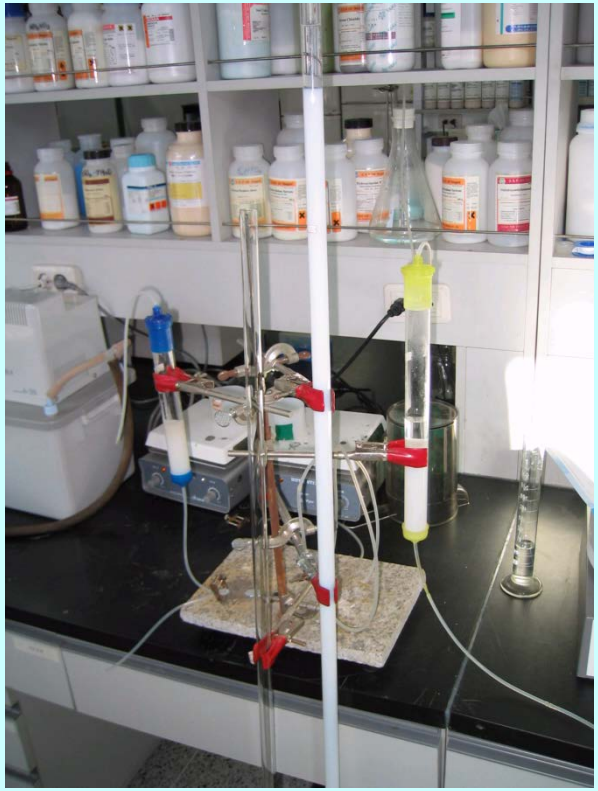
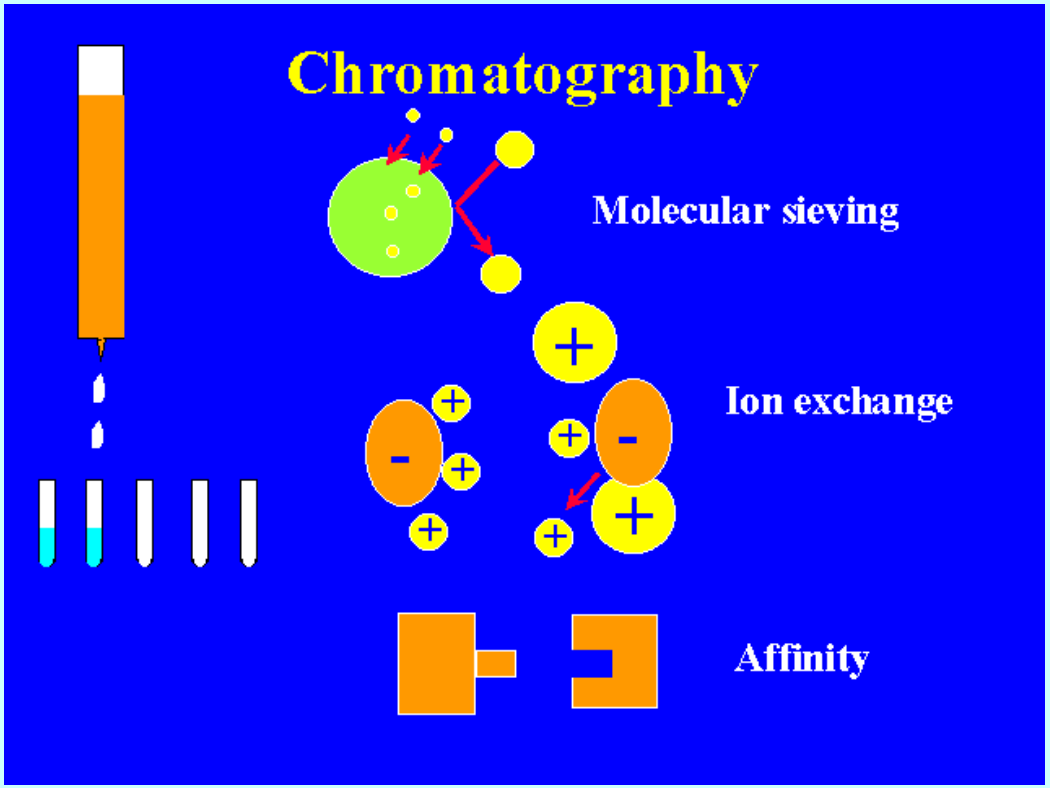
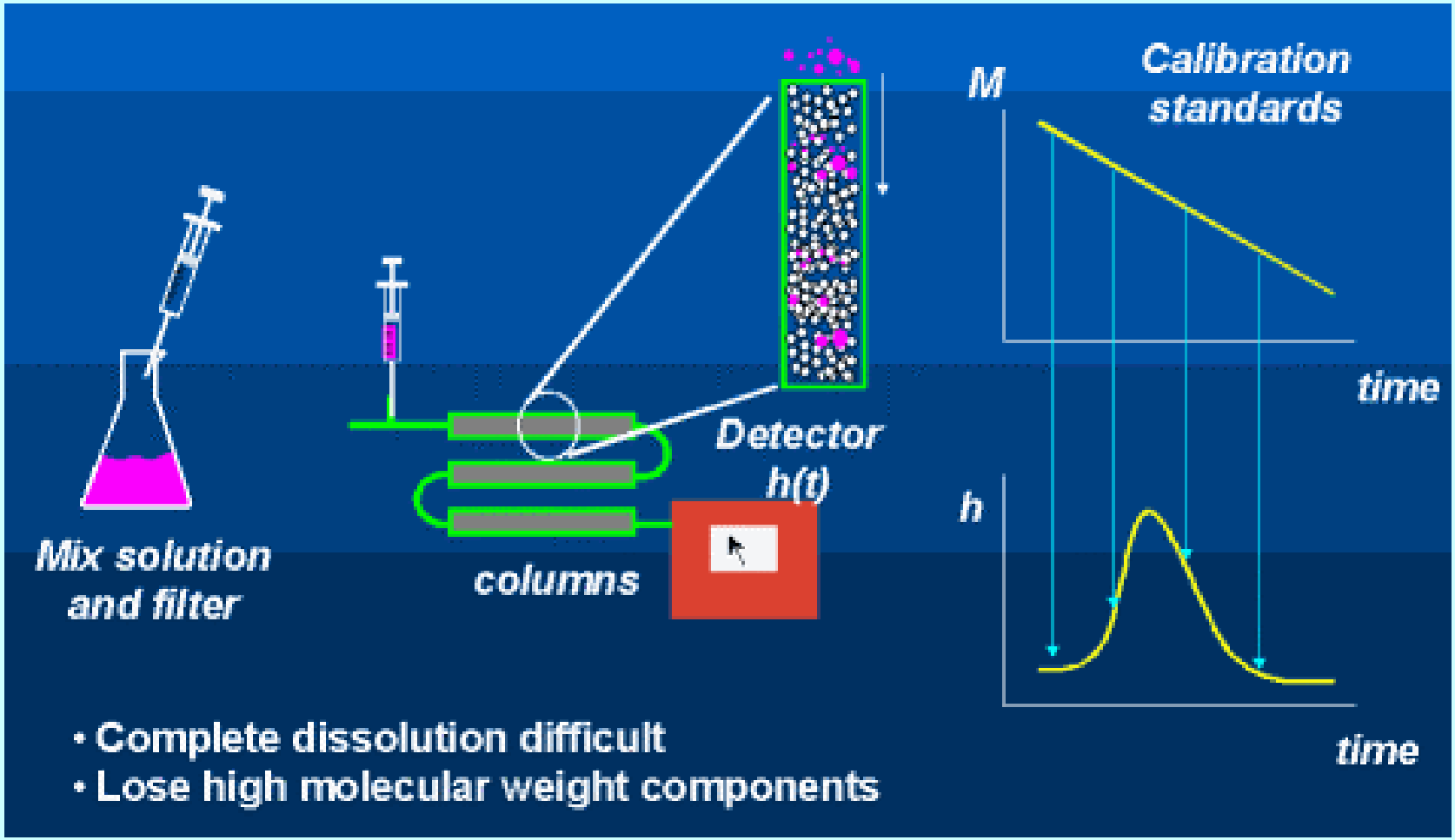
Chromatography

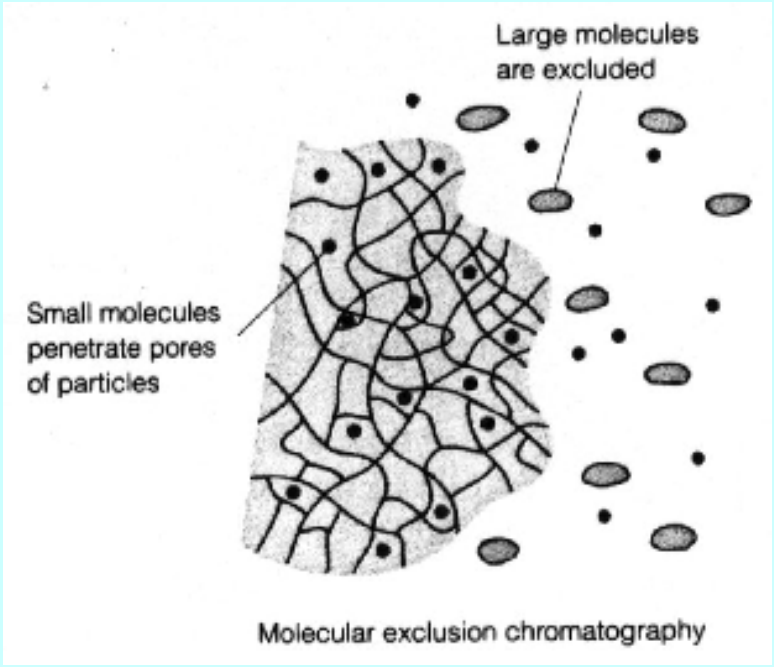




Principle of gel chromatography (size exclusion chromatography)



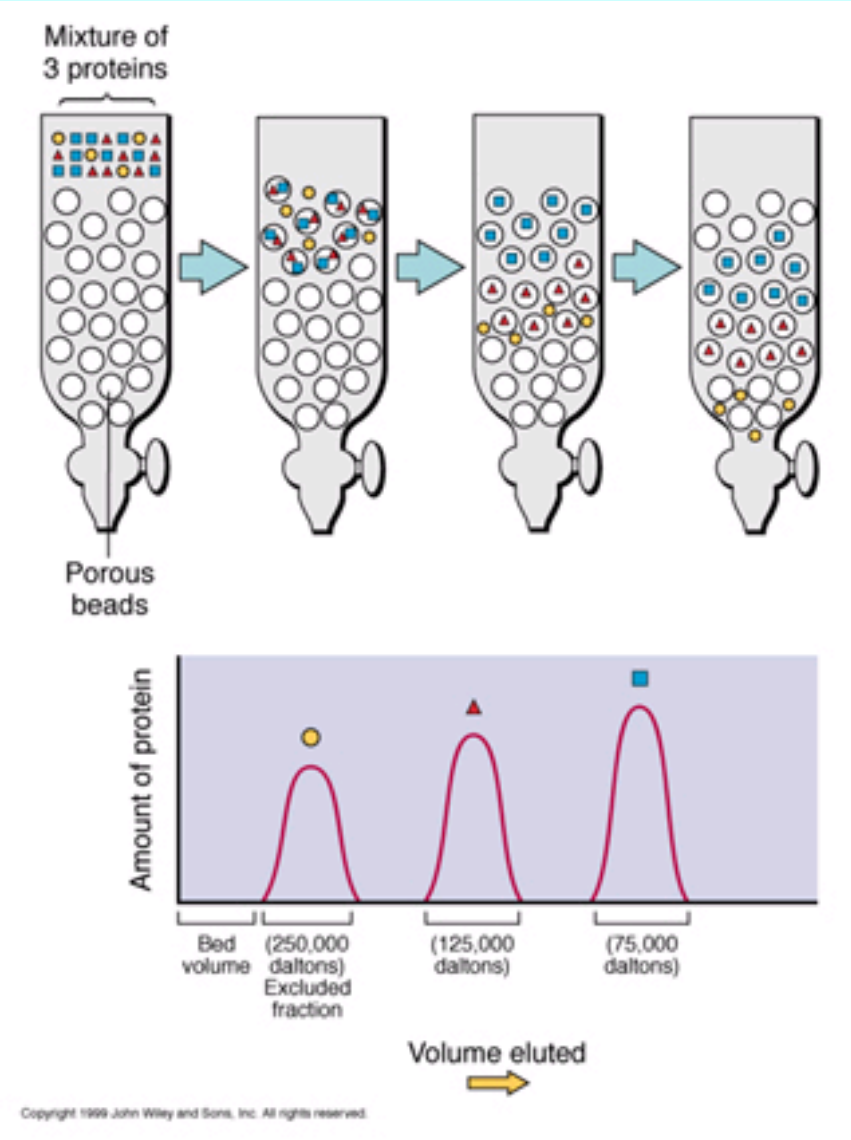
Gel-particle interaction - I



Molecular exclusion chromatography

Also known as gel permeation or gel filtration, this type of chromatography lacks an attractive interaction between the stationary phase and solute. The liquid or gaseous phase passes through a porous gel which separates the molecules according to its size. The pores are normally small and exclude the larger solute molecules, but allows smaller molecules to enter the gel, causing them to flow through a larger volume. This causes the larger molecules to pass through the column at a faster rate than the smaller ones.

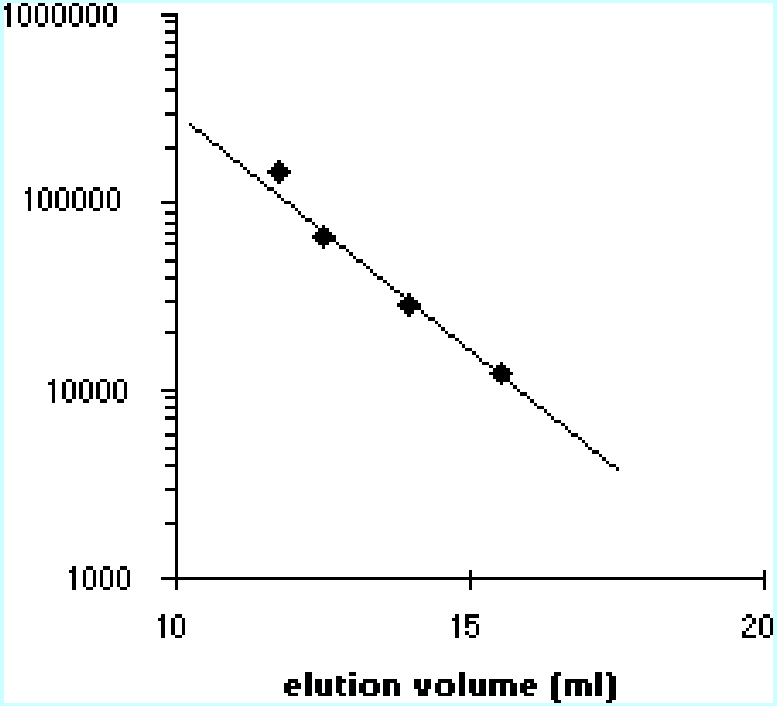
Gel-particle interaction - II



Principle of the separation:

Small proteins and salts are able to pass the pores of the gel. Therefore, these molecules have a higher column transit time. In consequence, high molecular weight proteins elute first (in the exclusion volume), followed by medium sized proteins, small proteins ans salts, respectively.

Determination of molecular weight



Molecular weight vs. elution volume. Plot of molecular weight of proteins against their elution volume from a gel filtration column. Proteins are yeast alcohol dehydrogenase [150 kilodaltons (kD)], bovine serum albumin (66 kD), carbonic anhydrase (29 kD), and cytochrome c (12.4 kD).

Examples of gel chromatography - I

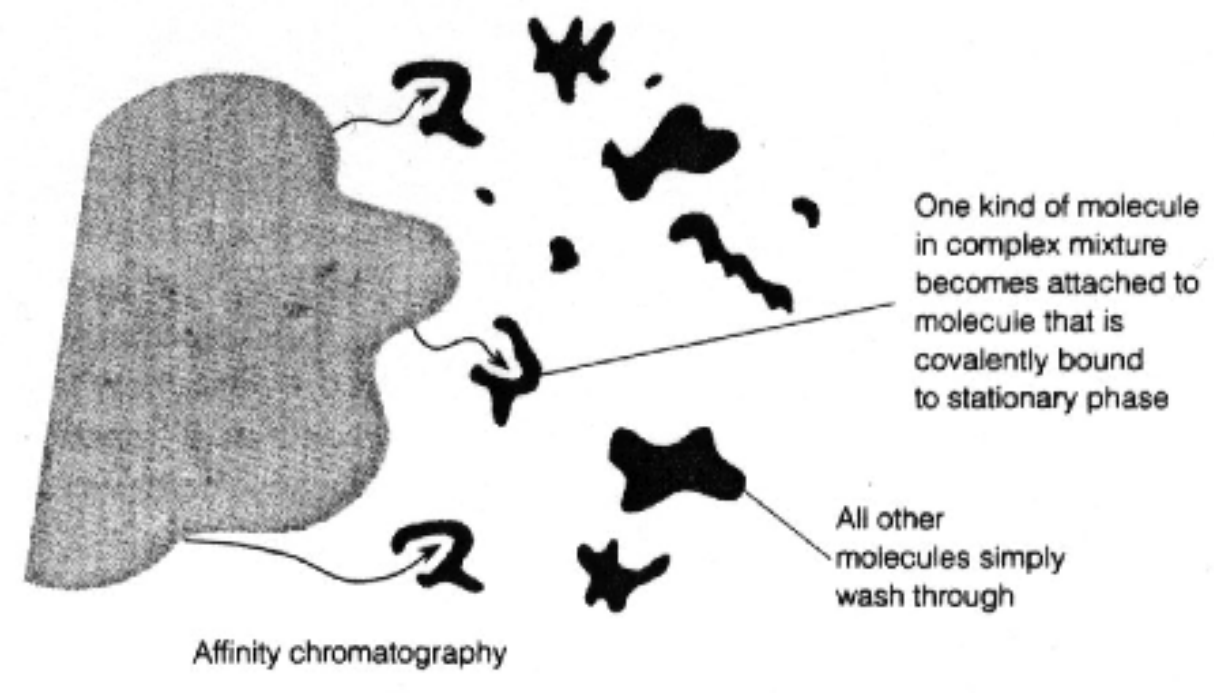
Desalting:

This experiment introduces gel exclusion chromatographic separation of dyes of different colors on the basis of their size and shape. This experiment contains materials for dye separation which include dye sample, elution buffer and plastic disposables. Columns may be rinsed and reused.

Examples of gel chromatography - II

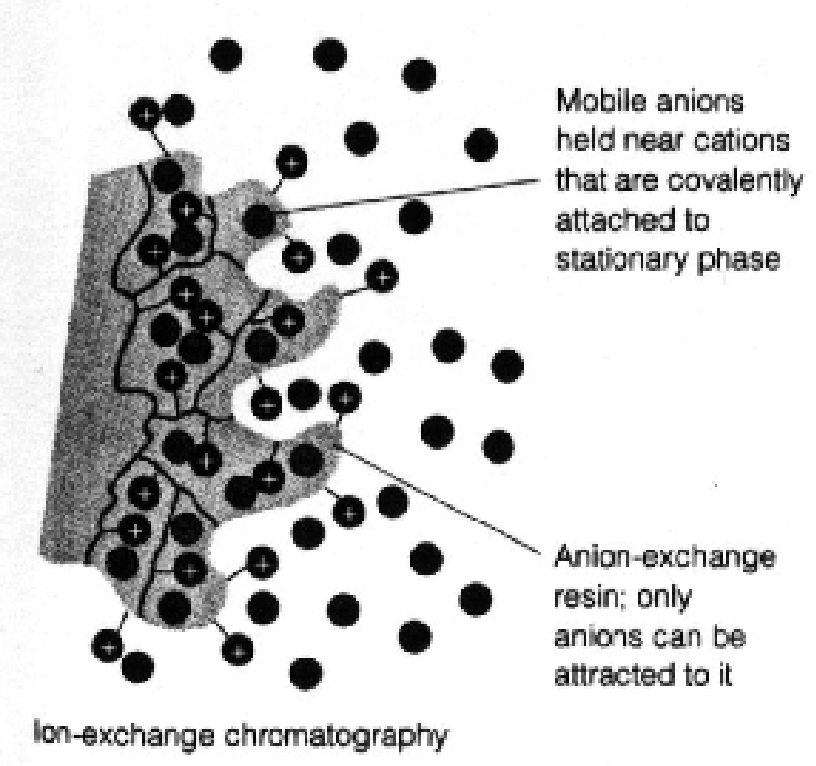
Laboratory-scale automated protein analytics and isolation

Affinity chromatography



This is the most selective type of chromatography employed. It utilizes the specific interaction between one kind of solute molecule and a second molecule that is immobilized on a stationary phase. For example, the immobilized molecule may be an antibody to some specific protein. When solute containing a mixture of proteins are passed by this molecule, only the specific protein is reacted to this antibody, binding it to the stationary phase. This protein is later extracted by changing the ionic strength or pH.

Ion exchange chromatography



Ion-exchange chromatography

In this type of chromatography, the use of a resin (the stationary solid phase) is used to covalently attach anions or cations onto it. Solute ions of the opposite charge in the mobile liquid phase are attracted to the resin by electrostatic forces.

Ionic properties of proteins

Amino acids - each has ist own ionic properties depending on its side chain

Proteins contain many amino acids - these contribute to the ionic properties of the protein

Important to know about the ionic properties of a protein because protein function/activity of a protein depends on ionic properties

Further, proteins can be purified on these properties

Use of ionical properties

Different proteins have a different net charge at a given pH value

This property can be used to separate proteins from each other e. g. by means of

electrophoresis

ion exchange chromatography

Ion exchange chromatography - I

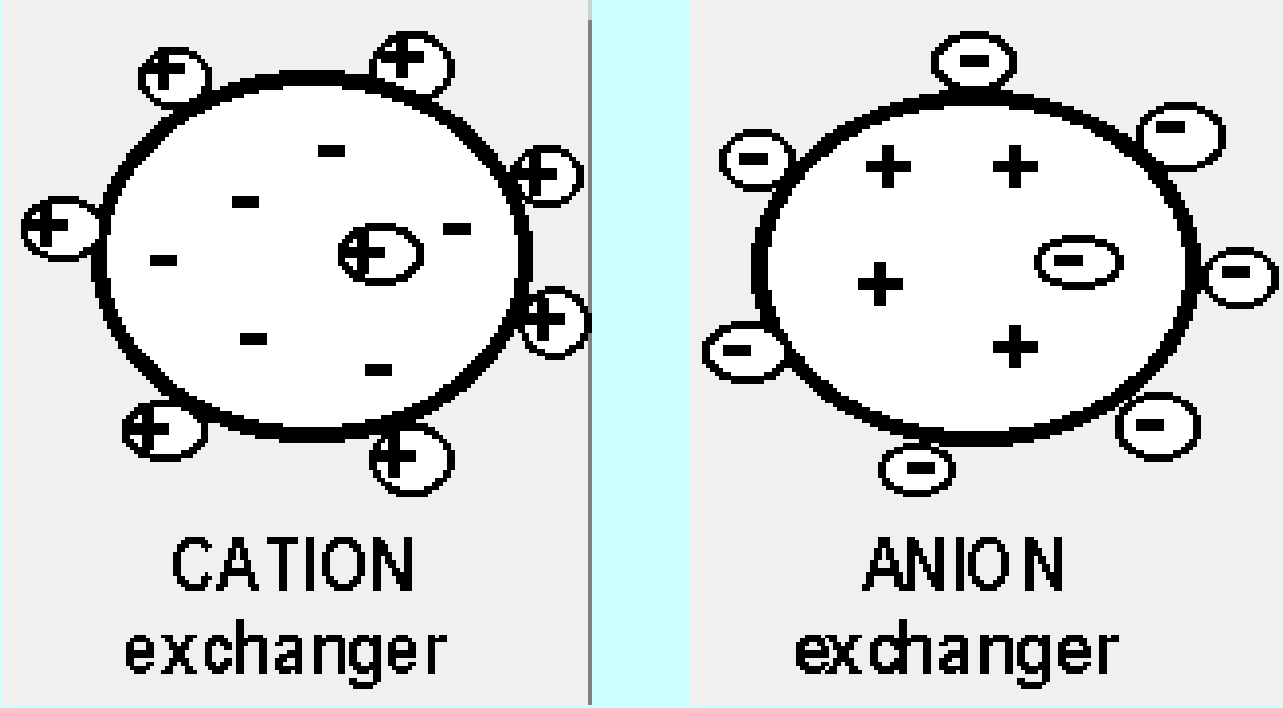
In ion exchange chromatography proteins are separated from each other on the basis of their electrical charge

Use ion exchange resins which are either:

- Polyanions - contain negatively charged groups and exchange cations (positively charged molecules) or
- Polycations - contain positively charged groups and exchange anions (negatively charged molecules)

Ion exchange resins are usually supported in a long glass tube or column

Ion exchange resins



Separation is achieved by ionic interactions between proteins in the buffer and the charged groups on the ion exchange resin.

Ion exchange chromatography - II

The stronger the interactions between the protein and the ion exchange resin, the more tightly the protein will be bound

In a column with proteins in a buffer flowing downwards through the ion exchange resin, the more tightly bound proteins will be retarded in their migration down the column

This forms the basis for separation of proteins

Ion exchange chromatography - III

Migration through an anion exchange resin depends on:

- Nature of the ionic matrix
- Ionic properties of the solute (protein)
- Properties of the solvent or buffer

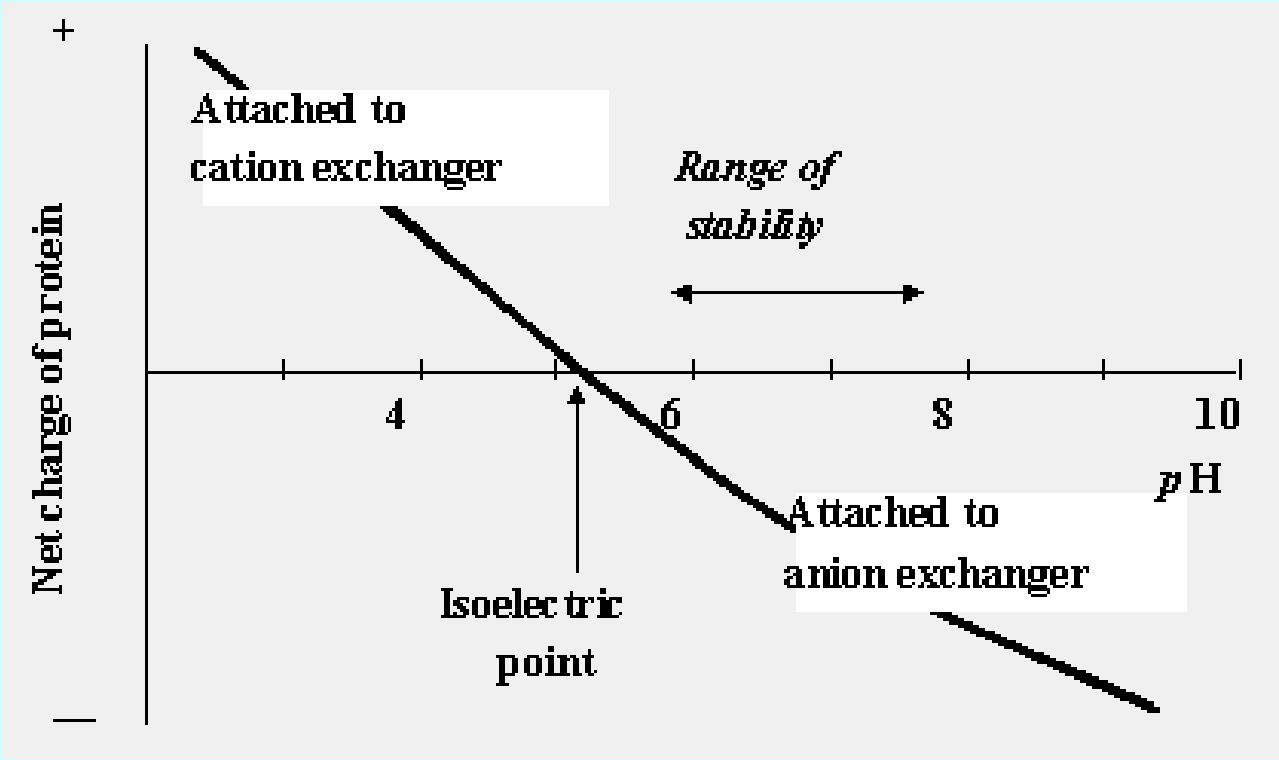
Can get different kinds of ion exchange resins:

- Synthetic resins for amino acids and peptides
- Cellulose, dextran and agaroses for proteins

Resins can differ in:

- Type of charge (+/-)
- Strength of charge (strong/weak)
- Capacity (number of exchangers)

Choice of ion exchange resin



Depends on the net charge of proteins at the chosen pH. Proteins are ampholytes and can bind to either type of exchanger.

The stability of protein within the pH range can be a limiting factor.

Strategy for chromatofocussing

Proteins are run into the column at a certain pH and are bound tightly by the ion exchange resin

Proteins are then eluted from the column with a pH gradient - e. g. a buffer of increasing pH passing through a cation exchange column

Proteins are then eluted from the column in the order of their isoelectric point (IEP)

Resolving power of chromatofocussing

Depends on slope of the pH gradient

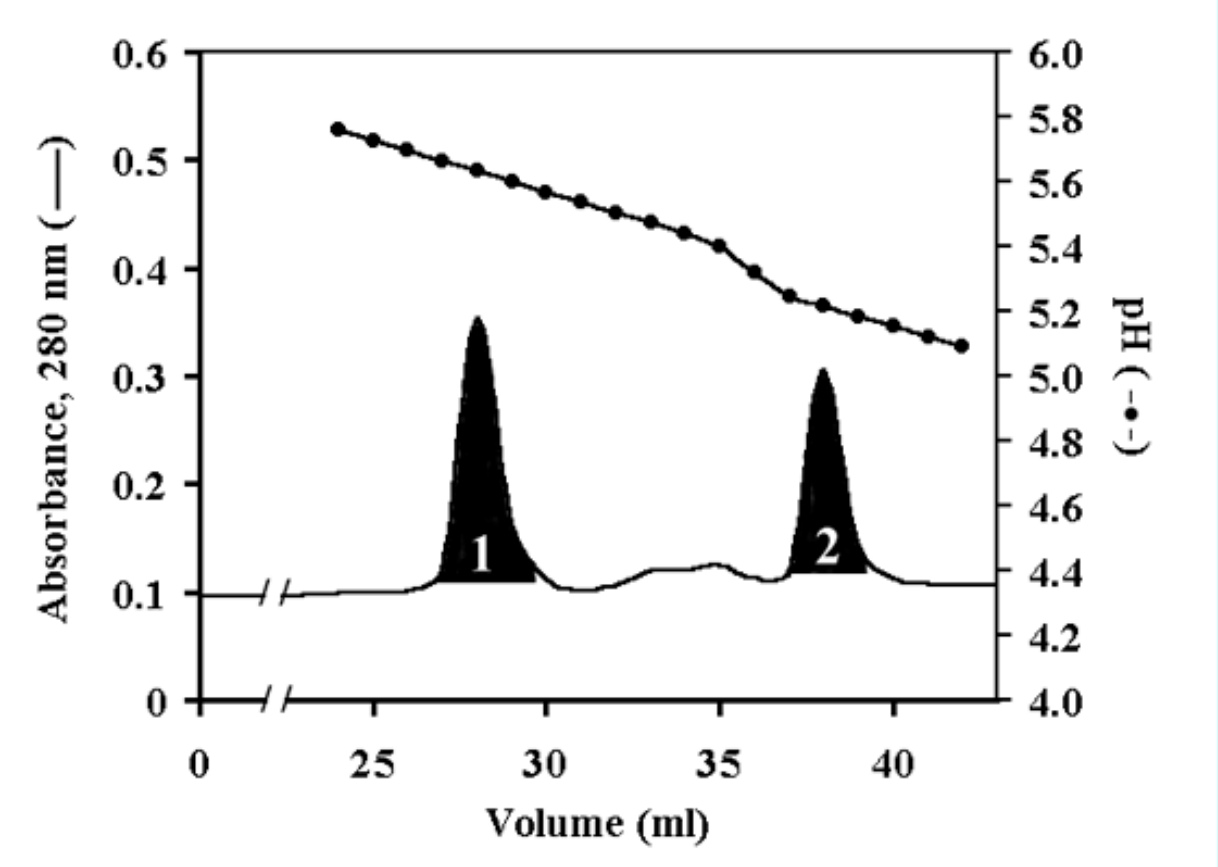
- Good to have the slope as flat as possible, i. e. gradual pH changes

Depends on the charge of the ion exchanger.

- Good to have a high charge difference between the resin and the solute, and low ionic strength

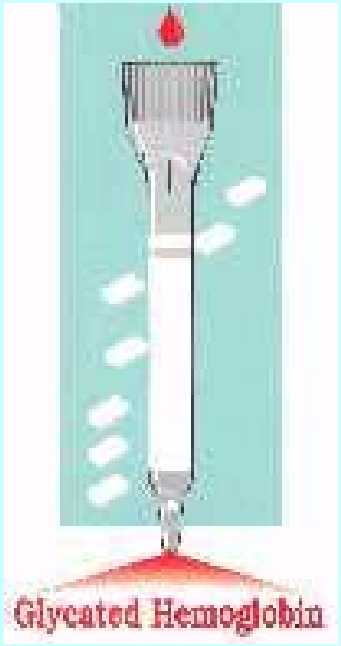
The column packing needs to be nice and regular

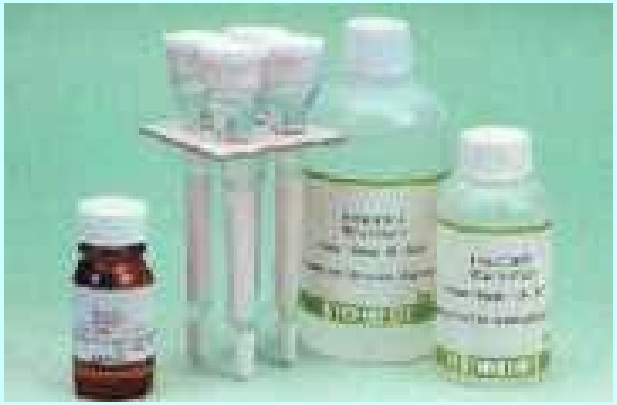
Example of chromatofocussing



Mono P HR 5/20 FPLC chromatofocusing elution profile of RBPs following fractionation by Sephadex G-75 and Superose 12 HR 10/30 FPLC (A). A pH gradient was employed using 0.025 M bis-Tris, pH 6.7 and polybuffer 74, pH 5.0. A 0.5 ml/min flow rate, 0.5 cm /min chart speed and 0.1 AUFS were used. Native-PAGE analysis of the corresponding peaks (B). Peak 1 (lane 1); peak 2 (lane 2).

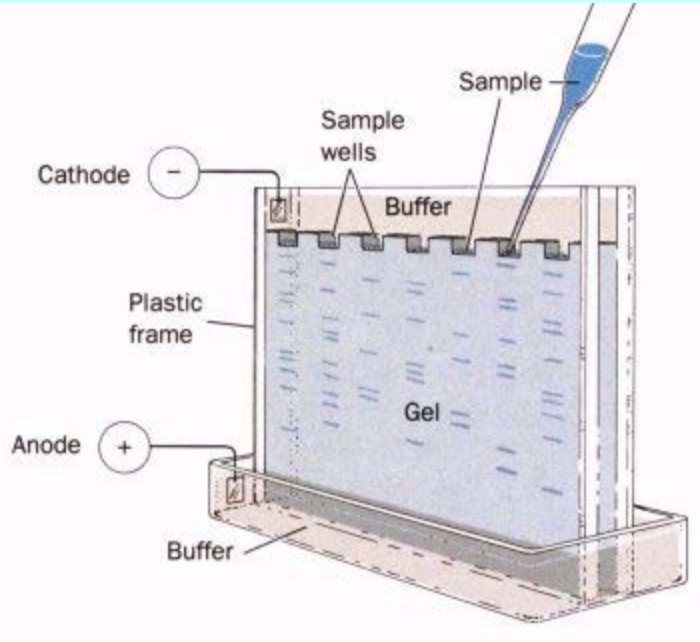
Clinical example - diabetes monitoring

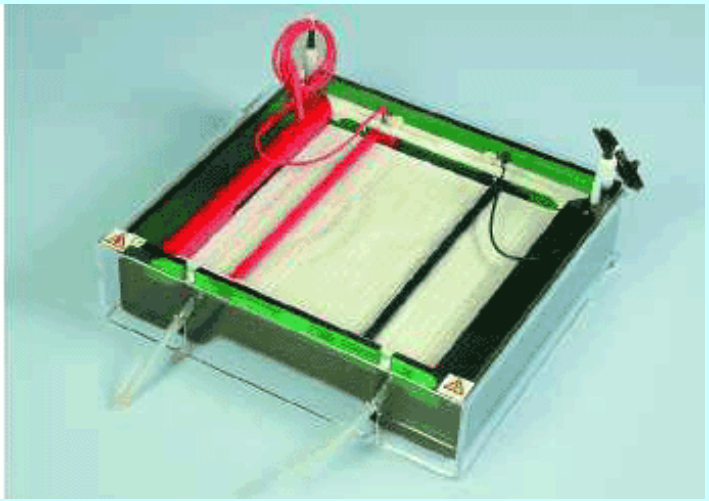




Glycosylated hemoglobin HbA_{1c} dosage is used for the biological monitoring of diabetic conditions. HbA_{1c}, a stable hemoglobin compound modified by the fixation of sugar, is an indicator of the average glucose level during the four to eight weeks prior to the taking of a sample. Measurement of HbA_{1c} is therefore an excellent indicator of the stability of a diabetic condition.
The HBA1C8 and HBA1C50 kits from Biomidi can be used to measure HBA_{1C} levels by micro-column ion-exchange chromatography. This method is very simple and quick and does not require expensive equipment.

Electrophoresis - I





Electrophoresis:

Electrophoresis is a separation technique that is based on the the mobility of ions in an electric field. Positively charged ions migrate towards a negative electrode and negatively-charged ions migrate toward a positive electrode. For safety reasons one electrode is usually at ground and the other is biased positively or negatively. Ions have different migration rates depending on their total charge, size, and shape, and can therefore be separated.

Electrophoresis - II

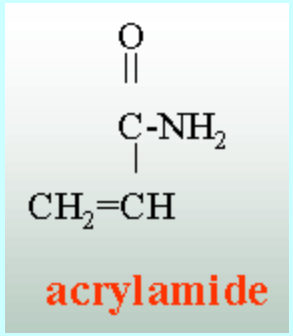
Most widely used media are polyacrylamide and agarose which can form gels with pores of a specified size

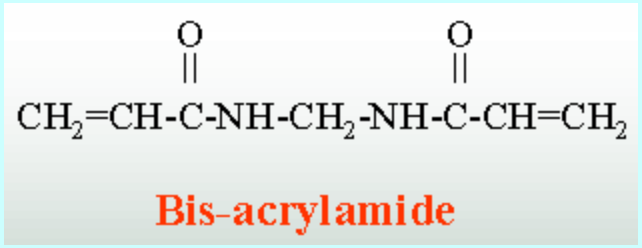
Gels will separate on a basis of size and charge

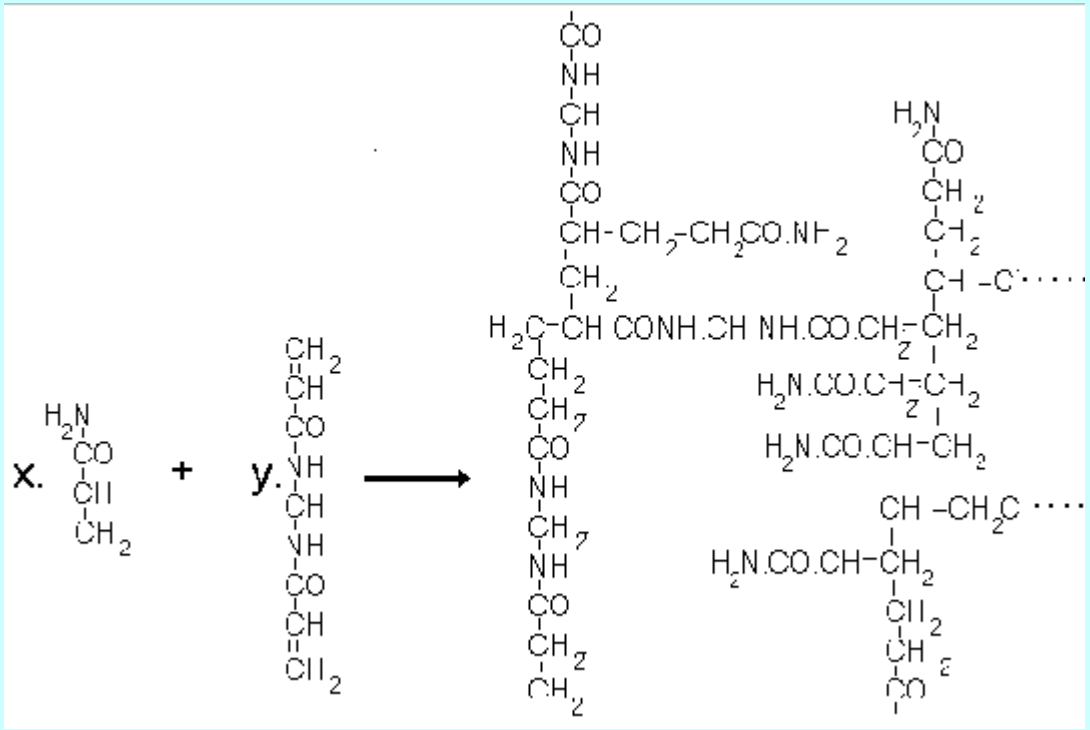
Unlike gel filtration, in gel electrophoresis larger molecules are retarded by the gel relative to smaller ones

Electrophoresis - III

Polyacrylamide gels are made by polymerization of acrylamide and bis-acrylamide.







Electrophoresis - IV

Polyacrylamide gel electrophoresis (PAGE):

Cross links between acrylamide and bisacrylamide are formed upon adding these reagents:

Ammonium persulfate

TEMED (tetramethylenediamine)

The pore size of the polyacrylamide gel is proportional to the concentration of acrylamide and the degree of cross-linking

Acrylamide is usually used at 3-15 % v/v, bisacrylamide at 5 %

Electrophoresis - V

Stacking gels (PAGE):

A „stacking“ gel is a large pored PAGE gel cast on the top of a separating, „running gel“.

The stacking gel has pH different from and a conductivity lower than that of the running gel and the running buffer.

Proteins tend to accelerate through the stacking gel, and then to compact before entering the small pored, high conductivity „running“ gel.

This compacting or „stacking“ results in greater resolution of protein separation.

Electrophoresis - VI

SDS-PAGE:

SDS = Sodium dodecylsulphate, a detergent

SDS binds tenaceously to proteins and causes them to assume a rod-like shape

Proteins bind SDS in the ratio of about 1 SDS molecule per 2 amino acids

The negatively charged SDS masks the charge on the protein so that all proteins have the same charge, and an extended rod-like conformation

As a result, proteins are separated by PAGE solely according to size

Molecular weight can be determined by accuracy of 5-10 %

Electrophoresis - VII

SDS-PAGE:

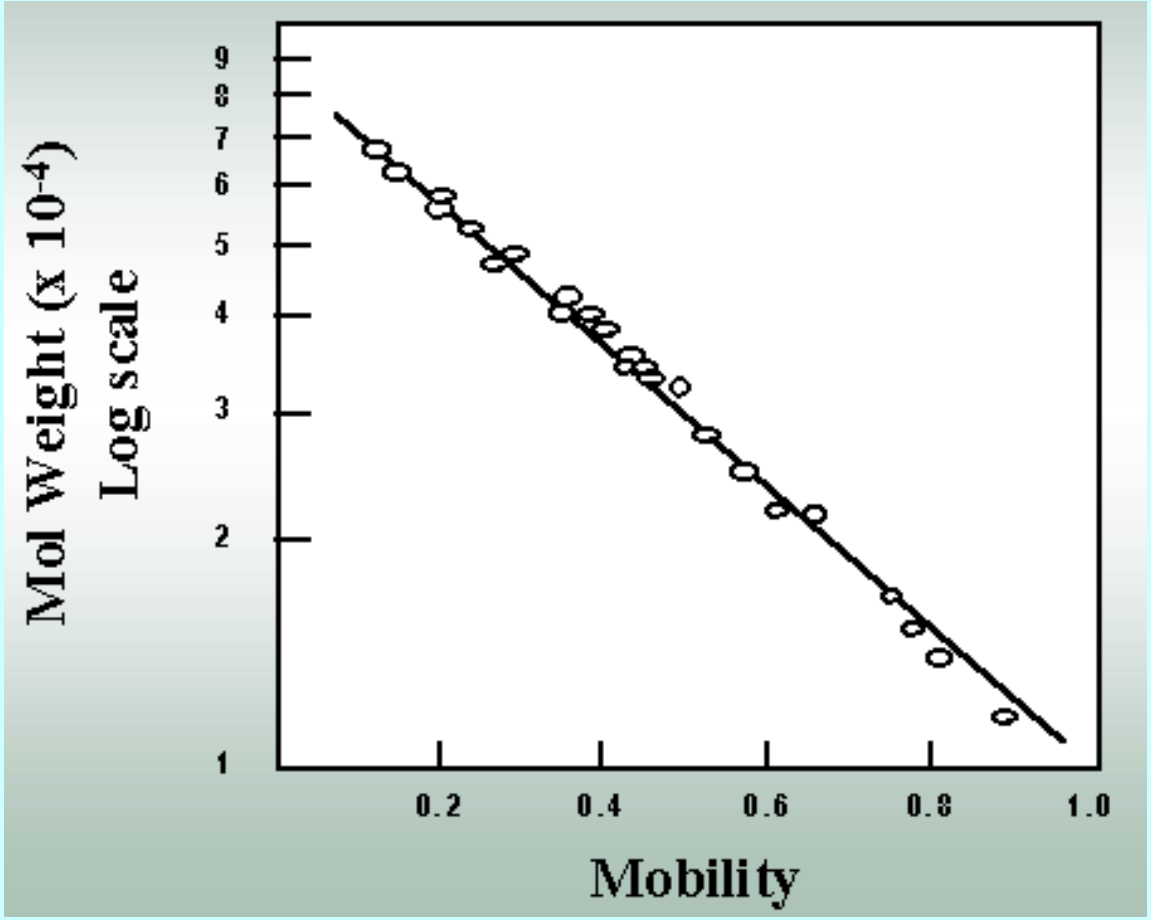
Many proteins contain more than 1 protein chain, i. e. have subunits

SDS-treatment disrupts the non-covalent interactions between these subunits

Therefore, the SDS-PAGE will give the molecular weight of individual subunits rather than that of the intact protein

To obtain sizes of subunits that are linked by disulfide-bridges, β-meraptoethanol is added to reduce the S-S to SH HS - this separates the subunits

Electrophoresis - VIII



SDS-PAGE size-mobility relationship

Electrophoresis - IX

Separation of proteins on the basis of their iso-electric point

Can be done preparatively as well as analytically

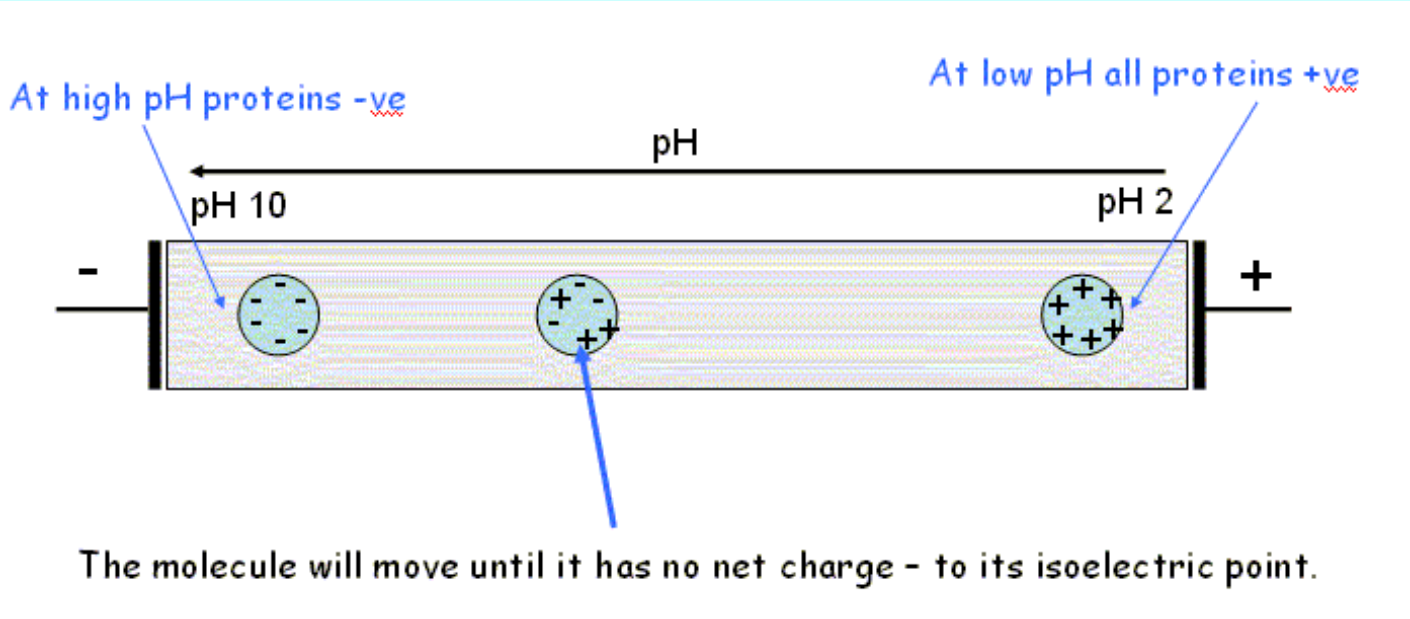
A pH gradient is set up in a gel or column with a solution of ampholytes (molecules that have both positive and negative charges)

These ampholytes are subjected to an electric current (electrophoresis)

Proteins are then applied and they migrate through the medium to a position corresponding to their respective isoelectric points (IP´s)

Once this point is reached, protein migration stops.

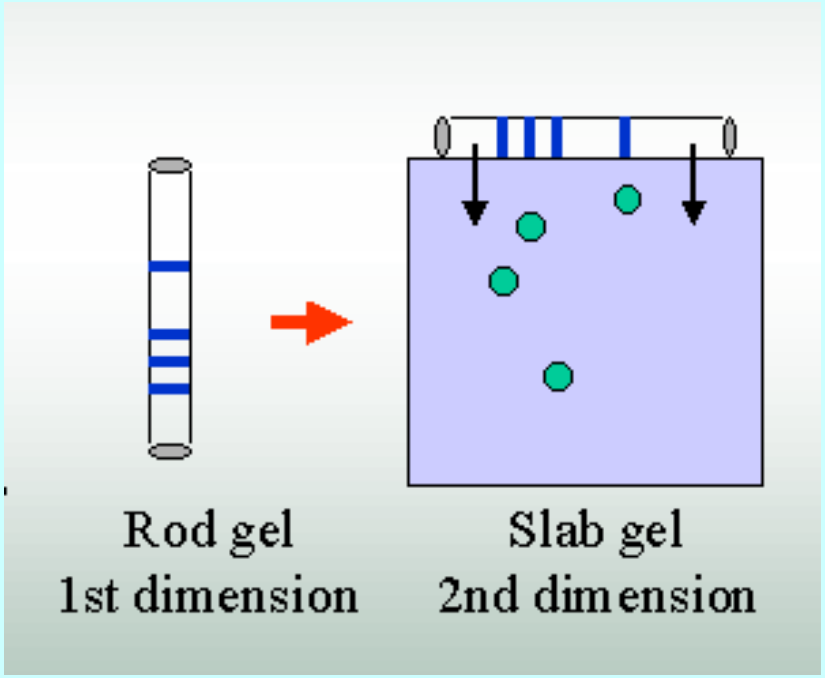
Electrophoresis - X



Isoelectric focussing is another form of electrophoresis that allows separation of protein by their isoelectric points. The isoelectric point (pI) of a protein is the pH at which it has no net charge: Since a protein bristles with many different ionisable groups, at low pH, it will tend towards being positively charged, and at high pH, towards being negatively charged. At the pI, the number of positive and negative charges balance, and the protein has no net overall charge. At this pH, the protein will be unaffected by an electric field. Isoelectric focussing uses a special gel that creates a fixed pH gradient. A current is then passed through the gel. A protein migrates through the gel according to its charge, but as it does so, the pH changes, and so too does the charge on the protein. Eventually the protein will come to rest at the point in the pH gradient corresponding to its pI. The gel may be left like this, or perpendicular current may be applied afterwards to separate the proteins in a second dimension by conventional PAGE techniques.

Electrophoresis - XI

Two-dimensional electrophoresis

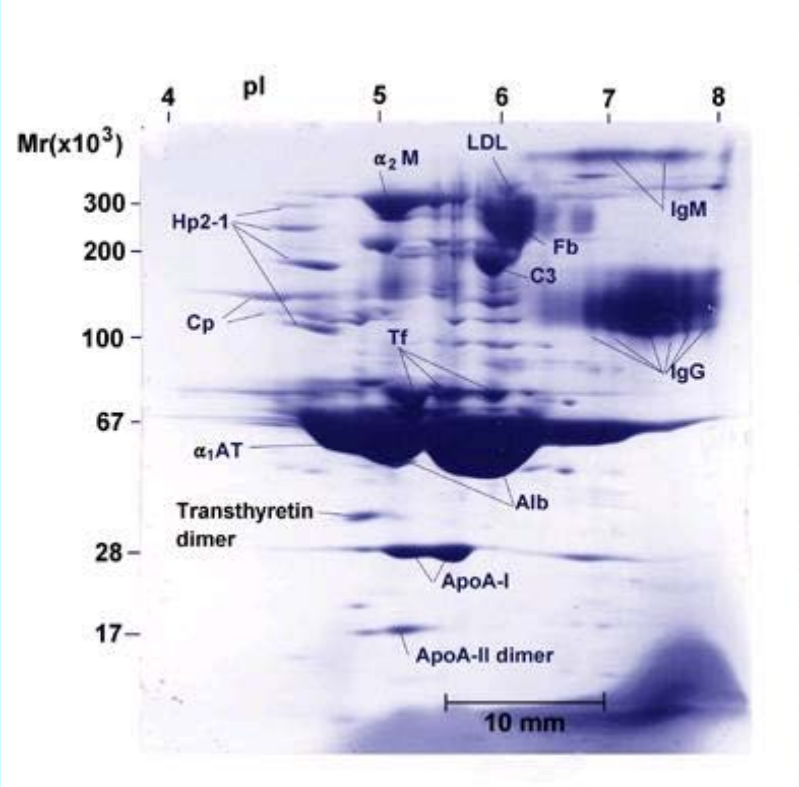


In two-dimensional electrophoresis proteins are separated in one dimension first using a rod gel, then in a second dimension using a slab gel.

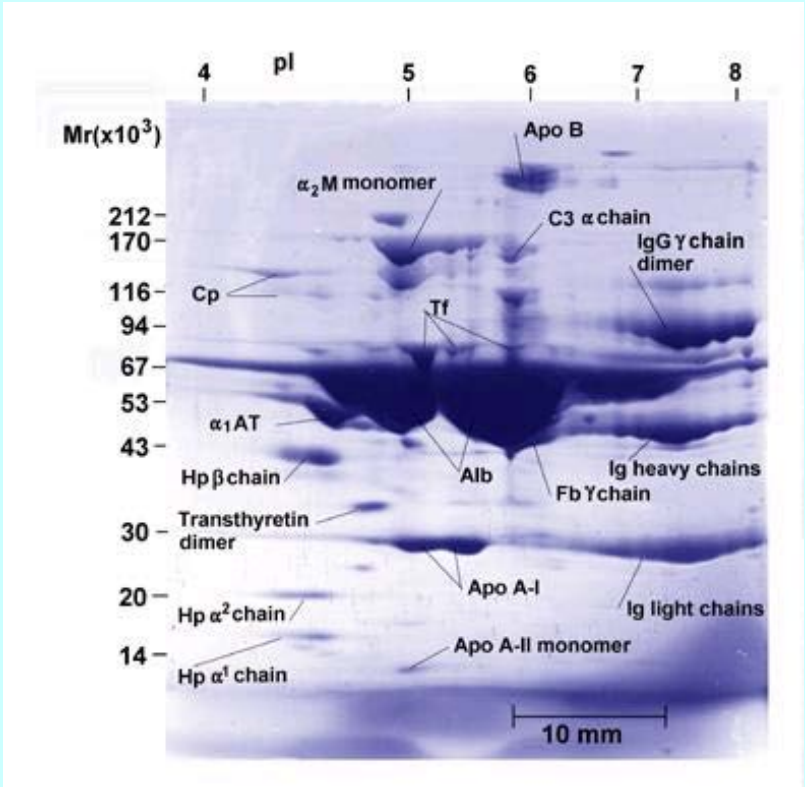
Use different separation techniques for each dimension - e. g. a first dimension isoelectric focussing for separation by charge and a second dimension SDS-PAGE for separation by size.

Electrophoresis - XII

Two-dimensional electrophoresis of serum



Native (without SDS and β-meraptoethanol



Native (without SDS and β-meraptoethanol

Diagnostic use of electrophoresis in clinical routine

Frequently used examples are:

Serum protein electrophoresis
Immunofixation electrophoresis of serum
Lactate dehydrogenase isoenzyme electrophoresis
Creatine kinase isoenzyme electrophoresis
Alkaline phosphatase isoenzymes
α_1-antitrypsin electrophoresis
Hemoglobin electrophoresis
Lipoprotein electrophoresis
Urine protein electrophoresis
Liquor protein electrophoresis

Some of these methods require specific detection reactions (e. g. Western blot or enzyme reaction after electrophoresis or immunofixation).

Electrophoretic and immunological methods

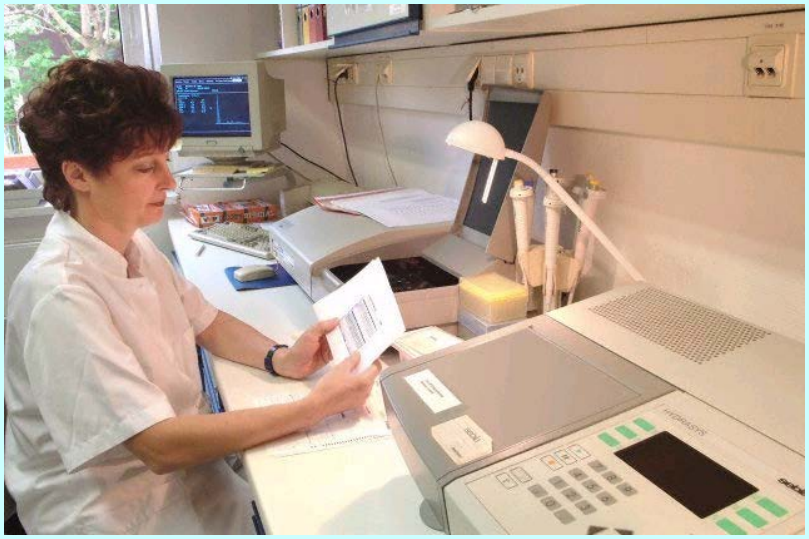
Electrophoretic and immunological methods are used for:

Characterization of protein mutations (e. g. hemoglobin)

Detection of atypical proteins in serum and/or urine (e. g. M-gradient, Bence-Jones proteins)

Quantification of serum proteins

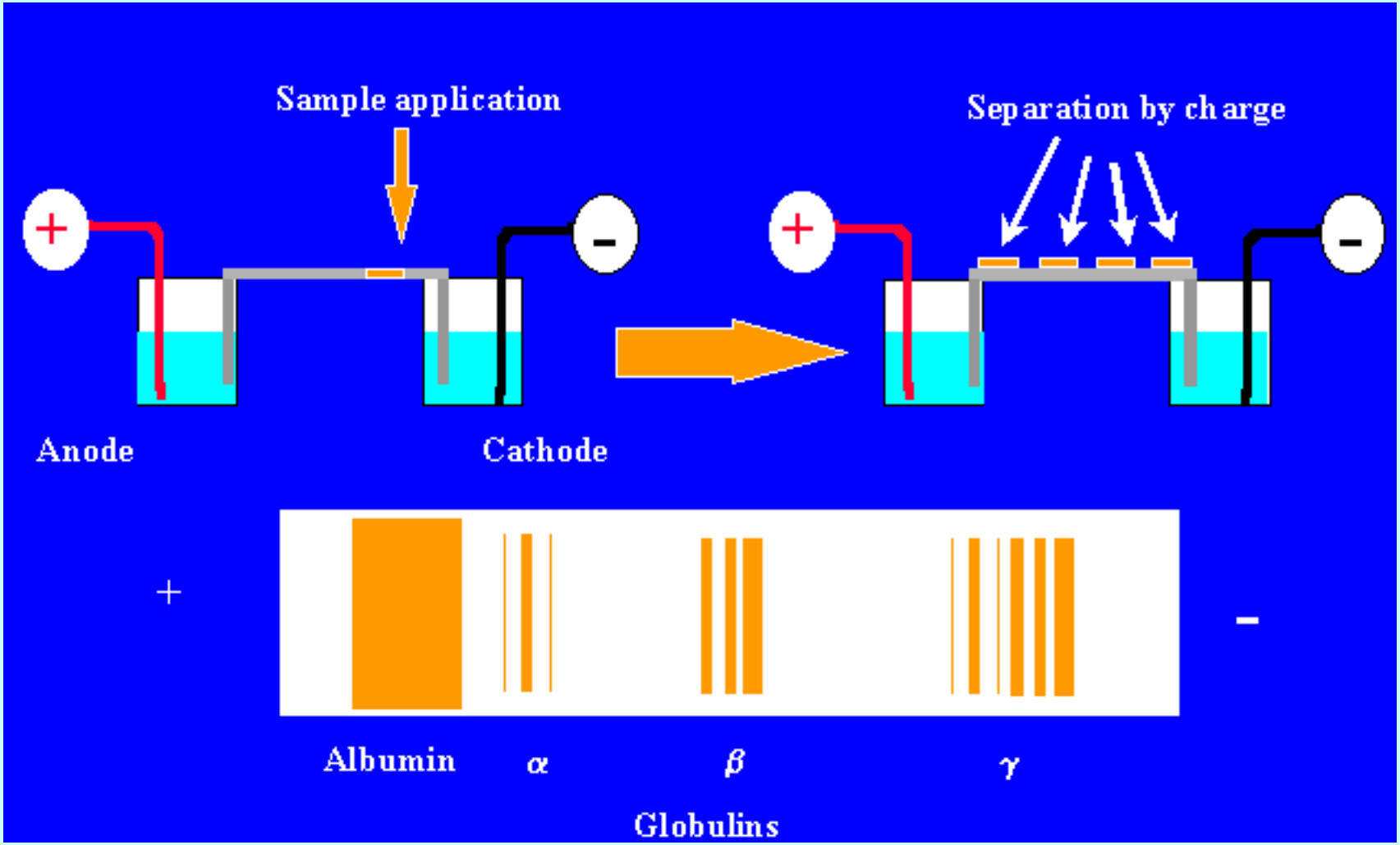
Serum protein electrophoresis - I



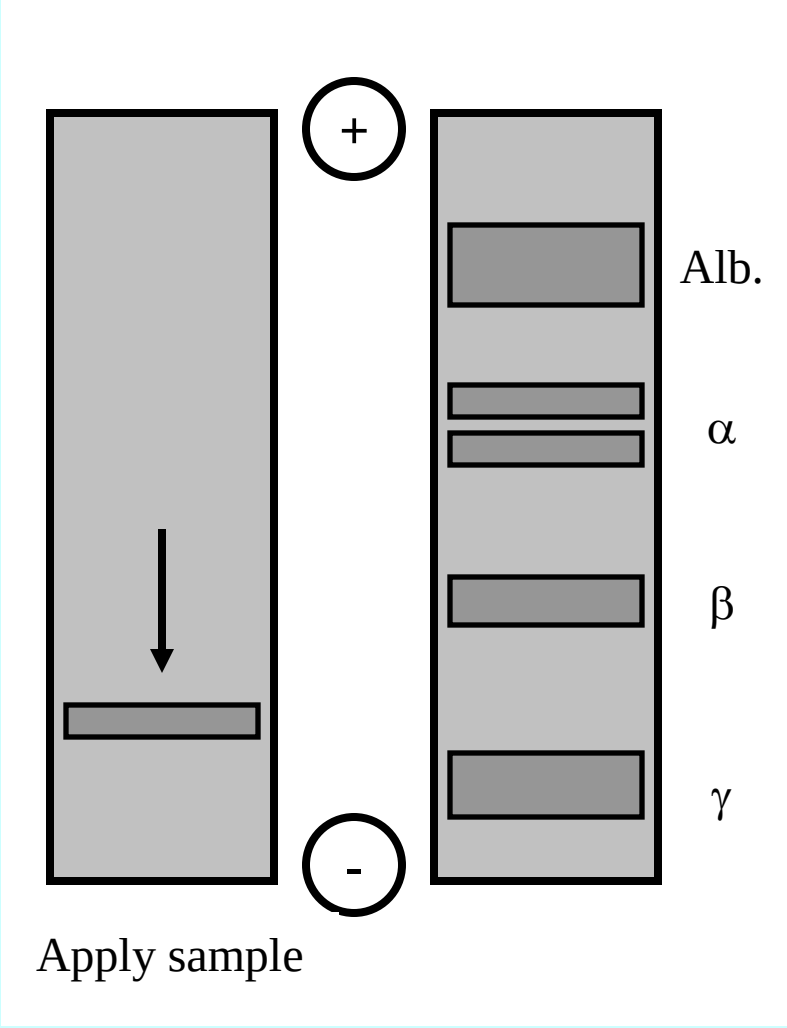
Left: Elektrophoresis system SEBIA HYDRYS and HYDRASYS (Protein electrophoresis, immunofixation, hemoglobin electrophoresis).

Right: Special electrophoresis (HELENA Lab, Rapid Electrophoresis REP System).

Serum protein electrophoresis - II

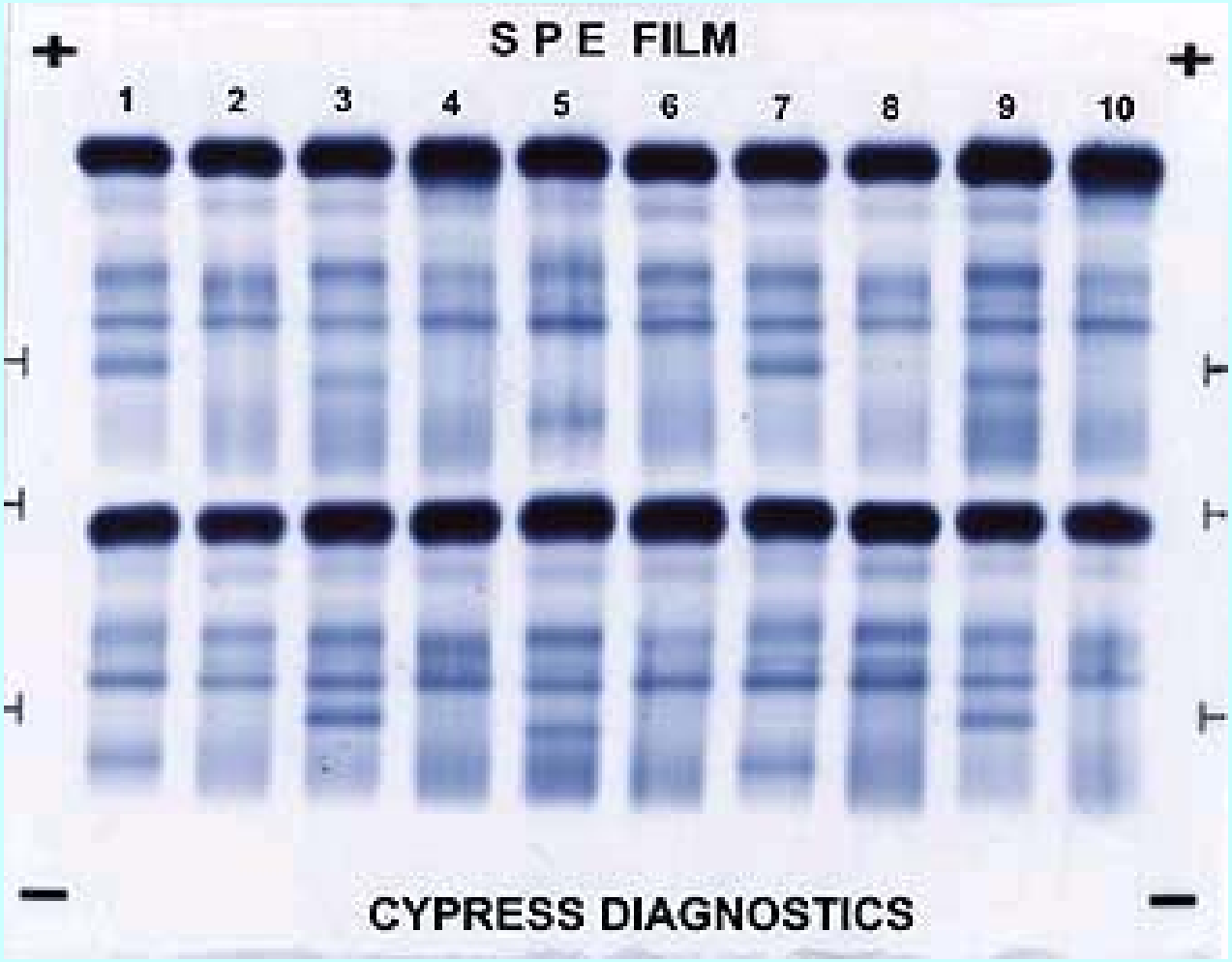


Serum protein electrophoresis - III



A drop of serum is applied in a band to a thin sheet of supporting material, like paper, that has been soaked in a slightly-alkaline salt solution. At pH 8.6, which is commonly used, all the proteins are negatively charged, but some more strongly than others. A direct current can flow through the paper because of the conductivity of the buffer with which it is moistened. As the current flows, the serum proteins move toward the positive electrode. The stronger the negative charge on a protein, the faster it migrates. After a time (typically 20 min), the current is turned off and the proteins stained to make them visible (most are otherwise colorless). The separated proteins appear as distinct bands.

Serum protein electrophoresis - IV



Albumin

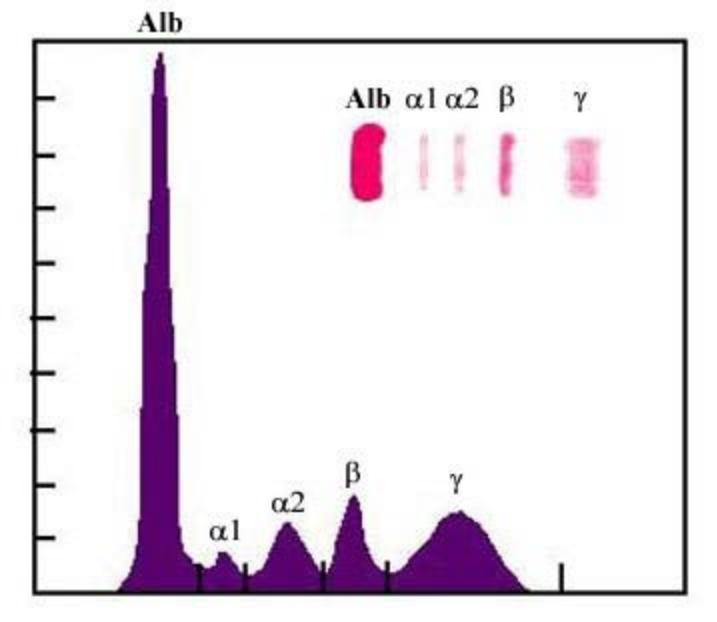
α_1-globulin

α_2-globulin

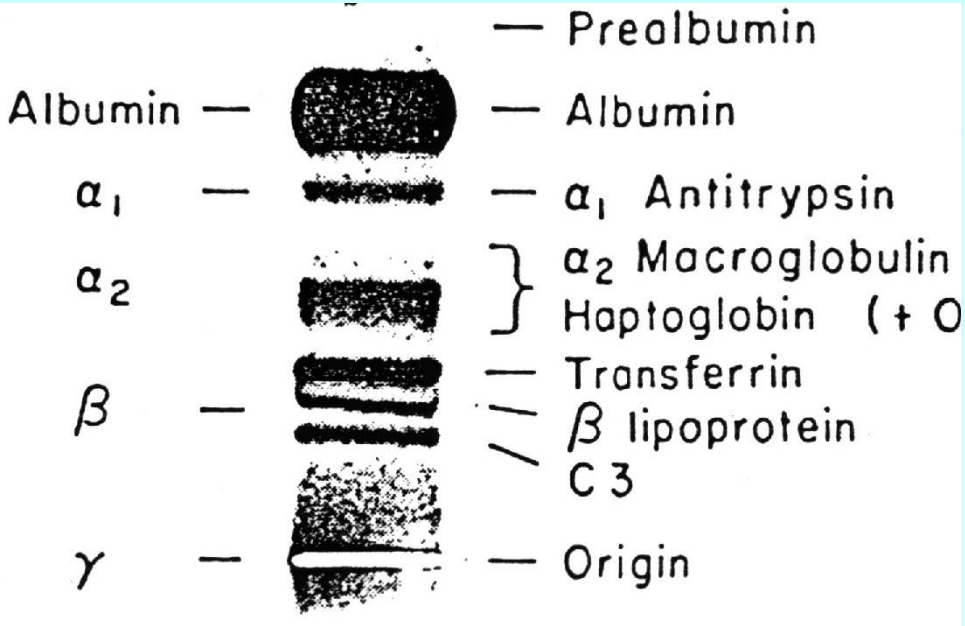
β-globulin

γ-globulin

Normal serum protein electrophoresis

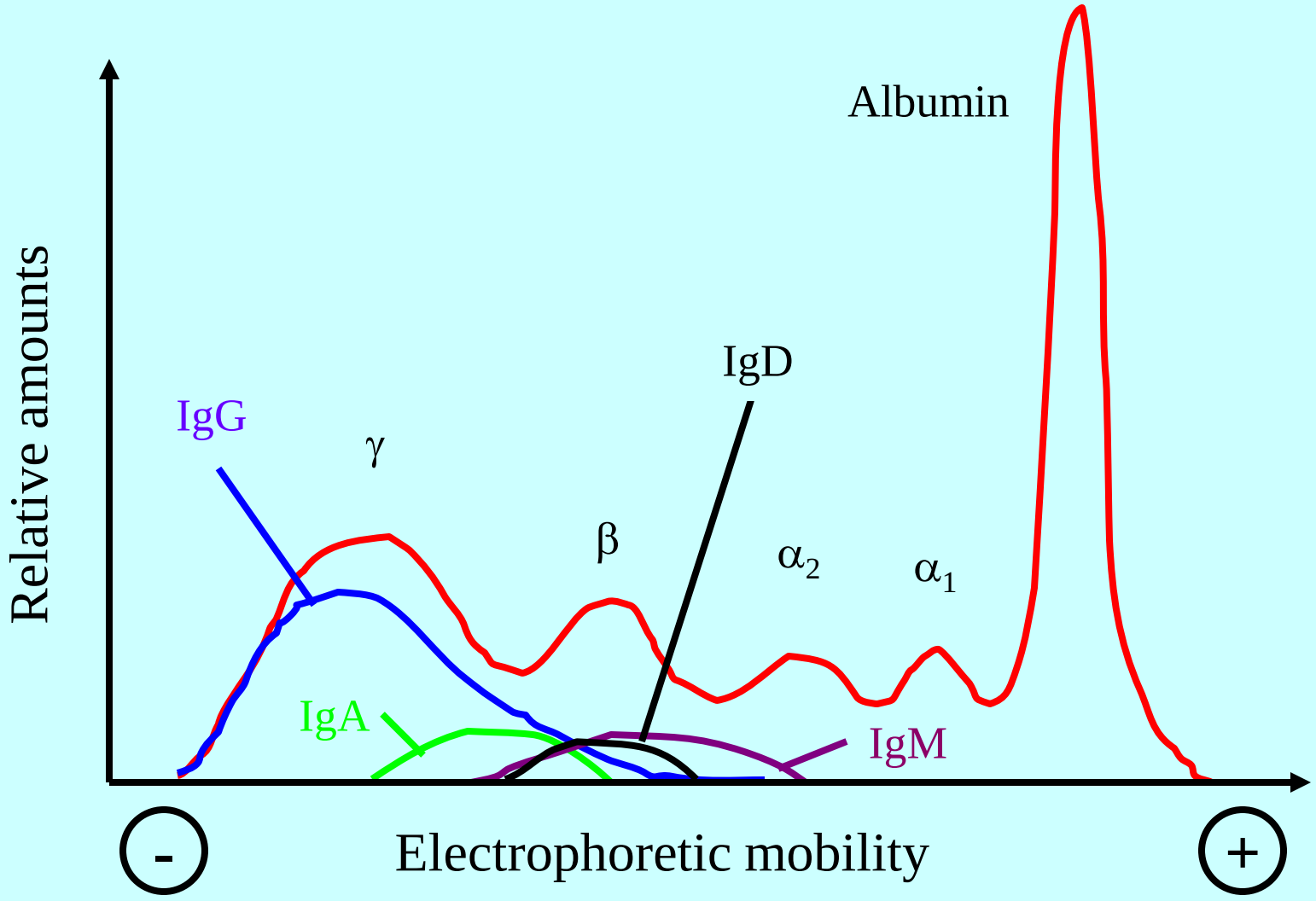


Density profile of a normal serum protein electrophoresis

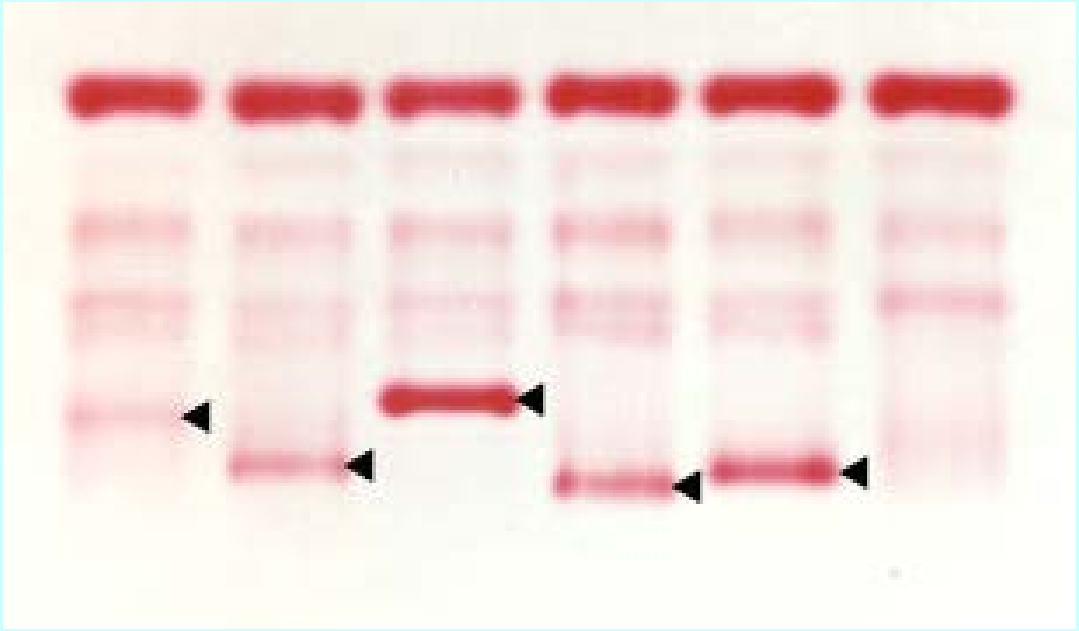


Distribution profile of some plasma proteins in the normal serum electrophoresis

Mobility of immunoglobulins in serum electrophoresis

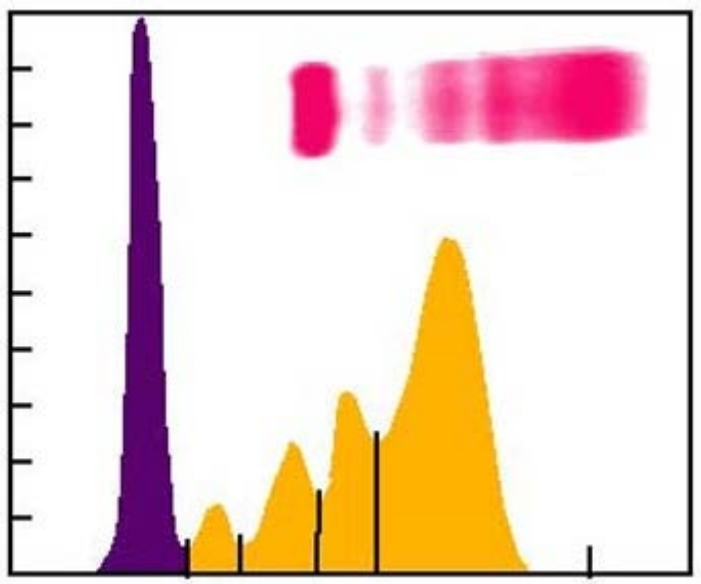


Serum electrophoresis in patients with multiple myeloma

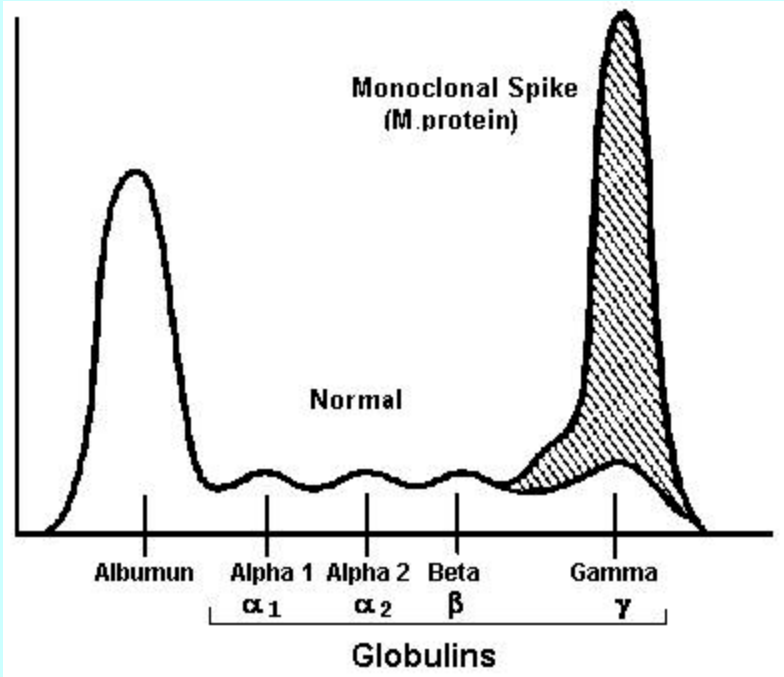


The electrophoresis shows the mobility pattern of samples from different patients with multiple myeloma (indicated samples) and a normal reference sample (right). The location and intensity of the M-gradient differs strongly in the 5 patients.

Pathological serum electrophoreses



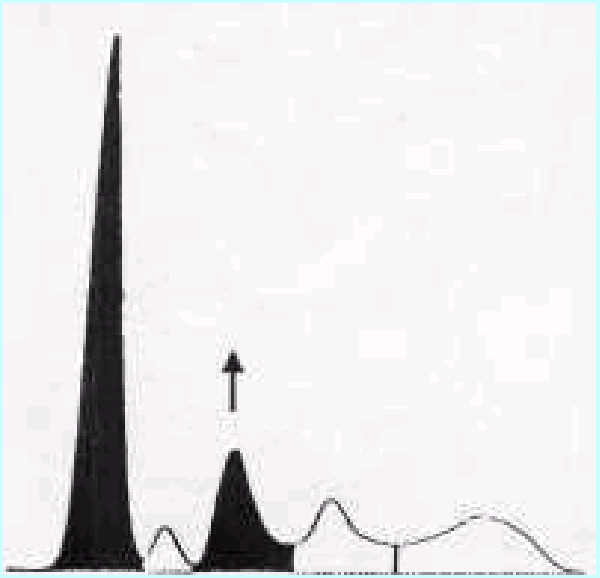
Polyclonal gammopathy usually occurs secondary to many chronic diseases. This patient was a 39 year old male with sarcoidosis. The sequential increase of the globulin fractions illustrated "sarcoid stepping." IFE excluded the possibility of a monoclonal protein.



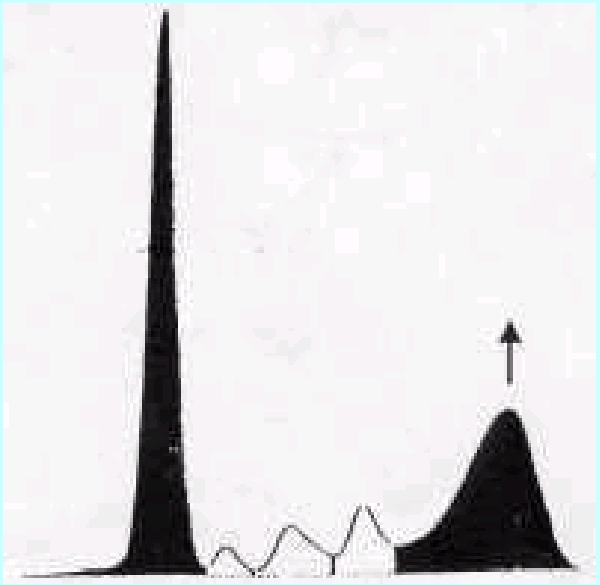
Monoclonal gammopathy (M-gradient) in a patient with multiple myeloma. The diagnosis can be proven by IFE which shows a monoclonal fraction of IgG chains.

Typical patterns of serum electrophoresis - I

Acute inflammation



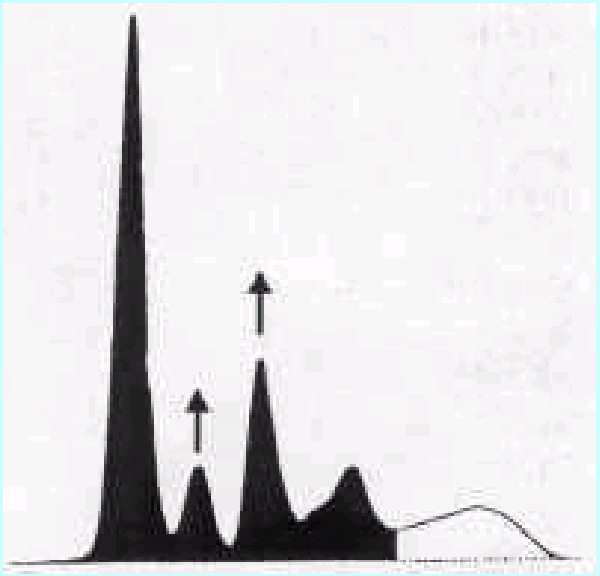
Late phase of acute inflammation or chronic inflammation



	Value	Reference			Value	Reference	
Albumin	51.3	58.0 - 72.0	%	Albumin	47.7	58.0 - 72.0	%
α_1-Globulin	3.7	1.5 - 4.0	%	α_1-Globulin	2.2	1.5 - 4.0	%
α_2-Globulin	15.7	5.0 - 10.0	%	α_2-Globulin	6.1	5.0 - 10.0	%
β-Globulin	12.1	7.0 - 13.0	%	β-Globulin	7.0	7.0 - 13.0	%
γ-Globulin	17.2	10.0 - 19.0	%	γ-Globulin	37.0	10.0 - 19.0	%
Total protein	79	66 - 83	g/l	Total protein	77	66 - 83	g/l

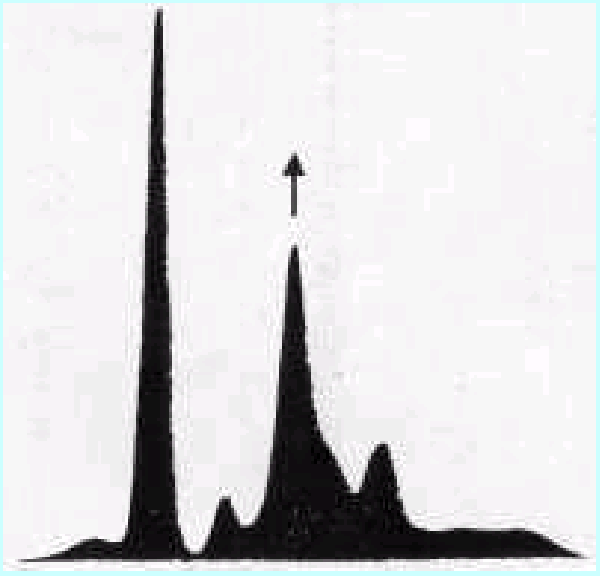
Typical patterns of serum electrophoresis - II

Necrotizing tumor



	Value	Reference	
Albumin	46.3	58.0 - 72.0	%
α_1-Globulin	7.9	1.5 - 4.0	%
α_2-Globulin	17.3	5.0 - 10.0	%
β-Globulin	14.0	7.0 - 13.0	%
γ-Globulin	14.5	10.0 - 19.0	%
Total protein	72	66 - 83	g/l

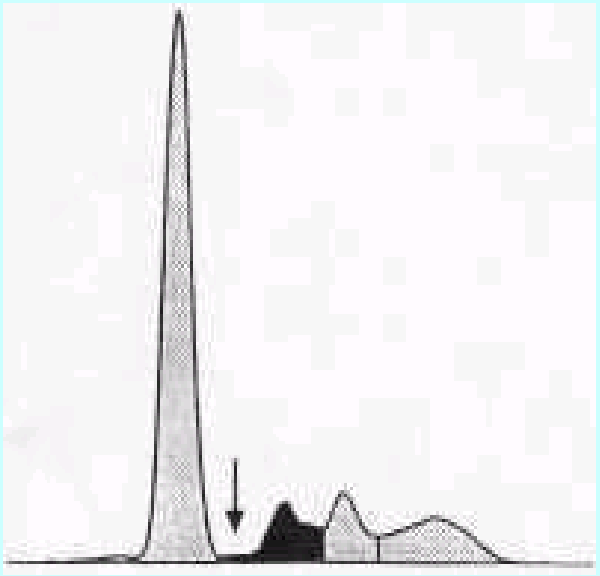
Nephrotic syndrome



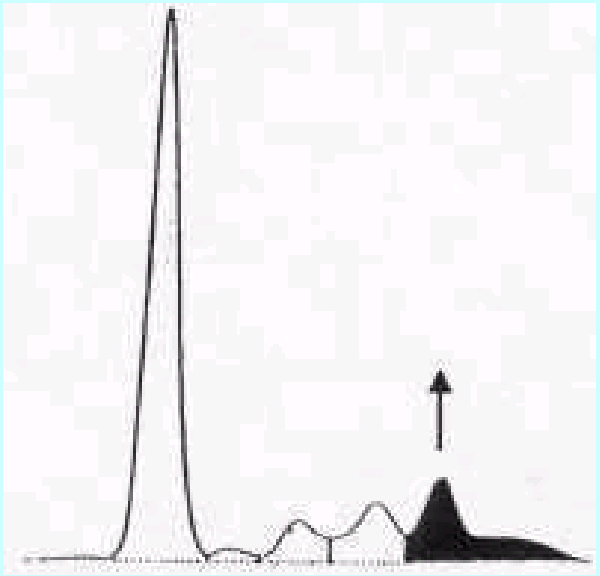
	Value	Reference	
Albumin	37.9	58.0 - 72.0	%
α_1-Globulin	4.6	1.5 - 4.0	%
α_2-Globulin	35.5	5.0 - 10.0	%
β-Globulin	13.5	7.0 - 13.0	%
γ-Globulin	8.5	10.0 - 19.0	%
Total protein	46	66 - 83	g/l

Typical patterns of serum electrophoresis - III

α_1-antitrypsin deficiency



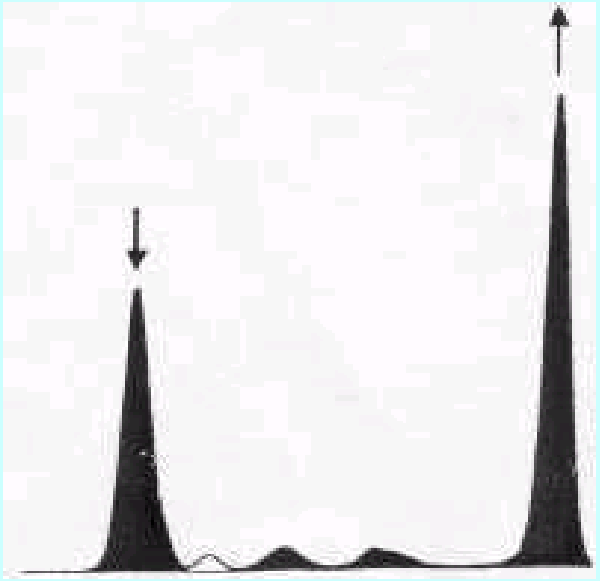
Monoclonal gammopathy



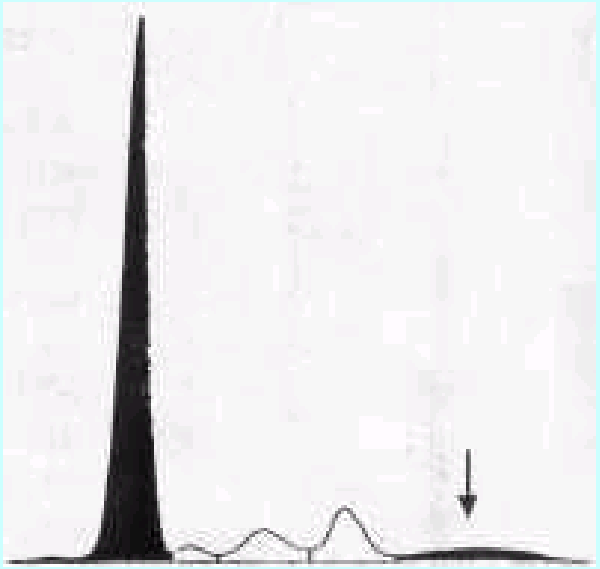
	Value	Reference			Value	Reference	
Albumin	65.0	58.0 - 72.0	%	Albumin	61.9	58.0 - 72.0	%
α_1-Globulin	0.7	1.5 - 4.0	%	α_1-Globulin	1.9	1.5 - 4.0	%
α_2-Globulin	10.2	5.0 - 10.0	%	α_2-Globulin	6.5	5.0 - 10.0	%
β-Globulin	9.3	7.0 - 13.0	%	β-Globulin	10.3	7.0 - 13.0	%
γ-Globulin	14.8	10.0 - 19.0	%	γ-Globulin	19.4	10.0 - 19.0	%
Total protein	74	66 - 83	g/l	Total protein	80	66 - 83	g/l

Typical patterns of serum electrophoresis - IV

Final stage of plasmocytoma



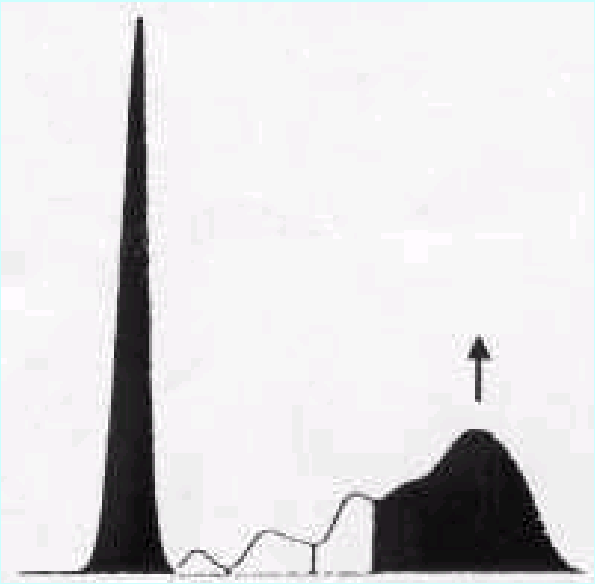
Antibody deficiency



	Value	Reference			Value	Reference	
Albumin	38.2	58.0 - 72.0	%	Albumin	73.0	58.0 - 72.0	%
α_1-Globulin	1.9	1.5 - 4.0	%	α_1-Globulin	2.6	1.5 - 4.0	%
α_2-Globulin	4.3	5.0 - 10.0	%	α_2-Globulin	7.8	5.0 - 10.0	%
β-Globulin	4.9	7.0 - 13.0	%	β-Globulin	9.7	7.0 - 13.0	%
γ-Globulin	50.7	10.0 - 19.0	%	γ-Globulin	6.9	10.0 - 19.0	%
Total protein	95	66 - 83	g/l	Total protein	65	66 - 83	g/l

Typical patterns of serum electrophoresis - V

Liver cirrhosis



Value	Reference		
Albumin	42.3	58.0 - 72.0	%
α_1-Globulin	1.9	1.5 - 4.0	%
α_2-Globulin	6.0	5.0 - 10.0	%
β-Globulin	8.1	7.0 - 13.0	%
γ-Globulin	41.7	10.0 - 19.0	%
Total protein	85	66 - 83	g/l

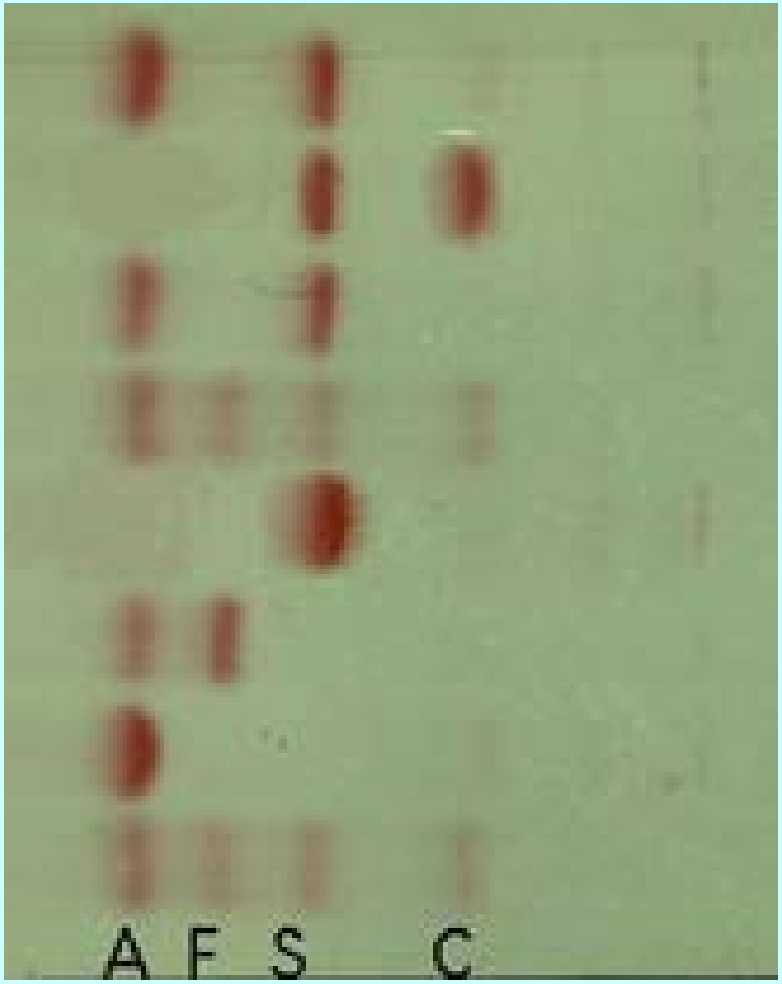
Changes of serum electrophoresis and additional tests - I

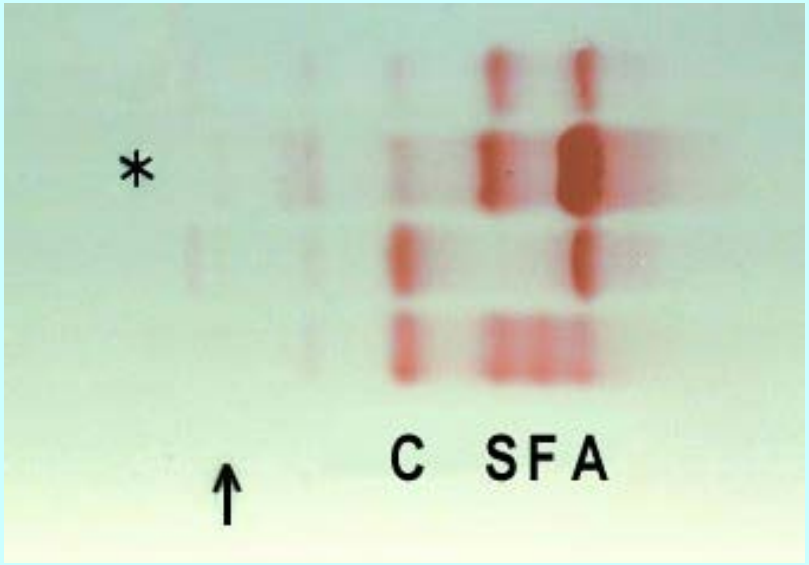
Disease	Alb.	α_1	α_2	β	γ	Additional tests
Acute inflammation Beginning nephrotic syndrome	↓		↑			Urine status, urine protein, urine protein electrophoresis, serum albumin, CRP, cholesterol, ESR, fibrinogen, blood cell count, ferritin
Late phase of acute inflammation, chronic inflammation	↓				↑	CRP, ESR, fibrinogen, ferritin, blood cell count, immunoglobulins
Necrotizing tumor	↓	↑	↑			CRP, ESR, fibrinogen, ferritin, blood cell count
Nephrotic syndrome	↓		↑			Immunoglobulins quantitative, total urine protein (24 hours), serum albumin, AT-III, cholesterol, triglycerides, aldosteron, creatinine clearance, electrolytes

Changes of serum electrophoresis and additional tests - II

Disease	Alb. α_1 α_2 β γ	Additional tests
α_1-antitrypsin deficiency		α_1-antitrypsin in serum, phenotyping of α_1-antitrypsin, genotyping of α_1-antitrypsin
Monoclonal gammopathy plasmocytoma, heavy chain disease, amyloidosis	 	Immunoglobulins, immunofixation in urine, Bence-Jones-protein in 24 hours urine
Antibody deficiency		Immunoglobulins quantitative, immunofixation in urine immunoglobulin subclasses
Liver cirrhosis	 	Immunoglobulins quantitative, Quick-value (INR) Fibrinogen, platelets, pseudocholine esterase, GOT (ASAT), GPT (ALAT), γ-GT, alkaline phosphatase, hepatitis serology, α_1-fetoprotein, ferritin, autoantibodies (AMA)

Hemoglobin electrophoresis





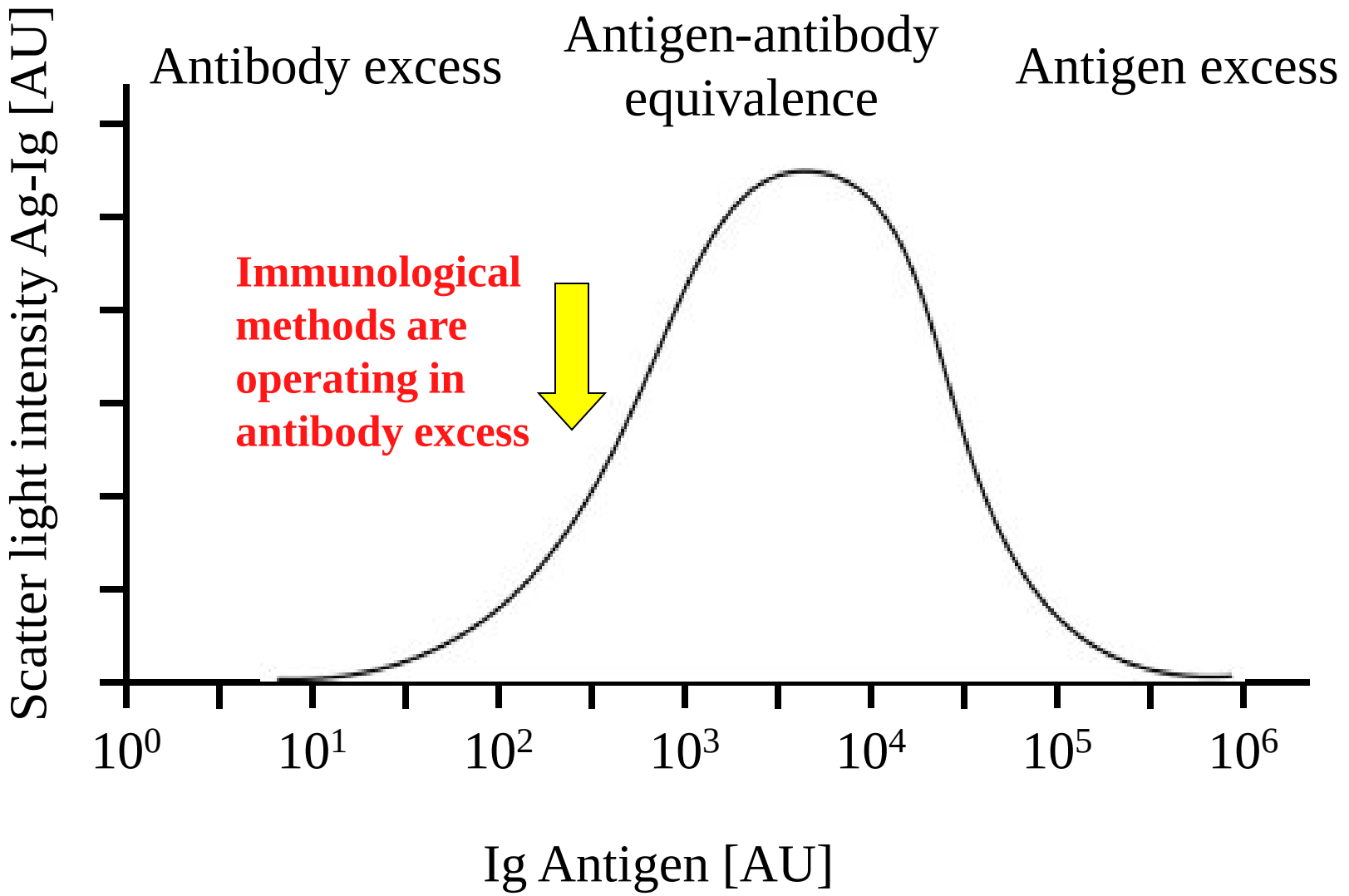
Top figure:

Healthy African-American child. Hb electrophoresis, cellulose acetate pH 8.4; the patient (*) appears to have Hb A and Hb S.

Lipid electrophoresis

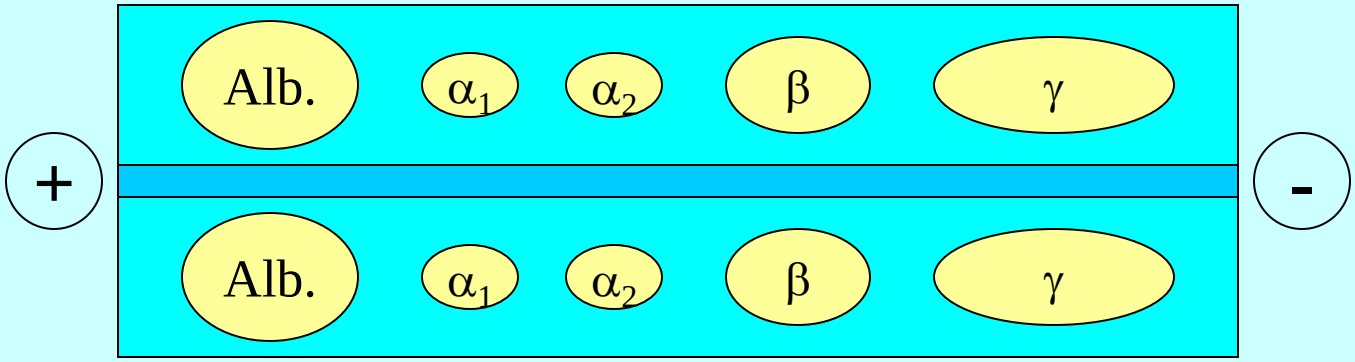
The MIDIGEL LIPO Kit will clearly separate alpha (HDL), pre-beta (VLDL), and beta (LDL) lipoproteins. During electrophoresis, the lipoproteins separate (in order of decreasing mobility) into 4 fractions: alpha, pre-beta, beta lipoproteins and chylomicrons. MIDIGEL LIPO provides excellent resolution of the beta-pre-beta-lipoprotein zone (the alpha-lipoprotein band is sometimes split into alpha-lipoproteins and pre-alpha-lipoproteins).

Heidelberger-Kendall-curve

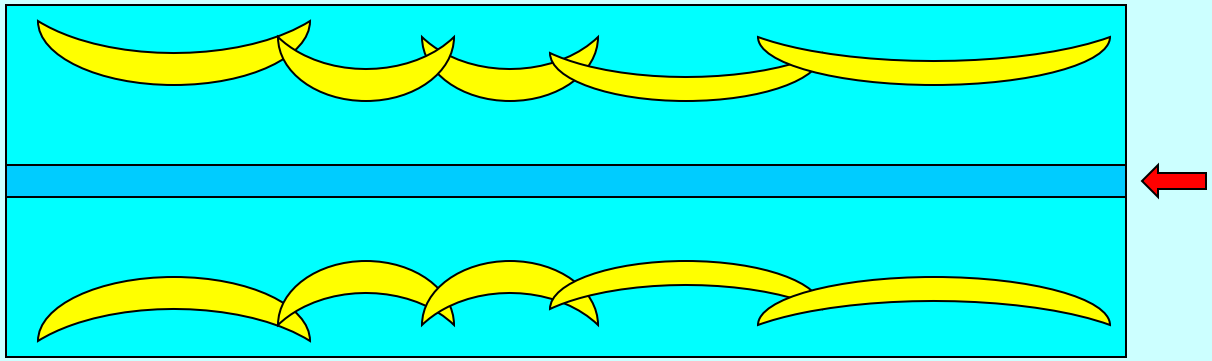


Immunoelectrophoresis a la Grabar and Laurell - I

1. Electrophoresis of human serum:

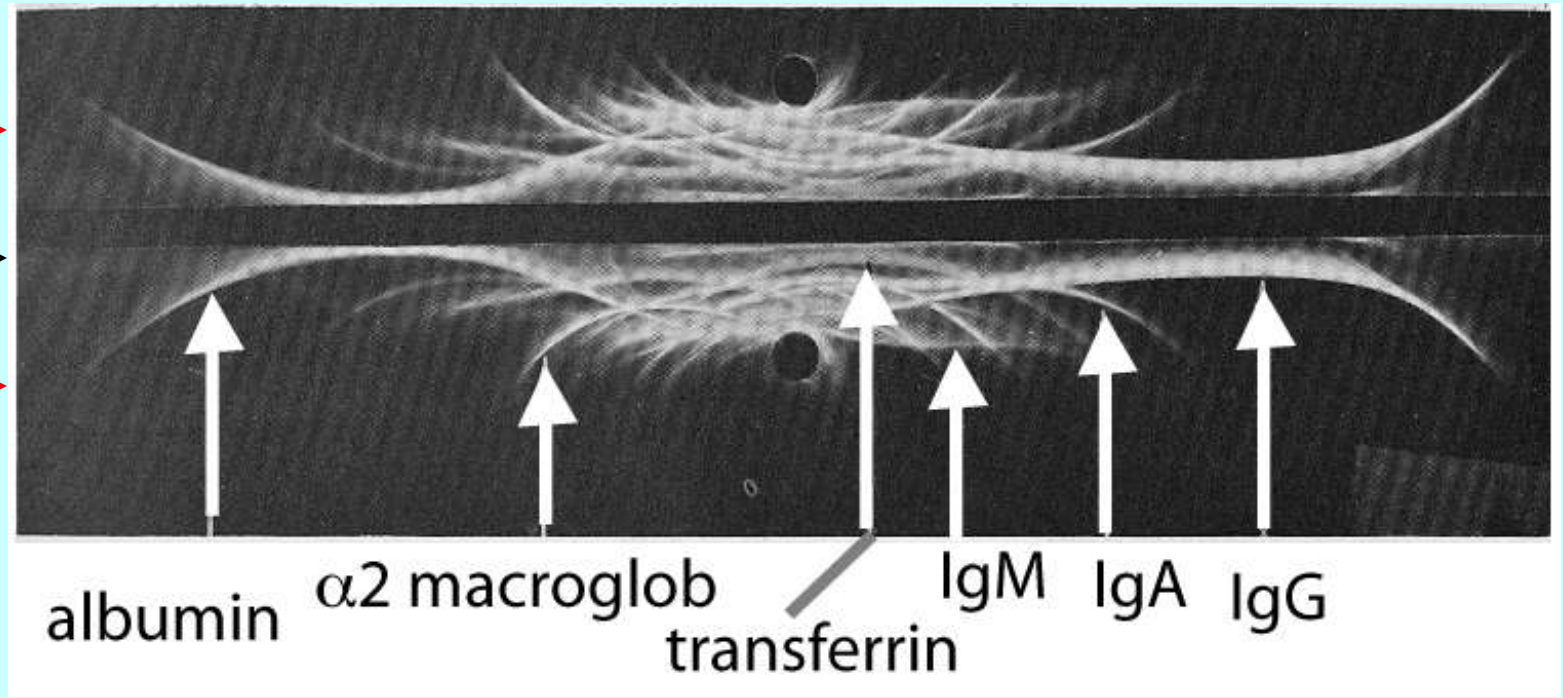


2. Immunoprecipitation:



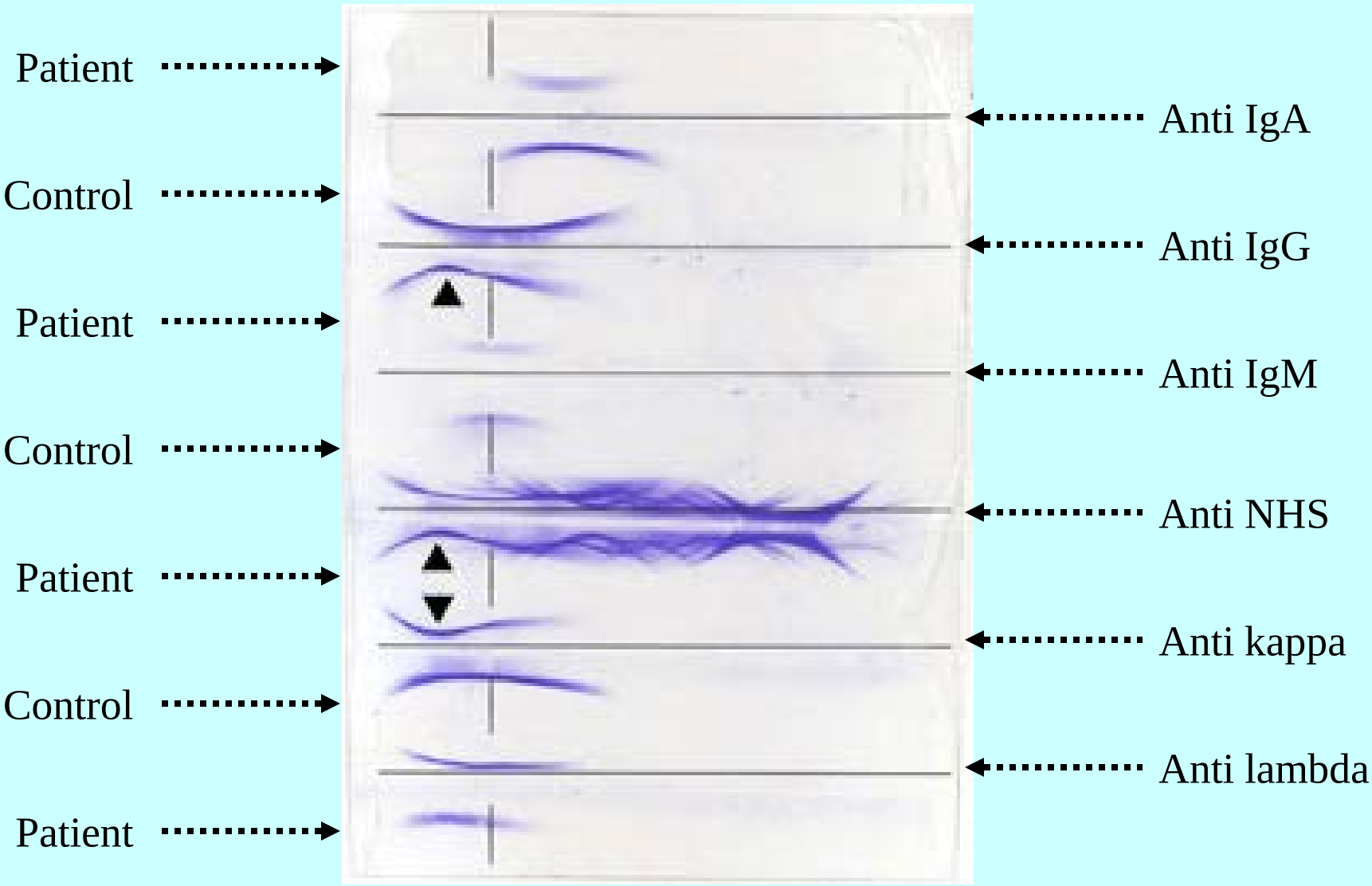
Polyspecific anti-human antiserum

Immunoelectrophoresis a la Grabar and Laurell - II

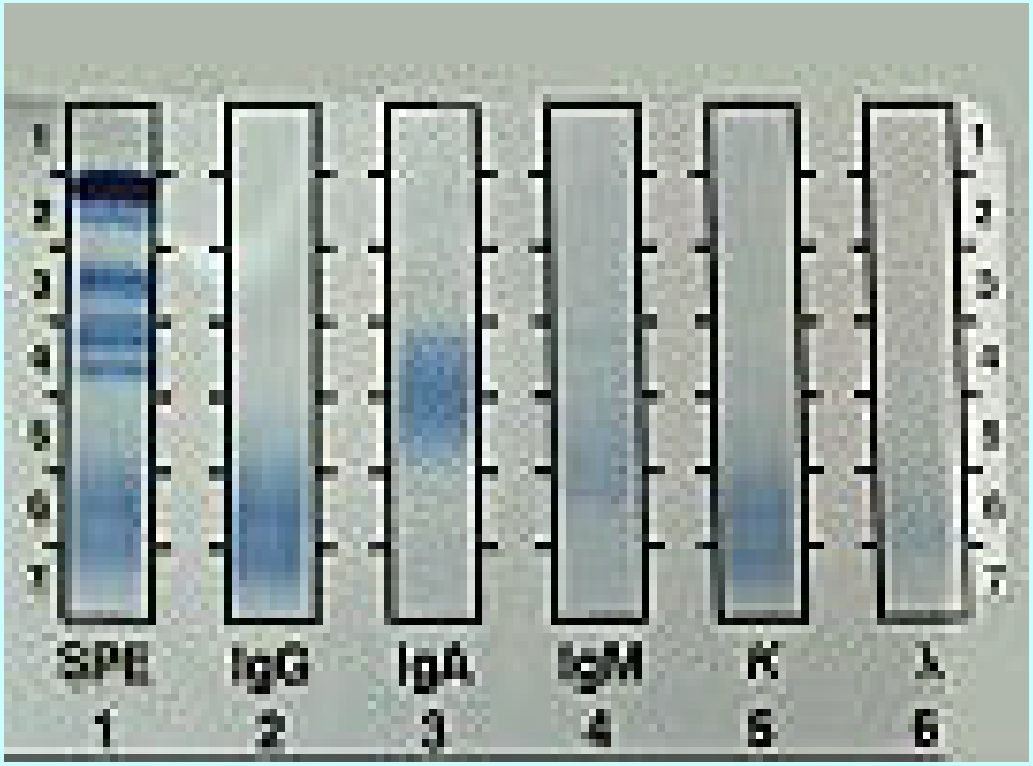


In a first step serum proteins are separated electrophoretically (red arrows). Then, the gel is incubated with antibodies against human serum proteins which were added into the indicated spline of the gel (black arrow). Antigen-antibody complexes are developing and can be detected by protein staining.

Pathological immunoelectrophoresis



Immunofixation



In a first step serum proteins are separated electrophoretically. Then, the different strips are incubated with (monoclonal) antibodies against human serum proteins (1), IgG (2), IgA (3), IgM (4), kappa (5) and lambda (5). After formation of immune complexes the gel is washed and stained for proteins. Immune complexes become visible after protein staining. The method allows the detection of specific immunoglobulins or their fragments in myeloma patients.

Agglutination tests - I

Reaction of a particulate antigen with antibodies

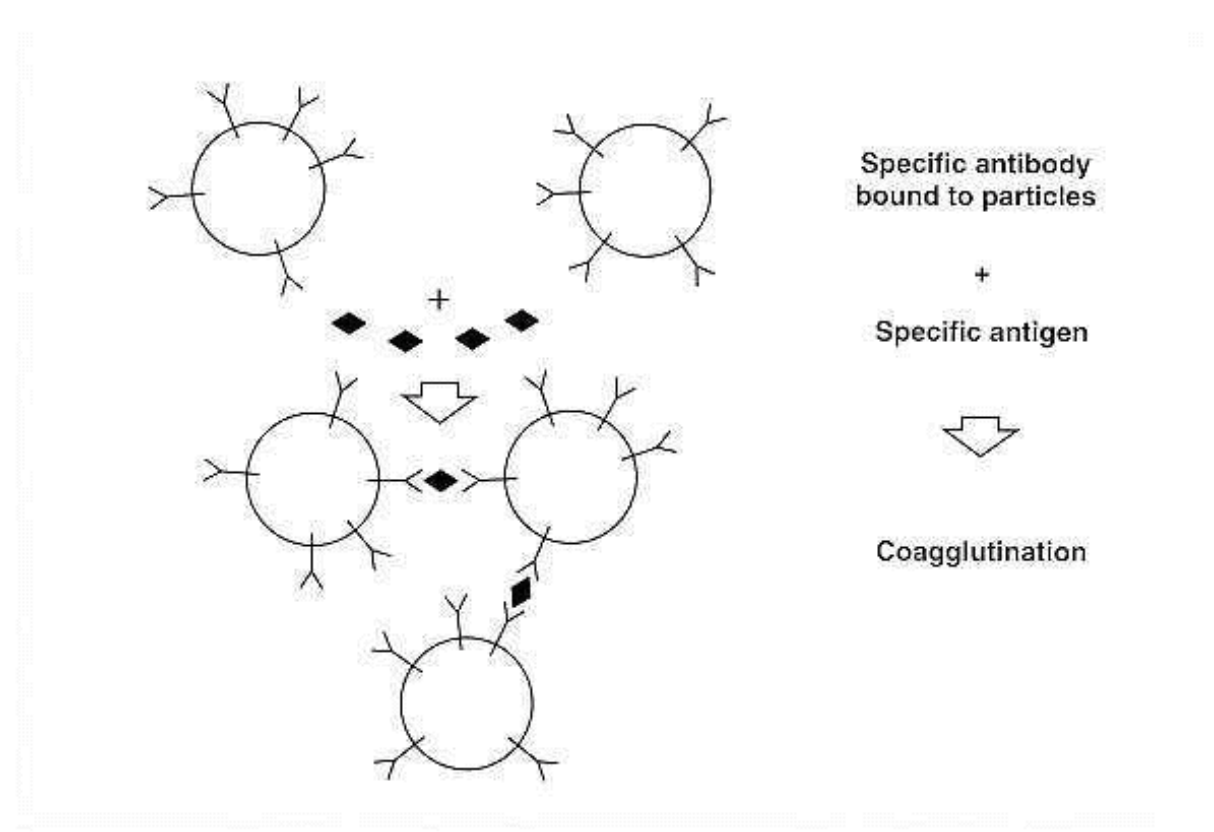
Form visible aggregates

Titer: Concentration of serum antibody

Direct agglutination

Latex agglutination tests

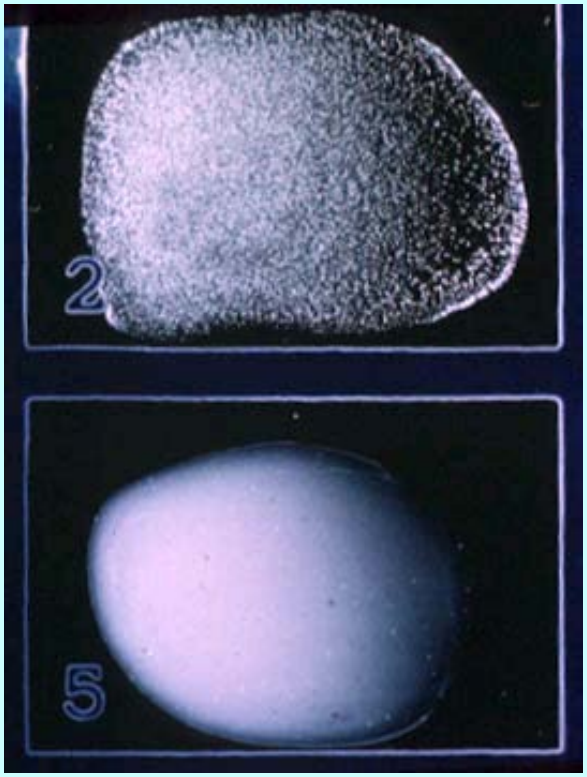
Agglutination tests - II



Latex agglutination tests (LATs) are very popular among laboratories around the world, because they are rapid (under 5 minutes), easy to perform and do not require any special tools or instruments. Moreover, they are cheaper than most other immunologic tests.

Agglutination tests - III

Latex Agglutination Test for Rheumatoid Factor



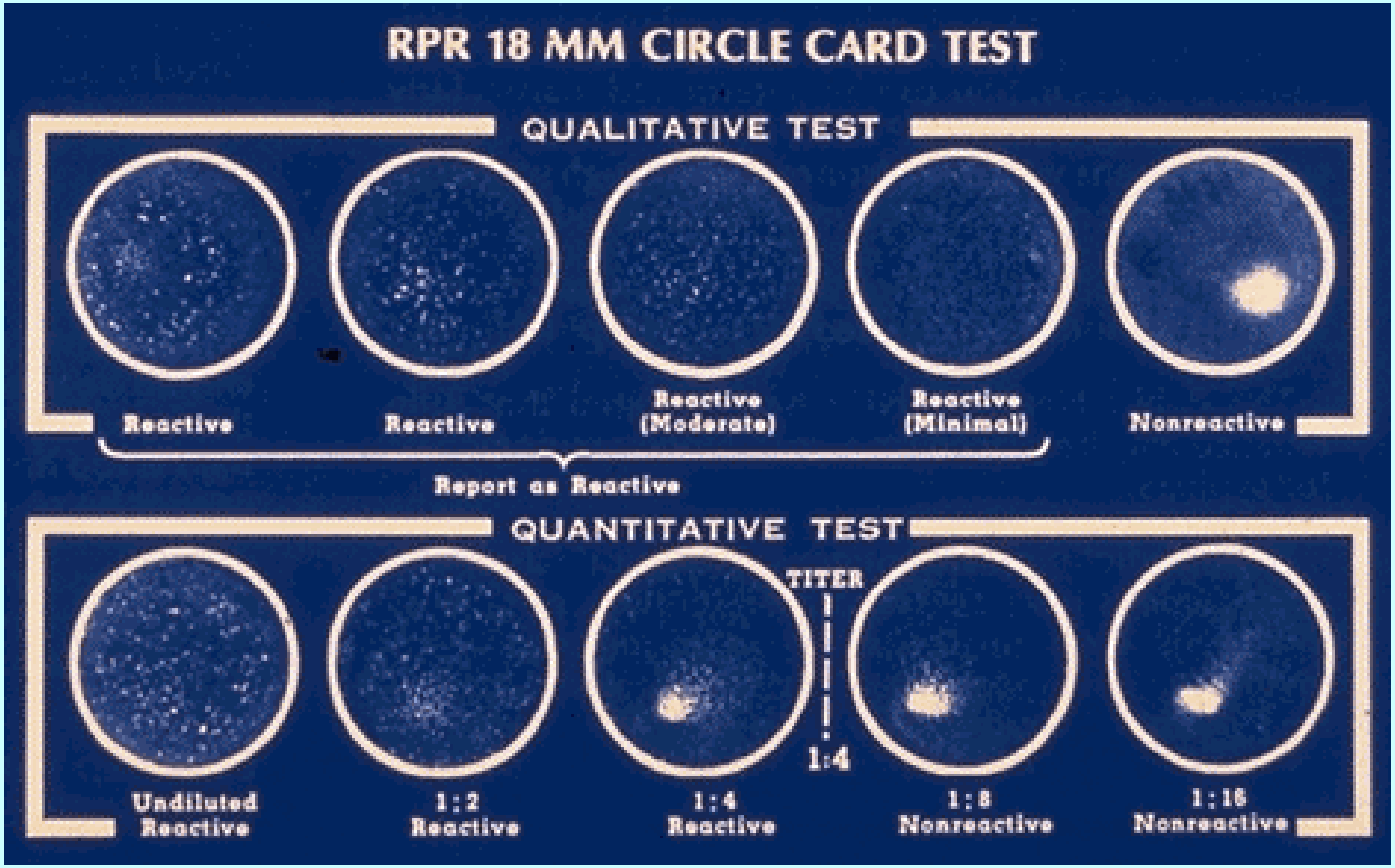
Two latex agglutination tests:

Top: Positive test (clumping of the latex particles)

Bottom: negative test

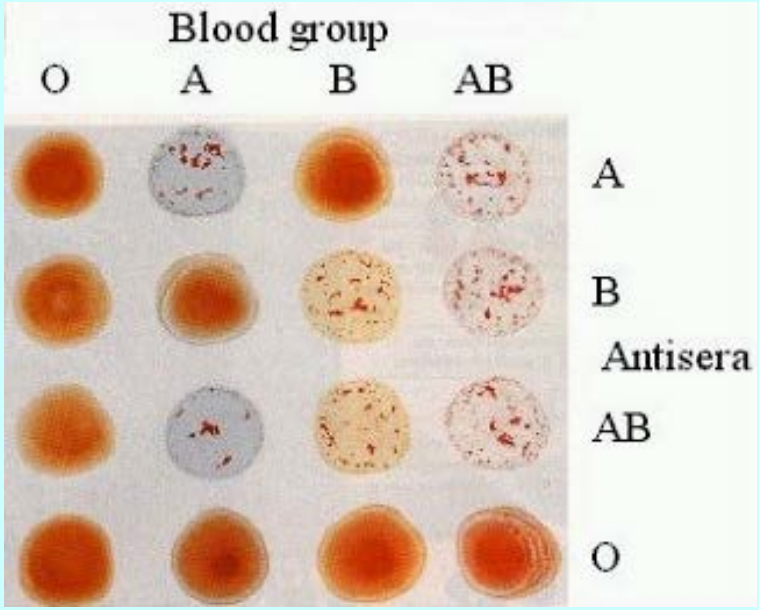
IgG is covalently attached to the latex particles in this test. The presence of rheumatoid factor (antibodies to IgG of the same animal) is detected by clumping of the latex particles.

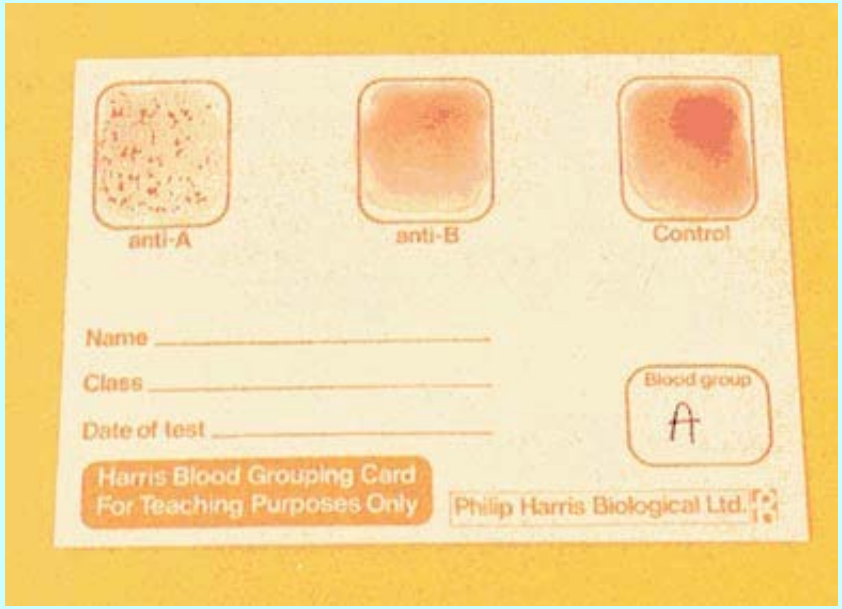
Agglutination tests - IV



The test can be used for qualitative assays and for quantitative determinations (e. g. antibody titers in serology).

Agglutination tests - V





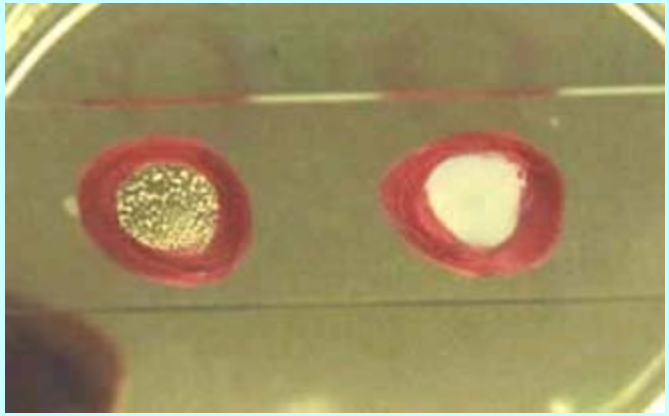
Blood group testing:

Left: Compatibility testing of blood.

Right: Bedside test prior to blood transfusion.

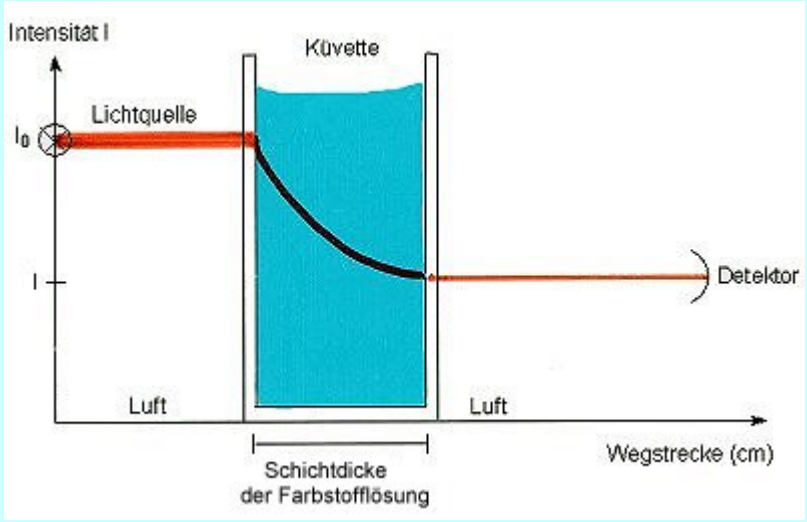
Agglutination tests - VI

Typing of salmonella strains



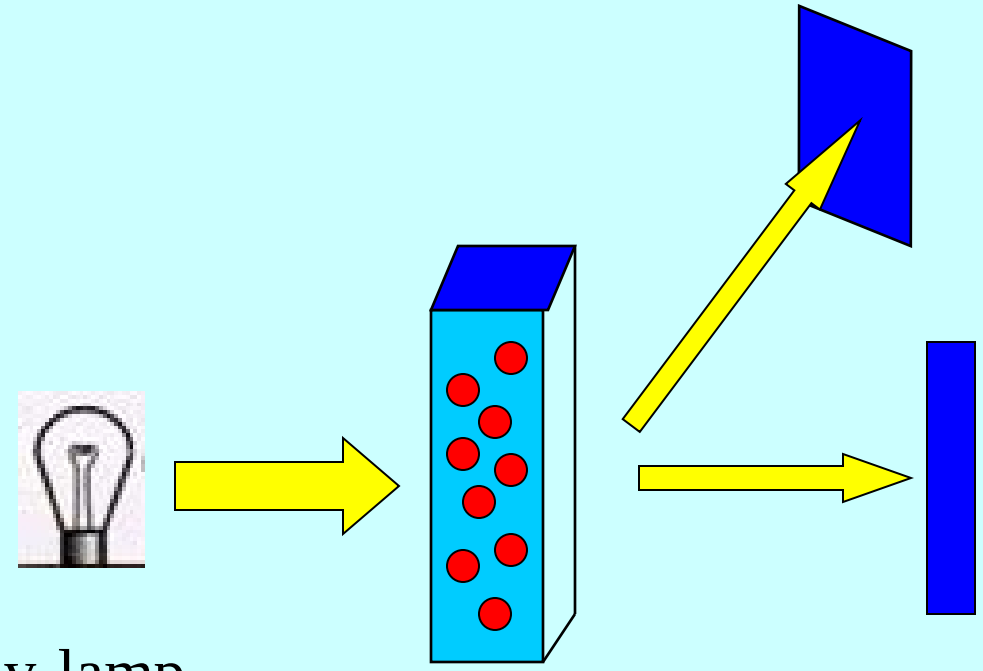
The test for cell wall ("O") and flagella („H“) antigens can be done on an isolate likely to be Salmonella. With a wax pencil, circular areas are marked off on the surface of a glass slide. These marks should be drawn heavily in order for the circular areas to contain cell suspensions which must not be allowed to run into each other or off the slide. After a drop of cells suspended in saline is placed in each circle, a drop of antiserum is subsequently added to each. (Alternatively, the antiserum drops can be placed on the slides first, and the cells can be suspended directly into the antiserum.) Where there is a reaction between antibodies in the antiserum and their homologous antigens on the cell wall of the bacteria, the cells will clump together ("agglutinate"), and the drop will appear to contain many small particles. The reaction is best observed from underneath the slide, and this can be accomplished with a mirror or by placing the slide in a petri dish and then holding the dish above eye level. In this photo, one drop is seen to contain agglutinated cells, and the other retains its original milky appearance.

The Beer-Lambert law



Wenn Licht durch ein farbiges Medium wandert, wird es absorbiert. Die Abnahme der Intensität I des Lichts längs der Schichtdicke d wird durch eine Abklingfunktion beschrieben.
Den absoluten Potenzterm nennen wir **Extinktion E** oder auch **Absorption A**.
$E_l = e_l \cdot c \cdot d$
Dieser Ausdruck ist bereits das **Lambert-Beersche Gesetz**.
In der Gleichung bedeuten
E_l dimensionslose Extinktion oder Absorption bei einer bestimmten Wellenlänge l
e_l molarer Extinktionskoeffizient (cm^2/mol)
c Konzentration des Stoffs (mol/cm^3)
d Schichtdicke der Messküvette (cm).
Man erhält das Gesetz auch durch Logarithmieren der Abklingfunktion. Damit wird deutlich, dass die Extinktion nichts anderes als das logarithmierte Verhältnis von Anfangsintensität I_0 zur nach dem Durchgang durch die Probe mit der Schichtdicke d gemessenen Intensität I ist.

Turbidimetry and nephelometry



Increase of sideward scatter light

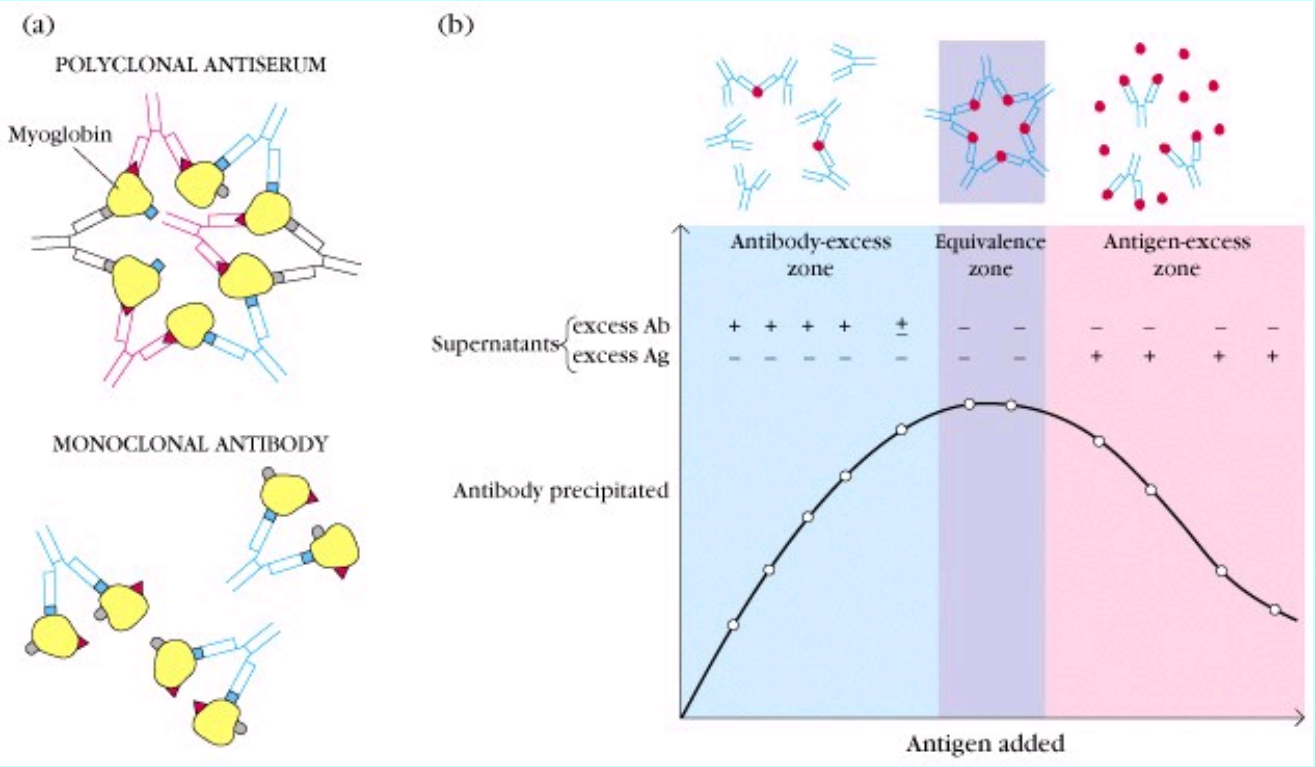
Increase of absorption

uv-lamp

Turbidimetry: Measurement of forward light absorption about two hours after begin of antigen-antibody reaction.

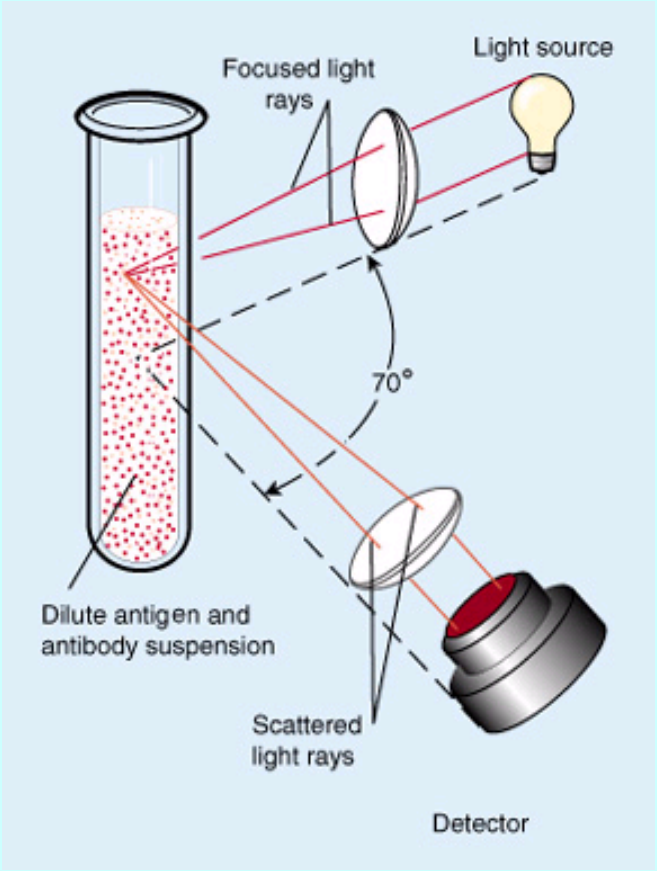
Nephelometry: Measurement of sideward light scatter after end of the antigen-antibody reaction or within the reaction (kinetic nephelometry).

Polyclonal and monoclonal antibodies in turbidimetry and nephelometry



Serum (IgG) antibodies are bivalent in their reactions with antigen and have the capacity to crosslink antigens; antigens are often multivalent in their interactions with antibodies; serum antibodies are typically polyclonal in nature; and antibodies are highly specific in terms of the structures they recognize on antigenic molecules.

Nephelometry

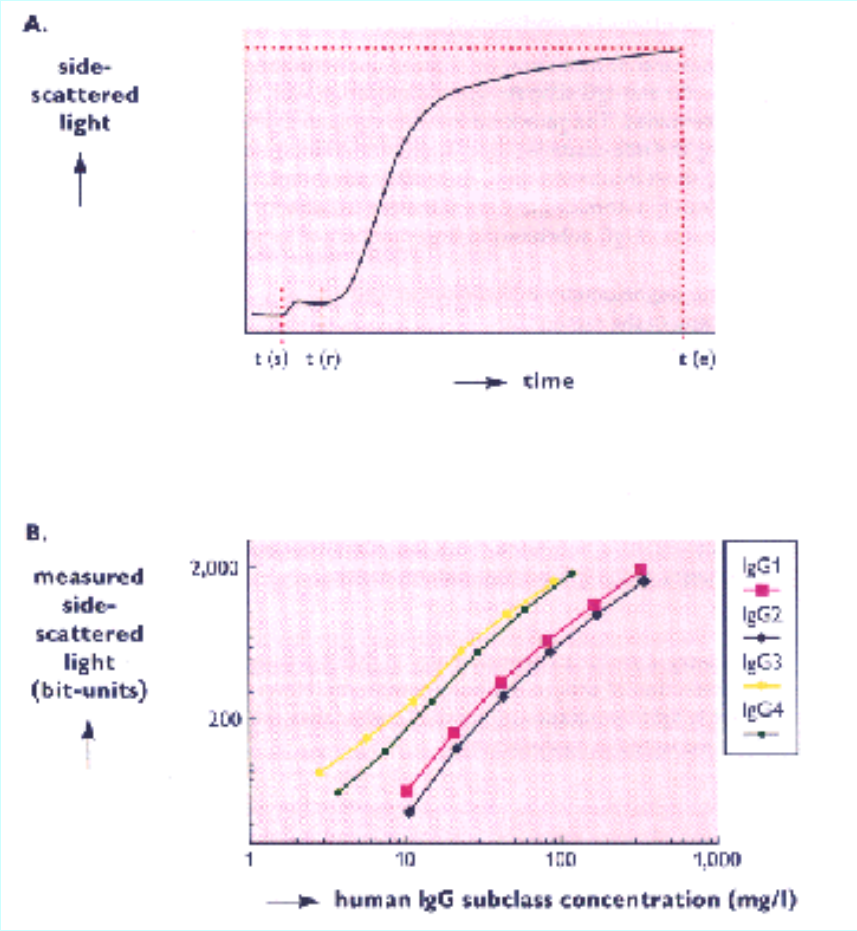


Nephelometry

This technique is widely used in clinical laboratories because it is relatively easily automated. It is based on the principle that a dilute suspension of small particles will scatter light (usually a laser) passed through it rather than simply absorbing it. The amount of scatter is determined by collecting the light at an angle (usually about 70 or 75 degrees).

Antibody and the antigen are mixed in concentrations such that only small aggregates are formed that do not quickly settle to the bottom. The amount of light scatter is measured and compared to the amount of scatter from known mixtures. The amount of the unknown is determined from a standard curve.

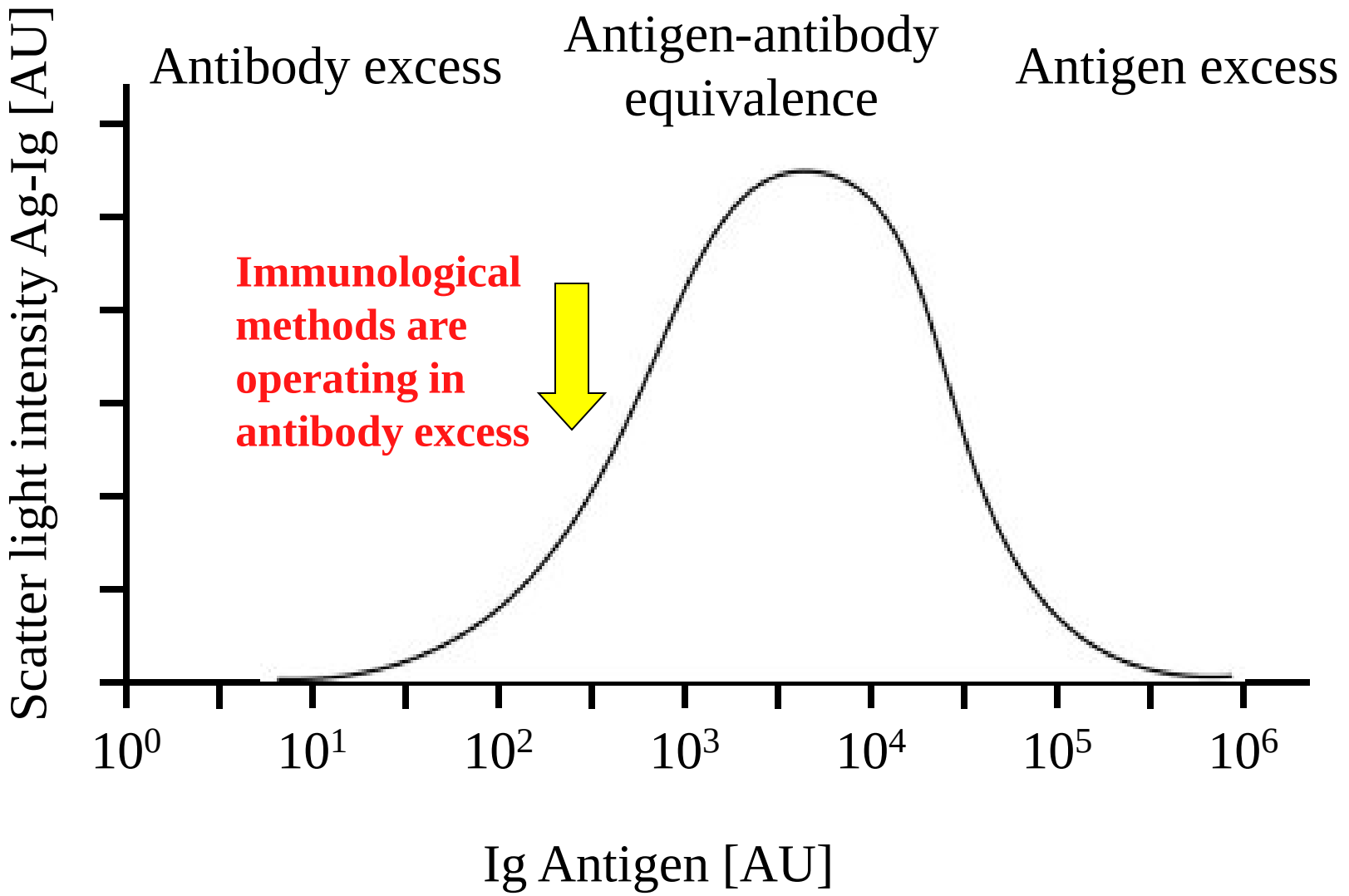
Reaction kinetics in turbidimetry and nephelometry



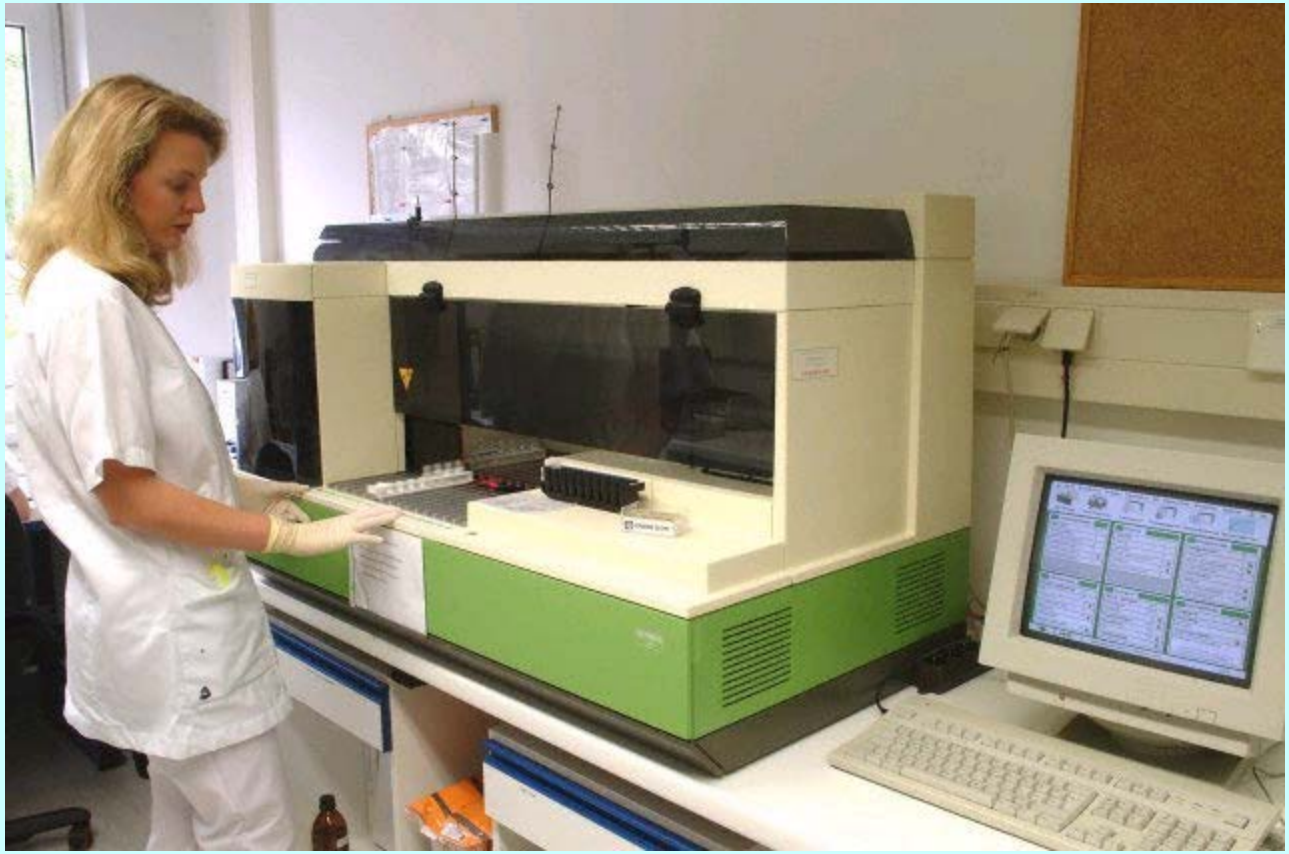
Top: The intensity of the antigen-antibody reaction depends on (1.) the concentrations of antigen and antibody, the affinity of the antibody to the specific epitpes, the type of antibody (e. g. subclass) the ionic strength of the solution (e. g. LISS) and the temperature. After calibration the kinetics of the reaction can be used for analytical purposes (kinetic nephelometry).

Bottom: Differences in the reaction of various IgG subclasses.

Heidelberger-Kendall-curve

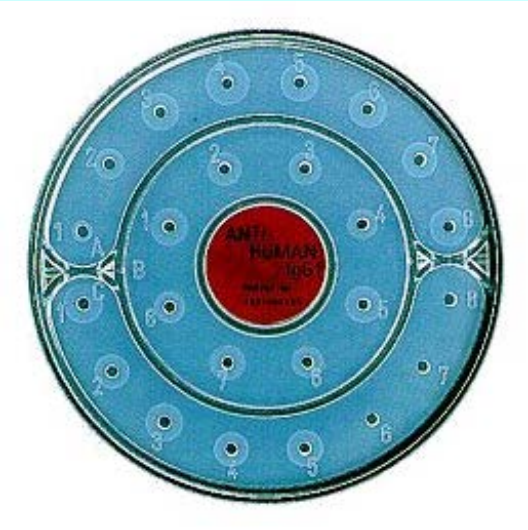


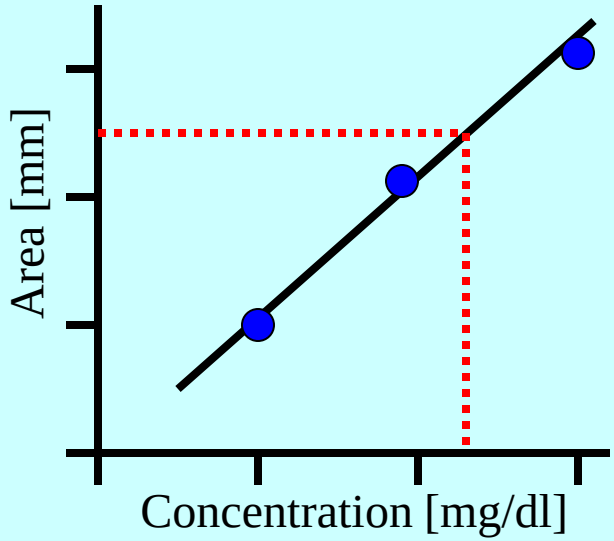
Nephelometry



Dade-Behring Nephelometer II

Radial immunodiffusion (Mancini) - I



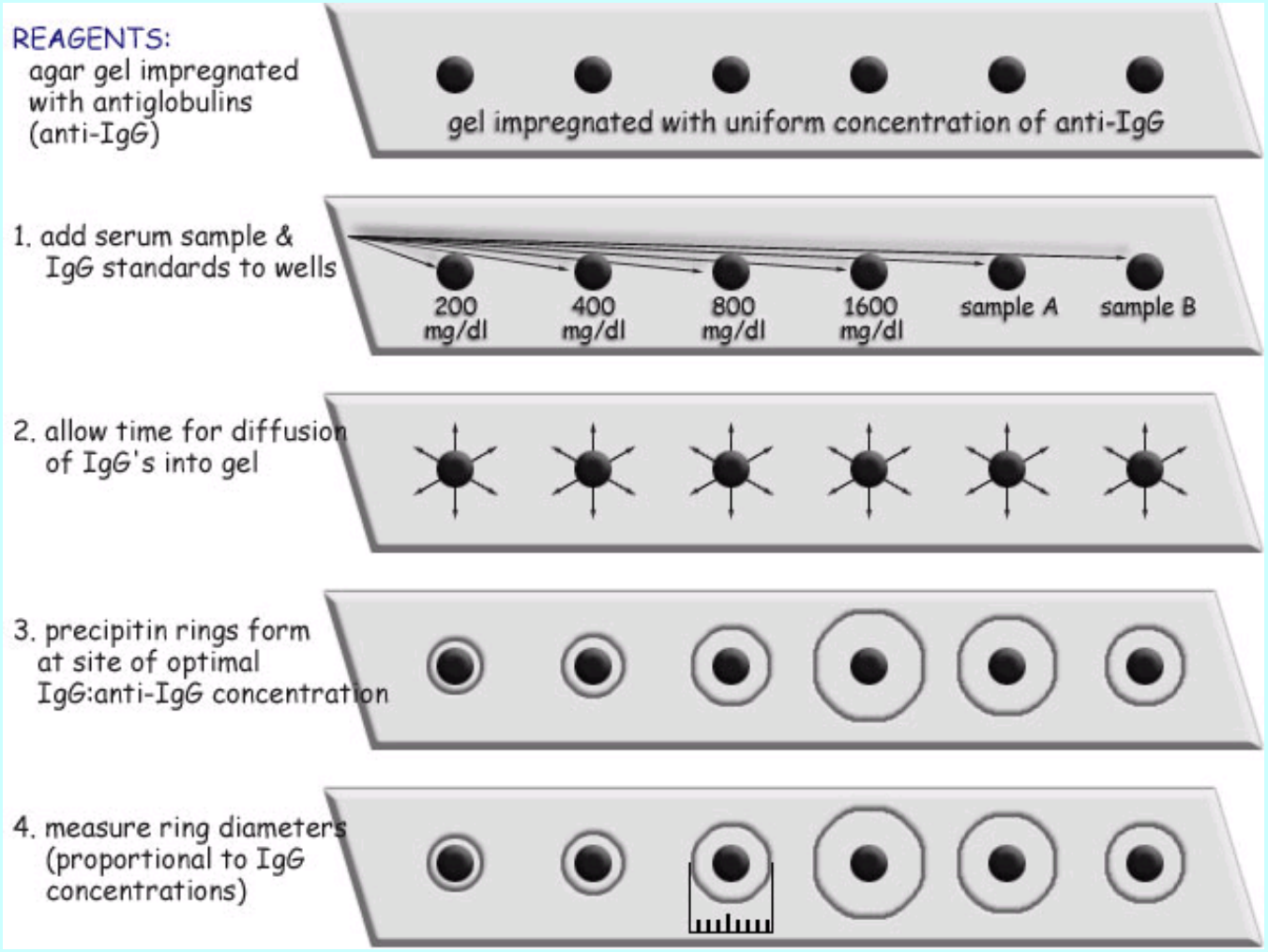


The RID assay is performed in (ready-for-use) agar plates, containing a specific antibody against ferritin. Test samples, standard sera and control sera are prepared and added to the plates. After 48-64 hours incubation at room temperature the diameters of the immunoprecipitation rings are measured. The ferritin concentrations in the test samples may be quantified in two ways:

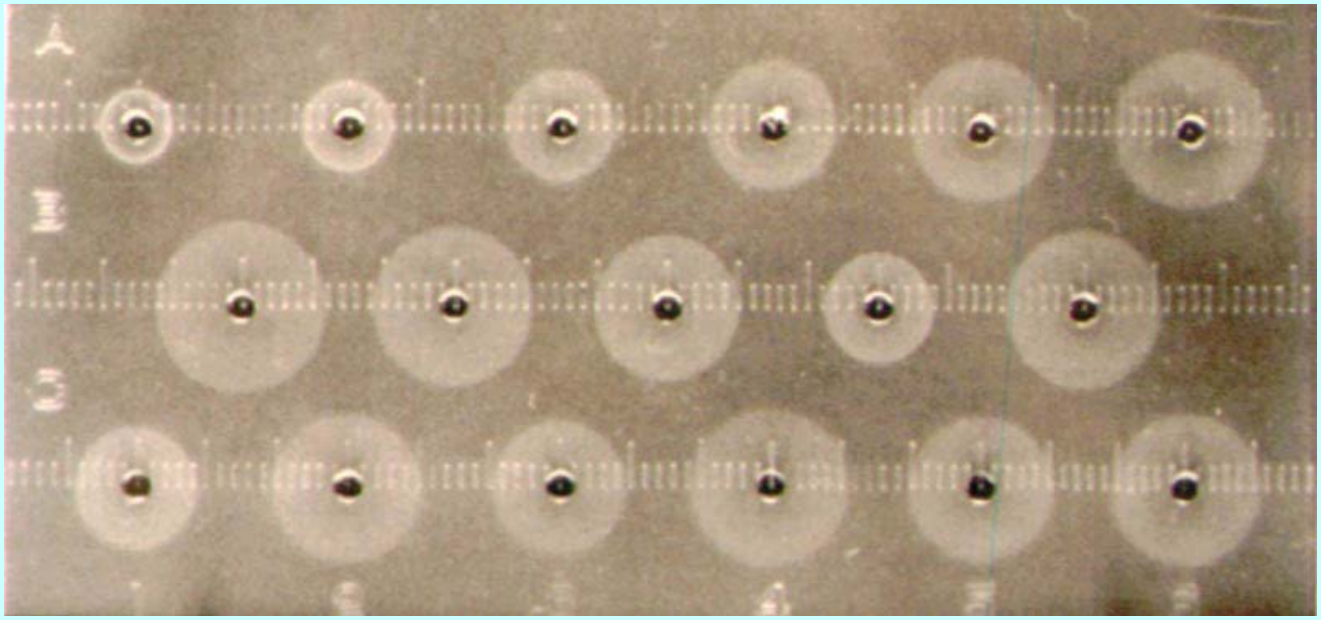
Calibration curve method: Ring diameters and concentrations of the standards are plotted and the values of the test sample are determined by interpolation.

Tabular method: Ring diameters of the calibration curve are listed and the values of the test sample are read from a table. It is not necessary to make a calibration curve. The control serum is assayed to check the validity of the calibration curves and also the accuracy of the test.

Radial immunodiffusion (Mancini) - II



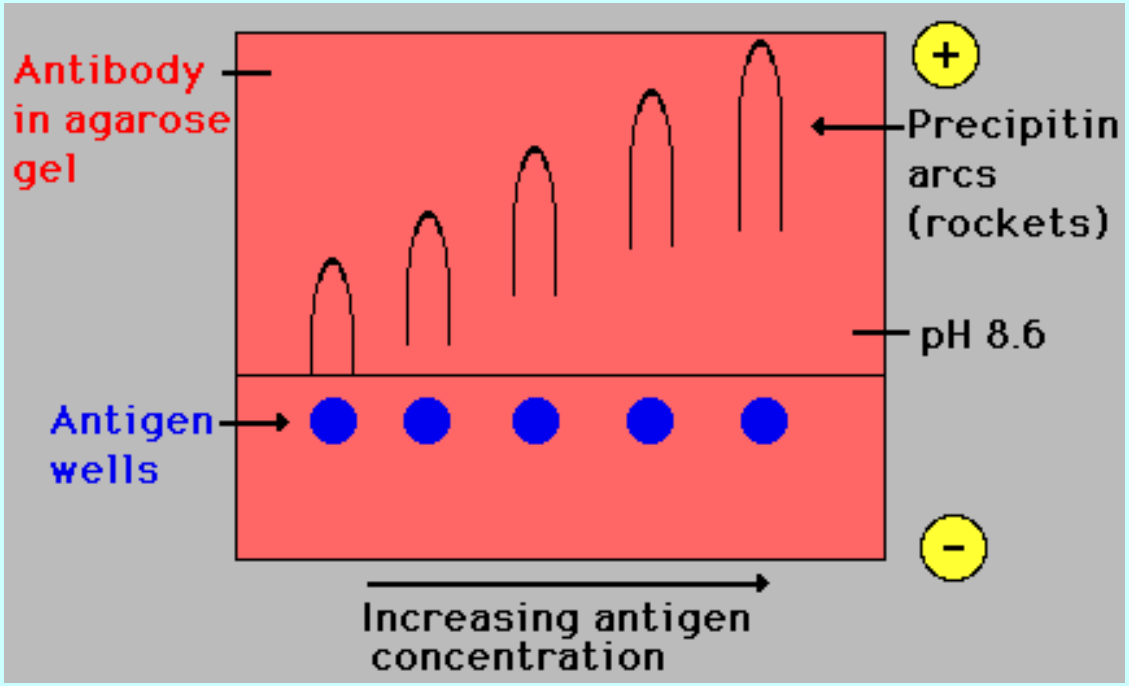
Radial immunodiffusion (Mancini) - III



This technique is used to determine the concentration of an antigen, in this case horse IgG. This assay (like Ouchterlony) is also carried out in a thin layer of agarose, but here an antibody specific for horse IgG is mixed into the agarose when the plate is prepared, and samples of horse serum are then placed into each of the small visible wells. As the IgG diffuses outward it forms a precipitate with the antibody present throughout the agarose; the area of the circle of precipitate can be measured and is directly related to the concentration of antigen (IgG) in the sample.

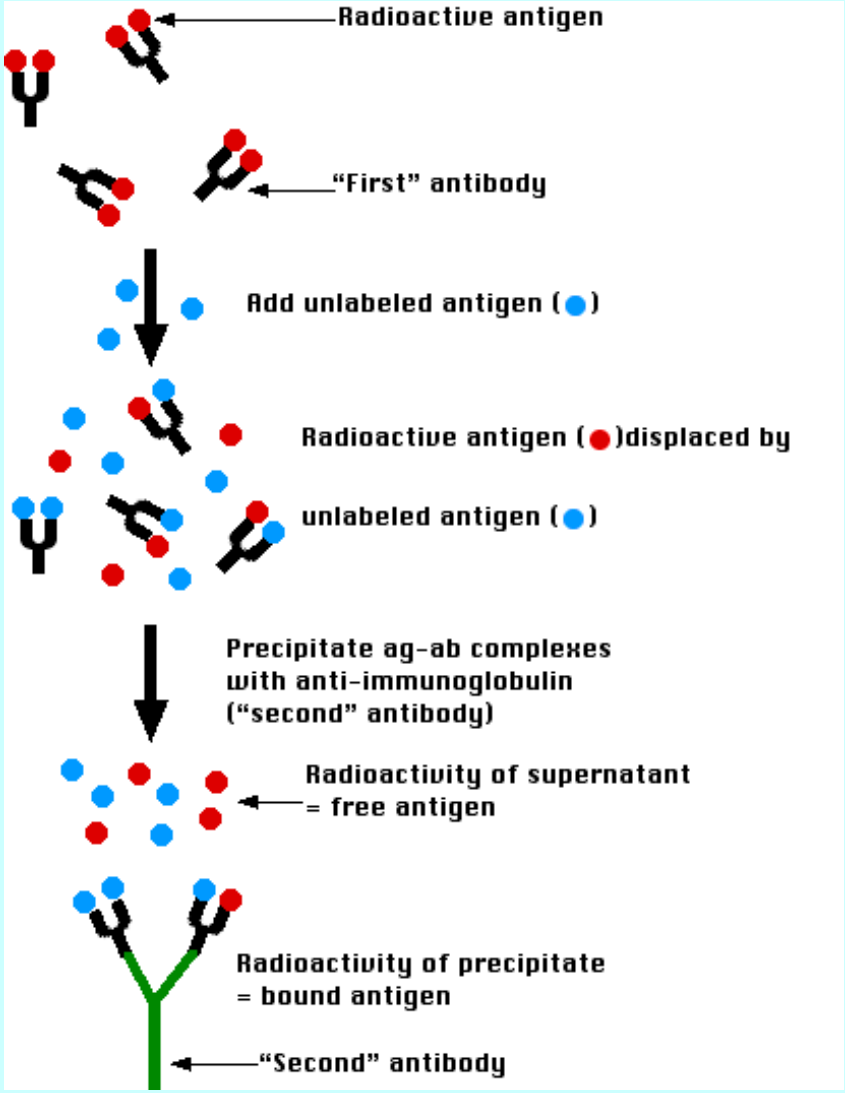
The top row of wells contains a set of standards of increasing concentration of horse IgG (from left to right), the other wells are test samples which can be compared with the standards to determine their concentration of IgG.

Rocket electrophoresis



Antigen is electrophoresed into gel containing antibody. The distance from the starting well to the front of the rocket shaped arc is related to antigen concentration.

Radioimmunoassay (RIA) - I

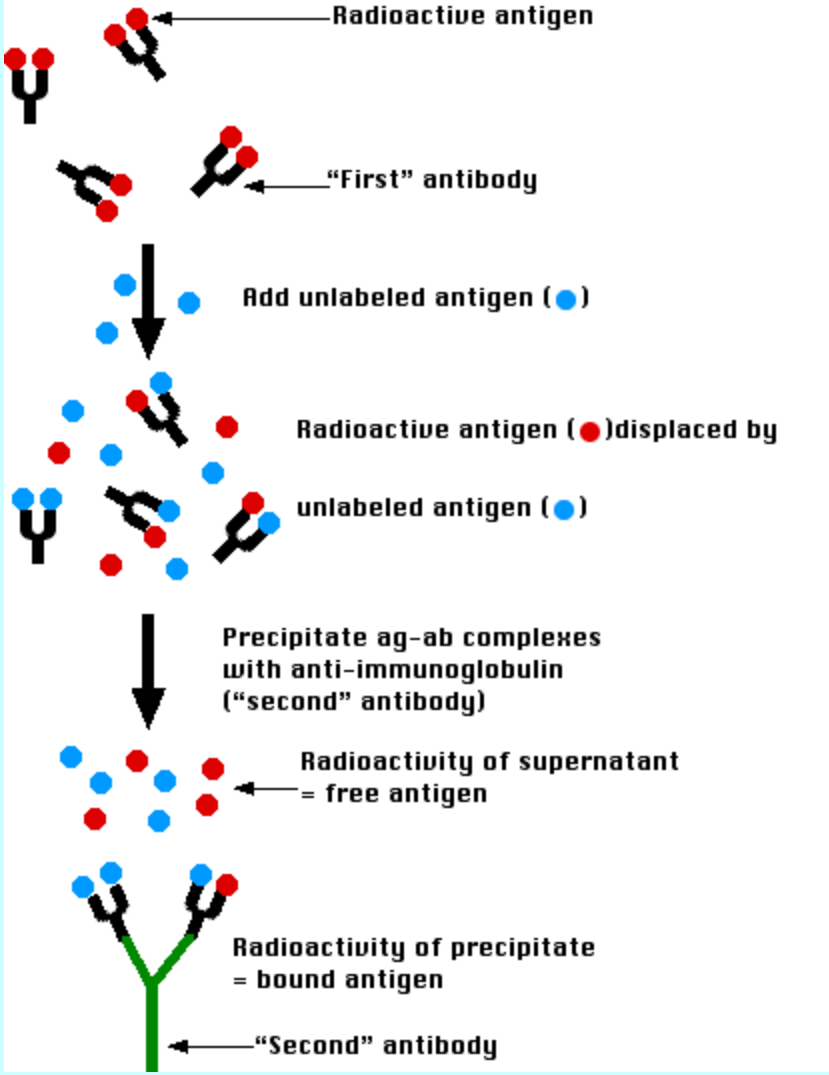


The technique was introduced in 1960 by Berson and Yalow as an assay for the concentration of insulin in plasma. It represented the first time that hormone levels in the blood could be detected by an in vitro assay.

The technique

A mixture is prepared of radioactive antigen and antibodies against this antigen. Because of the ease with which iodine atoms can be introduced into tyrosine residues in a protein, the radioactive isotopes ^{125}I or ^{131}I are often used.

Radioimmunoassay (RIA) - II



The technique (continued):

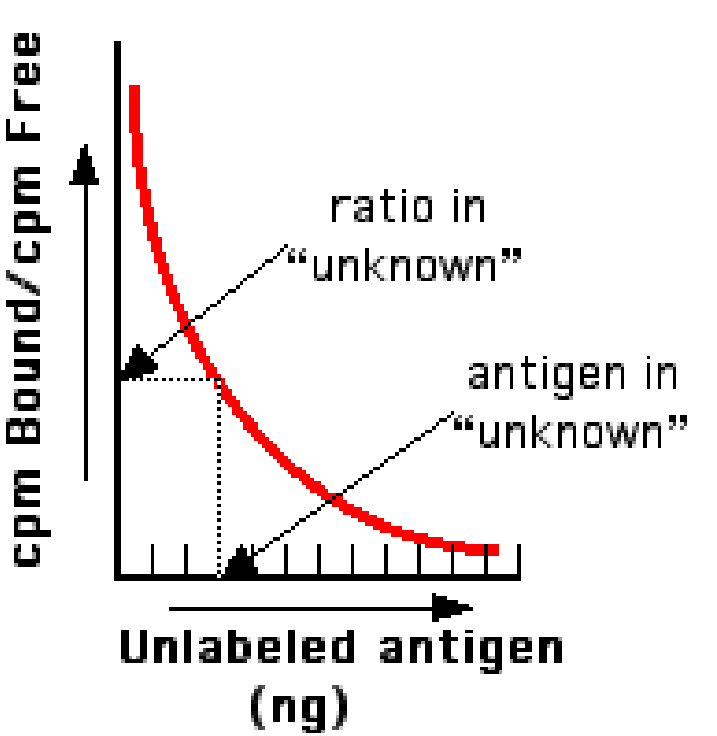
Known amounts of unlabeled ("cold") antigen are added to samples of the mixture. These compete for the binding sites of the antibodies.

At increasing concentrations of unlabeled antigen, an increasing amount of radioactive antigen is displaced from the antibody molecules.

The antibody-bound antigen is separated from the free antigen in the supernatant fluid, and the radioactivity of each is measured.

From these data, a standard binding curve, like this one shown in red, can be drawn.

Radioimmunoassay (RIA) - III



Calculation of the standard curve:

The samples to be assayed (the unknowns) are run in parallel. After determining the ratio of bound to free antigen in each unknown, the antigen concentrations can be read directly from the standard curve.

Radioimmunoassay (RIA) - IV

Separation of bound and free antigen:

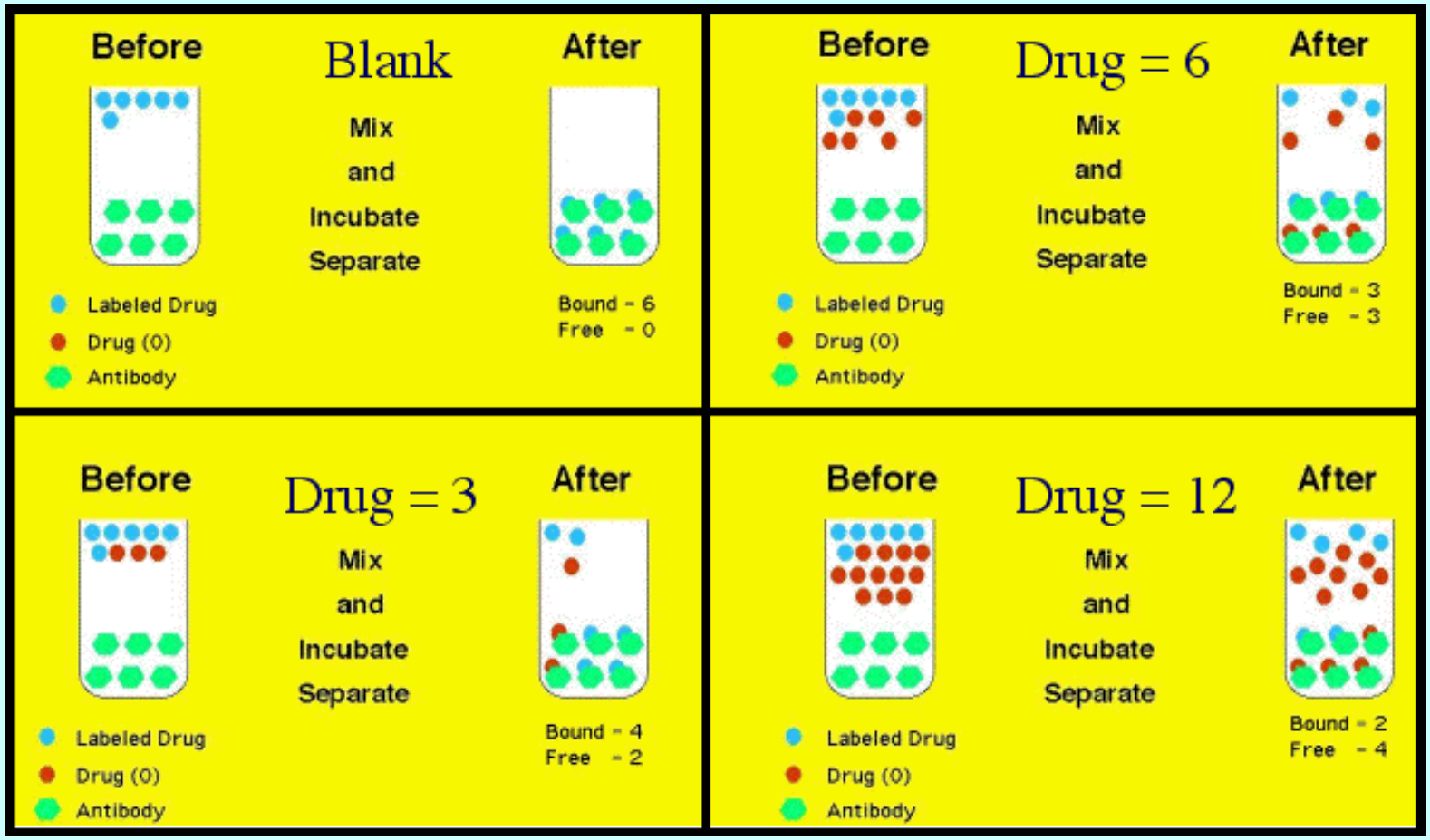
There are several ways of doing this:

(1.) Precipitate the antigen-antibody complexes by adding a "second" antibody directed against the first. For example, if a rabbit IgG is used to bind the antigen, the complex can be precipitated by adding an antirabbit-IgG antiserum (e.g., raised by immunizing a goat with rabbit IgG). This is the method shown in the diagram before.

(2.) The antigen-specific antibodies can be coupled to the inner walls of a test tube. After incubation, the contents ("free") are removed, the tube is washed ("bound"), and the radioactive of both is measured.

(3.) The antigen-specific antibodies can be coupled to particles, like Sephadex. Centrifugation of the reaction mixture separates the bound counts (in the pellet) from and the free counts in the supernatant fluid.

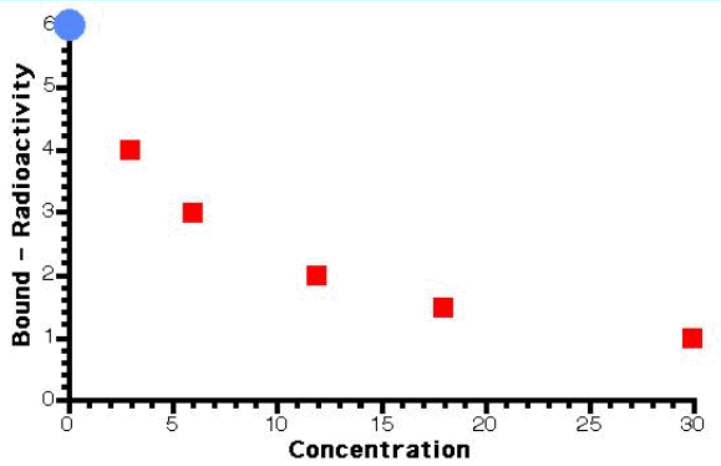
Radioimmunoassay (RIA) - V

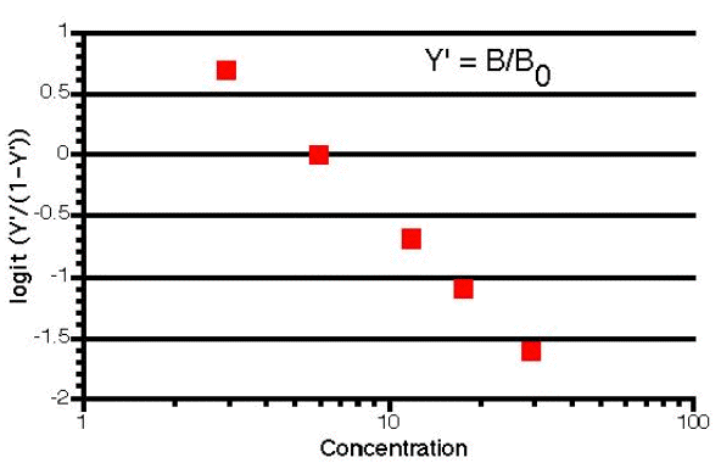


Example:

RIA before and after incubation; blank and three standard samples.

Radioimmunoassay (RIA) - VI





Bound and free drug concentrations

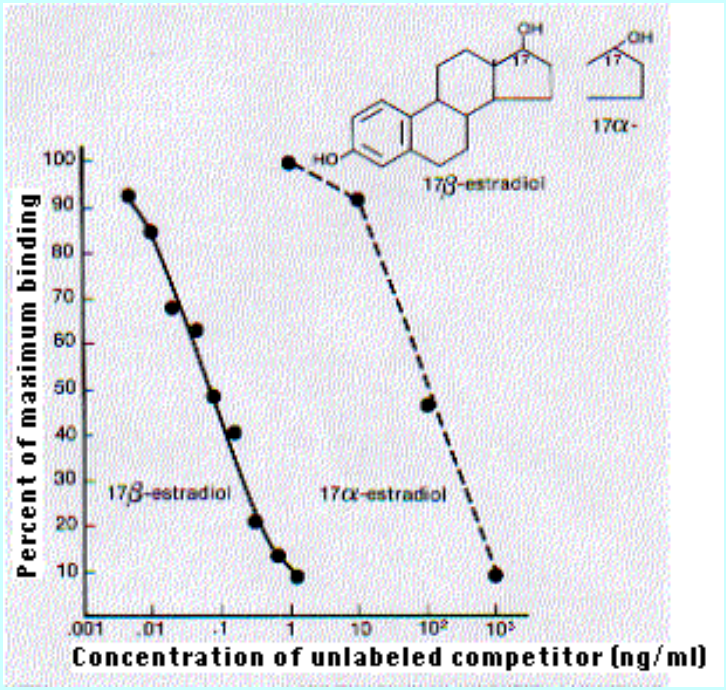
Total drug	Bound drug	Free drug drug
0	6	0
3	4	2
6	3	3
12	2	4

Top right: Table from the data given in the example before.

Top left: Plot of bound versus total drug concentration.

Bottom left: Logit versus log total C plot.

Radioimmunoassay (RIA) - VII



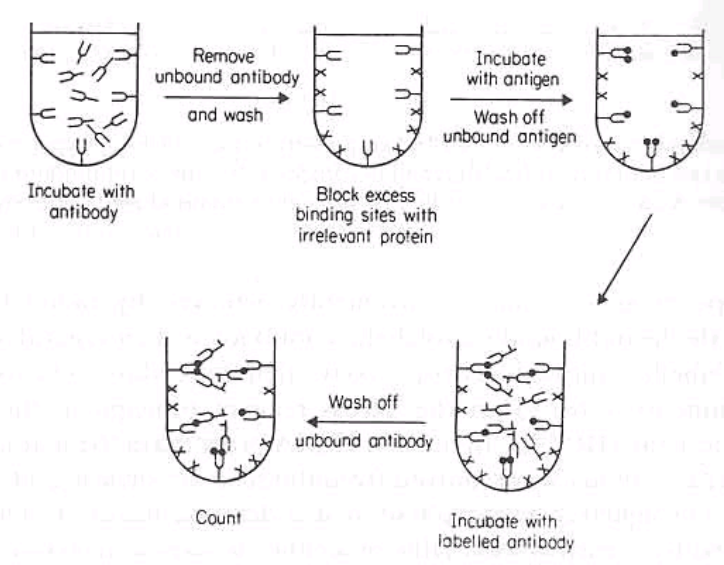
Sensitivity and specificity:

Radioimmunoassays are widely-used because of their great sensitivity. Using antibodies of high affinity ($K_0 = 10^8$–10^{11} M^{-1}), it is possible to detect a few picograms (10^{-12} g) of antigen in the tube.

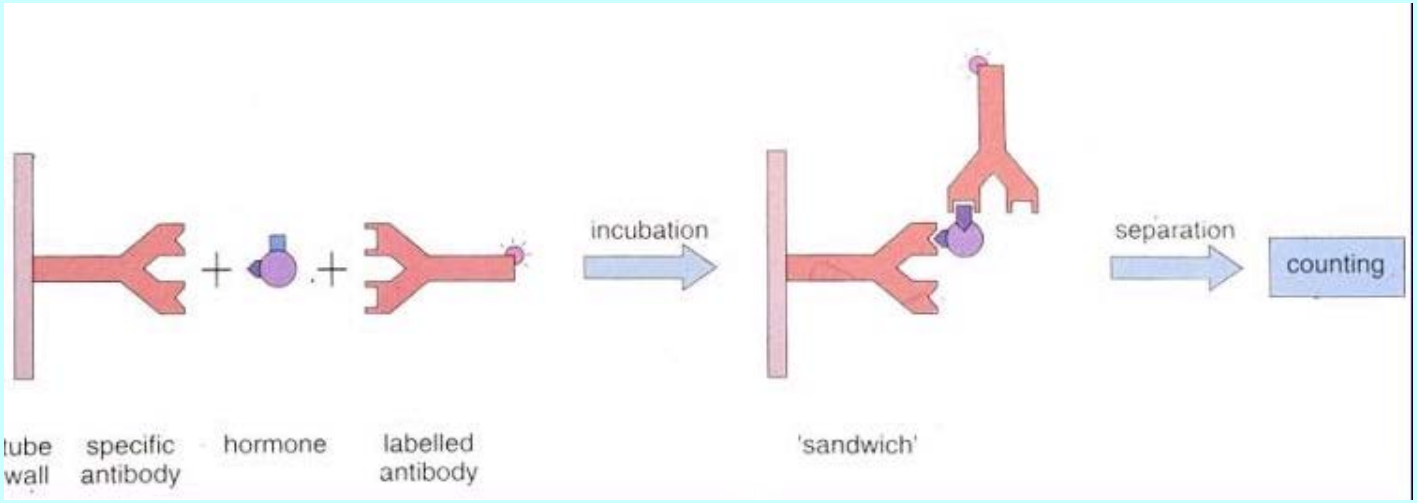
The specificity of antibodies can be so precise that they are able to discriminate between enantiomers of the same molecule. The major estrogen found in women of reproductive age is 17β-estradiol.

But as this graph shows, the shift of the hydroxyl group on carbon 17 from the beta position (extending above the plane of the molecule) to the alpha position (extending below) lowers by 1000-fold the affinity of the molecule for antibodies raised against 17β-estradiol.

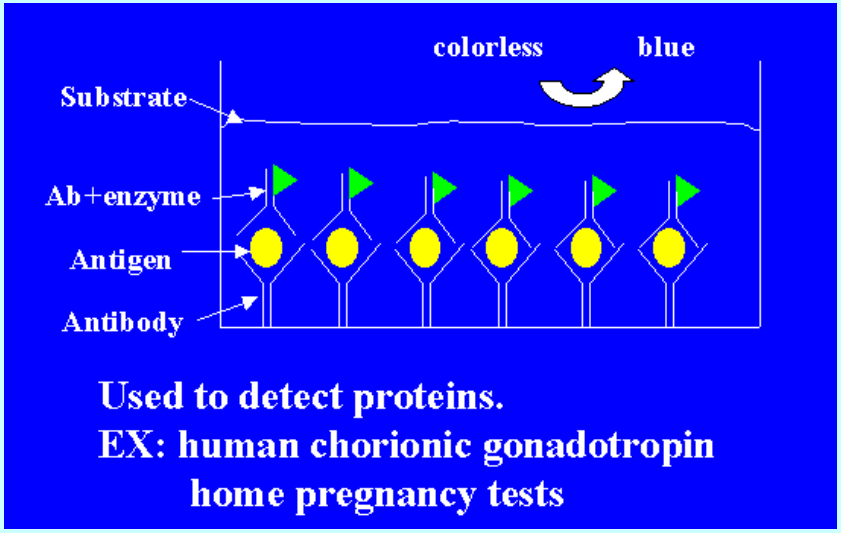
Immunoradiometric assay (IRMA)

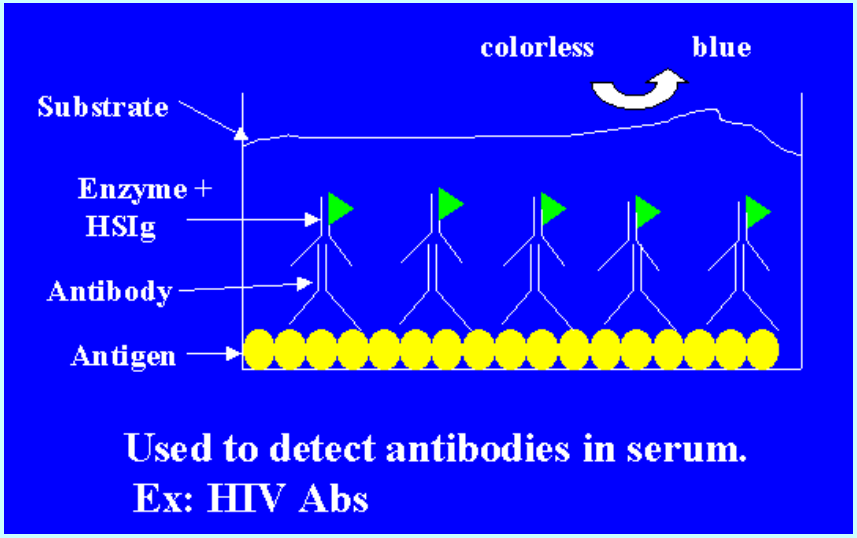


Principle of an IRMA: (1.) Primary antibody is bound to the tube surface; (2.) Excess binding sites are blocked (e. g. with BSA); (3.) Incubation with the sample - antigen is bound to the primary antibody; (4.) Labelled secondary antibody is added; (5.) Excess of labelled secondary antibody is removed by washing and the signal intensity is determined by a gamma-counter.



Non-competitive enzyme linked immuno sorbent assay (ELISA)





Well coated with primary antibodies against a protein

Protein (e. g. from serum) is captured

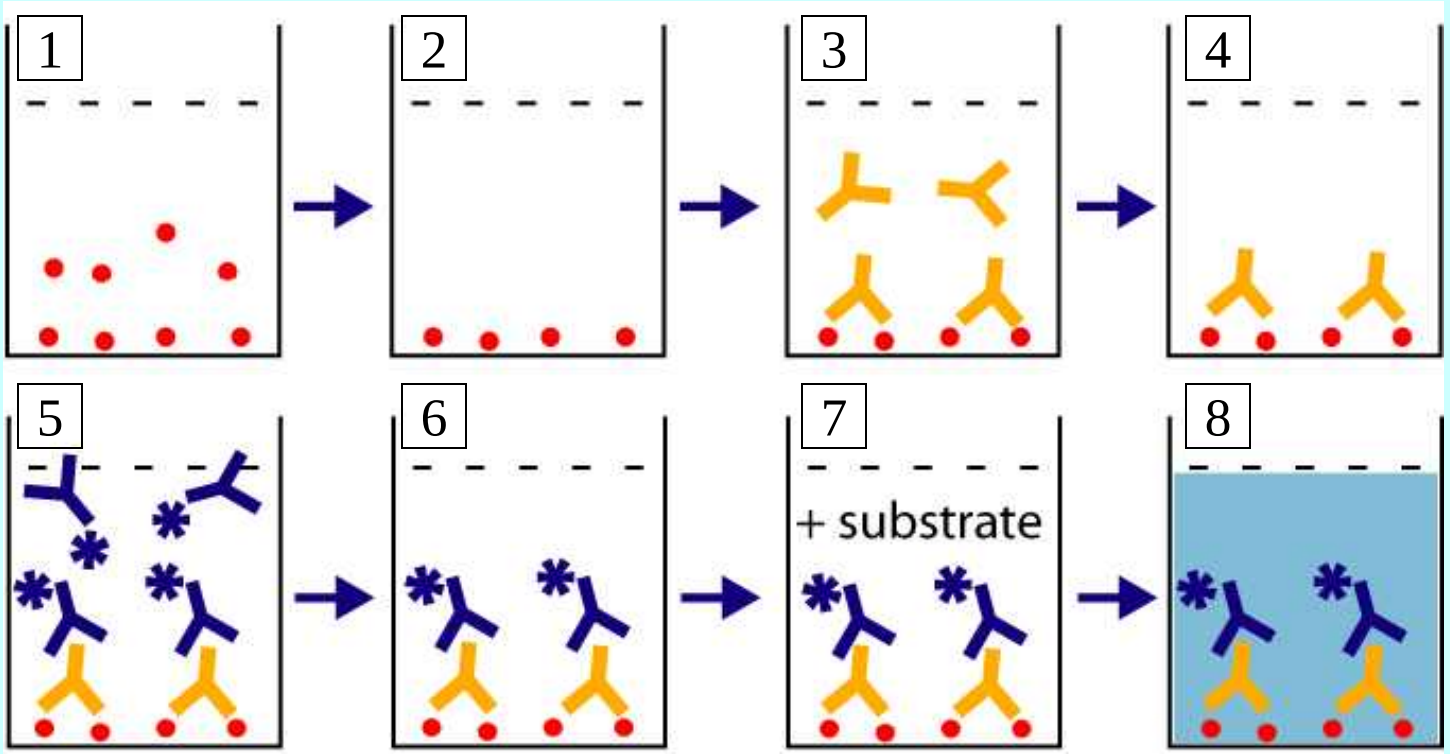
Secondary labelled antibody binds to bound proteins

Well coated with antigen (e. g. virus)

Antibodies against these antigen (e. g. from serum) are captured

Secondary labelled antibody binds to bound antibodies

Principle of an ELISA method



Principle of a sandwich elisa: (1.) Wells are coated with antigen; (2.) Excess of antigen is removed by washing; (3.) Antibodies from the sample are allowed to bind to the antigen; (4.) Unbound antibodies are removed by washing; (5.) Labelled secondary (detection) antibody is allowed to bind to the primary antibody; (6.) Excess of secondary antigen is removed by washing; (7.) Substrate solution is added and incubated; (8.) After end of incubation the colour reaction ist stopped.

Serological tests

Reactions between antigen and antibody, but one must be known to determine the other. The test can be qualitative or quantitative.

Neutralization - Antigen and antibody neutralize each other. This is used for identification of toxins, antitoxins, viruses, antibodies etc..

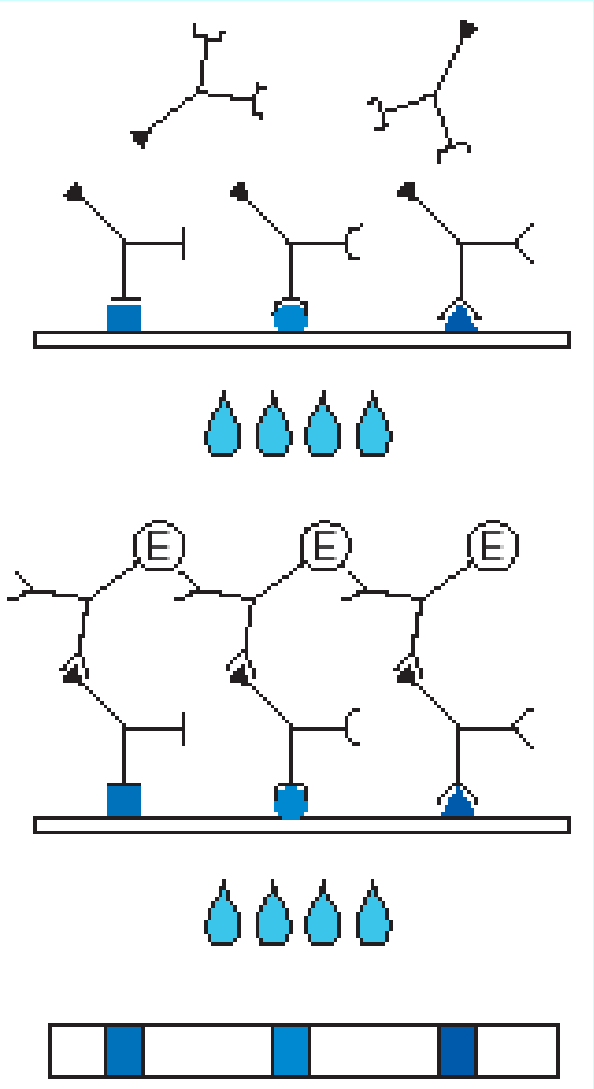
Precipitation - Antigen and antibody cross link at multiple sites to form a lattice that precipitates.

Agglutination - Antigen and antibody react on the surface of objects or cells causing clumping.

Immunodiffusion - Antigen and antibody diffuse and react to form lines of precipitate.

Westernblot - Specific detection of antigens (e. g. antibodies against bacterial or viral proteins; HIV confirmation test).

Strip immuno assays - I



1st Incubation:

A test strip loaded with Yersinia antigens is incubated with diluted serum or plasma in a dish for 1 h. Wash 3 times.

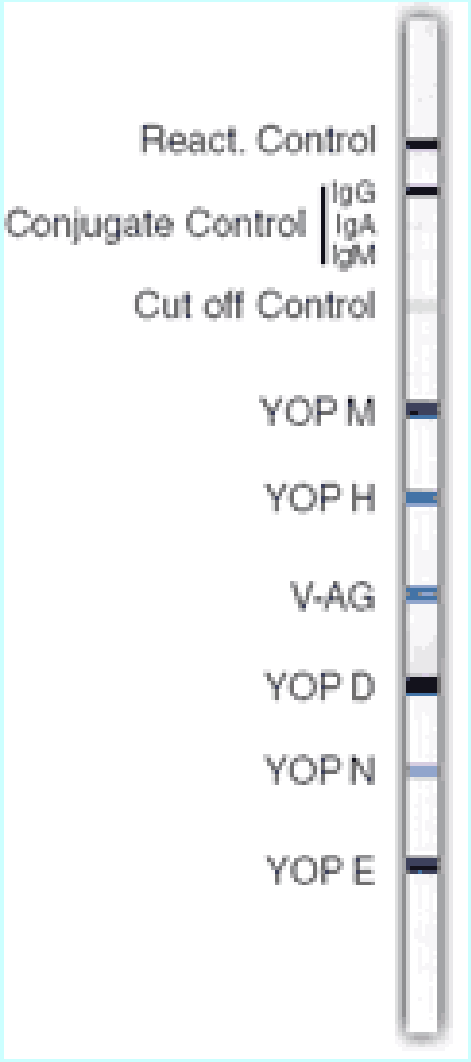
2nd Incubation:

Peroxidase conjugated anti-human antibodies (IgG, IgM or IgA specific) are added. Incubate for 45 min. Wash 3 times

3rd Incubation:

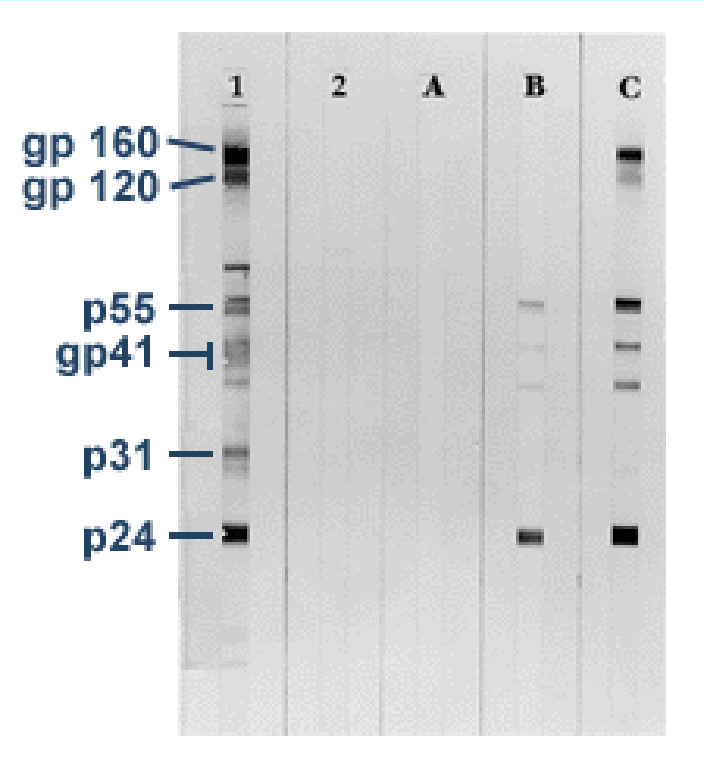
5 - 10 minutes after addition of the coloring solution, insoluble colored bands develop at the sites on the test strips occupied by antibodies.

Strip immuno assays - II



Strip-Immunoassay with antigens produced by recombinant techniques for the detection of IgG, IgM or IgA antibodies directed Y. enterocolitica and Y. pseudotuberculosis.

Strip immuno assays - III



Band pattern Interpretation:

Lane 1, HIV+ serum (positive control)
Lane 2, HIV- serum (negative control)
Lane A, Patient A
Lane B, Patient B
Lane C, Patient C

Western blot for HIV:

HIV, like any other virus, is composed of a number of different proteins. The Western Blot positive control lane contains proteins from patient sera as well as HIV proteins. HIV positivity can therefore only be confirmed by the presence of the following types of proteins:

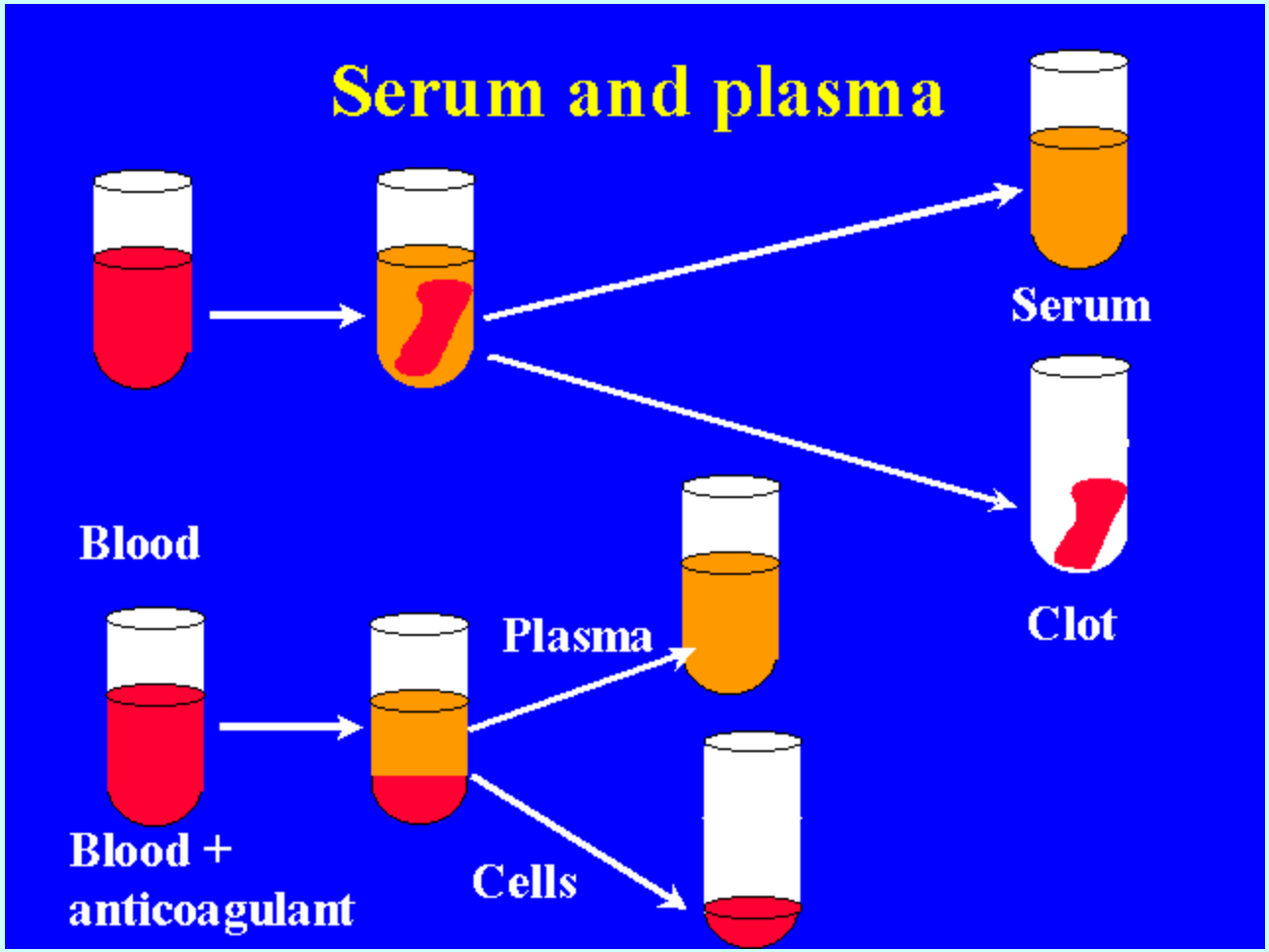
gp160	viral envelope precursor (env)
gp120	viral envelope protein (env) binds to CD4
p24	viral core protein (gag)
p31	Reverse Transcriptase (pol)

Band Pattern Interpretation

Background information on the HIV Western blot test. In 1987 the Centers for Disease Control along with several other organizations established criteria for serologic interpretation of HIV Western blot tests. The criteria are:

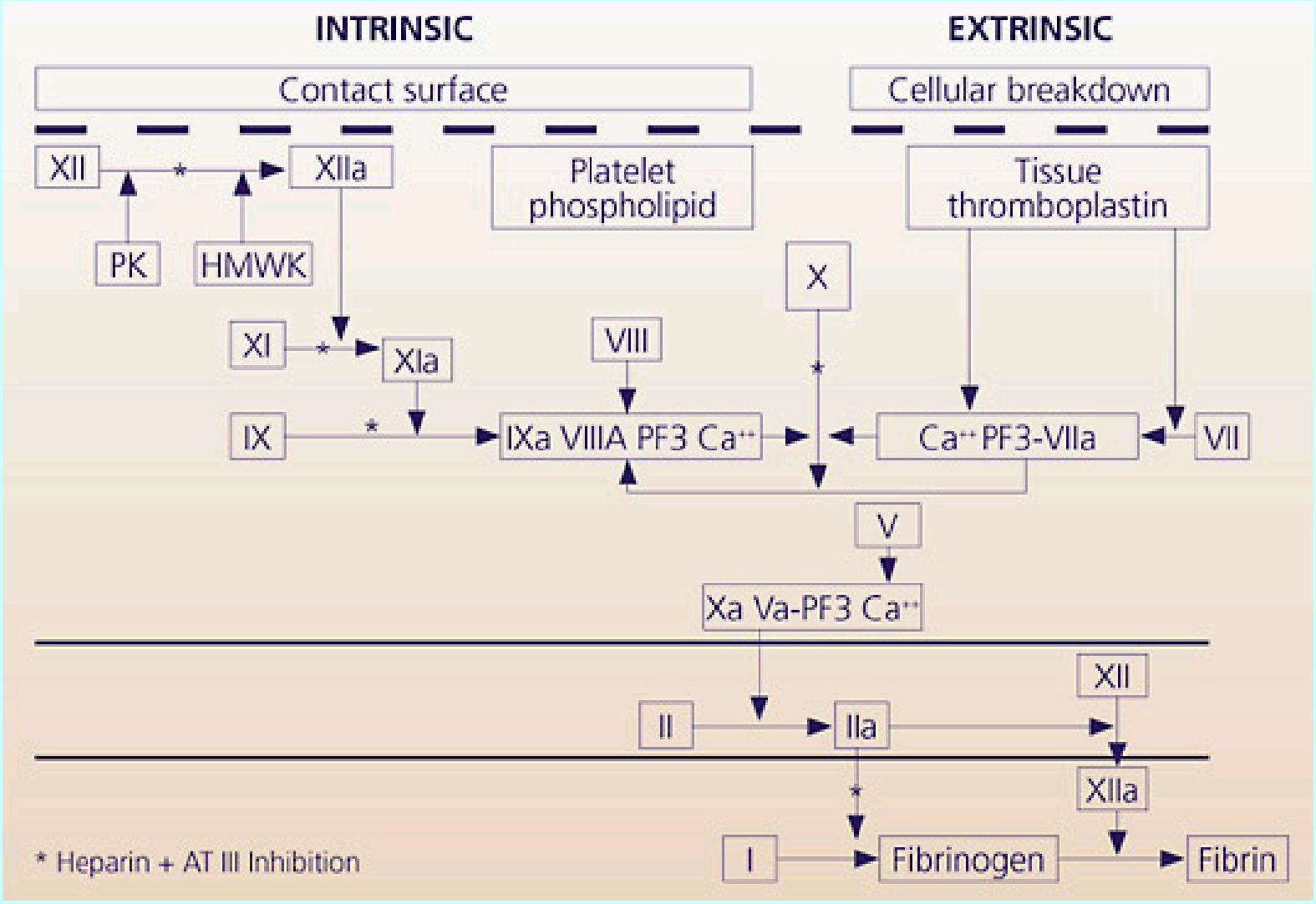
No bands present: Negative
Bands at either p31 OR p24 and bands present at either gp160 OR gp120: Positive
Bands present, but pattern does not meet criteria for positivity: Indeterminate.

Serum and plasma



Serum contains no relevant amounts of clotting factors (e. g. fibrinogen). In addition, some analytes may be found in increased concentrations because of hemolysis and secretion from platelets within the clotting process (e. g. hemoglobin, serotonine).

Clotting system

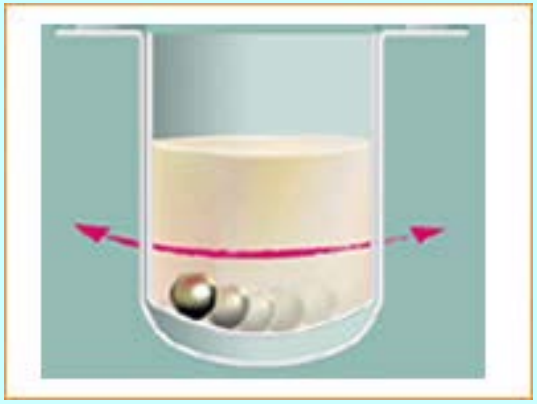


Analyzes of the clotting system

Analyzes of the clotting system are functional tests determining the activity of the extrinsic system, the intrinsic system or distinct factors (e. g. factor VIII).

Therefore, special analyzers ar required. However, immunological analyzes play also a role (e. g. factor VIII, fibrinogen).

Technical principles of clotting analyzers



Electromechanical measuring principle:

Coagulometric determinations based on electromechanical measuring principle. This method has no interference in lipemic, icteric and hemolytic samples and delivers reliable results also in the presence of blood substitution products

Photometric measuring principle:

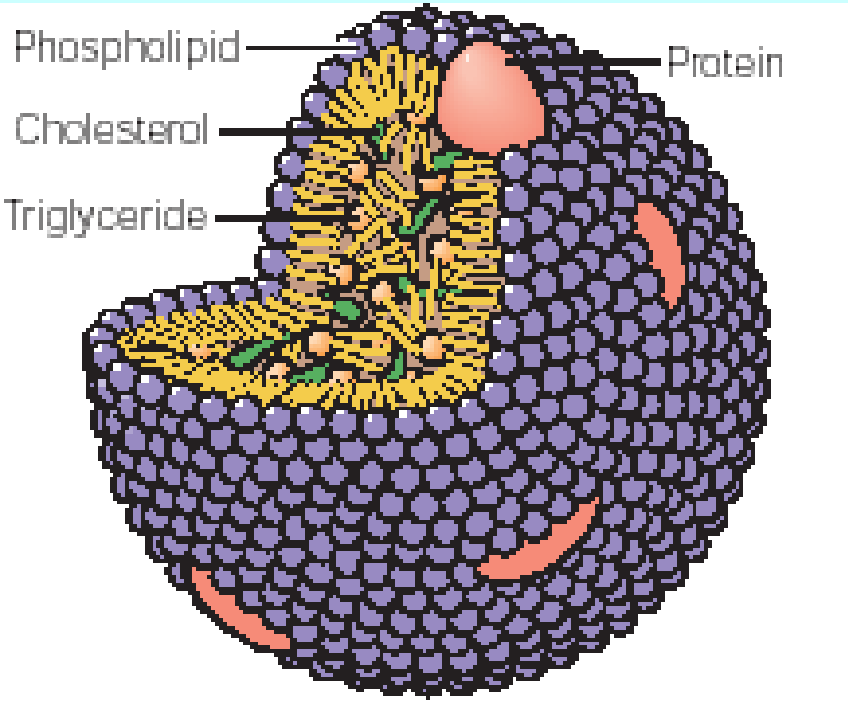
Photometric determinations with chromogenic substrates and for the quantification of specific antigen-antibody reactions (immunoassay)

Clotting analysis

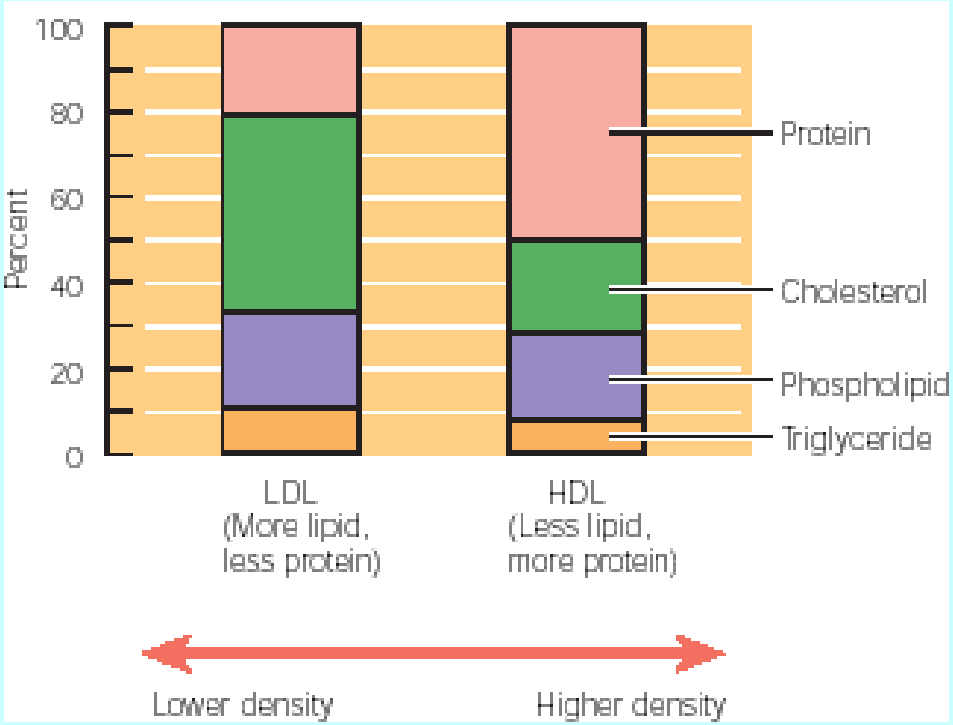
Left: Sigma AMGA

Right: Sigma AMAX

Lipoproteins



Model of a lipoprotein



LDL: Low density lipoprotein

HDL: High density lipoprotein

Lipoprotein analytics

Electrophoresis (α, pre-β, β-lipoproteins)

Clinical chemistry (e. g. cholesterol, triglycerides)

Immunological methods (e. g. ApoA-I, apoB)

Ultracentrifugation (VLDL, LDL, HDL)

Preparative ultracentrifugation of lipoproteins - I

Top left: Ultracentrifuge

Bottom left: Rotor for ultracentrifugation

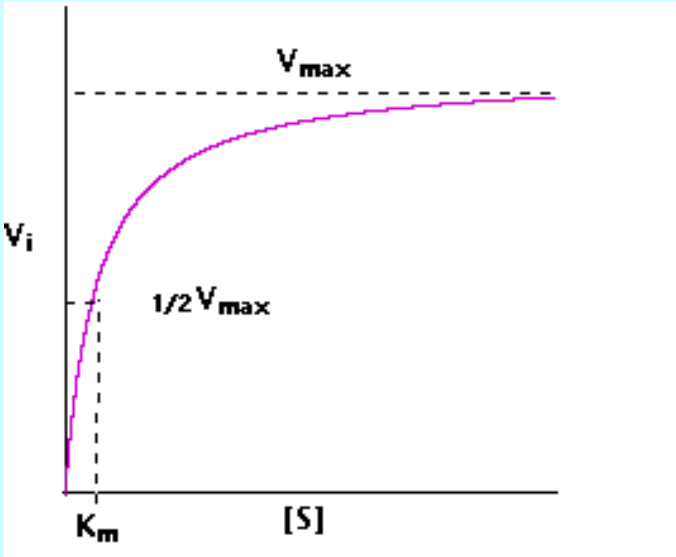
Right: Tubes for ultracentrifugation

Enzymes in clinical chemistry

Determination of substrate concentration (e. g. glucose) in biological fluids (e. g. serum) by means of enzymatic reactions.

Determination of enzyme activity (e. g. lactate dehydrogenase, LDH) in biological fluids (e. g. serum) by means of enzymatic reactions.

Mechanisms of enzyme activity - I

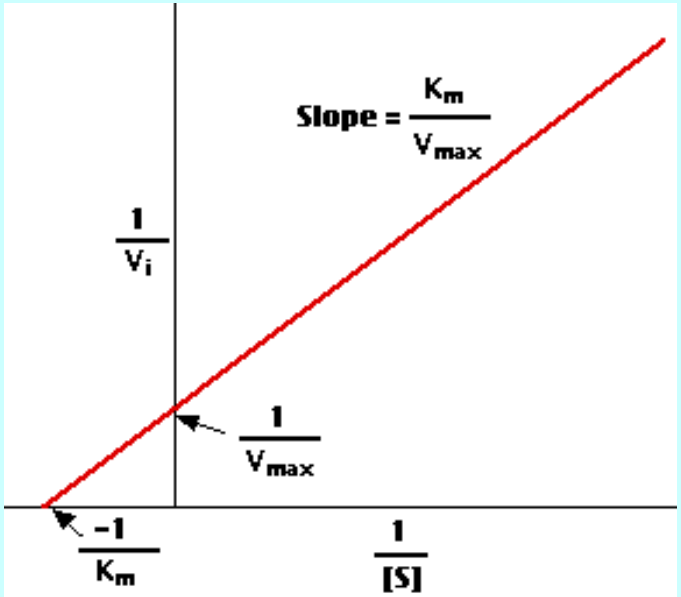


v_i: initial velocity (moles/time)

[S]: Substrate concentration (molar)

v_{max}: Maximum velocity

Km: Substrate concentration when v_i is one half v_{max}. (Michaelis-Menton constant)



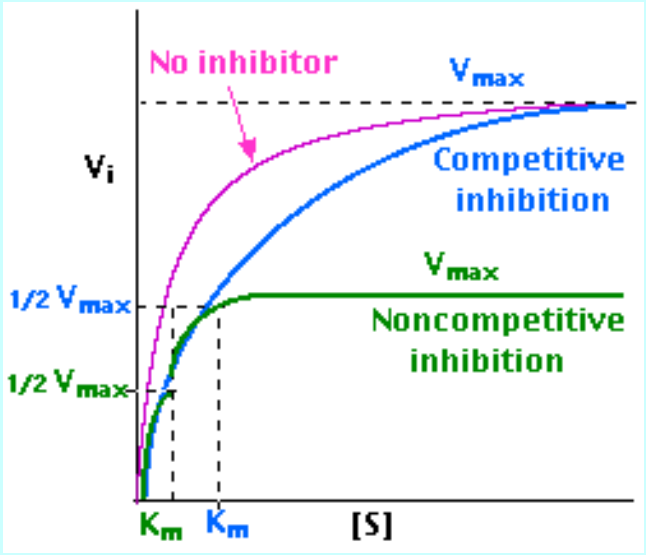
Plotting the reciprocals of the same data points yields a "double-reciprocal" or Lineweaver-Burk plot. This provides a more precise way to determine V_{max} and K_m.

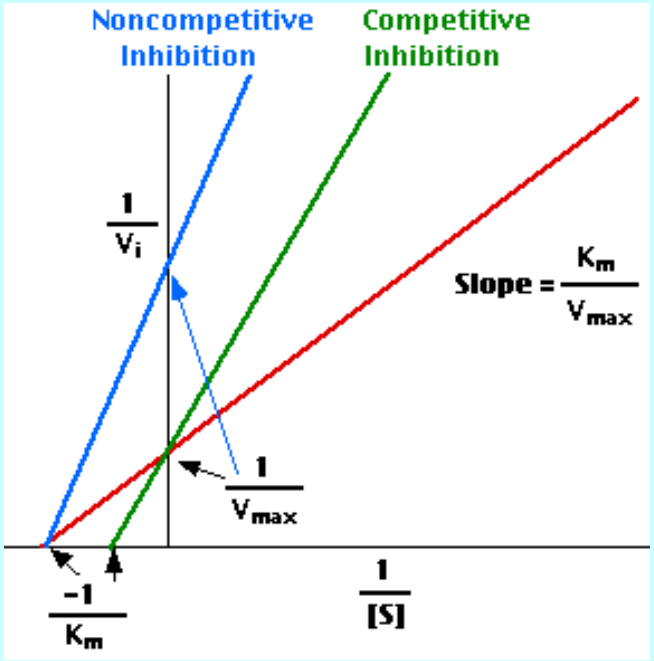
V_{max} is determined by the point where the line crosses the $1/V_i = 0$ axis (so the [S] is infinite).

Note that the magnitude represented by the data points in this plot decrease from lower left to upper right.

K_m equals V_{max} times the slope of line. This is easily determined from the intercept on the X axis.

Mechanisms of enzyme activity - II





Enzymes can be inhibited competitively, when the substrate and inhibitor compete for binding to the same active site or noncompetitively, when the inhibitor binds somewhere else on the enzyme molecule reducing its efficiency. The distinction can be determined by plotting enzyme activity with and without the inhibitor present.

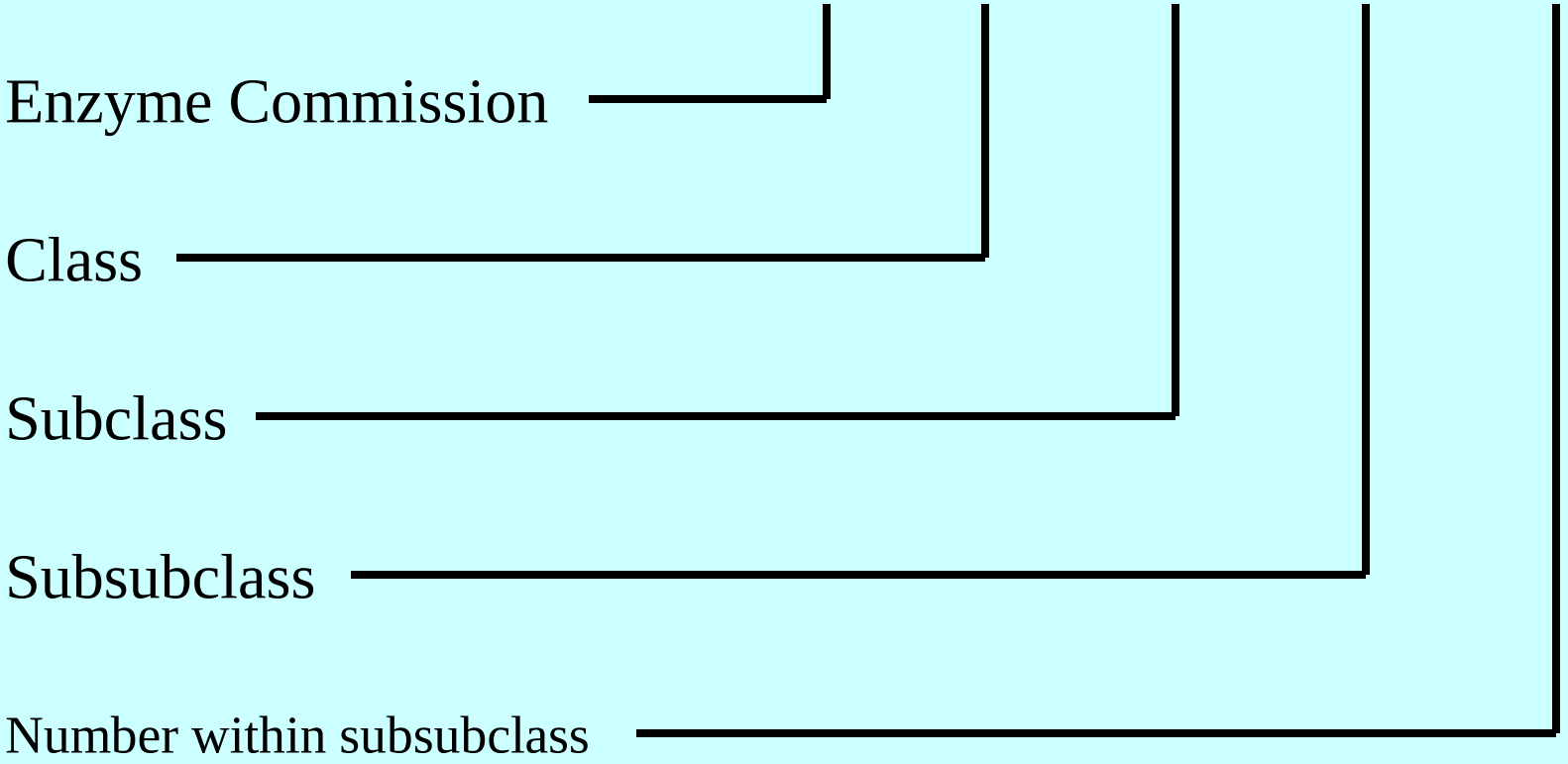
Competitive Inhibition: In the presence of a competitive inhibitor, it takes a higher substrate concentration to achieve the same velocities that were reached in its absence. So while V_{max} can still be reached if sufficient substrate is available, one-half V_{max} requires a higher [S] than before and thus K_m is larger.

Noncompetitive Inhibition: With noncompetitive inhibition, enzyme molecules that have been bound by the inhibitor are taken out of the game so enzyme rate (velocity) is reduced for all values of [S], including V_{max} and one-half V_{max} but K_m remains unchanged because the active site of those enzyme molecules that have not been inhibited is unchanged.

The Lineweaver-Burk plot displays these results.

International enzyme nomenclature – I

Hexokinase **EC 2. 7. 1. 1.**



International enzyme nomenclature – II

Class: 1. Oxidoreductases (e. g. LDH, GOD)

2. Transferases (e. g. ALAT, ASAT)

Subclasses: 2.1. C_1-groups

2.2. Aldehyde- or keto-groups

2.3. Acyl-groups

2.4. Glycosyl-groups

2.7. Phosphate-groups (e. g. hexokinase)

2.8. S-containing groups

3. Hydrolases (e. g. ALP)

4. Lyases (aldolase, pyruvatedecarboxylase)

5. Isomerases (e. g. phosphohexose-isomerase)

6. Ligases (e. g. acetyl-CoA-synthetase)

Determination of substrates by means of enzymatic reactions

Substrate concentration must be rate limiting (end point reaction).

Optimal pH-value and ionic strength of the reagent buffer.

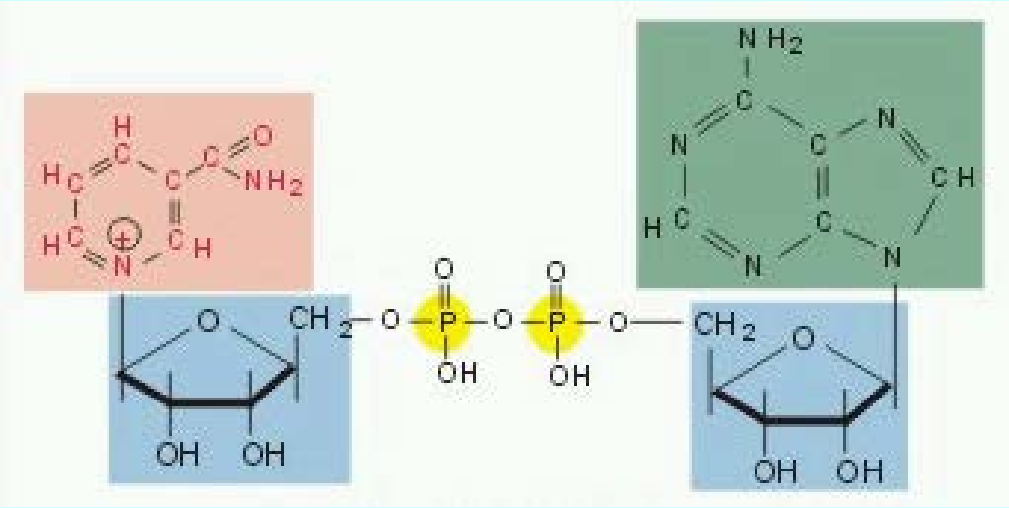
Enzymes and coenzymes (e. g. NADH, ATP) are added in excess.

Optimal temperature for ezyme kinetics (standardization not critical because reaction are endpoint reactions).

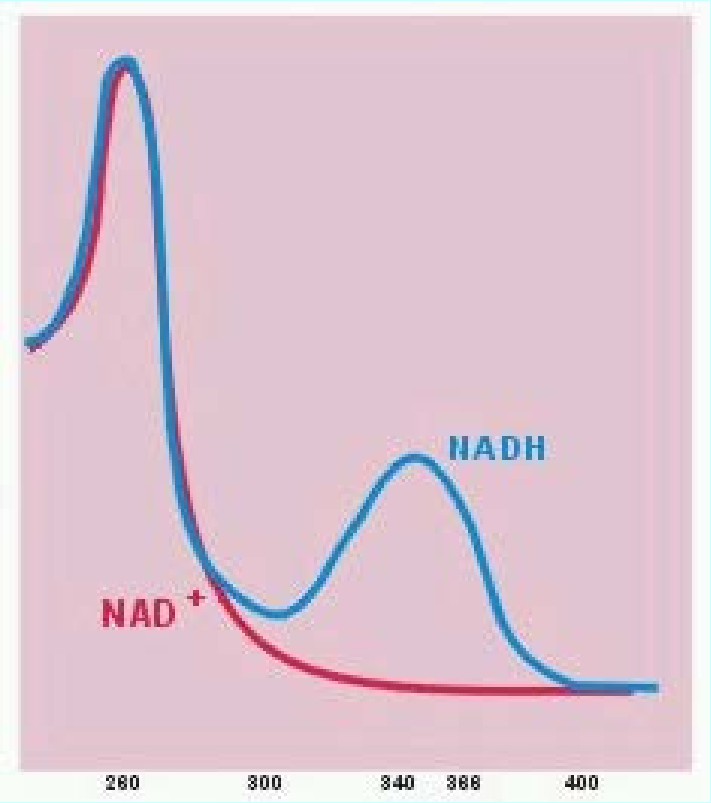
The increase of a (coloured) reaction product is determined by means of photometry and correlates to the concentration of the substrate.

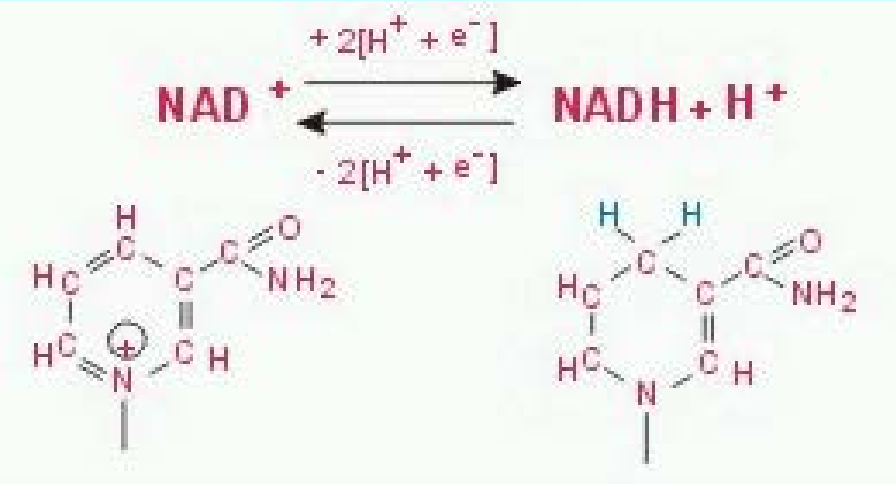
In some tests the decrease of a cosubstrate (NADH, NADPH) serves for the determination of substrate concentration.

The NAD/NADH-system



Nicotinamideadeninediculeotide (NAD)





Abscissa: Wavelength [nm]

Ordinate: Specific absorbance

Determination of enzymes by means of enzymatic reactions - I

Reaction rate assays: Rate is proportional to enzyme reactivity (or concentration).

Enzyme concentration must be rate limiting.

The activity is determined in [U/l] or [µmol/(l*s)].

Optimal pH-value and ionic strength of the reagent buffer.

Substrates and coenzymes (e. g. NADH) are added in excess.

Optimal temperature for ezyme kinetics (standardized on 37°C).

The increase of a (coloured) reaction product or the decrease of a cosubstrate is determined by means of photometry serves as a marker of the enzyme activity.

The absorbance is determined continuously (kinetic reaction).

Determination of enzymes by means of enzymatic reactions - II

Measurement of enzyme kinetics is more difficult than measurement of substrate concentration because the reaction proceeds to equilibrium.

Reaction products must be removed as fast as they are formed in order to avoid the inhibition of enzyme reaction by the end products.

Some tests for the determination of enzymes are based on an additional second enzymatic reaction. In these tests the second enzyme and the required cosubstrate are added in excess and the first enzyme (which will be measured) is the rate limiting step. Examples are ALAT (second enzyme: LDH) and ASAT (second enzyme: Malate dehydrogenase).

Enzymes in clinical chemistry

Determination of substrate concentration (e. g. glucose) in biological fluids (e. g. serum) by means of enzymatic reactions.

Determination of enzyme activity (e. g. lactate dehydrogenase, LDH) in biological fluids (e. g. serum) by means of enzymatic reactions.

In vivo assays for analysis of enzyme activity.

Diagnostics of enzyme activity in vivo

The activity of some enzymes is determined in the organism:

Diagnostics of infection with helicobacter pylori:

Labeled urea is orally administered. If a patient is infected with H. pylori bacterial urease will metabolize urea and labeled CO_2 is detected in expired air.

Diagnostics of intestinal malabsorption:

Labeled bile salts or sugars are orally administered. In cases of bacterial degradation of these compounds instead of intestinal absorption labeled degradation products (CO_2, H_2) are detected in expired air. Examples are:

Breath test for bile salt degradation

Xylose breath test

Lactose breath test

Lactulose breath test

Clinical enzymology

Isoenzymes, alloenzymes, macroenzymes
Organ/tissue distribution of enzymes
Release of cellular enzymes and their secretion into plasma
Clearance of plasma enzymes
Factors affecting plasma enzyme activity

Isoenzymes

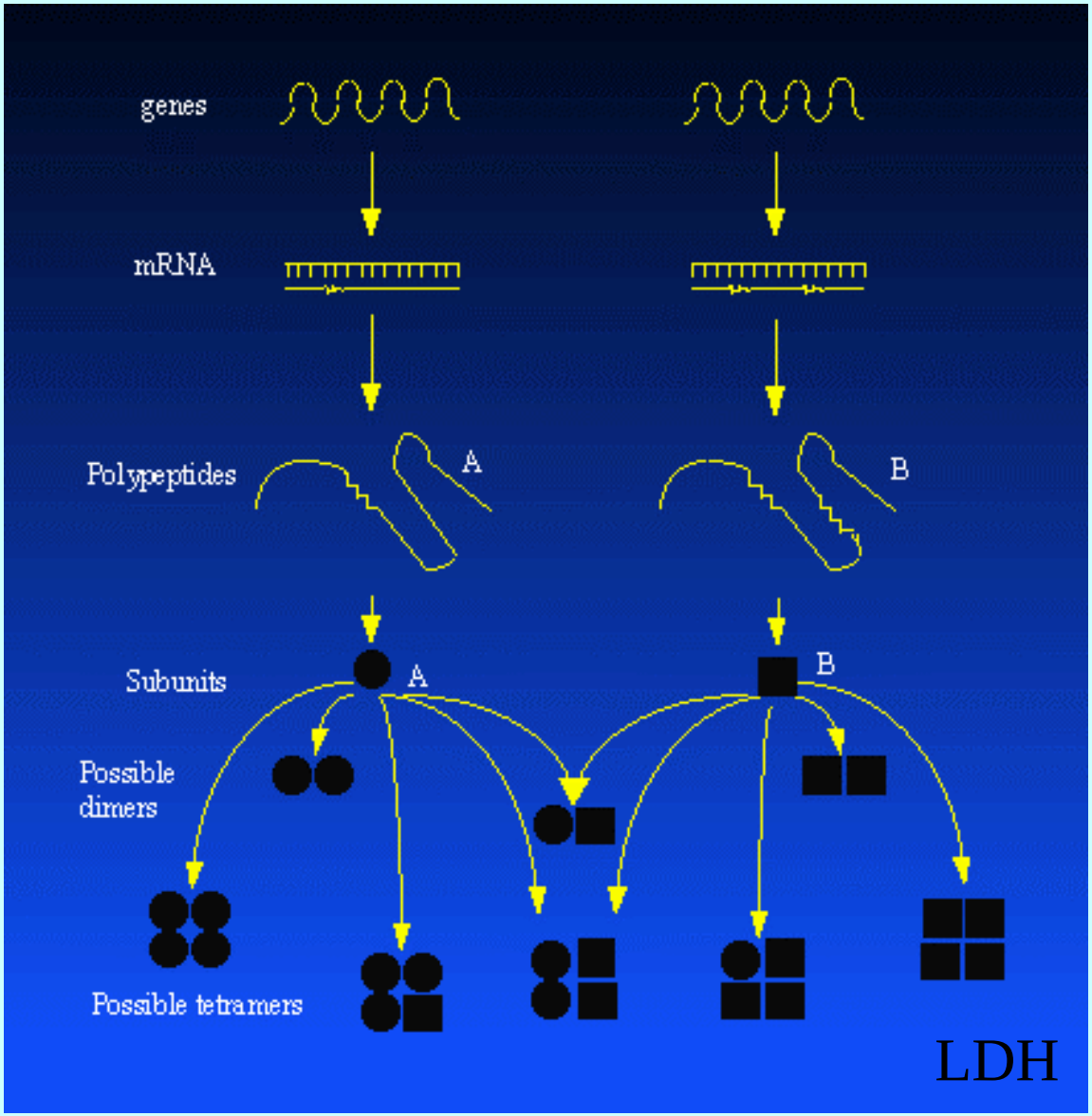
Isoenzymes are coded by different genetic loci.

As a consequence of evolutionary mutations the molecular structure (primary structure = sequence of amino acids) of isoenzymes is different.

Isoenzymes often differ in their substrate specificity.

The term „isoenzyme“ has been firstly introduced for the description of lactate dehydrogenase (LDH) isoenzymes.

Origin of isoenzymes



Lactate dehydrogenase

Examples of isoenzymes - I

Enzyme	Gene/Type	Isoform
Alkaline phosphatase	Unspecific	Liver, bone, kidney
	Placental	
	Intestinal	
	Fetal intestinal	Testes, thymus, lung
ASAT	Cytoplasmatic (ASAT-1)	
	Mitochondrial (ASAT-2)	
Amylase	Salivary	
	Pancreatic	
Cholinesterase		Alleles U, A, S, F, H, J, K
Creatine kinase (CK)	Musculature-type (M; CK-MM)	Hybride type: CK-MB
	Brain-type (B; CK-BB)	
	Mitochondrial (Mi; CK-MiMi)	

Examples of isoenzymes - II

Enzyme	Gene/Type	Isoform
Lactate dehydrogenase (LDH)	LDH-1 (H,H,H,H)	Kidney, heart, erythrocytes
	LDH-2 (M,H,H,H)	
	LDH-3 (M,M,H,H)	
	LDH-4 (M,M,M,H)	
	LDH-5 (M,M,M,M)	Musculature, liver

Macroenzymes - I

In macroenzymes the molecular structure has been modified after the synthesis of the enzyme.

There are two types of macroenzymes:

Type 1: Antibodies have bound to the enzyme resulting in the formation of an enzyme-immunoglobulin complex.

Type 2: Formation of oligomers in blood (e. g. mitochondrial CK-MiMi) or adsorption of enzymes to membranes or plasma components.

Note: Complexes of enzymes and their physiological inhibitors are no macroenzymes. Examples are inhibitors in the clotting system, the complement system and inflammatory enzymes (elastase, trypsin).

Macroenzymes - II

Macroenzymes type-I

Macroenzyme	Prevalence	Diagnosis	Immunoglobuline and Ig-specificity
ALAT	Case reports	Chron. liver disease	IgGκ, (IgGλ); specificity not reported
AP	0.1 - 0.4 %	Patients	IgGλ, (IgGκ, IgA); mostly isoenzyme specific
α-amylase	1.0-9.6 %	Patients (m>f)	IgA, IgG, κ, λ; rarely isoenzyme specific
ASAT	Case reports	Normals, patients	IgG, κ, λ, (IgA); in part isoenzyme specific
γ-GT	Case reports	Hepatobiliary disease	IgAλ; specificity not reported
CK	Up to 10 %	Normals, patients	IgA, IgG, κ, λ; BB specific
LDH	0.03-3.0 %	Normals, patients	IgA, IgG, (IgM), κ, (λ); mostly H- or M specific
Lipase	Case report	Hodgkin´s disease	IgG; lipase specific
Acidic phosphatase	Case reports	Patients	IgG; not reported

Macroenzymes - III

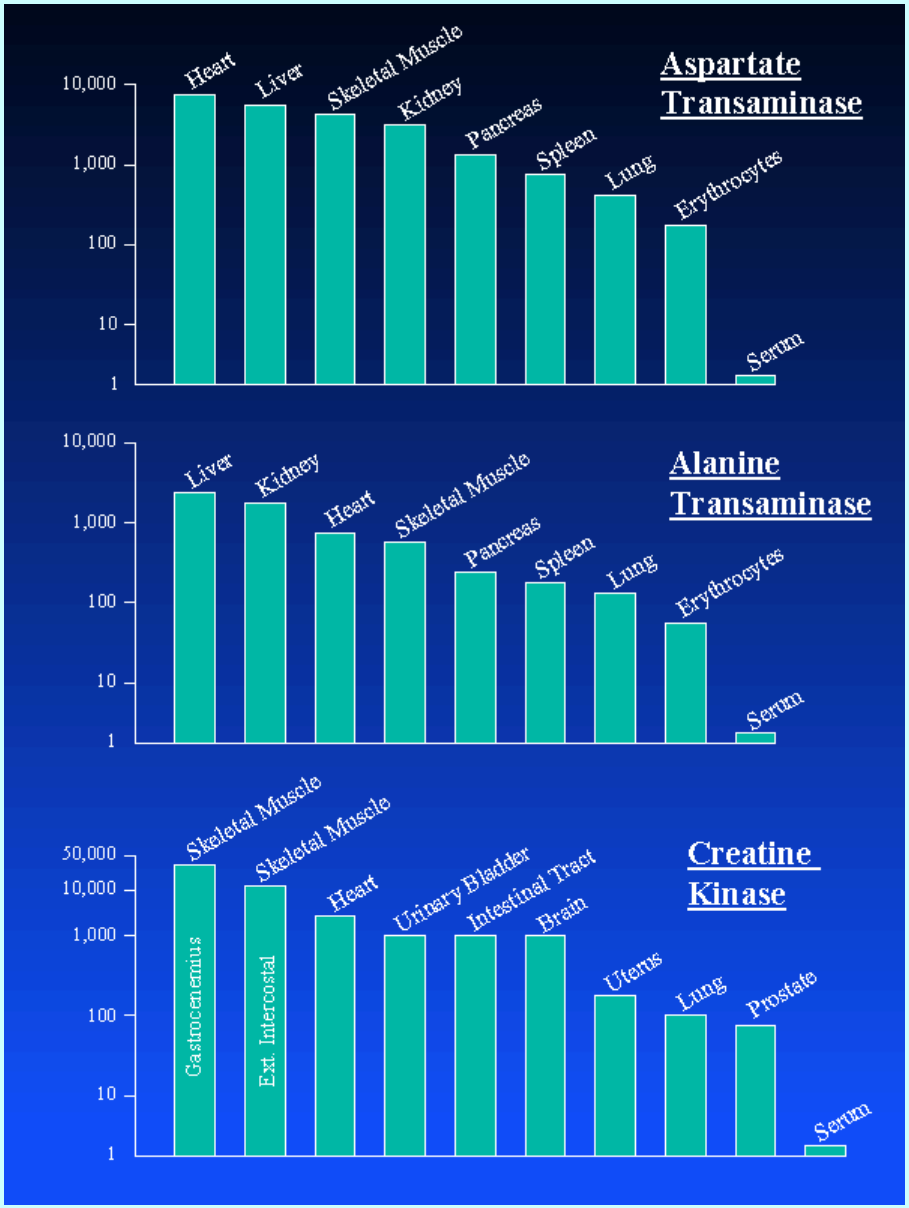
Macroenzymes type-II

Macroenzyme	Diagnosis	Nature
AP	Hepatobiliary diseases	Fragments of cell membranes; adhesion to lipoproteins
α-amylase	Iatrogen	Adhesion to derivatives of hydroxyethyl starch (plasma expander)
γ-GT	Hepatobiliary diseases	Fragments of cell membranes; adhesion to lipoproteins
CK	Severely ill patients; malig-nant diseases	Oligomerized mitochondrial CK
Leucine amino-peptidase (LAP)	Hepatobiliary diseases	Fragments of cell membranes; adhesion to lipoproteins

Clinical enzymology

Isoenzymes, alloenzymes, macroenzymes
Organ/tissue distribution of enzymes
Release of cellular enzymes and their secretion into plasma
Clearance of plasma enzymes
Factors affecting plasma enzyme activity

Organ/tissue distribution of enzymes - I



The concentration gradients between some human tissues and serum for aspartate transaminase, alanine transaminase, and creatine kinase. The concentration gradient axis is logarithmic.

Organ/tissue distribution of enzymes - II

Enzyme	Organ/tissue	Diagnostic indication
α-amylase	Pancreas salivary gland	Acute pancreatitis
ALAT	Liver	Parenchymatic liver diseases
ASAT	Liver, heart, musculature	Myocardial infarction, parenchymatic liver diseases, diseases of the skeleton musculature
Alkaline phosphatase	Liver, bone, intestine, kidney	Diseases of the skeleton, hepatobiliary diseases
Creatine kinase	Skeleton musculature, heart, intestinal musculature	Myocardial infarction, musculature diseases

Organ/tissue distribution of enzymes - III

Enzyme	Organ/tissue	Diagnostic indication
Cholinesterase	Liver	Intoxication with organophosphates Parenchymatic liver diseases
Glutamate dehydrogenase	Liver	Severe parenchymatic liver disease
γ-GT	Liver	Hepatobiliary diseases, alcoholism
LDH	Liver, heart, musculature, erythrocytes, platelets, lymph nodes	Parenchymatic liver diseases, myocardial infarction, hemolysis, ineffective erythro-poiesis, lymphoma
Lipase	Pancreas	Acute pancreatitis

Clinical enzymology

Isoenzymes, alloenzymes, macroenzymes
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Release of enzymes from cells - I

The intracellular concentration of enzymes (in cytoplasm or linked to mitochondria) is much higher than their concentration in plasma (1000-times to 10000 times). This allows the determination of enzyme activity for diagnostic purposes.

The mechanism of enzyme release from intact cells is not yet clear. However, the release of enzymes under pathological conditions is based on:

direct impairment/destruction of cell membrane (e. g. viruses or chemicals)

hypoxia, anoxia, ischemia of the tissue.

Release of enzymes from cells - II

The pattern of enzyme release from cells (height and time course of enzyme activity) depends on:

the gradient of intra- vs. extracellular enzyme activity

intracellular distribution of the enzyme (e. g. cytoplasm or mitochondria; in the liver also lobular distribution)

type and cause of the tissue or organ lesion

extent and duration of tissue hypoxia

organ perfusion and actual metabolic activity

Release of enzymes from cells - III

Increased plasma enzyme activity can also be observed without tissue lesion:

Increased activity of osteoclasts in the growth period (increased plasma activity of alkaline phosphatase (AP).

Enzyme induction by chemical stimuli (e. g alcohol, phenytoin and barbiturates cause an increase of γ-GT activity).

Note: These are only few typical examples.

Clinical enzymology

Isoenzymes, alloenzymes, macroenzymes
Organ/tissue distribution of enzymes
Release of cellular enzymes and their secretion into plasma
Clearance of plasma enzymes
Factors affecting plasma enzyme activity

Clearance of enzymes in plasma

An important criterium of enzyme diagnostics in clinical routine is the observation of enzyme activity as a function of the time (e. g. time interval after organ lesion).

The time-course of plasma enzyme activity depends on the release of the enzyme (type and severity of organ lesion) and the plasma half-life time of the enzyme.

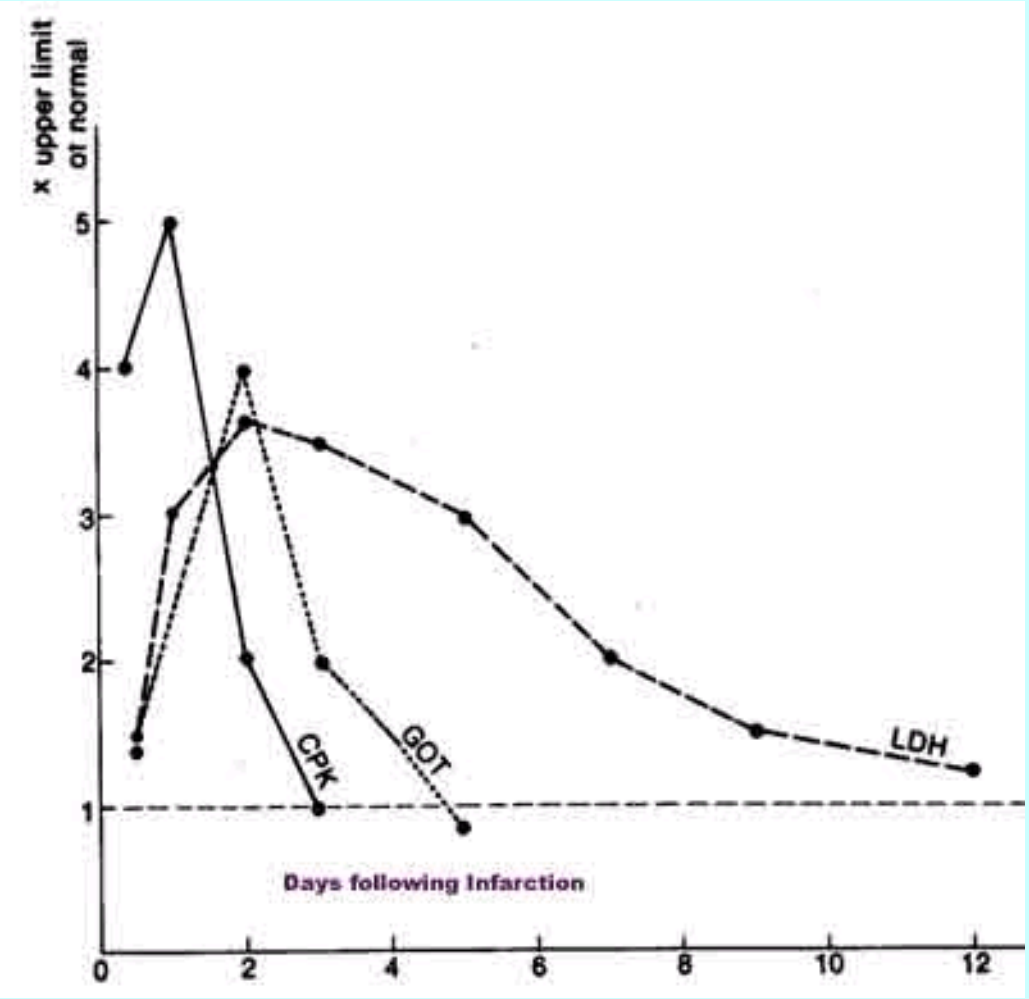
Plasma enzymes are mostly metabolized by the reticuloendothelial system.

Very high values of enzyme activity may indicate macroenzymes.

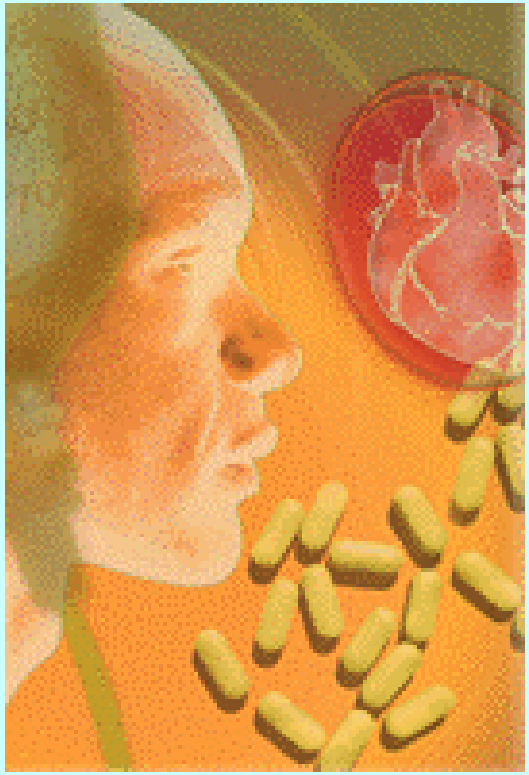
Half-life times of plasma enzymes

Alkaline phosphatase (ALP) (bone)	1 – 2 days
ALP (liver)	7 days
ALP (intestine)	<0.5 hours
α-amylase	9 – 18 hours
ALAT	50 hours
ASAT	12 – 14 hours
Cholinesterase	4 – 10 days
Creatine kinase (CK)	12 hours
CK-MM	20 hours
CK-MB	10 hours
CK-BB	3 hours
GLDH	16 – 18 hours
γ-GT	3 – 4 days
LDH-1 (heart, erythrocytes)	4 – 5 days
LDH-5 (liver, body musculature)	8 – 10 hours
Lipase	7 – 14 hours

Time course of enzymes after myocardial infarction



What is therapeutic drug monitoring?



Therapeutic Drug Monitoring (TDM) enhances safety and efficacy of drugs and lowers the costs of the therapy by measurement of plasma concentrations.

It can also be used for the detection of drug abuse.

Requirements for Therapeutic Drug Monitoring (TDM)

Therapeutic drug monitoring of any drug requires:

A well-defined relationship between drug levels and activity, toxicity or resistance.

An accurate, sensitive and efficient drug assay.

Rapid distribution between the site of drug measurement ant the site of drug action.

Reversible interaction at the effect site.

A relatively long drug half-life, making measurement feasible.

Significant inter-individual variability in pharmacokineticsmaking measurement necessary.

Difficult or inefficient clinical monitoring of efficacy, toxicity or resistance

Indications for Therapeutic Drug Monitoring (TDM) - I

TDM is particularly useful for drugs belonging to the following categories:

Where the drug has a narrow therapeutic index (e. g. digoxin, aminoglycosides, theophylline, anticonvulsants).

Where the pharmacological effect is difficult to quantitate (e. g. salicylates, lithium).

Where drugs are used as prophylactic agents (e. g. anticonvulsants, antiarrhythmics).

Where there is an unpredictable relationship between drug dosage and plasma concentration (e. g. phenytoin, salicylate).

Indications for Therapeutic Drug Monitoring (TDM) - II

TDM is not useful or unnecessary:

Unnecessary when dosage need not be individualised (e. g. penicillin V).

Unnecessary when intensity of pharmacological effects can be clinically quantitated (e. g. hypoglycemics, anticoagulants, antihypertensives)

Useless when plasma concentration is not predictably related to intensity of pharmacological effects (e. g. anticoagulants).

Not yet useful because concentration-effect relationship remains undefined (e. g. antidepressants).

Indications for Therapeutic Drug Monitoring (TDM) - III

At present, TDM is most useful for:

Cardiac glycosides (Digoxin)

Antiarrhythmics (Lidocaine, procainamide)

Anticonvulsants (Phenytoin, carbamazepine, barbiturates)

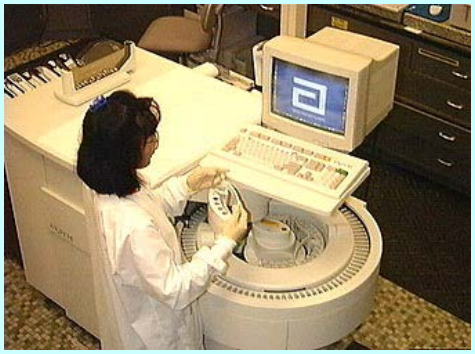
Lithium

Theophylline

Aminoglycoside antibiotics (Gentamicin, tobramycin)

Salicylate

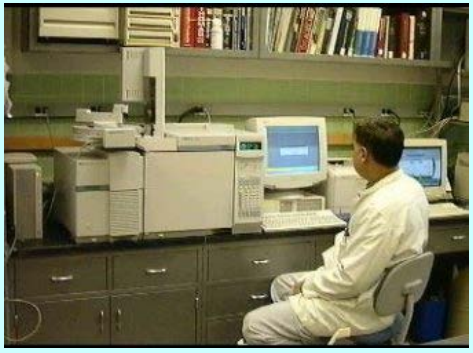
Methods used for Therapeutic Drug Monitoring (TDM) - I



Fluorescence Polarisation Immunoassay (FPIA)

Radioimmunoassay (RIA)

High Performance Liquid Chromatography (HPLC)



Gas Chromatography Mass Spectrometry (GC/MS)

Enzyme Linked Immuno Sorbant Assay (ELISA)

Head Space Mass spectrometry

Methods used for Therapeutic Drug Monitoring (TDM) - II

Gas Chromatography Infrared Spectroscopy (GC/FTIR)

HPLC Mass Spectrometry

UV/Vis spectrometry

Principle of the Fluorescence polarisation Immunoassay (FPIA) - I

Fluorescence Polarization (FP) assays are homogeneous, single-step assays ideally suited for high-throughput screening of large numbers of samples.

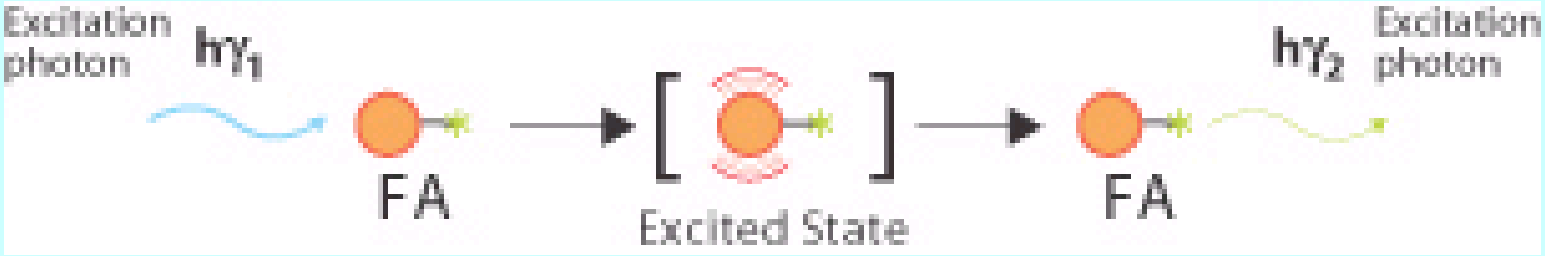
All FP assays employ a large molecular species, or binding partner (BP), in conjunction with a small, low molecular weight fluorescent analyte (FA).

When the large BP molecule is an antibody, the assay is referred to as a fluorescence polarization immunoassay (FPIA).

FP can increase from approximately 50 to around 250 mP units following the addition of the BP to a suitable FA.

Principle of the Fluorescence polarisation Immunoassay (FPIA) - II

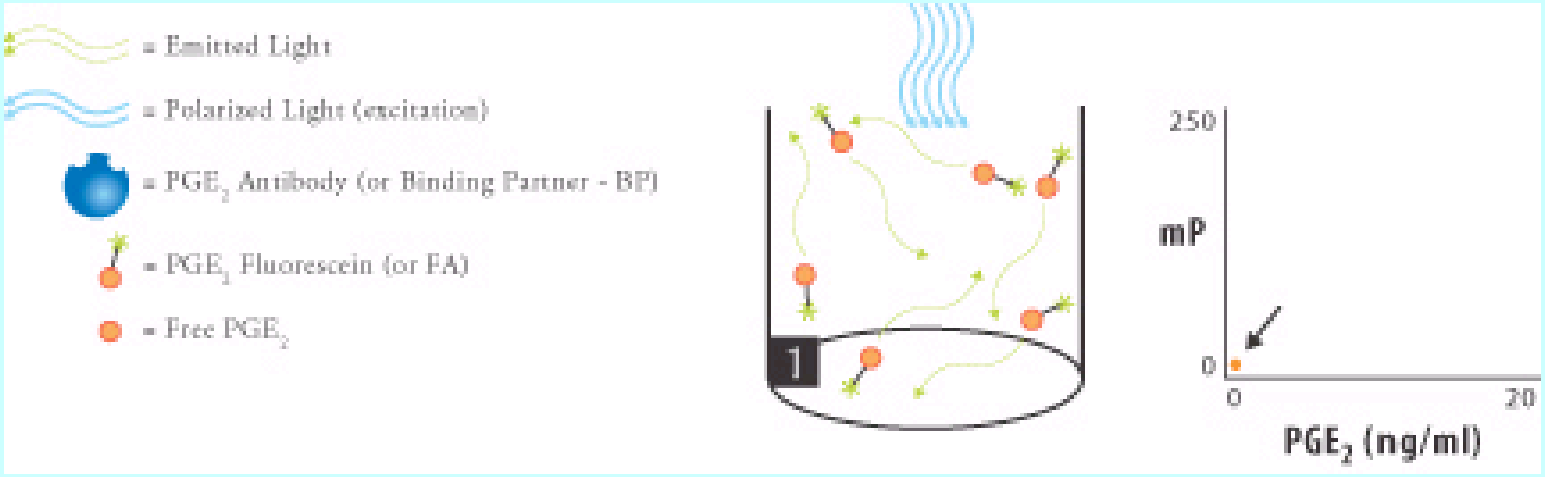
Fluorescence is by definition the ability of a molecule to absorb the energy of an incoming (excitation) photon and then re-emit most of this energy as a new, slightly less energetic (emission) photon.



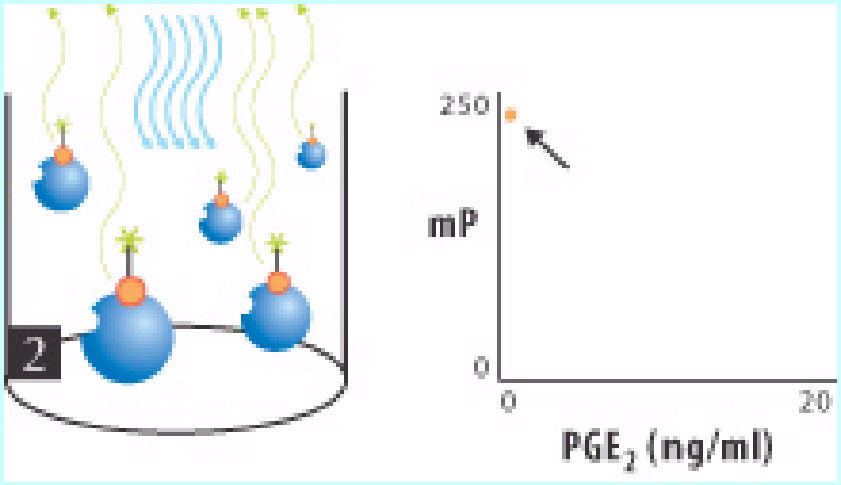
A small fluorescent molecule will rotate appreciably during the very small interval of time between absorption of a photon and emission of the fluorescence photon.

Principle of the Fluorescence polarisation Immunoassay (FPIA) - III

If the excitation light is polarized, this rotation will result in complete randomization of the plane of the emitted light. Thus, small fluorescent molecules depolarize an excitation pulse of polarized light (1).



Principle of the Fluorescence polarisation Immunoassay (FPIA) - IV



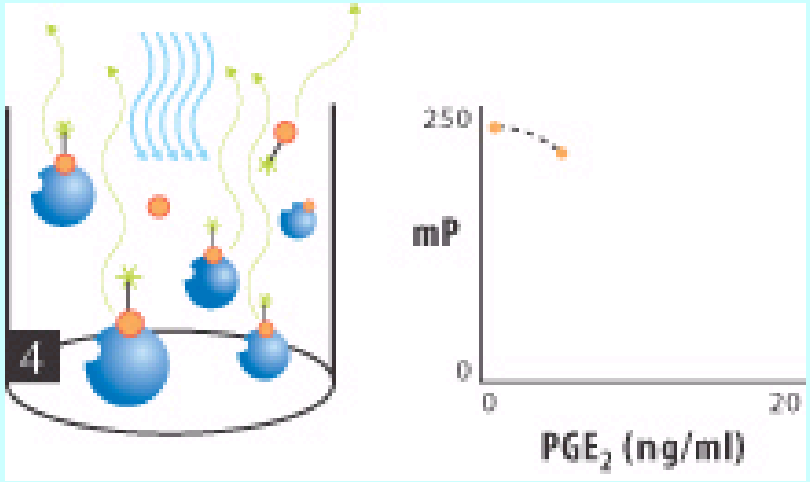
Large fluorescent molecules (MW > 100000) do not rotate appreciably in the same small interval of time. They will therefore emit light that retains some of the polarization of the polarized excitation light. This polarization is quantified as milli-polarization units, or mP. A fluorescence polarization reader is required to make this measurement. When a small fluorescent molecule becomes tightly bound to a large one, as in the binding of Prostaglandin E_2 antibody to PGE_2-fluorescein, the rotational speed of the small molecule is abruptly reduced to that of the entire complex as a whole (2).

Principle of the Fluorescence polarisation Immunoassay (FPIA) - V

Therefore, PGE_2-fluorescein bound to its antibody represents a large fluorescent molecule, which exhibits a high degree of fluorescence polarization (FP). A microplate well filled with the PGE_2-fluorescein: antibody complex will give a high FP reading. The PGE_2 FPIA is based on the competition of free PGE_2 in the samples or standards for the high affinity binding site of a PGE_2 monoclonal antibody occupied by a fluorescently labeled PGE_2-fluorescein conjugate. Addition of a small amount of natural, unlabeled PGE_2 will result in a competition between the unlabeled PGE_2 and the PGE_2-fluorescein for the antibody (3).

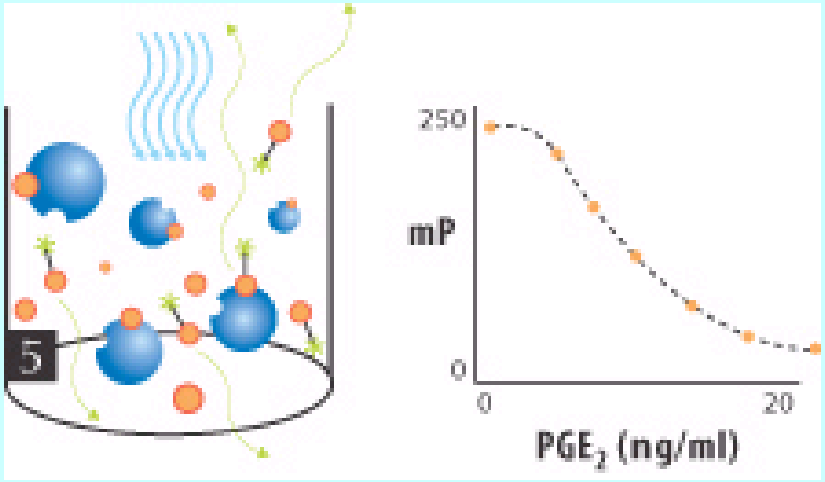
Principle of the Fluorescence polarisation Immunoassay (FPIA) - VI

Some of the fluorescent PGE_2-fluorescein will be released from the antibody, and will resume its intrinsic, rapid rate of rotation. This will cause a detectable loss of FP in the well (4).

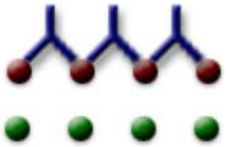
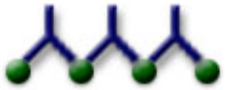
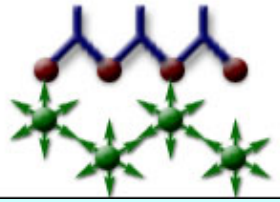


Principle of the Fluorescence polarisation Immunoassay (FPIA) - VII

The addition of large amount of PGE_2 (5-10 ng/ml) will result in a much larger reduction in the mP of the well (5). Plotting mP *versus* PGE_2 concentration allows the construction of a standard curve with a broad dynamic range. This is similar to, but not strictly analagous to, the sigmoidal dose-response curve in a traditional solid phase EIA.



Principle of the Fluorescence polarisation Immunoassay (FPIA) - VIII

REAGENTS:	POSITIVE SAMPLE	NEGATIVE SAMPLE
Ag (in the specimen)  Ab  Allow time to react	Ag is present; Ab's bind 	no Ag is present; Ab's do not bind 
REAGENTS: fluorescein-labeled Ag (tracer)  Allow time to react	Ab's do not bind to Ag-tracer 	Ab's bind to Ag-tracer 
PROCEDURE: illuminate with polarized light measure polarized fluorecscence emissions POSITIVE: no polarized emissions NEGATIVE: polarized emissions	rotation of free Ag-tracer: non-polarized fluorescence 	no rotation of bound Ag-tracer: polarized fluorescence