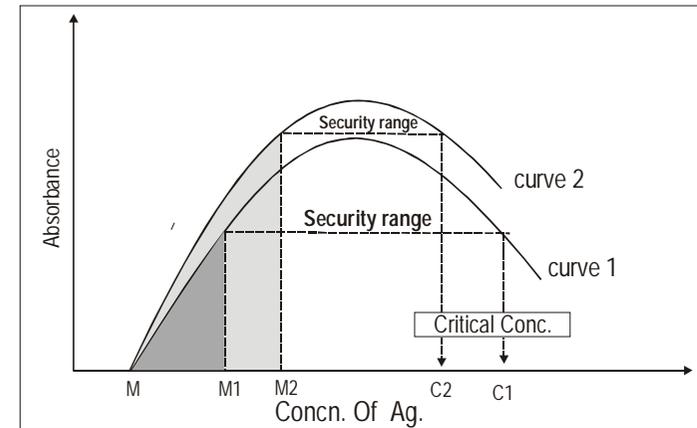
Security range:

The **critical point** (C_x in fig. 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. 13) at which erroneous interpolation begins. The interval between the signal of highest standard and the signal of the critical concentration can be referred as the **security range** of the assay system for the analyte.

Reagent optimisation:

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 a 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 (refer fig 14).

Fig.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

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

The fig 15 shows the effect of increasing the antibody concentration on the security ranges while keeping constant, the measuring range of the dose response curve.
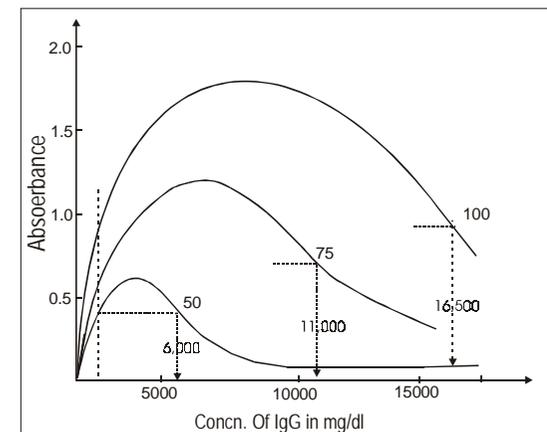
Fig.15 Effect of increasing antibody concentration on security range.
Where 50, 75 and 100 ul are volumes of antibody and 6000, 11000 and 16500 are their respective critical concentrations.

When Ab concentration used is 50 μl, the security range obtained is around 5000 mg/dl. As you increase the antibody concentration to 75μl the security range increases to > 10,000 mg/dl. With a further increase of 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 antibody concentration. If antibody concentration 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 – 25 times the lowest calibrator value can be possibly optimized, with a security range still giving a warning upto 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 optimised, 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.
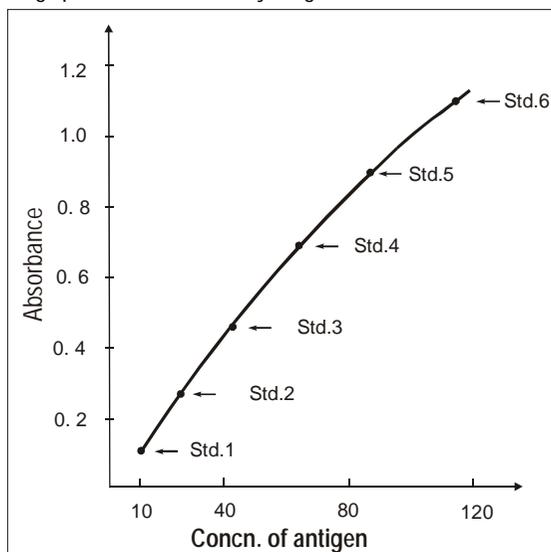

Fig. 16 Standard Curve

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.

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

INSTRUMENTATION FOR TURBIDEMETRY

The development of automated instruments for the clinical laboratory began in the 1950's at the same time as the demand for test such as RF, 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 mode of measuring absorbance.

1. 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 non-enhanced antibody reagents are proteinacious 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 immunecomplexes 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.

Further 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 the amount of activation buffer and sample will have to be used.

2. 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.
- Semi-automated analyzers
- Automated analyzers

Semi automated analyzers
Instruments with an absorbance linearity of 2.0 are suitable for turbidimetric estimations of both particle enhanced and non-particle 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 programmed with a facility to store the calibration curve utilising 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.
Centrifugal analyzers
Static instruments (non-centrifugal analyzers)

1. Centrifugal analyzers
In these analyzers the cuvettes are arranged in circle (rotor) that can be rotated at a velocity about 1000 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.

2. Static Instruments
In these instruments the cuvettes are mostly arranged in a circle (rotor), and this is a 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.