SEMESTER VI

17BTU601A MOLECULAR DIAGNOSTICS 3H - 3C

Total hours/week: L:3 T:0 P:0 Marks: Internal: 40 External: 60 Total: 100

Scope: Molecular diagnostics deals with the molecular techniques for identification of diseases.

Objective: This paper will enable the students to learn applications of enzymes and immuno assays to identify the microbial and autoimmune diseases.

UNIT-I

Enzyme Immunoassays: Comparison of enzymes available for enzyme immunoassays, conjugation of enzymes. Solid phases used in enzyme immunoassays. Homogeneous and heterogeneous enzyme immunoassays. Enzyme immunoassays after immuno blotting. Enzyme immuno histochemical techniques. Use of polyclonal or monoclonal antibodies in enzymes immuno assays. Applications of enzyme immunoassays in diagnostic microbiology.

UNIT-II

Molecular methods in clinical microbiology: Applications of PCR, RFLP, Nuclear hybridization methods, Single nucleotide polymorphism and plasmid finger printing in clinical microbiology Laboratory tests in chemotherapy: Susceptibility tests: Micro-dilution and macro-dilution broth procedures. Susceptibility tests : Diffusion test procedures. Susceptibility tests: Tests for bactericidal activity. Automated procedures for antimicrobial susceptibility tests.

UNIT-III

Diagnosis and Standardization: Automation in microbial diagnosis, rapid diagnostic approach including technical purification and standardization of antigen and specific antibodies.

UNIT-IV

Diagnostic immunology: Concepts and methods in idiotypes. Anti idiotypes and molecular mimicry and receptors. Epitope design and applications .Immunodiagnostic tests. Immuno-florescence. Radioimmunoassay.

UNIT-V

GC, HPLC, Electron microscopy, flow cytometry and cell sorting. Transgenic animals.

References

1. Willey, J.M., Sherwood, L.M., & Woolverton, C.J. (2008). *Prescott, Harley and Klein's Microbiology* (7th ed.). McGraw Hill Higher Education.
2. Goering, R., Dockrell, H., Zuckerman, M., & Wakelin, D. (2007). *Mims' Medical Microbiology* (4th ed.). Elsevier.
3. Ananthanarayan, R., & Paniker, C.K.J. (2005). *Textbook of Microbiology* (7th ed.). University Press Publication.
4. Brooks, G.F., Carroll, K.C., Butel, J.S., & Morse, S.A. (2007). Jawetz, Melnick and *Adelberg's Medical Microbiology* (24th ed.). McGraw Hill Publication.
5. Joklik, W.K., Willett, H.P., & Amos, D.B. (1995). *Zinsser Microbiology* (19th ed.). Appleton-Centuary-Crofts publication.

KARPAGAM ACADEMY OF HIGHER EDUCATION

(Deemed to be University Established Under Section 3 of UGC Act 1956)

Coimbatore – 641 021

LECTURE PLAN

DEPARTMENT OF BIOTECHNOLOGY

STAFF NAME	**: Dr. SELVAKUMAR S**		
SUBJECT NAME	**: MOLECULAR DIAGNOSTICS**	SUB.CODE	**: 17BTU601A**
SEMESTER	**: VI**	CLASS	**: III B.Sc. (BT)**

S.No.	Lecture Duration Period	Topics to be Covered	Support Material/Page Nos.
		Unit - I	
1.	1	Comparison of enzymes available for enzyme immunoassays, conjugation of enzymes	T1: 273-279
2.	1	Solid phases used in enzyme immunoassays	T1: 280-283
3.	1	Homogeneous and Heterogeneous enzyme immunoassays	T1: 283-287
4.	1	Enzyme immunoassays after immuno blotting	T1: 288-290
5.	1	Enzyme immuno histochemical techniques	T1: 291-294
6.	1	Use of polyclonal or monoclonal antibodies in enzymes immuno assays	T2: 301
7.	1	Applications of enzyme immunoassays in diagnostic microbiology	T1: 301-307
8.	1	Unit test	-
Total No of Hours Planned for Unit I			08 hr
		Unit – II	
9.	1	Applications of PCR, RFLP	T3: 182-183
10.	1	Nuclear hybridization methods, Single nucleotide polymorphism	T3: 291-298
11.	1	Plasmid finger printing in clinical microbiology Laboratory tests in chemotherapy	T3: 302-305
12.	1	Susceptibility tests: Micro-dilution and macro-dilution broth procedures.	T3: 312-317
13.	1	Susceptibility tests : Diffusion test procedures.	T3: 312-317
14.	1	Susceptibility tests: Tests for bactericidal activity.	T3: 312-317
15.	1	Automated procedures for antimicrobial susceptibility tests.	T3: 312-317
16.	1	Unit test	-
Total No of Hours Planned for Unit II			08 hr
		Unit – III	
17.	1	Automation in microbial diagnosis	T2: 102-105
18.	1	Rapid diagnostic approach including technical purification	T2: 231-234
19.	1	Continue: Rapid diagnostic approach including technical purification	T2: 231-234
20.	1	Standardization of antigen	T5: 432-433

21.	1	Continue: Standardization of antigen	T5: 432-433
22.	1	Standardization specific antibodies	T5: 437-441
23.	1	Continue: Standardization specific antibodies	T5: 437-441
24.	1	Unit test	-
Total No of Hours Planned for Unit III			08 hr
UNIT-IV			
25.	1	Concepts and methods in idiotypes	T1: 321-324
26.	1	Anti idiotypes and molecular mimicry and receptors	T3: 124-126
27.	1	Continue: Anti idiotypes and molecular mimicry and receptors	T3: 124-126
28.	1	Epitope design and applications	T3: 128-130
29.	1	Immunodiagnostic tests	T5: 320-331
30.	1	Immuno-florescence	T5: 320-331
31.	1	Radioimmunoassay	T5: 320-331
32.	1	Unit test	-
Total No of Hours Planned for Unit IV			08 hr
UNIT-V			
33.	1	GC	T4: 210-212
34.	1	HPLC	T4: 210-212
35.	1	Electron microscopy	T4: 358-359
36.	1	Flow cytometry	T2: 542
37.	1	Cell sorting	T4:327-329
38.	1	Transgenic animals	T4:330-331
39.	1	Unit test	-
40.	1	Previous year ESE Question papers discussion	-
Total No of Hours Planned for Unit V			08 hr
Total Planned Hours			40 hr

REFERENCES

T1: Willey, J.M., Sherwood, L.M., & Woolverton, C.J. (2008). *Prescott, Harley and Klein's Microbiology* (7th ed.). McGraw Hill Higher Education.

T2: Goering, R., Dockrell, H., Zuckerman, M., & Wakelin, D. (2007). *Mims' Medical Microbiology* (4th ed.). Elsevier.

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T4: Brooks, G.F., Carroll, K.C., Butel, J.S., & Morse, S.A. (2007). Jawetz, Melnick and *Adelberg's Medical Microbiology* (24th ed.). McGraw Hill Publication.

T5: Joklik, W.K., Willett, H.P., & Amos, D.B. (1995). *Zinsser Microbiology* (19th ed.). Appleton- Centuary-Crofts publication.

UNIT-I

SYLLABUS

Enzyme Immunoassays: Comparison of enzymes available for enzyme immunoassays, conjugation of enzymes. Solid phases used in enzyme immunoassays. Homogeneous and heterogeneous enzyme immunoassays. Enzyme immunoassays after immuno blotting. Enzyme immuno histochemical techniques. Use of polyclonal or monoclonal antibodies in enzymes immuno assays. Applications of enzyme immunoassays in diagnostic microbiology.

Enzyme immunoassay

- Enzyme immunoassay (EIA) is now widely used as a diagnostic tool in various industries as well as medicine.
- They can also be used as analytical tools for detecting particular antigens or antibodies in a certain sample during biomedical research.

The history

- Rosalyn Sussman Yalow and Solomon Berson are credited with the development of the first immunoassays in the 1950s. Yalow accepted the Nobel Prize for her work in immunoassays in 1977.
- Immunoassays became simpler to perform and more popular when techniques for chemically linked enzymes to antibodies were demonstrated in the late 1960s.
- In 1983, Professor Anthony Campbell at Cardiff University replaced radioactive iodine used in immunoassay with an acridinium ester that makes its own light: chemiluminescence.

How does an enzyme immunoassay (EIA) test work?

- During EIA the process uses enzyme labelled antibodies and antigens to detect the small biological molecules required.
- The technique makes use of the basic immunology concept that an antigen binds a specific antibody.
- Such antigen molecules, which can be identified in a fluid sample, include molecules such as peptides, hormones and proteins.

- The enzymes which are commonly used in this process include glucose oxidase and alkaline phosphase.
- The antigen in the fluid is allowed to bind to a particular antibody which is then detected with another enzyme-coupled antibody.
- A particular colour change occurs or fluorescence which indicates the presence of the antigen.
- The amount of the particular biological molecule, which is being looked for and is present in the sample, is based on the colour or fluorescence observed.

EIA Techniques and Procedures

- There are several methods which are used to detect very low concentrations of the biological molecules including the 'indirect and 'sandwich' techniques.

Enzymes available for enzyme immunoassays

Enzymes used in ELISAs include **horseradish peroxidase (HRP), alkaline phosphatase (AP) or glucose oxidase**. These enzymes allow for detection often because they produce an observable color change in the presence of certain reagents. In some cases these enzymes are exposed to reagents which cause them to produce light or Chemiluminescence.

Horseradish Peroxidase

- Horseradish Peroxidase is a metalloenzyme that exists in the root of the horseradish plant.
- There are a large number of peroxidase isoenzymes of horseradish with the most common being the C type.

Introduction

- Horseradish peroxidase uses hydrogen peroxide to oxidize both organic and inorganic compounds.

- Horseradish peroxidase along with other heme peroxidases are brightly colored especially under the near-ultraviolet light.
- This property of heme peroxidases make them useful for attaching to "transparent" proteins so that they can be seen under different wavelengths.
- The heme group that is in horseradish peroxidase is simpler than those in mammalians and therefore makes it an excellent starting point in the in-depth study of heme peroxidases and their functions.

Structure

- Horseradish peroxidase C has two metal centers, one of iron heme group and two calcium atoms.
- The heme group has a planar structure with the iron atom held tightly in the middle of a porphyrin ring which is comprised of four pyrrole molecules.
- Iron has two open bonding sites, one above and one below the plane of the heme group.
- The heme group has a histidine (enzyme) attached in the proximal histidine residue (His170) which is located below the heme group in Figure 1.
- The second histidine residue in the distal side of the heme group, above the heme group, is vacant in the resting state.
- This site is open for hydrogen peroxide to attach during reduction-oxidation reactions. An oxygen atom will bond to this vacant site during activation. The iron atom's sixth octahedral position is considered the active site of the enzyme.
- During the enzyme reaction, the bonding of the hydrogen peroxide to the iron atom creates an octahedral configuration around the iron atom. Other small molecules can also bond to the distal site, creating the same octahedral configuration.

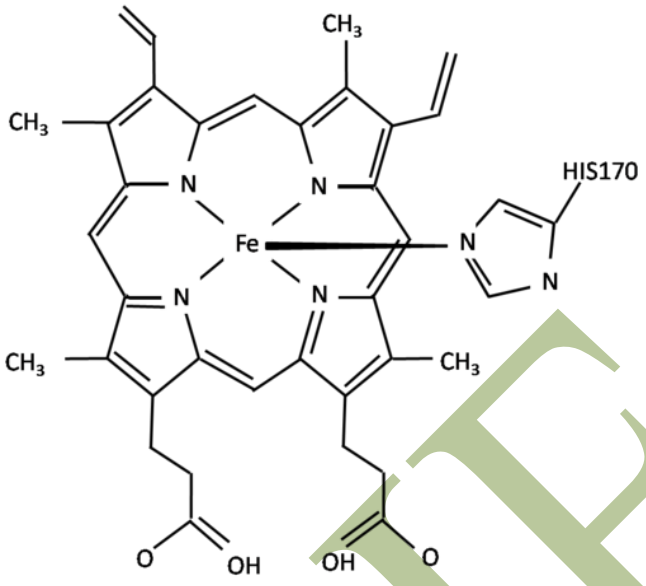


Figure 1: Structure of iron heme group in horseradish peroxidase. There is a histidine (enzyme) attached to the proximal histidine residue ("under" heme group plane).

Alkaline phosphatase

Structure

- **Alkaline phosphatase** (**ALP**, **ALKP**, **ALPase**, **Alk Phos**), or **basic phosphatase**, is a homodimeric protein enzyme of 86 kilodaltons.
- Each monomer contains five cysteine residues, two zinc atoms and one magnesium atom crucial to its catalytic function, and it is optimally active at alkaline pH environments.

Function

- ALP has the physiological role of dephosphorylating compounds.
- The enzyme is found across a multitude of organisms, prokaryotes and eukaryotes alike, with the same general function but in different structural forms suitable to the environment they function in.
- Alkaline phosphatase is found in the periplasmic space of *E. coli* bacteria.

- This enzyme is heat stable and has its maximum activity at high pH. In humans, it is found in many forms depending on its origin within the body – it plays an integral role in metabolism within the liver and development within the skeleton.
- Due to its widespread prevalence in these areas, its concentration in the bloodstream is used by diagnosticians as a biomarker in helping determine diagnoses such as hepatitis or osteomalacia.

Application

- The level of alkaline phosphatase in the blood is checked through the ALP test, which is often part of routine blood tests.
- The levels of this enzyme in the blood depend on factors such as age, sex, blood type.
- Blood levels of alkaline phosphatase also increase by two to four times during pregnancy. This is a result of additional alkaline phosphatase produced by the placenta. Additionally, abnormal levels of alkaline phosphatase in the blood could indicate issues relating to the liver, gall bladder or bones.
- Kidney tumors and infections as well as malnutrition have also shown abnormal level of alkaline phosphatase in blood.
- Alkaline phosphatase levels in a cell can be measured through a process called "The scoring method". This is a technique used where a sample of the enzyme is extracted from the inside of blood cells and is analyzed and compared for varying enzyme activity.

Enzyme-conjugates

Alkaline phosphatase and horseradish peroxidase conjugated to Streptavidin, or directly to antibodies, are useful enzymes in immunoassays.

Enzymes

Alkaline Phosphatase (ALP) and Horseradish Peroxidase (HRP) are commonly used enzymes in immunoassays. Since ALP and HRP catalyze different chemical reactions, different substrates are required for these enzymes. When working with assays involving

HRP, avoid using buffers with the preservative sodium azide since it inactivates the enzyme.

Enzyme conjugates in immunoassays

In immunoassays, ALP and HRP are used as enzyme conjugates. This means that the enzymes have been conjugated (chemically bound) to other molecules. Streptavidin is often used for this purpose due to its high affinity for biotin. ALP or HRP coupled to Streptavidin are often referred to as Streptavidin-ALP and Streptavidin-HRP, respectively. ALP and HRP can also be directly conjugated to an antibody, thereby enabling its use as a detection antibody in immunoassays.

Addition of a substrate to enzyme conjugates initiates chemical reactions that subsequently will result in a product. ELISA substrates generate soluble products, while ELISpot substrates generate precipitating products. Several different soluble and precipitating substrates are available for both ALP and HRP enzymes.

Solid phases used in enzyme immunoassays

- an antigen is bound to a solid surface
- recognized specifically by an antibody conjugated with an enzyme
- incubation with an enzyme substrate gives a measureable product

Basic immunological protocol

Immobilization of antigen/antibody to solid surface:

96-well (or 384-well) polystyrene plates (microplates)

Addition of reagents:

Specific binding mostly via antigen-antibody reaction

Washing steps:

Separation bound from nonbound material and elimination of non-specifically bound materials

Detection basics (scheme)

A/ Mostly: via functional enzyme linked

- directly to the primary antibody or
- indirectly through a secondary antibody

Commonly used enzymes

- horseradish peroxidase (HRPO)
- alkaline phosphatase (AP)
- others: β-galactosidase, acetylcholinesterase and catalase

B/ Substrate: chromogenic, fluorogenic and chemiluminescent

- to be chosen according to
  - required assay sensitivity
  - instrumentation available for signal-detection
- (spectrophotometer, fluorometer or luminometer)

C/ Involvement of biotin

- biotin-avidin interaction (biotin-labelled primary antibody, avidin-enzyme/tag)
- biotin-antibiotin interaction (antibiotin-enzyme conjugate)

Detection: enzyme labels

Alkaline phosphatase (AP)

- Mr: large (140 kDa) protein
- catalytic function: hydrolysis of phosphate groups
- pH optimum: basic pH range (pH 8-10)
- inhibitors: cyanides, arsenate, Pi, divalent cation chelators (e.g. EDTA)

Horseradish peroxidase (HRPO)

- Mr: small (40 kDa) protein

- catalytic function: oxidation of substrates by hydrogen peroxide
- pH optimum: a near-neutral pH
- inhibitors: cyanides, sulfides and azides

Comparison:

antibody-HRPO conjugates are superior to antibody-AP conjugates:

- specific activities
- high activity (high turnover rate)
- good stability
- sensitivity can be increased: poly-HRPO conjugated 2. antibodies
- low cos

Detection: substrates

The intensity of signal is directly proportional to the amount of the tested antigen (antibody)

Chromogenic/colorimetric substrates: direct visualization

Requirement: standard absorbance plate readers

Drawback: less sensitive than fluorescent/chemiluminescent substrates

Fluorescent substrates/tags: signal emission after excitation

High sensitivity

Drawback: requirement for a fluorometer (correct excitation beam)

Chemiluminescent substrates: signal emission

Highest sensitivity

Requirement: luminometer plate reader (or e.g. digital camera systems)

Drawback: signal intensity can change: fading (signal decay with time)

Detection: special substrates

Chromogenic/colorimetric substrates

Alkaline phosphatase (AP)

PNPP (p-nitrophenyl phosphate, disodium salt)

- yellow, water-soluble product
- light absorption: 405 nm

Horseradish peroxidase (HRPO)

ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt)

- green, water-soluble product
- light absorption: 410 nm and 650 nm
    - less sensitive than the next two
    - colour development is slow (about 20 minutes, could be advantageous)

OPD (o-phenylenediamine dihydrochloride)

- yellow-orange, water-soluble product
- light absorption: 492 nm

TMB (3,3',5,5'-tetramethylbenzidine)

- blue, water-soluble product
- light absorption: 370 nm and 652 nm (after sulfuric/phosphoric acid: 450 nm)
    - very sensitive; colour development is fast

Fluorescent/chemiluminescent substrates/tags

Fluorescent tags -- horseradish peroxidase (HRPO)

QuantaBlu Fluorogenic Substrate

- rel. large linear detection range, stable reaction

- Emax/Amax of 420 nm/325 nm

QuantaRed enhanced chemifluorescent substrate

- rel. large linear detection range, stable reaction
- Emax/Amax of 585 nm/570 nm
    - most sensitive tag
    - fluorescent reaction product (resorufin) is rel. stable (4 h)

Chemiluminescent substrates -- horseradish peroxidase (HRPO)

SuperSignal ELISA Pico Chemiluminescent Substrate

SuperSignal ELISA Femto Maximum Sensitivity Substrate

    - incase of proper optimalization - the lower detection limit:
- 1 -10 orders of magnitude lower than with common colorimetric substrates

Homogeneous and heterogeneous enzyme immunoassays

Heterogeneous Assays

- A conjugate useful in determining the amount of antigen or antibody in a liquid sample, said conjugate having a marker, an immunoreactive component (i.e. antigen or antibody) bound to the marker and an insolubilizing binding component which is also bound to the marker.
- The insolubilizing binding component portion of the conjugate will react with an insolubilizing receptor to form a solid product of conjugate and receptor unless the conjugate reacts with the corresponding antigen or antibody to be analyzed in which event the conjugate will not react with the insolubilizing receptor.
- The conjugate will be added to a liquid sample containing an unknown amount of, for example, an antibody. A known amount of the corresponding antigen is also added which reacts with both the conjugate and antibody. After the reaction is complete, the liquid sample is contacted with the insolubilizing receptor.

- Since only the free conjugate reacts with the insolubilizing receptor the amount of antibody originally in the liquid sample can be determined by measuring the activity of the marker in the precipitate.

Example

Enzyme Linked Immunosorbent Assay (ELISA)

- Enzyme Linked Immunosorbent Assay (ELISA) is a very sensitive immunochemical technique which is used to access the presence of specific protein (antigen or antibody) in the given sample and it's quantification. It is also called solid-phase enzyme immunoassay as it employs an enzyme linked antigen or antibody as a marker for the detection of specific protein.
- An enzyme conjugated with an antibody reacts with a colorless substrate to generate a colored reaction product. A number of enzymes have been employed for ELISA, including alkaline phosphatase, horseradish peroxidase, and B-galactosidase.

Principle of ELISA

- ELISA is a plate-based assay technique. Along with the enzyme- labelling of antigens or antibodies, the technique involves following three principles in combination which make it one of the most specific and sensitive than other immunoassays to detect the biological molecule:
- An immune reaction i.e. antigen-antibody reaction.
- Enzymatic chemical reaction i.e. enzyme catalyzes the formation of colored (chromogenic) product from colorless substrate.
- Signal detection and Quantification i.e. detection and measurement of color intensity of the colored products generated by the enzyme and added substrate.

Types of ELISA

- A number of variations of ELISA have been developed, allowing qualitative detection or quantitative measurement of either antigen or antibody.

  1. Indirect ELISA

2. Sandwich ELISA

3.Competitive ELISA

1. Indirect ELISA

- The indirect ELISA detects the presence of antibody in a sample. The antigen for which the sample must be analyzed is adhered to the wells of the microliter plate. The primary antibody present in the sample bind specifically to the antigen after addition of sample.
- The solution is washed to remove unbound antibodies and then enzyme conjugated secondary antibodies are added. The substrate for enzyme is added to quantify the primary antibody through a color change. The concentration of primary antibody present in the serum directly correlates with the intensity of the color.

Advantages

- A wide variety of labeled secondary antibodies are available commercially.
- Versatile because many primary antibodies can be made in one species and the same labeled secondary antibody can be used for detection.
- Maximum immunoreactivity of the primary antibody is retained because it is not labeled.
- Sensitivity is increased because each primary antibody contains several epitopes that can be bound by the labeled secondary antibody, allowing for signal amplification.

Disadvantages

- Cross-reactivity might occur with the secondary antibody, resulting in nonspecific signal.
- An extra incubation step is required in the procedure.

2. Sandwich ELISA

- The sandwich ELISA is used to identify a specific sample antigen. The wells of microliter plate are coated with the antibodies. Non-specific binding sites are blocked using bovine serum albumin. The antigen containing sample is applied to the wells. A specific primary antibody is then added after washing.
- This sandwiches the antigen. Enzyme linked secondary antibody is added that binds primary antibody. Unbound antibody-enzyme conjugates are washed off. The substrate for enzyme is introduced to quantify the antigens.

Advantages

- High specificity because the antigen / analyte is specifically captured and detected.
- Suitable for complex (or crude/impure) samples as the antigen does not require purification prior to measurement.
- Flexible and sensitive, both direct or indirect detection methods can be used.

3. Competitive ELISA

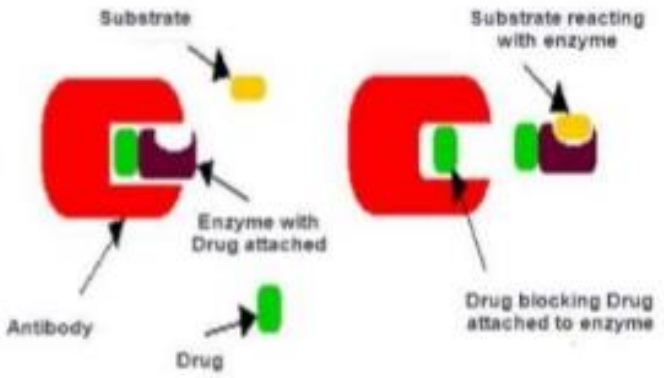
- This type of ELISA depends on the competitive reaction between the sample antigen and antigen bound to the wells of microliter plate with the primary antibody.
- First, the primary antibody is incubated with the sample. This results in the formation of Ag-Ab complex which are then added to the wells that have been coated with the same antigens. After an incubation, unbound antibodies are washed off. The more antigen in the sample, more primary antibody will bind to the sample antigen. Therefore there will be smaller amount of primary antibody available to bind to the antigen coated on well. Secondary antibody conjugated to an enzyme is added, followed by a substrate to elicit a chromogenic signal. Concentration of color is inversely proportional to the amount of antigen present in the sample.

Advantages

- It is highly sensitive even when the specific detecting antibody is present in relatively small amounts

Homogenous enzyme immunoassay

- Homogeneous methods have been generally applied to the measurement of small analytes such as abused and therapeutic drugs.
- Since homogeneous methods do not require the separation of the bound Ab-Ag* from the free Ag*, they are generally much easier and faster to perform



Enzyme multiplied immunoassay technique

- Enzyme multiplied immunoassay technique (EMIT) is a common method for qualitative and quantitative determination of therapeutic and recreational drugs and certain proteins in serum and urine.
- It is an immunoassay in which a drug or metabolite in the sample competes with an drug/metabolite labelled with an enzyme, to bind to an antibody. The more drug there is in the sample, the more free enzyme there will be, and the increased enzyme activity causes a change in color.
- Determination of drug levels in serum is particularly important when the difference in the concentrations needed to produce a therapeutic effect and adverse side reactions is small, the therapeutic window. EMIT therapeutic drug monitoring tests provide accurate

information about the concentration of such drugs such as immunosuppressant drugs and some antibiotics.

- The EMIT platform represents one of the earliest described homogeneous immunoassay platforms. It is based upon the ability of an antibody to inhibit the activity of an enzyme when bound to an Ag that has been attached the enzyme .
- When free Ag is present in a sample, the free Ag competes with the Ag-conjugated enzyme for antibody binding with increasing Ag leading to increasing enzymatic activity While the first EMIT assay used bacterial lysozyme and measured the change in turbidity of a killed bacterial cell suspension, this enzyme system was problematic for a number of reasons, including the inability to use serum due to the propensity to agglutinate the bacteria.
- Alternative enzyme systems were sought and modern EMIT assays typically employ glucose-6-phosphate dehydrogenase (G6PDH). The use of G6PDH makes EMIT assays easily adaptable to large-scale clinical analyzers using the absorbance change at 340 nm between the reaction cofactor NAD+ and its reduced form, NADH. EMIT assays are currently available for most drugs commonly monitored in the clinical laboratory.

Enzyme immunoassays after immuno blotting

Western Blotting

- Western Blotting (also called immunoblotting) is a technique used for analysis of individual proteins in a protein mixture (e.g. a cell lysate).
- In Western blotting (immunoblotting) the protein mixture is applied to a gel electrophoresis in a carrier matrix (SDS-PAGE, native PAGE, isoelectric focusing, 2D gel electrophoresis, etc.) to sort the proteins by size, charge, or other differences in individual protein bands.
- Antibodies are used to detect target proteins on the western blot (immunoblot).
- The antibodies are conjugated with fluorescent or radioactive labels or enzymes that give a subsequent reaction with an applied reagent, leading to a coloring or emission of light, enabling detection.

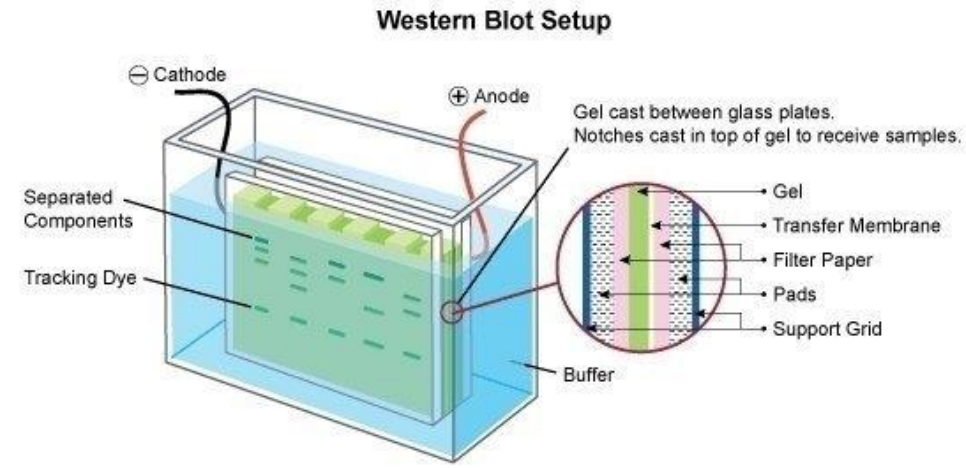


Diagram 1: *Illustration of Western Blot Setup.*

Blotting

- Following the separation of the protein mix the polypeptide bands are transferred to a membrane carrier.
- For this purpose the membrane is attached to the gel and this so-called sandwich is transferred to an electrophoresis chamber.
- It is possible that some of the SDS is washed out, and the protein partially re-naturates again, i.e. regains its 2D- and 3D structure. However, the applied electric charge causes the proteins to travel out of the gel vertically to the direction they traveled in on the gel, onto the membrane.
- The protein bands are thereby bound to the membrane. The "blotted" bands are now available to be treated further (e.g. for detection of specific proteins with specific antibodies).

Immunodetection

- The identification of specific antibodies is possible after the separation and blotting of proteins. Specific antibodies (mono- or polyclonal) bind to "their" band of proteins. Unspecifically binding antibodies are removed by washing with detergent-containing buffers.

- Primary antibodies are usually applied first, which are then recognized by a secondary antibody. The secondary antibody is conjugated with colour, radioactivity or an enzyme for detection. Biotin-conjugated antibodies are also used for this purpose.
- It can occasionally be advantageous to use polyclonal primary antibodies as such antibodies recognize several epitopes, contrary to monoclonal antibodies that are restricted in their binding affinity.
- Analysis of the western blot is then carried out using a variety of different imaging systems (e.g. luminescence, color reaction, autoradiography).

Immunohistochemistry (IHC)

Introduction

Immunohistochemistry (IHC) is a method for detecting antigens or haptens in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues. The antibody-antigen binding can be visualized in different manners. Enzymes, such as Horseradish Peroxidase (HRP) or Alkaline Phosphatase (AP), are commonly used to catalyze a color-producing reaction.

IHC is widely used in many research and clinical laboratories because this technique makes it possible to visualize the distribution and localization of specific cellular components within cells and in proper tissue context. There are numerous IHC methods that can be used to localize antigens. The method selected should include consideration of parameters such as the specimen types and assay sensitivity.

In this processes were contacted by following methods

1. Sample Preparation
2. Fixation
3. Tissue Sectioning
4. Paraffin Embedding
5. Inactivation and Blocking
6. Antigen Retrieval
7. Detection

8. Chromogens, Counterstains and Mounting Media

1. Sample Preparation

Sample collection and preparation play an important role in IHC as the antigen exhibition and location are largely depend on the quality of tissue sample.

2. Selection of Fixing Solution

Below is a list of commonly used fixing solutions. You may need to test whether a specific type of solution is appropriate for your detected antigens because there is no standard fixing solution for different kinds of antigen immobilization.

Acetone and Alcohol

- These two types of solutions, which are primary fixing solutions, play a role of precipitating sugars and fat as well as maintain the immunologic competence.
    - Alcohol is ineffective to maintain low molecular weight protein, polypeptide and cytoplasmic proteins. However, it can be mixed with glacial acetic acid, ethyl ether, chloroform and formaldehyde.
    - Acetone is often used for frozen tissue and cytological smears because it has a strong penetrability and dehydration property.

3. Tissue Sectioning

Frozen

- The most important feature for this type of tissue section is to keep antigen's immune-competence completely, especially for the cell surface antigen. Both fresh and fixed tissues can be processed as frozen tissues. However, the tissues must be dried (or primary fixed) and stored at low temperature.

Paraffin-Embedded

- Paraffin-embedded tissue section is normally sliced by a rotary microtome to give a thickness of 2-7 μm. With proper treatment, the section reveals clear tissue structure and

exact antigen location to enable high medical-value pathology researches and retrospective studies. This section type can be stored at 4°C for long term use.

4. Paraffin Embedding

- Five major steps are involved in paraffin embedding: fixation, dehydration, transparentizing, immersion and embedding.

5. Inactivation and Blocking

Inactivation

- When either the horseradish peroxidase (HRP) or alkaline-phosphatase (AP) system is applied for IHC, activation of endogenous enzymes should be blocked or inhibited to avoid producing non-specific binding.

Blocking

- Residual sites on the tissue section may bind to secondary antibody and produce follow-up false positive results. Therefore, serum from the same species as the secondary antibody is commonly used for blocking. Animal's autoantibody in the serum can bind to the sites in advance. Blocking should be done at room temperature for 10-30 min (avoid excessive blocking).

6. Antigen Retrieval

- Formaldehyde fixation usually generates methylene bridges which cross-link proteins and therefore mask the epitope of interest. It is essential to unmask the antigen epitopes in order to allow the antibodies to bind, either by heat (Heat Induced Epitope Retrieval: HIER) or enzymatic digestion (Proteolytic Induced Epitope Retrieval: PIER).

7. Detection

- IHC detection methods vary and are based on the nature of analyze reporting and binding chemistry, among other factors. Three methods are described here: immunofluorescence (IF), Enzymatic and Affinity.

8. Chromogens, Counterstains and Mounting Media

Chromogens for HRP

DAB

- DAB (3,3'-Diaminobenzidine) is typically used as a signal enhancer in conjunction with the HRP-based immunostaining systems. The dark brown end-product derived from DAB is insoluble in water and alcohol, stable and suitable for long-term storage. In addition, the end-product could be observed under a light microscope or processed with OsO4 for observation under electron microscopy. Hematoxylin, methyl green and methyl blue are the compatible counterstains. Since DAB may cause skin and bladder cancers, it is advised that personal protective equipment should be used and skin/mucosa should be avoided.

Chromogens for AP

BCIP/NBT

- Used in conjunction, BCIP (5-Bromo-4-Chloro-3-Indolyl-Phosphate)/NBT (Nitro Blue Tetrazolium) is a widely accepted chromogenic substrate used in the AP-based immunostaining systems. After exposing to AP, the substrate changes to bluish violet or black violet. The end-product derived from BCIP/NBT is insoluble in alcohol. Nuclear fast red and brilliant green are the suitable counterstains for BCIP/NBT.

Counterstains

- After staining the target antigen by IHC, a secondary stain is usually applied to provide contrast that helps the primary stain more distinct. While many of these stains show specificity for discrete antigens or cellular compartments, other stains will deliver the staining of a whole cell.

Mounting Media

- A mounting medium may be used to attach a coverslip or may itself be used to replace the coverslip. Generally, the medium selection depends on a few factors including the

chemical compatibility with chromogen and counterstain as well as the preservation period.

Use of polyclonal or monoclonal antibodies in enzymes immuno assays.

Monoclonal-Polyclonal Sandwich Immunoassay

In a typical microtiter plate sandwich immunoassay, a monoclonal antibody is adsorbed onto a plastic microtiter plate.

When the test sample is added to the plate, the antibody on the plate will bind the target antigen from the sample, and retain it in the plate. When a polyclonal antibody is added in the next step, it also binds to the target antigen (already bound to the monoclonal antibody on the plate), thereby forming an antigen 'sandwich' between the two different antibodies.

This binding reaction can then be measured by radio-isotopes, as in a radio-immunoassay format (RIA), or by enzymes, as in a enzyme immunoassay format (EIA or ELISA) attached to the polyclonal antibody. The radio-isotope or enzyme generates a color signal proportional to the amount of target antigen present in the original sample added to the plate. Depending on the immunoassay format, the degree of color can be detected and measured with the naked eye (as with a home pregnancy test), a scintillation counter (for an RIA), or with a spectrophotometric plate reader (for an EIA).

- Step 1: Monoclonal antibodies adsorbed onto the well of a plastic microtiter plate with coating buffer (no sample added).
- Step 2: Addition of a sample (such as human blood, diluted appropriately) to the well of the microtiter plate. The target antigen binds to the antibody adsorbed on the plate, retaining the antigen in the well.
- Step 3: Binding of an enzyme-conjugated polyclonal antibody to the target antigen (bound to the monoclonal antibody on the plate), thereby forming an antigen 'sandwich' between the two different antibodies.
- Step 4: Addition of a colorimetric substrate for detection of the enzyme-conjugated polyclonal antibodies will generate a color signal proportional to the amount of target antigen present in the original sample added to the plate.

Antigen-Down Immunoassay

In an antigen-down immunoassay, the analyte is coated onto a 96-well microtiter plate (rather than an antibody) and used to bind antibodies found in a sample. When the sample is added (such as human serum), the antigen on the plate is bound by antibodies (IgE for example) from the sample, which are then retained in the well. A species-specific antibody (anti-human IgE for example) labeled with HRP is added next, which, binds to the antibody bound to the antigen on the plate. The higher the signal, the more antibodies there are in the sample. Antigen-down assays can be configured as rapid tests and are often used to diagnose allergy conditions – routinely a patient's blood is tested against different allergens to see if the person has antibodies to that allergen.

Competitive Inhibition Immunoassay

In addition to the Monoclonal-Polyclonal (Mo-Po) Antibody Sandwich format, many immunoassays are structured in a competitive inhibition format. Competitive inhibition assays are often used to measure small analytes because competitive inhibition assays only require the binding of one antibody rather than two, as in standard ELISA formats. Because of the high probability for steric hindrance occurring when two antibodies attempt to bind to a small molecule at the same time, a sandwich assay format may not be feasible. Therefore a competitive inhibition assay would be preferable.

In a sequential competitive inhibition assay, the sample and conjugated analyte are added in steps like a sandwich assay, while in a classic competitive inhibition assay, these reagents are incubated together at the same time. In a sequential competitive inhibition assay format, a monoclonal antibody is coated onto a 96-well microtiter plate. When the sample is added, the MoAb captures free analyte out of the sample. In the next step, a known amount of analyte labeled with either biotin or HRP is added. The labeled analyte will then also attempt to bind to the MoAb adsorbed onto the plate, however, the labeled analyte is inhibited from binding to the MoAb by the presence of previously bound analyte from the sample. This means that the labeled analyte will not be bound by the monoclonal on the plate if the monoclonal has already bound unlabeled analyte from the sample. The amount of unlabeled analyte in the sample is inversely proportional to the signal generated by the labeled analyte. The lower the

signal, the more unlabeled analyte there is in the sample. A standard curve can be constructed using serial dilutions of an unlabeled analyte standard. Subsequent sample values can then be read off the standard curve as is done in the sandwich ELISA formats. The classic competitive inhibition assay format requires the simultaneous addition of labeled (conjugated analyte) and unlabeled analyte (from the sample). Both labeled and unlabeled analyte then compete simultaneously for the binding site on the monoclonal capture antibody on the plate. Like the sequential competitive inhibition format, the colored signal is inversely proportional to the concentration of unlabeled target analyte in the sample. Detection of labeled analyte may be made by using a peroxidase substrate such as TMB, which can be read on a microtiter plate reader.

Rapid Immunoassay

In addition to microtiter plates, immunoassays are also configured as rapid tests, such as a home pregnancy test. Like microtiter plate assays, rapid tests use antibodies to react with antigens and can be developed as MoAb-PoAb sandwich formats, competitive inhibition formats, and antigen-down formats. With a rapid test, the antibody and antigen reagents are bound to porous membranes, which react with positive samples while channeling excess fluids to a non-reactive part of the membrane. Rapid immunoassays commonly come in 2 configurations: a lateral flow test where the sample is simply placed in a well and the results are read immediately; and a flow through system, which requires placing the sample in a well, washing the well, and then finally adding an analyte-colloidal gold conjugate and the result is read after a few minutes. One sample is tested per strip or cassette. Because rapid tests are faster than microtiter plate assays, require little sample processing, are often cheaper, and generate yes/no answers without using an instrument, they often used in the field by non-laboratory people testing whole samples. However, rapid immunoassays are not as sensitive nor can they be used to accurately quantitate an analyte. (Self-monitoring of blood glucose levels by diabetics is considered quantitative rapid testing, however, immunoassay technology is not used for these tests.) All rapid immunoassay tests can be converted to a microtiter plate assay, but not all microtiter plate assays can be converted to a rapid test.

Applications of enzyme immunoassays in diagnostic microbiology

ELISA and its clinical implications

ELISA can be performed to evaluate either the presence of antigen or the presence of antibody in a sample, it is a useful tool for determining serum antibody concentrations (such as with the HIV test or West Nile virus).

It has also found applications in the food industry in detecting potential food allergens, such as milk, peanuts, walnuts, almonds, and eggs and as serological blood test for coeliac disease.

ELISA can also be used in toxicology as a rapid presumptive screen for certain classes of drugs.

ELISA tests used to detect various kind of diseases, such as malaria, Chagas disease, and Johne's disease. ELISA tests also are used as in in vitro diagnostics in medical laboratories.

The other uses of ELISA include:

- Detection of Mycobacterium antibodies in tuberculosis
- Detection of rotavirus in feces
- Detection of hepatitis B markers in serum
- Detection of enterotoxin of *E.Coli* in feces
- Detection of HIV antibodies in blood samples

Western blot methods and its clinical implications

The western blot technique has evolved from identification of a specific protein in a complex mixture to the direct detection of protein in a single cell. This technique has become an important analytical tool for clinical research. Western blotting is a valuable tool to study regulatory signaling processes and confirmatory serum diagnosis of HIV. A study on stem cell signaling and differentiation, as well as drug response in tumor cells, have been studied using advanced single cell western blotting technique. Also single cell western blotting could analyze cell-to cell variations in approximately 2000 cells simultaneously within complex populations of cells. With the integration of intact cell imaging, the technique allows the identification of protein expression. Western blotting and 2-DE gel separation together with the spotting of protein by peptide mass fingerprint helped to analyze clinically relevant

Helicobacter pylori (H. pylori) in related gastric disease conditions (chronic gastritis, duodenal ulcer). Western blotting is commonly used for the clinical diagnosis of various parasitic and fungal diseases including echinococcosis, toxoplasmosis, and aspergillosis. The database of H. pylori (low expressed and membrane proteins) was created through the application of one-dimensional or 2-DE/MALDI-mass spectrometry techniques. In a similar manner, the Simple Western technique was employed for the analysis of 15 valent pneumococcal vaccine PCV15-CRM197. Due to its high sensitivity and automation, the Simple Western method may be extended to analyze serotypes of other polysaccharide protein conjugate vaccines. The assay was successfully used in a recent study, for the reliable serodiagnosis of Farmer's lung disease (FLD), a pulmonary disorder caused by inhalation of antigenic particles. Thus, this technique can be exploited for rapid routine diagnosis of FLD in clinics. Different molecular and immunological methods were used for clinical diagnosis of Tuberculosis meningitis, a chronic disease of the central nervous system. However, each of these techniques has their own limitations. It was then that immunoreactivity to Mycobacterium tuberculosis antigens performed by western blotting helped in early and sensitive diagnosis. Western blotting helped to detect a new subgroup of human lymphotropic retroviruses (HTLV), in patients with the acquired immunodeficiency syndrome (AIDS). Western blotting has also been used as a test for variant Creutzfeldt-Jakob Disease and some forms of Lyme disease. It has been sometimes used as a confirmatory test for Hepatitis B and Herpes Type 2 infections. Western blots have also been used to confirm feline immunodeficiency status in cats. Recently, LD Bio Diagnostics (France) developed a commercial kit to carry out immunoblotting for the clinical diagnosis of chronic aspergillosis The commercial kit was found to be sensitive and can analyze hundreds of samples from patients with aspergillus disease. Thus, the clinical applications of western blotting technique will continue to progress as further advancements are made to improve sensitivity and reproducibility of the western blot.

Capillary and microchip electrophoresis

Few major limitations of western blotting are time-consuming nature, requirement of a relatively large amount of sample (usually 10-20 µg/assay) and detection of only one protein at a time. Moreover, it requires detection of housekeeping proteins or total proteins on the

membrane to normalize the target bands detected. To address these issues, a hybrid between capillary electrophoresis for SDS-PAGE and conventional blotting for the western part referred to as capillary and microchip electrophoresis (MCE) based western blotting was developed. In brief, multiple injections of the same protein samples are loaded in separate tracks on a microchip and are captured on a PVDF membrane for immunoassay. Using only 400 ng of Jurkat cell lysate sample MCE was applied to measure 11 different proteins including ERK1/2, MEK1/2 AKT, STAT3, phosphor-ERK1, phospho-ERK2, and β-tubulin. Compared to conventional western blotting technique, MCE has a higher sensitivity with better resolution that allows measurement of multiple target proteins from a single cell lysate sample. This approach eliminates blocking steps and requires shorter analysis time (approx. 8 min for electrophoretic resolution). MCE allows the performance of parallel multiplexed assays of a group of proteins using a small sample amount.. This method is still being developed and further improvements to this technique would allow significant improvements in multiplexing protein detection.

Automated microfluidic protein immunoblotting and single cell-resolution western blotting

Another automated microfluidic protein immunoblotting technique were developed to save time, avoid multiple assay steps and limit equipment and reagents requirements. This automated protein immunoblotting is a programmable controlled technique (i.e.voltage control and pressure) that combines PAGE with blotting in one device. The technique allows the integrated assay steps (PAGE, transfer and in-gel blotting) to be viewed using an epifluorescence microscope equipped with a charge-coupled device camera. In this method photo patterning (photochemical etching) of polyacrylamide gels is done on microfluid glass devices that act as a platform to integrate the multiple assay steps. The method is rapid and was employed to detect free prostate specific antigen in the human seminal fluid sample in less than 5 min. This method is economical and lowers the consumption of reagents as the glass chips that are used are reusable after simple chemical treatments. The technique is still in process of development to further improve sensitivity and enable protein quantitation.

Single cell-resolution western blotting

It was developed to detect individual cell-to cell variations in protein expression between cells. The microdevice used for this assay consists of a thin layered polyacrylamide gel with micro wells. The micro wells in the gel layer are loaded with single cell protein samples and are lysed chemically in each well to obtain single cell lysate to be resolved on PAGE. Subsequently, the proteins are immobilized on the same gel using ultraviolet (UV) light and are probed with antibodies for immunoblotting. Multiplexing of a single cell is achieved by incubating immobilized proteins with different antibodies using stripping/ re-probing protocols. From a single cell, multiplexed data (more than 10 proteins) was obtained within a time of 4-6 hrs.

Simple Western

In 2010 a new instrument, Simple Western™, which utilized a microchip capillary electrophoresis (CE)-SDS combined with immunoprecipitation, but separated proteins based upon size instead of charge was reported. Simple is based on CE SDS where the separated proteins are attached to the wall of capillary by a proprietary photo (ultraviolet) activated chemical crosslink. Each capillary contains separation gel matrix and stacking matrix proteins are separated based upon size using high voltage. Subsequent blotting is done automatically by washing the capillary to remove the gel matrix, then incubating and washing the capillary with primary and secondary antibodies conjugated with horseradish peroxidase. The horseradish peroxide is detected by its signal generated upon exposure to luminol and an electropherogram trace or a virtual gel image displayed. The system combines immunoassay, detection, and data analysis in one machine.

DigiWest

DigiWest, is a modified version of the western blot method which increases the throughput of western blotting. The DigiWest method separates proteins by electrophoresis and transfers proteins to blotting membrane as usually done for western blots. The next step is to biotinylated (using amino-reactive biotinylation reagents) proteins on the membrane and cut the membrane into 96 strips to create 96 different molecular weight fractions that are on the membrane. The proteins on these strips are eluted into wells in 96 well plates that contain neutravidin-coated Luminex beads. Each well contains a different distinct bead set with

different color codes. After pooling the beads, the components of the entire lane are now reconstructed with different color codes corresponding to the molecular weight of the immobilized proteins. Using a small aliquot of the bead pool, new wells are then incubated with antibodies overnight and then phycoerythrin-labelled secondary antibodies added to detect the primary antibodies used. Detection of the phycoerythrin is done using a Luminex FLEXMAP 3D instrument. The advantages of this method are the increased throughput of the samples, lower amounts of lysate require/target detection, and lower amounts of antibody required.

Micro-loader

This method involves a funnel-like structure sample micro-loader device to load samples. The device is attached to the top of polyacrylamide gel and filled with 4% stacking gel solution through the outlet of the tips via capillary action. The protein in a sample that travels through the transfer pipette is concentrated by electrophoresis. The incorporation of micro-loader device in gels improved both protein separation and resolution. This technique was able to detect number of proteins and phosphoproteins in each sample by loading only 1.5 µg of protein per lane. This technique has the advantage of being relatively simple to do and would be very useful to measure protein expression and phosphorylation in samples that are limited.

Thin-film direct coating with suction-western blotting (TDCS)

TDCS is a capillary-tube based approach designed to reduce antibody consumption and time required for western blotting. This is a highly sensitive and rapid detection method for quantitative analysis of multiple antigen-antibody interactions. The operational time for TCDS is much shorter (about 5 min) than conventional western blotting with increased signal-to noise ratio. In this method proteins are resolved on SDS-PAGE by electrophoresis and are transferred to PVDF membrane for 1 hour using 10 mM 3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS) transfer buffer (pH 11) with 15% methanol. The PVDF membrane is placed on a stage and allowed to air dry for 10 min. Thereafter, the primary antibody solution (0.1 -0.2 µl) is added to the coater (super light-and-slim slot die coater or capillary tube) mounted on a translational stage for coating the membrane via capillary

force/liquid pump. The coating process is less than one minute and is programmable. The gap between the coater and the PVDF membrane is controlled. The PVDF membrane is then incubated for 2-10 min followed by subsequent washing steps for less than one minute with suction (~30 cm Hg) and again coated with secondary antibody using a similar coating process. Thus, this technique is fast and enables quantitative analysis of multiple protein interactions.

SYLLABUS

Molecular methods in clinical microbiology: Applications of PCR, RFLP, Nuclear hybridization methods, Single nucleotide polymorphism and plasmid finger printing in clinical microbiology Laboratory tests in chemotherapy: Susceptibility tests: Micro-dilution and macro-dilution broth procedures. Susceptibility tests: Diffusion test procedures. Susceptibility tests: Tests for bactericidal activity. Automated procedures for antimicrobial susceptibility tests.

Polymerase chain reaction (PCR)

Principle:

Polymerase chain reaction is method for amplifying particular segments of DNA. It is an enzymatic method and carried out invitro. PCR technique was developed by Kary mullis in 1983. PCR is very simple, inexpensive technique for characterization, analysis and synthesis of specific fragments of DNA or RNA from virtually any living organisms.

Steps or procedures:

PCR consists of three basic steps.

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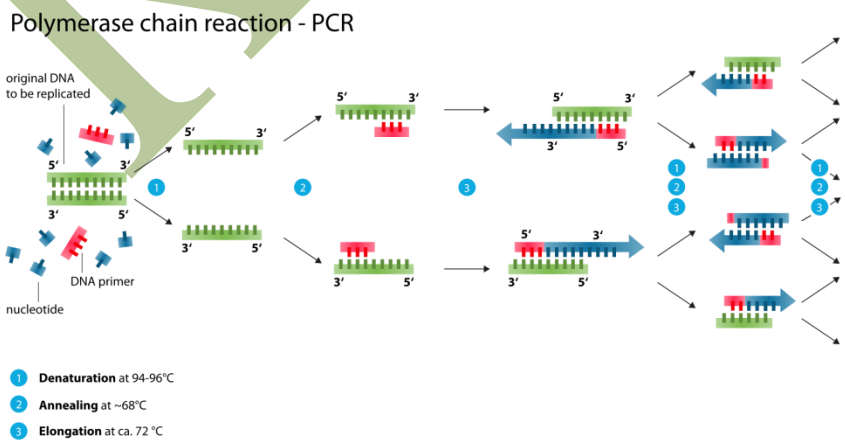
PCR consists of three basic steps.



1. Denaturation:

- Two strand of DNA separates (melt down) to form single stranded DNA
- This step is generaly carried out at 92C-96C for 2 minutes.

2. Annealing:

- Annealing of primer to each strand is carried out at 45C-55C

3. Extension:

- DNA polymerase adds dNTPs complementary to templates strands at 3'end of primer.
- It is carried out at temperature of 72^{o}C.
- *These three steps are repeated 20-30 times in an automated thermocycler that can heat and cool the reaction mixture in tube within very short time. This results in exponential accumulation of specific DNA fragments.*
- *The doubling of number of DNA strands corresponding to target sequences can be estimated by amplification number associated with each cycle using the formula.*
- *Amplification=2n, where n=no. of PCR cycle.*
- *PCR can amplify a desired DNA sequences of any origin hundred or millions time in a matter of hour, which is very short in comparison to recombinant DNA technology.*
- *PCR is especially valuable because the reaction is highly specific, easily automated and very sensitive.*
- *It is widely used in the fields like- clinical medicine for medical diagnosis, diagnosis of genetic diseases, forensic science; DNA finger printing, evolutional biology*

Factors affecting PCR

i. Primer

- PCR reaction needs two primer, a forward and a reverse primer
- Primer are synthesized oligonucleotide usually ranging from 15-30 bases long
- Primers are designed such that at 3'end they donot have more than two bases complementary to each other as this results in PRIMER-DIMER formation.

- The G+C contents is in the range of 40-60%
- The melting temperature (Tm) of both forward and reverse primer is usually the same.
- Low concentration of primer results in poor yield while high concentration may results in non specific amplification. Hence optimal concentration of primer is needed ie 0.1-1µ

ii. Amount of Template DNA

- Optimal amount of template DNA usually in nano gram. Higher concentration inhibit or results in non specific amplification.
- Taq DNA polymerase:
- Taq DNA polymerase is 94 KD thermostable DNA polymerase isolated from Thermus aquaticus.
- Optimal temperature for activity of Taq polymerase is 72° but it can tolerate high temperature and donot affects by denaturating temperature of 94°C.
- Taq DNA polymerase have both 5'-3' polymerase activity and 5'-3' exonuclease activity. But it lacks 3'-5' exonuclease activity (proof reading activity).

Types of PCR

1. Standard PCR:

- Nested PCR
- Random amplified polymorphic DNA
- Long PCR
- Restriction fragment length polymorphism (RFLP)
- Amplified fragment length polymorphism (AFLP)
- Multiplex PCR
- Single cell PCR
- Fast cycling PCR

- In situ PCR
- High fidelity PCR
- Asymmetric PCR
- Repetitive sequence based PCR
- Overlap extension PCR
- Assemble PCR
- Mini primer PCR
- Solid phase PCR
- Touch Down PCR

2. Reverse transcriptase Polymerase chain reaction (RT-PCT): for RNA

- One step RT-PCR
- Two step RT-PCR

3. Real time PCR: for DNA or RNA

- Dye binding to ds DNA
    - Fluorescent probes

Application:

1. Forensic science: DNA finger printing, paternity testing and criminal identification
2. Diagnosis: Molecular identification of microorganisms
3. Evolution study: evolutionary biology
4. Fossil study: paleontology
5. Gene cloning and expression
6. Gene sequencing
7. Vaccine production by recombinant DNA technology
8. Drug discovery
9. Mutation study

10. Human genome project

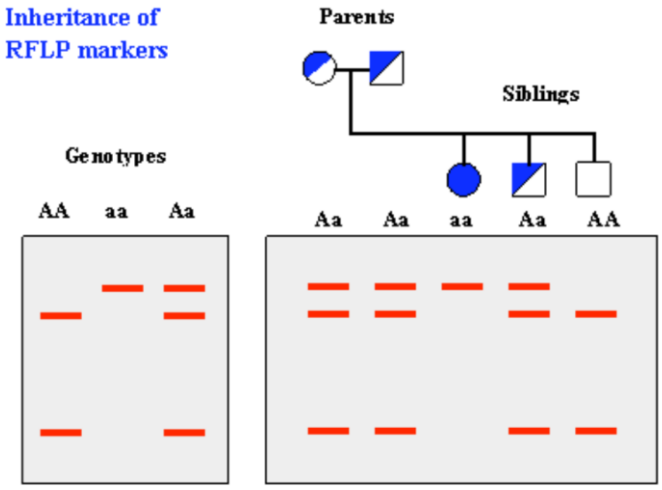
RFLPs (restriction fragment length polymorphisms)

Review of basic techniques:

The use of restriction fragment length polymorphisms (RFLPs) and variable length tandem repeats (VNTRs) as genetic markers and tools in DNA fingerprinting is heavily dependent on the use of Southern blotting with appropriate restriction endonucleases and carefully selected probes.

Use of RFLPs as genetic markers:

When a specific cloned DNA probe is used to analyze a Southern blot of human (or other) DNA, a limited number of restriction fragments of specific and characteristic lengths will be identified. Because single base mutations can either create additional restriction sites or destroy pre-existing sites, DNA preparations from different individuals frequently exhibit different patterns of size distribution of restriction fragments that hybridize with a particular probe. These differences are called restriction fragment length polymorphisms (RFLPs). In many cases, the genetic polymorphisms that generate RFLPs will have no obvious genetic effect because they are located in introns or involve "silent" mutations that convert a codon to different codon specifying the same amino acid. However, they are inherited as codominant Mendelian markers and are extremely useful in studies of human genetic linkage.



Annonymous probes:

The special advantage of RFLPs as genetic markers is that they do not need to have any special properties other than the existence of the restriction endonuclease that responds to the presence or absence of a particular cut site and the availability of a probe that can be used to visualize the fragments. Any random clone, including sequences located in introns or between genes, that happens to emerge during "shotgun" cloning can potentially be used as a probe. Probes of this sort that do not correspond to any known genes are referred to as anonymous probes. Many useful RFLPs are identified with anonymous probes.

Human linkage markers: It is difficult to find suitable linkage markers for human genetic linkage studies. The total number of known genes is still rather small (although it is now growing rapidly because of the human genome project). In addition, many of the genetic loci have been identified only in terms of relatively rare alleles that cause disease phenotypes, with the vast majority of the population carrying the wild-type alleles that do not differ from one individual to another.

Codominant expression:

RFLP haplotypes (RFLPs carried on single chromosomes in a genome) are stable genetic markers that are inherited in a codominant manner, often with a relatively high frequency of alternative alleles in healthy individuals. This allows them to be used in all types of genetic

studies, including analysis of their linkage to the genes responsible for human genetic diseases. Because of their usefulness, large numbers of human RFLPs have been studied in detail, including the chromosomal locations of the DNA sequences responsible for the polymorphisms.

Linkage to RFLP haplotypes:

Because most human genetic diseases are initially identified only by the disease phenotype, demonstration of linkage to a specific RFLP haplotype is frequently the first step toward identifying the chromosome that carries the disease gene. In addition, a close linkage (identified by a high lod score) can localize the disease gene to a specific region of the chromosome. This in turn provides the starting point for studies leading to the isolation and cloning of the specific gene that is responsible for the disease. Six different examples of identification and cloning of genes responsible for inherited human diseases are presented below, each of which employed a somewhat different experimental approach.

Some examples:

Neurofibromatosis:

Type 1 neurofibromatosis is an autosomal dominant condition associated with a wide range of nervous system defects, including benign tumors and learning disabilities. As described in the textbook (pages 464-465), a search for linkage to specific RFLPs localized the candidate gene to a region near the centromere of human chromosome 17. After the gene was localized as much as possible, chromosome walking was undertaken until a candidate gene was encountered. Its involvement in the disease was verified by sequencing studies that showed mutations in individuals afflicted with the disease. The overall process that led to the discovery of the NF1 gene is called positional cloning. The wild-type gene appears to function in intracellular signal transduction, and more specifically in down-regulating cellular reproduction.

Marfan syndrome:

A rather different approach was taken to identify the gene that is defective in Marfan syndrome, an autosomal dominant condition that causes alterations in connective tissue. Particular attention was given to genes coding for proteins known to function in various types of connective tissue. A protein known as fibrillin, which is found in tissues affected by Marfan syndrome was identified as a likely candidate. The gene for fibrillin had already been cloned and mapped to the long arm of human chromosome 15. RFLP studies verified a linkage between the inheritance of Marfan syndrome and markers on chromosome 15. Cloning of the fibrillin gene from individuals with Marfan syndrome then verified the substitution of a proline for arginine at position 239 in the protein. The textbook describes this as the candidate gene approach.

Huntington disease:

The search for the gene responsible for Huntington disease (also known as Huntington's chorea) was described in a previous textbook as an example of the use of RFLPs. Huntington disease is an autosomal dominant degenerative brain disease that usually does not exhibit any obvious symptoms prior to middle age. There is then a progressive loss of motor coordination, accompanied by uncontrolled spontaneous movements, ultimately resulting in death, but only after a prolonged period of increasingly severe symptoms.

Nuclear Acid Hybridization Method

Nuclear Acid Hybridization Method:

The DNA double helix consists of two strands of nucleotides twisted around each other and held together by hydrogen bonding between the bases. If a solution of DNA is heated, the input of energy makes the molecules vibrate and the hydrogen bonds start coming apart.

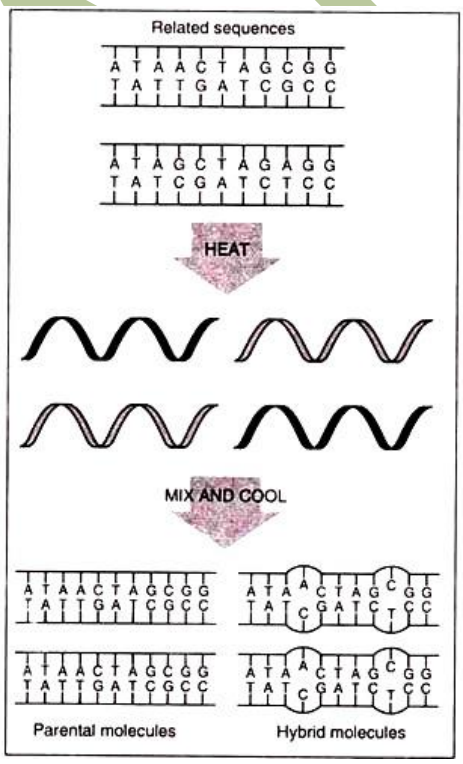
If the temperature is high enough, the DNA comes completely apart into two separate strands. This is known as denaturation or “melting”. Since the GC base pair has three hydrogen bonds compared to two holding AT together, GC base pairs are stronger than AT base pairs. Therefore, as the temperature rises, AT pairs come apart first and regions of DNA with lots of GC base pairs melt at higher temperatures.

The melting temperature of a DNA molecule is defined as the temperature at halfway point on melting curve. The halfway point is used because it is more accurate than trying to guess precisely where melting is complete.

Melting is followed by measuring the UV absorption, since disordered DNA absorbs more UV light. Overall, the higher the proportion of GC base pairs, the higher the melting temperature of a DNA molecule.

If denatured DNA is cooled again, the single DNA strand will recognize partners by base pairing and double stranded DNA will re-form. This is referred to as annealing. For proper annealing, DNA must be cooled slowly to allow time for single strand to find the correct partners.

Consider two completely different. DNA molecules. If they are mixed, melted and then cooled to re-anneal the single strand, each single strand will recognize and pair with its original complementary strand.

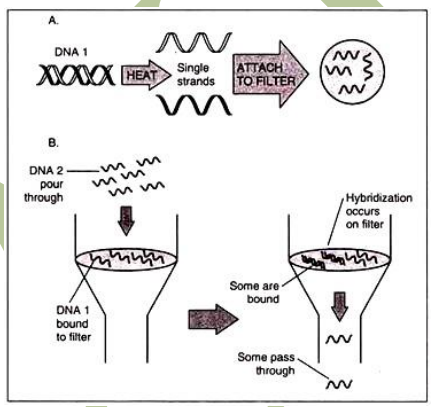


Suppose on the other hand, two closely related DNA molecules are used. Although the sequences may not match perfectly, nonetheless, if they are similar enough, some base pairing will occur. The result will be formation of hybrid DNA molecules.

The followings are uses of Nucleic acid hybridization:

(a) To Test the Frequency of Relatedness between Two DNA Molecules:

To do this, a sample of first DNA molecule is heated to melt it into single-stranded DNA. The single strands are then attached to suitable filter. Next, the filter is treated chemically to block any remaining sites that would bind DNA. Then, after melting, a solution of second DNA molecule is poured through the filter.



Some of the single strands of DNA molecule No. 2 will base pair with the single strands of DNA molecule No. 1 and will stick to the filter. The more closely related the two molecules are, the more hybrid molecules will be formed and the higher the proportion of molecule No. 2 will bound by the filter.

For example, if the DNA for a human gene, such as haemoglobin, is fully melted and bound to a filter, then DNA for the same gene but from different animals could be tested. We might expect Gorilla DNA to bind strongly, frog DNA to bind weakly and mouse DNA to be intermediate.

(b) To Isolate Genes for the Process of Cloning:

Suppose we already have the human haemoglobin gene and want to isolate the corresponding Gorilla gene. First, the human DNA is bound to the filter as before. Then gorilla DNA is cut into short segments with a suitable restriction enzyme.

The gorilla DNA is heated to be melted into single strands and poured over the filter. The DNA fragment that carries the gorilla gene for haemoglobin will bind to human haemoglobin gene and remain stuck to the filter. Other unrelated genes will not hybridize. This approach allows isolation of new genes provided a related gene is available for hybridization.

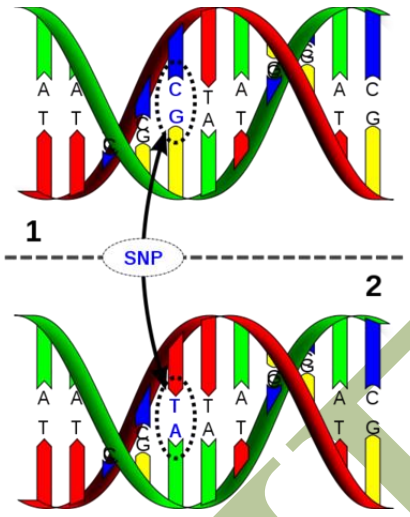
(c) To Search for Identical or Similar Sequences in Experimental Sample of Target Molecules:

A wide range of method based on hybridization is used for analysis in molecular biology. The basic idea in each case is that a known DNA sequence acts as a "probe". Generally, the probe molecule is labelled by radioactivity or fluorescence for ease of detection.

The probe is used to search for identical or similar sequences in experimental sample of target molecules. Both the probe and target DNA must be treated to give single-stranded DNA molecules that can hybridize to each other by base pairing. In previous example, the probe DNA would be the human haemoglobin DNA since the sequence is already known. The gorilla DNA would be sample of target molecules.

Single nucleotide polymorphisms (SNPs)

A **single-nucleotide polymorphism** (**SNP**, pronounced *snip*) is a DNA sequence variation occurring when a single nucleotide adenine (A), thymine (T), cytosine (C), or guanine (G]) in the genome (or other shared sequence) differs between members of a species or paired chromosomes in an individual. For example, two sequenced DNA fragments from different individuals, AAGC**C**TA to AAGC**T**TA, contain a difference in a single nucleotide. In this case we say that there are two *alleles*: C and T. Almost all common SNPs have only two alleles.



Within a population, SNPs can be assigned a minor allele frequency — the lowest allele frequency at a locus that is observed in a particular population. This is simply the lesser of the two allele frequencies for single-nucleotide polymorphisms. There are variations between human populations, so a SNP allele that is common in one geographical or ethnic group may be much rarer in another.

Types of SNPs

Single nucleotides may be changed (substitution), removed (deletions) or added (insertion) to a polynucleotide sequence. Single nucleotide polymorphisms may fall within coding sequences of genes, non-coding regions of genes, or in the intergenic regions between genes. SNPs within a coding sequence will not necessarily change the amino acid sequence of the protein that is produced, due to degeneracy of the genetic code.

A SNP in which both forms lead to the same polypeptide sequence is termed *synonymous* (sometimes called a silent mutation) — if a different polypeptide sequence is produced they are *nonsynonymous*. A nonsynonymous change may either be missense or nonsense, where a missense change results in a different amino acid, while a nonsense change results in a premature stop codon. SNPs that are not in protein-coding regions may still have consequences for gene splicing, transcription factor binding, or the sequence of non-coding ribonucleic acid (RNA).

Listing SNPs

A confirmed SNP Z282 (in green equals positive + or confirmed by SNP testing) chart from FTDNA. This screenshot shows the various colors used for designating SNPs.

SNPs, in particular the key marker SNP used in Hapologroups, are designated by color or code in genetic genealogy.

A non-tested or estimated SNP is usually designated in a black or the standard color of the text or without a symbol of tested confirmation (+) or non-confirmation (-) code.

A tested SNP that is confirmed by SNP testing is usually designated in a green colored text or with a plus symbol (+) after the SNP designation. It is often referred to as derived or given a positive (+) code.

A tested SNP that is not confirmed or that is negative (-) is usually in red text.

SNPs pending testing, or assumed negative have different colors, but do not use the code of either positive (+) or negative (-). Different colors can be used by various testing companies, so it is important to confirm the designations within the charts given.

Use and importance of SNPs

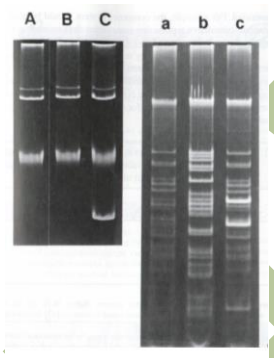
Variations in the DNA sequences of humans can affect how humans develop diseases and respond to pathogens, chemicals, medication, vaccines, and other agents. SNPs are also thought to be key enablers in realizing the concept of personalized medicine. However, their greatest importance in biomedical research is for comparing regions of the genome between cohorts (such as matched cohorts with and without a disease).

Plasmid finger printing

- Plasmid fingerprinting identifies microbial species or similar strains as related strains often contain the same number of plasmids with the same molecular weight.
- Plasmid of many strains and species of *E. coli*, *Salmonella*, *Camylobacter* and *Psseudomonas* has demonstrated that this methods is more accurate than phenotypic

methods such as biotyping, antibiotic resistance patterns, phage typing and serotyping.

- The procedure involves: The bacterial strains are grown, the cells lysed and harvested. The plasmids are separated by agarose gel electrophoresis.
- The gels are stained with EtBr and the plasmids located and compared.



Laboratory tests in chemotherapy

A healthy body constantly replaces cells through a process of dividing and growing. When cancer occurs, cells reproduce in an uncontrolled manner.

As a part of the body produces more and more cells, they start to occupy the space that useful cells previously took up.

Chemotherapy drugs interfere with a cancer cell's ability to divide and reproduce.

A single drug or combination of drugs can do this.

Treatment can either:

- attack cancer cells throughout the body or
- target specific sites or processes

What does chemotherapy do?

Chemotherapy drugs can:

- prevent cell division
- target the cancer cells' food source (the enzymes and hormones they need to grow)
- trigger apoptosis, or the "suicide" of cancer cells

Some emerging treatments aim to stop the growth of new blood vessels that supply a tumor in order to starve it. Some scientists are concerned that this strategy might encourage the growth and spread of cancer in some cases.

Why use chemotherapy?

A doctor may recommend chemotherapy:

- to shrink a tumor before surgery
- after surgery or remission, to remove any remaining cancer cells and delay or prevent a recurrence
- to slow disease progression and reduce symptoms in the later stages, even if a cure is unlikely

What to expect?

Chemotherapy is an invasive treatment that can have severe adverse effects both during the therapy and for some time after. This is because the drugs often target both cancer cells and healthy cells.

How long does chemo last?

The doctor will make a plan with the individual that specifies when treatment sessions will occur and how many they will need.

A course of treatment can range from a single dose on one day to a few weeks, depending on the type and stage of cancer.

Those who need more than one course of treatment will have a rest period to allow their body to recover.

A person might have treatment on one day, followed by a week's rest, then another one-day treatment followed by a three-week rest period, and so on. A person may repeat this several times.

Some people may find talking to a counselor about the mental and emotional aspects of cancer and chemotherapy helpful.

Blood tests

Blood tests assess the person's health and ensure that they will be able to cope with possible side effects.

Liver health: The liver breaks down chemotherapy chemicals and other drugs. Overloading the liver could trigger other problems. If a blood test detects liver problems before treatment, the person may have to postpone treatment until it recovers.

Low count of red or white blood cells or platelets: If these blood counts are low before treatment, the person may need to wait until they reach healthy levels before starting chemotherapy.

It is important to have regular blood tests during the treatment period to ensure that blood and liver functions remain as healthy as possible and monitor the effectiveness of the treatment.

How is the dose given?

Most people will receive chemotherapy in a clinical setting, but sometimes a person can take it at home.

Ways of taking chemotherapy include:

- by mouth, as tablets, liquid or capsules
- intravenously, as an injection or infusion
- topically, onto the skin

In some cases, a person may be able to take the medication at home. However, they will need to make regular visits to the hospital to check their health and how they are responding to treatment.

The person must take the dose exactly as the doctor prescribes. If they forget to take a dose at the right time, they should call their doctor immediately.

Sometimes, a person will need a continuous dose. This means that they may have to wear a pump that delivers the drug slowly for several weeks or months. They can wear the pump as they go about their daily life.

Types

Types of chemotherapy include:

Alkylating agents: These affect the DNA and kill the cells at different stages of the cell life cycle.

Antimetabolites: These mimic proteins that the cells need to survive. When the cells consume them, they offer no benefit, and the cells starve.

Plant alkaloids: These stop the cells from growing and dividing.

Antitumor antibiotics: These stop the cells from reproducing. They are different from the antibiotics people use for an infection.

Susceptibility tests

The introduction of various antimicrobials for treating variety of infections showed the necessity of performing antimicrobial susceptibility testing as a routine procedure in all microbiology laboratories. In laboratories it can be made available by using antibiotic disk which will diffuse slowly into the medium where the suspected organism is grown. The basic principle of the antibiotic susceptibility testing has been used in microbiology laboratories over 80 years. Various chemical agents such as antiseptics, disinfectants, and antibiotics are employed to combat with the microbial growth. Among these, antibiotics are generally

defined as the substances produced by the microorganism such as Penicillium, which has the ability to kill or inhibit the growth of other microorganisms, mainly bacteria. Antimicrobial susceptibility tests (ASTs) basically measures the ability of an antibiotic or other antimicrobial agent to inhibit the invitro microbial growth.

There are many different procedures that microbiologists use to study the effects of various antimicrobial agents in treating an infection caused by different microorganisms. Mueller Hinton Agar is considered as best for the routine susceptibility testing since it is has batch-to-batch reproducibility, low concentration of inhibitors of sulphonamide, trimethoprim and tetracyclines and produce satisfactory results for most of the non-fastidious pathogens. Fastidious organisms which require specific growth supplements need different media to grow for studying the susceptibility patterns.

The Kirby Bauer test is a qualitative assay whereby disks of filter paper are impregnated with a single concentration of different antibiotics or any chemicals that will diffuse from the disk into the agar. The selected antibiotic disks are placed on the surface of an agar plate which has already been inoculated with test bacteria. During the incubation period, the antibiotics/chemicals diffuse outward from the disks into the agar. This will create a concentration gradient in the agar which depends on the solubility of the chemical and its molecular size. The absence of growth of the organism around the antibiotic disks indicates that, the respected organism is susceptible to that antibiotic and the presence of growth around the antibiotic disk indicates the organism is resistant to that particular antibiotic. This area of no growth around the disk is known as a zone of inhibition, which is uniformly circular with a confluent lawn of growth in the media.

The diameters of the zone of inhibition are measured (including disk) using a metric scale or a sliding caliper. The measured zone diameter can be compared with a standard chart for obtaining the susceptible and resistant values. There are zone of intermediate resistance which means that the antibiotic may not be sufficient enough to eradicate the organism from the body.

Factors affecting Antibiotic Susceptibility Testing

Many conditions can affect the accuracy of the AST results, which is described in detail below.

pH

pH of the medium is an important factor which influences the accuracy of the antibiotic susceptibility testing. If the pH of the medium is too low than the desired pH, certain drugs such as amino glycosides, quinolones and macrolides lose their potency, on the other hand, antibiotic classes such as tetracyclines appear to have excess activity a lower Ph and the vice versa happens in the case of the higher pH.

Moisture

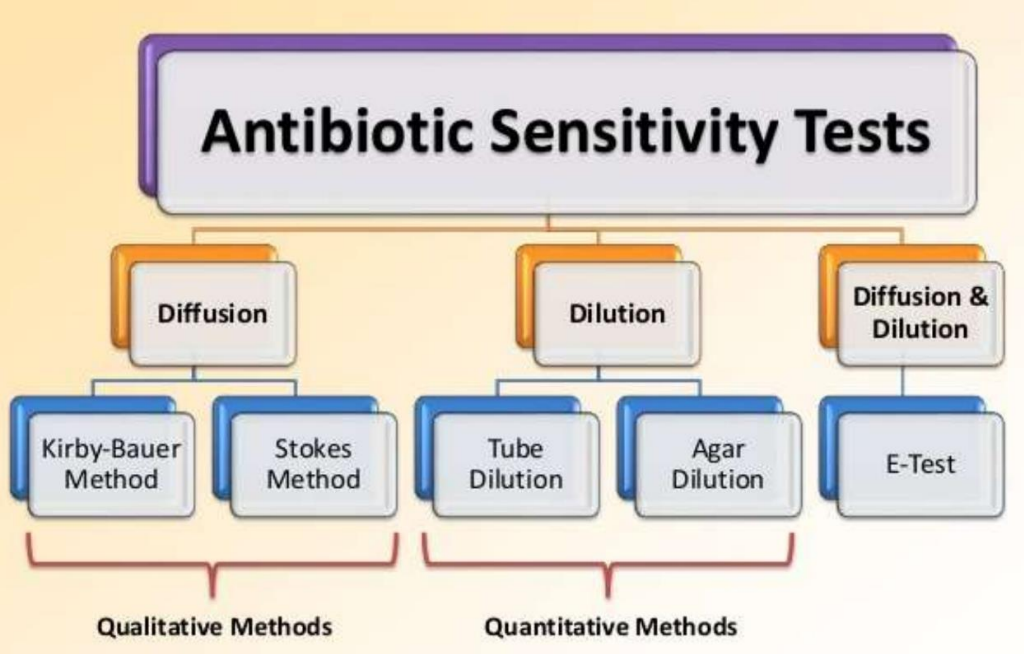
The presence of moisture content on the medium can counter act with accuracy of the susceptibility testing. It is important to remove the excess moisture present in the agar surface, by keeping it in the laminar flow hood for few minutes.

Effects of medium components

If the media selected for the antibiotic susceptibility contains excessive amounts of thymine or thymidine compounds, they will reversibly inhibit the action of certain antimicrobial agents such as trimethoprim groups. This reversible inhibition yields smaller or less distinct or even no zones and will be misinterpreted as resistant antibiotics. MHA is low in thymine and thymidine content and it can be used successfully to study the susceptibility of antibiotics. Also the medium containing excessive cation reduces the zone size, while low cation content results in unacceptably large inhibition zones.

Amount of organism

The amount of the organism used for the susceptibility testing is standardized using a turbidity standard. This is obtained by a visual approximation using McFarland standard of 0.5 or else it can be determined by using a spectrophotometer with Optical density of 1 at 600 nm wavelength.



CONVENTIONAL AST

DISK DIFFUSION METHOD:

• Simplest and most convenient

• Widely used everywhere

• Developed by kirby, sherrris, bauer and turk in 1966

Types:

1. Kirby Bauer method

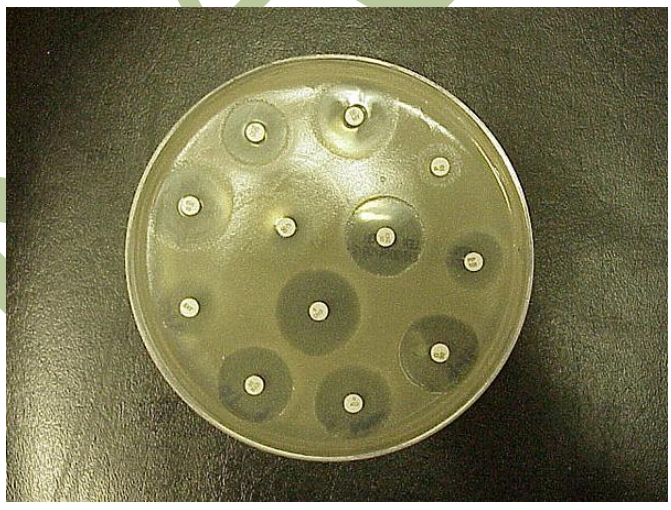
2. Stokes method

Kirby-Bauer Method

A true antibiotic is an antimicrobial chemical produced by microorganisms against other microorganisms. Mankind has made very good use of these antimicrobials in its fight against infectious disease. Many drugs are now completely synthetic or the natural drug is

manipulated to change its structure somewhat, the latter called semisynthetics. Bacteria respond in different ways to antibiotics and chemosynthetic drugs, even within the same species. For example, Staphylococcus aureus is a common normal flora bacterium found in the body. If one isolated this bacterium from 5 different people, the 5 isolates would likely be different strains, that is, slight genetically different. It is also likely that if antibiotic sensitivity tests were run on these isolates, the results would vary against the different antibiotics used.

The Kirby-Bauer test for antibiotic susceptibility (also called the *disc diffusion test*) is a standard that has been used for years. First developed in the 1950s, it was refined and by W. Kirby and A. Bauer, then standardized by the World Health Organization in 1961. It has been superseded in clinical labs by automated tests. However, the K-B is still used in some labs, or used with certain bacteria that automation does not work well with. This test is used to determine the resistance or sensitivity of aerobes or facultative anaerobes to specific chemicals, which can then be used by the clinician for treatment of patients with bacterial infections. The presence or absence of an inhibitory area around the disc identifies the bacterial sensitivity to the drug.



The basics are easy: The bacterium is swabbed on the agar and the antibiotic discs are placed on top. The antibiotic diffuses from the disc into the agar in decreasing amounts the further it

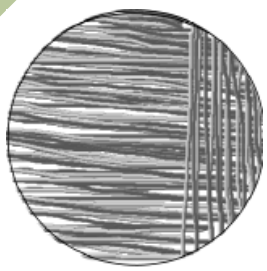
is away from the disc. If the organism is killed or inhibited by the concentration of the antibiotic, there will be **NO growth** in the immediate area around the disc: This is called the **zone of inhibition** (Figure 9.1). The zone sizes are looked up on a standardized chart to give a result of sensitive, resistant, or intermediate. Many charts have a corresponding column that also gives the **MIC** (minimal inhibitory concentration) for that drug. The **MIC** is currently the standard test run for antibiotic sensitivity testing because it produces more pertinent information on minimal dosages.

The **Mueller-Hinton medium** being used for the Kirby-Bauer test is very high in protein.

MATERIALS NEEDED: per table

- 2 Mueller-Hinton agar plates
- 24 hr old cultures (likely to be Staph, E. coli, Bacillus subtilus, Enterococcus fecalis) sterile swabs
- Antibiotics
- Ethanol
- Forceps
- ***Pseudomonas aeruginosa*** Kirby-Bauer plate for demo
- **guidelines chart** for interpretation of antibiotic susceptibility

THE PROCEDURES



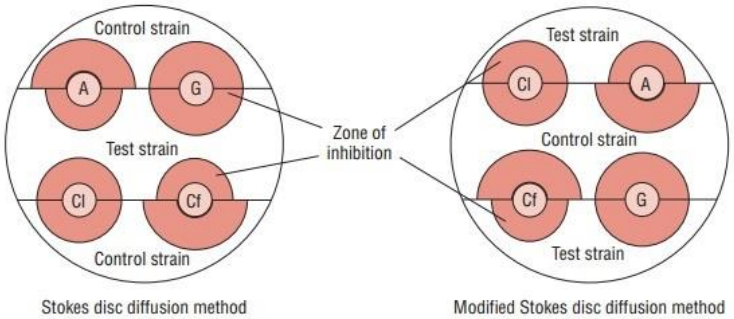
Swab a Mueller-Hinton plate with **ONLY 2** of the bacteria (tables will run different combinations of the 4 bacteria). Dip a sterile swab into the broth and express any excess moisture by pressing the swab against the side of the tube.

Swab the surface of the agar completely (**you do not want to leave any unswabbed agar areas at all**). In the pictures below, you can see what happens when the plate is not swabbed correctly with even coverage of the bacterium over the entire agar.

After completely swabbing the plate, **turn it 90 degrees and repeat the swabbing process**. (It is not necessary to re-moisten the swab.) Run the swab around the circumference of the plate before discarding it in the discard bag.

Allow the surface to dry for about 5 minutes before placing antibiotic disks on the agar.

Stokes Disc Diffusion Technique varies from **Kirby Bauer disc diffusion** in the use of both control and test strain on a same plate. Stokes disc diffusion technique is not as highly standardized as the Kirby-Bauer technique and is used in laboratories particularly when the exact amount of antimicrobial in a disc cannot be guaranteed due to difficulties in obtaining discs and storing them correctly or when the other conditions required for the Kirby-Bauer technique cannot be met.



Schematic diagram showing Stokes method of antibiotic sensitivity.

Comparative disc diffusion techniques based on Stokes method is still in wide use in majority of laboratories in UK, to determine antibiotic susceptibility. The stokes' method allows each

individual **isolate to be compared with a sensitive control of the same or similar species** which is subjected to the same technical conditions of medium, incubation time, atmosphere, temperature and disc content. As control and test organisms are adjacent on the same plate the difference between respective zone sizes can be measured directly.

Procedure

- At least three to five well-isolated colonies of the same morphological type of both test and control strains are selected from an agar plate culture. The top of each colony is touched with a loop, and the growth is transferred into a tube containing 4 to 5 ml of a suitable broth medium, such as tryptic soy broth.
- The broth culture is incubated at 37°C until it achieves or exceeds the turbidity of the **0.5 McFarland standard** (usually 2 to 6 hours).
- The turbidity of the actively growing broth culture is adjusted with sterile saline or broth to obtain a turbidity optically comparable to that of the 0.5 McFarland standard. This results in a suspension containing approximately 1 to 2 x 10^8 CFU/ml for *E.coli* ATCC 25922. To perform this step properly, either a photometric device can be used or, if done visually, adequate light is needed to visually compare the inoculum tube and the 0.5 McFarland standard against a card with a white background and contrasting black lines.
- Optimally, within 15 minutes after adjusting the turbidity of the inoculum and control strains suspension, sterile cotton swabs are dipped into each of the adjusted suspension. The swabs should be rotated several times and pressed firmly on the inside wall of the tube above the fluid level. This will remove excess inoculum from the swab.
- A dried **Müeller-Hinton agar** plate is divided into 3 halves.
- The dried surface of a Müeller-Hinton agar plate is inoculated by streaking the **control strains evenly across the upper and lower thirds of the plate**, and the **test strains between the control**, leaving a **distance of not more than 5mm** on each side of the control strain.

- Allow the inocula to dry for few minutes with the lid.
- Place antimicrobial discs in the gap between the test and control strain using a sterile forcep and press gently.
- Within 30 mins of applying the discs, incubate the plates aerobically at 35-37 °C for 18-24 hrs.

Inoculation of Test Plates

NOTE: Extremes in inoculum density must be avoided. Never use undiluted overnight broth cultures or other unstandardized inocula for streaking plates.

Interpretation of results

Measure the radius of the inhibition zone from the edge of the disc to the edge of the zone.

- **Sensitive (S)**: Zone radius is wider than or equal to, or not more than 3mm smaller than the control.
- **Intermediate (I):** Zone Radius is > 2 mm but smaller than the control by > 3mm.
- **Resistant (R)**: No zone of inhibition or zone radius measures 2mm or less

Advantages of Stokes method:

- The control strain and test strain can be checked on the same plate.
- More reliable for the quality testing of discs.
- The effect of variation of environmental condition like temperature, time affect both simultaneously thus minimizing error
- Errors due to using too heavy or light inoculums will be detected.

Minimum inhibitory concentration: Dilution methods

Minimum inhibitory concentration (MIC) is determined when a patient does not respond to treatment thought to be adequate, relapses while being treated or when there is immunosuppression.

Dilution methods can be carried out in 2 ways

A. Broth dilution

Broth dilution testing allows the option of providing both quantitative (MIC) and qualitative (category interpretation) results. MIC can be helpful in establishing the level of resistance of a particular bacterial strain and can substantially affect the decision to use certain antimicrobial agents.

Broth dilution can again be performed by 2 ways

1. Macro dilution: Uses broth volume of **1 ml** in standard test tubes.
2. Microdilution: Uses about **0.05 to 0.1 ml** total broth volume and can be performed in a microtiter plate or tray.

The procedure for both macro and microdilution are same except the volume of the broth.

B. Agar dilution

MIC of an antibiotic using broth dilution method is determined by using the following procedure

1. Preparation of antibiotic stock solution
2. Preparation of antibiotic dilution range
3. Preparation of agar dilution plates
4. Preparation of inoculum
5. Inoculation
6. Incubation
7. Reading and interpreting results

Preparation of antibiotic Stock solution.

Antibiotic stock solution can be prepared by commercially available antimicrobial powders (with given potency). The amount needed and the diluents in which it can be dissolved can be calculated by using either of the following formulas to determine the amount of antimicrobial powder (1) or diluent (2) needed for a standard solution:

$$(1)\text{Weight (mg)} = \frac{\text{Volume (mL)} \cdot \text{Concentration (µg/mL)}}{\text{Potency (µg/mg)}}$$

or

$$(2)\text{Volume (mL)} = \frac{\text{Weight (mg)} \cdot \text{Potency (µg/mg)}}{\text{Concentration (µg/mL)}}$$

Prepare antimicrobial agent stock solutions at concentrations of at least 1000 µg/mL (example: 1280 µg/mL) or 10 times the highest concentration to be tested, whichever is greater.

Because microbial contamination is extremely rare, solutions that have been prepared aseptically but not filter sterilized are generally acceptable. If desired, however, solutions may be sterilized by **membrane filtration**. Dispense small volumes of the sterile stock solutions into sterile glass, polypropylene, polystyrene, or polyethylene vials; carefully seal; and store (*preferably at −60 °C or below, but never at a temperature warmer than −20 °C and never in a self-defrosting freezer).* Vials may be thawed as needed and used the same day.

Preparation of antibiotic dilution range

- Use sterile 13- x 100-mm test tubes to conduct the test. If the tubes are to be saved for later use, be sure they can be frozen.
- Close the tubes with loose screw-caps, plastic or metal closure caps, or cotton plugs.

- Prepare the final two fold (or other) dilutions of antimicrobial agent volumetrically in the broth. A minimum final volume of 1 mL of each dilution is needed for the test.

Note: For, microdilution,only 0.1 ml is dispensed into each of the 96 wells of a standard tray.

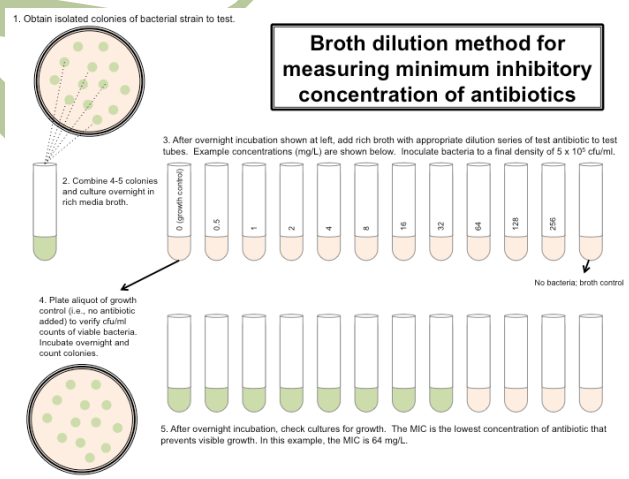
Preparation of inoculum

Prepare the inoculum by making a direct broth suspension of isolated colonies selected from an 18- to 24-hour agar plate *(use a non-selective medium, such as **blood agar**).*

Adjust the suspension to achieve a turbidity equivalent to a **0.5 McFarland turbidity standard**. This results in a suspension containing approximately 1 to 2 x 10^8 colony forming units (CFU)/mL for *Escherichia coli* ATCC®a 25922.

Compare the inoculum tube and the 0.5 McFarland standard against a card with a white background and contrasting black lines.

Optimally within 15 minutes of preparation, dilute the adjusted inoculum suspension in broth so, after inoculation, each tube contains approximately 5 x 10^5 CFU/mL.**Note:** *This can be accomplished by diluting the 0.5 McFarland suspension 1:150, resulting in a tube containing approximately 1 x 10^6 CFU/mL. The subsequent 1:2 dilution in step 3 brings the **final inoculum to 5 x 10^5 CFU/mL.***



Broth dilution method for measuring minimum inhibitory concentration of antibiotics.

Inoculation

Within 15 minutes after the inoculum has been standardized as described above, add 1 mL of the adjusted inoculum to each tube containing 1 mL of antimicrobial agent in the dilution series (and a positive control tube containing only broth), and mix.

This results in a 1:2 dilution of each antimicrobial concentration and a 1:2 dilution of the inoculums.

Incubation:

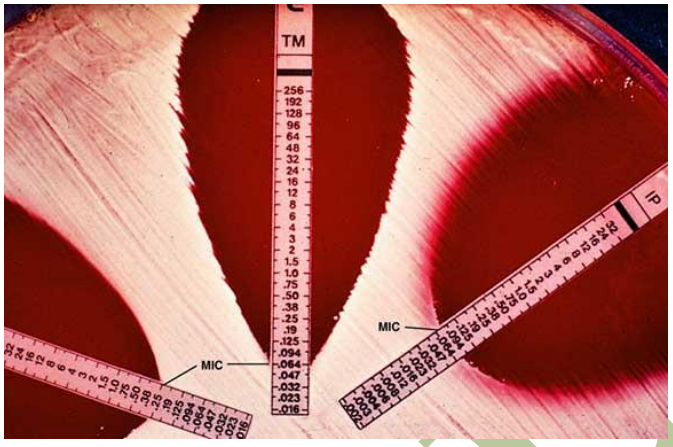
Incubate the inoculated tubes at 35 ± 2 ºC for 16 to 20 hours in an ambient air incubator. To maintain the same incubation temperature for all cultures, do not stack microdilution trays more than four high.

Interpretation

Compare the amount of growth in the wells or tubes containing the antimicrobial agent with the amount of growth in the growth-control wells or tubes (no antimicrobial agent) used in each set of tests when determining the growth end points. For a test to be considered valid, acceptable growth (≥ 2 mm button or definite turbidity) must occur in the growth-control well.

E (Epsilometer) Test

The E-test has been developed to provide a direct quantification of antimicrobial susceptibility of microorganisms. E-test is a laboratory test used to determine minimum inhibitory concentration (MIC) and whether or not a specific strain of bacterium or fungus is susceptible to the action of a specific antibiotic. The principle of E test was first described in 1988 and was introduced commercially in 1991 by AB BIODISK.



E test strip is a non-porous plastic strip immobilized with predefined continuous and stable gradient of 15 antibiotic concentrations on one side and printed with an MIC scale on the other side of nylon strips.

E-test has an extensive range of over 100 antimicrobial references that can be classed into 4 categories:

- Antibiotics
- Antifungal
- Anti-mycobacterial
- Resistance phenotype testing

Principle of E-Test

E test is a quantitative technique that is based on combination of concept of both dilution and diffusion principle for susceptibility testing. E test strip is placed on to an inoculated agar plate; there is an immediate release of antibiotics from the plastic carrier surface into the agar surface. After incubation, bacterial growth becomes visible, symmetrical inhibition ellipse along the strip is seen. The MIC value is read from the scale in terms of µg/ml where the ellipse edge intersects the strip.

Purpose of E-Test

1. Determine the MIC of fastidious, slow-growing or nutritionally deficient micro-organisms, or for a specific type of patient or infection.
2. Detect low levels of resistance.
3. Confirm/ detect a specific antimicrobial resistance phenotype such as ESBL, MBL, and AmpC.
4. Confirm an equivocal Antimicrobial Susceptibility Test result.
5. Test an antimicrobial not performed in routine use or a new, recently introduced antimicrobial agent.

Procedure of E-Test

1. Remove the E-test package from the freezer (-20°C) and kept at room temperature at least 30 minutes before the test performed.
2. Emulsify several well-isolated test strain colonies from an overnight agar plate in saline.
3. Vortex for 15 second.
4. Adjust the suspension turbidity to 0.5 McFarland standards. For mucoid organisms, adjust suspension to 1 McFarland standard. *(NOTE: Add more colonies to increase the turbidity or more saline to decrease the turbidity).*
5. Soak a sterile cotton swab into the inoculum suspension and remove the excess fluid by pressing it against the inside wall of the test tube.
6. Streak the swab over the entire agar surface in 3 directions by rotating the plate 60º on Mueller Hinton Agar (MHA). (*NOTE: For Haemophilus species use Haemophilus Test Medium Agar (HTM) and for S. pneumonia and viridians streptococci use Mueller Hinton Blood Agar (MHBA*)).
7. Allow the plate to dry for 5-15 minutes so that the surface is completely dry before applying E test gradient strip.
8. Place the E test strip on agar plate with MIC scale facing upward and the concentration maximum nearest the rim of the plate.
9. Incubate the plate as follows:
    1. *Haemophilus* species- CO2, 35ºC for 18 hours

2. *Streptococcus pneumoniae*– CO2, 35ºC for 20-24 hours
3. *Staphylococcus aureus* and *Enterococcus* species for Methicilllin and Vancomycin- O2, 35ºC for 24 hours
4. Others- O2, 35ºC for 24 hours.

10. After incubation observe zone of inhibition.

Result interpretation of E-Test

1. Read the MIC value at the point where ellipse intersects the scale/E-test strip. (*NOTE: Do not read the plate if the culture appears mixed or if the lawn of growth is too light or too heavy*).
2. If the MIC value between the standard two-fold dilution is seen, always round up to the highest value.
3. Read the MIC value at complete inhibition of all growth including isolated colonies.
4. If the intersect differs on either side of the strip, read the MIC as the greater value. Ignore any growth at the edge of the strip.
5. Interpret E-test MIC results as Susceptible, Intermediate or Resistant.

Precaution of E-Test

1. Aseptic procedures and precautions against microbiological hazards should be used when handling bacterial specimens.
2. E-test should be used strictly according to the procedures described.
3. Although based on a simple procedure, E-test should only be used by trained personnel.
4. Read results only if a good inhibition ellipse is visible.

Advantages of E-Test

1. Easy to perform and requires minimal training for test performance, however, end points can be difficult to determine for the azoles against *Candida*
2. Contamination can be easily recognized.
3. Can be easily set up for a small number of clinical isolates.

4. Adequate method to detect potentially resistant strains to Amphotericin B.

Limitation of E-Test

1. E-test is not suitable for *Cryptococcus neoformans*.

Automated procedures for antimicrobial susceptibility tests

There is a variety of methods available to clinical microbiology laboratories for antimicrobial susceptibility testing, disc diffusion techniques remain the most widely used for routine tests. In the past, instrumentation has had little impact on disc diffusion tests, but because of increased interest in using routinely derived susceptibility data for resistance surveillance, the potential of automated zone readers has recently attracted attention. The requirements of surveillance and, particularly in large busy laboratories, consideration of the potential for labour saving has also brought the use of automated systems under review. The manual determination of full MICs by agar or macro/microbroth dilution methods is infrequently undertaken in most clinical laboratories but instrumental systems for setting up and reading such tests are available. Agar-incorporation breakpoint methods are used for routine testing in several clinical laboratories in the UK and instrumental systems for inoculating and reading plates are widely used.

Instrumentation for disc diffusion susceptibility testing Studies in the 1960s, particularly the international collaborative study of Ericsson & Sherris,1 brought into focus the problems of variables in susceptibility testing methods, especially those affecting the performance of disc diffusion methods. These studies indicated the need for standardization. Standardization has centred around reducing variability by defining performance limits for growth medium (including removal of antagonists and appropriate supplementation for bacteria with fastidious growth requirements), incubation conditions, inoculum concentration, disc content (in the case of disc diffusion), setting of interpretative criteria (breakpoint MICs and inhibition zone diameters) and establishment of quality control parameters. Measurement of zone sizes is a significant variable that has received less attention despite being subjective and markedly

affected by lighting conditions. More recently, there has been a growing interest in the use of instrumentation for reading disc diffusion tests. Several systems available that use a camera or scanner to capture an image of the plate and then use image analysis software to measure zone sizes. Automated zone readers should reduce operator variability in reading plates and error in transcription of results, and provide automatic interpretation of zone diameters. Zone readers interfaced to laboratory information management systems (LIMS) would enable efficient data handling, including the storage and retrieval of epidemiological information in that accumulated zone diameters could be used locally for detailed examination of resistance and transferred electronically for regional or national resistance surveillance. Some systems also include so-called "expert" software to improve the quality of interpretation by the filtering of results according to a set of rules. Systems known to us include Accuzone (AccuMed International Inc., West lake, OH, USA), Aura Image (Oxoid, Basingstoke, UK), BIOMIC (Giles Scientific, New York, NY, USA), BioVideobact (Launch, Longfield, UK), Mastascan Elite (Mast, Bootle, UK), Osiris (Bio-Rad, Hemel Hempstead, UK), ProtoZONE (Don Whitley Scientific, Shipley, UK), and SIRSCAN (Becton Dickinson, Oxford, UK). They differ in the way data are input to identify specimens and select appropriate tests, in how the results are presented and in the flexibility and sophistication of the software. The systems appear to perform reasonably well with organisms that grow well on plain agar, but less well with tests on media containing blood. With these system it is advisable for an operator to check the on-screen image of the plate to ensure that zones have been read correctly. All systems allow rapid manual adjustment of zone sizes if necessary. If plates are set up and read with reproducibility, accuracy and close attention to quality control performance, it is suggested that results from routine qualitative disc diffusion techniques may be used to approximate the MIC by the application of appropriate computer algorithms. The attraction of this approach is the possibility of combining routine disc diffusion susceptibility data with MIC data generated by other systems as part of a "real-time" national or international surveillance programme as currently exemplified by the MRL TSN system. Instrumentation in other areas of disc diffusion tests has been limited to simple mechanical disc dispensers (available from disc manufacturers) for application of discs to inoculated plates, and simple

turbidimetric devices (available from disc manufacturers) or spectrophotometers for reading the density of suspensions of organisms when standardizing inocula.

Instrumentation for agar dilution methods

One of the earliest applications of instrumentation in susceptibility testing was the use of multipoint inoculum replicating systems for the application of multiple bacterial strains to a series of agar plates in MIC or breakpoint susceptibility tests. These devices deliver approximately 1µL spots of inoculum, depending on the size of the inoculator pins, and up to 96 test organisms per plate, depending on the plate size and pin arrangement. The device originally described in 1959 was simply mechanical, and although systems widely used today are motorized, plates are still fed manually. The use of image analysis systems to read agar incorporation breakpoint plates pre-dates the use of image analysis for reading zone diameters, although there are no published data on performance of the earlier readers for breakpoint plates. One of the recently developed zone readers, the Mastascan Elite, can also read breakpoint plates, but there are no published data on performance at present.

Instrumentation for broth dilution systems

Broth methods of susceptibility testing have been adapted to instrument-based systems with various degrees of mechanization and automation. At the lower end of sophistication endpoints are read visually and results are manually entered, and computer programs are simply used as an aid in recording, storage and analysis of data and in the generation of hard-copy reports. Interfacing with LIMS is possible and "expert" quality filters may be applied to the results before reporting. At the upper end of development, a small number of automated systems is available commercially and these incorporate microprocessor-controlled robotics for the manipulation of test cultures during incubation, and instrument-based growth detection by turbidometric or fluorometric methods before data handling and reporting. Some of these instruments have been adapted and validated as short-incubation systems (4-10 h depending upon the species and antimicrobial agent) and most can be used for identification as well as susceptibility testing.

SYLLABUS

Diagnosis and Standardization: Automation in microbial diagnosis, rapid diagnostic approach including technical purification and standardization of antigen and specific antibodies.

Automation in microbial diagnosis

While automation in clinical chemistry and hematology has moved gradually and in step with laboratory demand, clinical microbiology has not traditionally seen this trend. In 1992 Daniel Fung characterized the requirements for automation in microbiology by looking back at the historical development of rapid methods and automation then listing key questions to help better understand what makes an ideal automated microbiology system. Since then there has been a move to automate areas of microbiology such as blood culture, microbial detection and susceptibility however specimen processing and culture work-up remain manual tasks in most laboratories. While there have been dramatic improvements in efficiency with conventional testing methods such as Gram staining, culture, phenotyping and susceptibility time to final result is still a limiting factor. On average routine diagnostics can take anywhere from 48-72 hours. This delay can result in incorrect antibiotic therapy, prevents early targeted therapy and can promote nosocomial infections to more resistant organisms. Why has it taken until only recently for the microbiology laboratory to move towards total laboratory automation? Frankly change is hard and due to the complexity of testing, cost, and the need for the human factor, the historical perspective has been that, automating microbiology was too much of a challenge to undertake. However times are changing and there is a quiet revolution3 afoot in microbiology. The industry itself is changing with increasing test volumes, laboratory consolidation and 24/7 service in many laboratories. Trained microbiology technologists are few—less students choosing medical laboratory technology combined with an increase in the median age of the average tech—less replacements to fill the ranks. Quality requirements and an increased demand for faster turnaround times challenge every laboratory. In addition, Matrix-assisted laser desorption ionization time-of-flight mass spectrophotometry (MALDI-TOF MS) has revolutionized the clinical microbiology laboratory. MALDI-TOF spectral fingerprints could be obtained from whole

bacterial cells without pre-treatment, MALDI-TOF has been used to characterize and identify microorganisms from bacteria, fungi and viruses4. MALDI-TOF fits well in a laboratories automation workflow—procedures are relatively simple and reproducible at a low cost.

Automation solutions, and unique approach to the traditional Microbiology workflow fits well in Microbiology labs with all levels of automation from none to TLA. The Alifax® HB&L system using a pre-enrichment step and use of 0.5 McFarland Monitor offers a greater percentage of organism identification plus faster susceptibility results when combined with MALDI-TOF.

How urine testing in Microbiology (which represents over 4 million samples tested per year in Canada) is made more efficient through the use of a novel urine screening solution with the Alifax Sidecar® system. Additionally some more special applications in Microbiology such as bacterial detection in sterile human biological fluids (Blood, CSF) and show how the Alifax HB&L® system fits well into a positive blood culture analysis workflow.

Invasive and life threatening infections such as meningitis, pericarditis, peritonitis, septic arthritis, empyema and pneumonia are traditionally diagnosed by culture of body fluids. Traditional testing methods used to diagnose these infections involve culture on solid medium or in media such as thioglycolate or brain heart infusion broth. Overall these methods require up to 5 days for results. Examples such as eye infections (endophthalmitis) result in severe inflammation. These infections are a complication of intraocular or cataract surgery where the risk of bacterial or fungal infection is greater with transplantation of contaminated corneas.

Other infections such as catheter-related bloodstream infections are difficult to diagnose and represent a leading cause of health care associated infections with a high morbidity and mortality rate. Prosthetic and osteoarticular infections are complex and difficult to diagnose. Eradication of the organism is a challenge as the site of the infection is bone. Early diagnosis helps as this allows clinicians to carry out prolonged and targeted therapy. Hospital acquired pneumonia and ventilatorassociated pneumonia are the second most common cause of

nosocomial infections resulting in high morbidity and mortality in the ICU setting. Faster diagnosis (6-12 hrs vs. 5 days) leads to higher survival rates.

The rapid analysis of human biological liquids is crucial to the patient for whom the timely correct diagnosis and the beginning of an adequate therapy represents the best prognosis. In addition to community acquired infections, hospital acquired infections have a high Public Health impact by increasing morbidity and mortality rates and costs through prolonged hospital stays and additional diagnostic and treatment costs. The Alifax® Human Biologicals Liquid Culture test improves patient management by reducing hospital time, diagnostic analysis and therapeutic treatment. Enhanced liquid culture media combined with a specific supplement (DEB) has been developed to detect aerobic bacteria and significant micro-organisms such as Haemophilus influenza, Neisseria meningitidis as well as samples characterized by extremely low bacterial counts.

Light Scattering Technology Applied to Bacterial Culture

In order to perform rapid culture and sensitivity using either the DEB or Culture kit the following systems may be used:

- HB&L - Semi-Automated
- Alfred 60AST - Fully Automated
- Sidecar - Fully Integrated

Each system offers high sensitivity and specificity and use patented light scattering technology to monitor bacterial replication activity from inoculum step in culture broth. In addition a graphical representation of the kinetic bacteria growth curve is shown in real-time. Using the built in McFarland Monitor the turbidity levels in positive samples are monitored during bacterial culture test. A visual and audible alert advises when the sample reaches suitable bacterial concentration at 0.5 McFarland to perform direct susceptibility testing. Using the same sample we can obtain immediate identification of 100% of the gram positive and gram negative pathogens using Maldi-TOF technology.

Due to the optimization of the broth it is possible to perform bacterial culture in sterile and non-sterile samples such as respiratory fluids, cerebrospinal fluid and pleural fluid offering a sensitivity of 1 CFU/ml in 6 hours.

Broths are provided in sterile vials with a pierceable screw cap. Samples are incubated at 37C and constantly mixed to avoid sedimentation, flotation and growth anomalies.

Only live bacteria are detected while interference from non-replicating substances such as erythrocytes, leucocytes, dead cells and salts present in the sample are eliminated during the initial zero reading.

Advantages:

- One test with two results—bacterial culture result + 0.5 McFarland Sample
- Positive samples may be immediately tested with a customized antibiotic panel following therapeutic treatment
- Use of a bacterial culture in a logarithmic phase reduces stress conditions which can occur when bacteria reach the stationary phase
- Urinary Tract infections (UTI's) are considered to be one of the most common human bacterial infections second only to respiratory infections. Women are especially prone to UTI's, 1 in 5 will develop a UTI during their lifetime. The incidence of UTI's increases with the presence of diabetes, malformations of the urinary tract system as well as with age. UTI's are also the most common nosocomial infections mostly linked to urethral catheters and invasive diagnostic procedures. In the bacteriology laboratories urine samples represent a significant workload, but 60-80% of these have negative culture. The development of a rapid and reliable procedure for urine screening for bacteriuria can increase laboratory efficiency and improve the management of the patient1. The Alifax® Urine Culture Screen protocol used with either the Alifax Sidecar, Alfred 60AST or HB&L offer automation and high sensitivity and specificity. Using patented light scattering technology, bacterial replication activity is monitored in real time. Strong positive samples are flagged after only 45 minutes incubation. Three analytical protocols are available—Fast, Standard

and Boric Acid. A specific eugonic broth guarantees optimized aerobic pathogen growth curve. Only live bacteria are detected. Laboratories have seen a sensitivity of 99.8% and a negative predictive value of 99.9% when compared to Petri dish culture.

First Automated Streaking System Integrated With Rapid Bacteria Culture

Alifax® presents the first system totally automated for rapid bacterial growth integrated to a streaking system. The system is composed of two units: Alfred60AST and Sidecar. Alfred60AST detects the presence of bacteria and their resistance to drugs in a few hours with high sensitivity and specificity. Alfred60 AST monitors the growth phases of bacteria from the inoculum step into specific culture broths providing real time growth curves and quantitative bacterial results in CFU/ml. Broth turbidity level is detected by the McFarland Monitor and as the sample reaches the 0.5 McFarland it is buffered into the refrigerated area where it is ready to be tested with a customized antibiotic panel. Sidecar is an automated streaking system able to stock 240 Petri dishes chosen among a maximum of 12 different media. Streaked plates are incubated at 37°C for the requested analysis time in the system built in incubator. Only positive samples are streaked automatically.

Alifax Sidecar Advantages:

- Negative samples are reported in one day
- Drastic reduction of streaked samples
- Material and reading time saving
- Reduction of the use of broad-spectrum antibiotics
- Decrease of the spread of bacterial resistance.

100% BACTERIAL IDENTIFICATION FROM POSITIVE BLOOD CULTURE

Blood culture is considered the gold standard in detecting pathogenic microorganisms present in blood. Many factors such as collection time, sample volume, possible contamination and number of samples may limit the actual clinical value. From a positive blood culture an additional two days are needed on average to obtain a specific antibiotic susceptibility of the isolated bacteria which results in a delay in treatment of patients with targeted antibiotic

therapy. In cases of serious bacterial infections the timely administration of an effective antibiotic therapy is associated with an increase in disease resolution and subsequent patient survival. For this reason, the microbiology laboratory has to provide "clinically useful results" in order to guide the clinician to choose the most appropriate antibiotic therapy as soon as possible. Rapid Antimicrobial Susceptibility Test (AST) results facilitate effective treatment, reduce the number of laboratory tests ordered, days of hospitalization and Public Health costs. In addition, they are useful to monitor the evolution of resistant bacteria.

The Alifax® Antimicrobial Susceptibility Test (AST) when used with either the Alfred 60AST, HB&L or Sidecar fits easily into a laboratory routine. Positive samples are flagged when the turbidity reaches the 0.5 McFarland by McFarland Monitor. Monomicrobial samples can then be tested with a customized antibiotic panel providing results within a few hours. Using the same sample we can obtain immediate identification of 100% of the gram positive and gram negative pathogens using Maldi-TOF technology. The results are expressed in percentage of resistance to the antibiotic and classified as Resistant, Intermediate or Sensitive. Alifax® AST is used on positive blood cultures and provides results in 3 hours.

What is Rapid Diagnostic Test (RDT)?

Rapid diagnostic tests (RDTs) are diagnostic assays designed for use at the point-of-care (POC), and can be adapted for use in low-resource settings. An RDT is low-cost, simple to

operate and read, sensitive, specific, stable at high temperatures, and works in a short period of time. RDTs are already in use for several neglected diseases.

Overview

Rapid diagnostic tests (RDTs) are a type of point-of-care diagnostic, meaning that these assays are intended to provide diagnostic results conveniently and immediately to the patient while still at the health facility, screening site, or other health care provider. Receiving diagnosis at the point of care reduces the need for multiple visits to receive diagnostic results, thus improving specificity of diagnosis and the the chances the patient will receive treatment, reducing dependence on presumptive treatment, and reducing the risk that the patient will get sicker before a correct diagnosis is made. Rapid tests are used in a variety of point-of care-settings—from homes to primary care clinics or emergency rooms -- and many require little to no laboratory equipment or medical training.

RDTs are particularly important in low-resource settings, where:

- Harsh environmental conditions combined with limited access to electricity and refrigeration preclude the use of sensitive equipment
- Technology, equipment, and training required for more complicated laboratory tests are lacking
- Many patients cannot travel easily to the clinic to follow-up on results that take a long time

RDTs can be especially useful with patient samples that can be collected by minimally trained health personal, such as community health workers.[1] Body fluids that can be collected non-invasively, such as nasal swabs, urine, saliva, and tears, are preferred as these are most amenable to collection with only minimal training. However, capillary blood collection techniques, such as those used for malaria RDTs, demonstrate that innovation in sample collection can be used to improve the utility of RDTs in low resource settings.

Rather than one specific type of technology, rapid diagnostic tests can be built in a variety of platforms, each with their own benefits and limitations.[2] The vast majority of RDTs in use

today for neglected diseases are based on immunoassay technology due to its relative simplicity. These tests generally involve the interaction of a fixed reagent of either target antigen or antibody that is linked to some type of visible detector, that then reacts with a patient sample. Other types of technologies, such as nucleic-acid amplification, may be too expensive and require too much advanced technology to be applicable as a point of care test.

Rapid diagnostic tests have particular value as epidemiological tools, in addition to use as diagnostics. They enable a rapid screening of a potentially affected population, and can be used, as is the case with lymphatic filariasis, as a test of cure to determine when a mass drug administration has been successful. RDTs are less necessary for diseases that are generally accurately diagnosed syndromically, but could prevent over-prescription of antibiotics if used to differentially diagnose fever or diarrhea, respectively.

Common RDT Platforms

There are several different platforms commonly used to build rapid diagnostic tests. The relative utility of common RDT platforms is summarized below.

Lateral flow tests are the simplest type of RDT, requiring only very minimal familiarity with the test and no equipment to perform, since all of the reactants and detectors are included in the test strip. In a lateral flow test, the sample is placed into a sample well and migrates across the zone where the antigen or antibody is immobilized. The results are read after a certain amount of time has passed. Another type of RDT, a flow-through test, obtains results even faster than lateral flow tests, but requires an added wash and buffer step, which can limit its portability and stability.

An agglutination test works very simply by observation of the binding of carrier particles and target analytes into visible clumps, seen either through a microscope or with the naked eye. However, if the binding of the particles is weak, the results of the test can be inconclusive.

Dipstick format RDTs (with binding sites to test for multiple antigens) work by placing the dipstick in a sample. The dipstick is then washed and incubated to prevent non-specific

analyte binding. These additional steps can limit their usability in low-resource point of care settings.

Microfluidics, or "labs on a chip" are an emerging area of rapid diagnostic development. Using electrochemical sensors, these tests would include all detectors and reactants in a single portable cassette. For more information, see the Diagnostics Innovation Map Report. The limitations and advantages of each of these test types is summarized in the table below. For more information about the mechanisms of these tests, see PATH's RDT Info website.

Strengths and Weaknesses of RDTs and Common RDT Platforms

The general strengths of RDTs include their ease of use, minimum training requirements, rapid results, and limited need for instrumentation/infrastructure. The general weaknesses of RDTs including their subjective interpretation of readout, low throughput, often limited sensitivity relative to laboratory or reference tests, and need for quality control mechanisms. The following table summarizes the strengths and weaknesses of several RDT platforms:

Test	Strengths	Weaknesses	Examples
Lateral flow tests (immunochromatographic strip tests)	Rapid (5-15 min) Can be adapted for multiple sample types Easiest to use	Results are qualitative Less sensitive than an ELISA	Malaria RDTs, home pregnancy tests
Agglutination assays	Single-step Low cost per test Rapid results	Low sensitivity and ambiguous results in weak reactions Some tests require training and/or a microscope to read results Some cross-reactions can cause sensitivity problems	HIV latex agglutination assays, Leishmaniasis DAT
Flow-through	Very rapid (3-5 min)	Requires more training to perform than lateral flow Less sensitive at	*E. coli* detection

		antigen detection compared to lateral flow	
Solid-phase ("dipstick" assays)	One strip can test for multiple parameters	Requires several intermediate steps, some training	HIV "comb" test
Microfluidic chips, immunosensors, "labs on a chip"	Rapid Requires no manipulation	Potentially prohibitively expensive	Largely hypothetical at this point in time

Existing Products

RDTs as Non-Neglected Tropical Disease Diagnostics

Rapid diagnostic tests are commercially available for detection of multiple non-neglected diseases and conditions, including pregnancy, blood sugar in diabetic patients, and strep throat. The simplicity of these assays often allows them to be used at home or by minimally trained health care works in both the developed and developing worlds. In the United States, the use of rapid diagnostic tests by doctors is limited as current Medicaid/Medicare reimbursement schemes favor the use the centralized laboratories for diagnosis.

RDTs as Neglected Tropical Disease Diagnostics

One of the particular problems facing the production and implementation of RDTs in low-resource settings is the lack of an evaluative process to determine their real efficacy in the field. For example, the WHO recently issued a policy statement to recommend that rapid serological tests for TB not be useddue to a lack of sensitivity and specificity. WHO-TDR and FIND now have lot-testing programs for RDTs for neglected diseases, such as malaria and HIV, to improve standards and recommendations for use of RDTs in the field.

In order to address the ongoing challenge of quality assurance for rapid test, the WHO now independently evaluates RDTs for several diseases, including HIV and malaria, in order to evaluate them for pre-qualification. As with pre-qualification for drugs or vaccines, this designation is the WHO's method of validating the quality of specific diagnostic assays and

manufacturers for those in countries without rigorous and stringent diagnostic approval processes.

NTD	Test	Notes
HIV	Many types	There are agglutination assays, solid-phase tests, and lateral flow tests for HIV.
Malaria	Over 120 RDTs commercially available from over 60 companies Lateral flow, mostly based on presence of histidine-rich protein II (HRPII)	Largely qualitative—less helpful in endemic areas. Difficulty differentiating malaria subtypes. HRPII remains circulating in blood for several weeks after cure, so false positives are possible
Leishmaniasis	Direct Agglutination Test is field gold standard	Use of a fixed antigen in this test means that a 'test of cure' for leishmaniasis is still lacking
Oncocherciasis	Ov-16 rapid immunochromatographic card test	Not yet available in the field, laboratory only Antibody-detection test, so unable to determine whether an infection is active
Schistosomiasis	MAb-based urinary dipstick test	Only available for *S. haematobium*
Lymphatic Filariasis	Circulating Filariasis Antigen (CFA) Immunochromatographic card test	Only detects *W. bancrofti* Potentially prohibitively expensive

Purification and standardization of antigen and specific antibodies

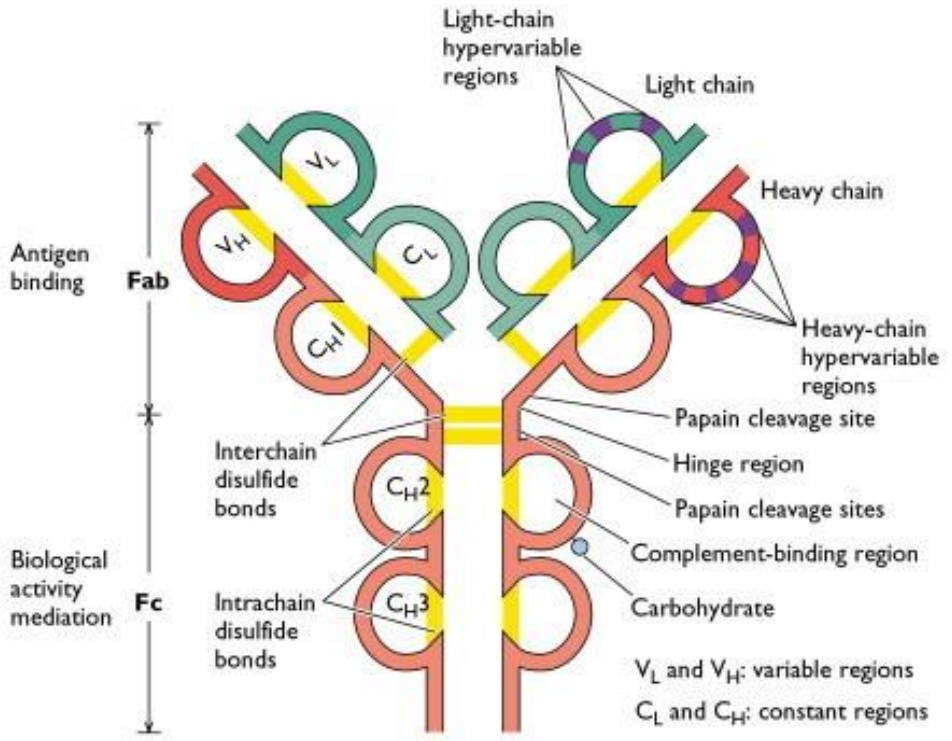
Scope and objective.

Biological scientists have used antibodies for many years to study proteins; but, as is the case with protein purification and recombinant DNA technology, the ways antibodies are produced and used has led to an increasingly powerful technology. The purpose of this section is to discuss the way these antibodies are produced and how they can be used. We will skirt the basic biology of the immune system as much as possible, but we must note that the discovery of how organisms have developed methods to generate millions of potential

antibodies reacting with millions of potential antigens is an intellectual triumph that would bring delight to any biological scientist. The combination of using multiple alleles which undergo specific recombination events and are further modified by somatic cell mutation is a fascinating story and the way the body controls this process will certainly be an object of study for decades to come, but these topics are appropriate to a course devoted to immunology.

Antibodies.

Actually include several classes of molecules some of which like the IgA are designed for secretion in the bodily fluids while others, like the IgM are designed to be expressed on the cell surface. The antibody that is most useful in biological studies is the IgG class, a protein molecule that is made and secreted and can recognize specific antigens. The IgG is composed of two subunits including two "heavy" chains and two "light" chains. These are assembled in a symmetrical structure and each IgG has two identical antigen recognition domains. The antigen recognition domain is a combination of amino acids from both the heavy and light chains. The molecule itself is roughly shaped like a "Y" and the regions of the extreme tips of the "Y" are the antigen recognition domains. Limited protease digestion of the antibody produces two 'Fab' fragments, each of which has a single antigen binding site. Thus, an intact IgG can cross-link two antigens, which may have important biological implications, while a Fab fragment could not do this. For example, cross linking cell surface antigens with an IgG can cause a change in their distribution on the cell surface or stimulate internalization. The stem of the "Y" is not involved in recognition of antigens and is fairly constant among the various classes of antibodies. This region is called the Fc region and is important because it itself can be used as an antigen that is recognized by other antibodies and because it binds with high affinity to the A protein which is a protein that can be isolated from Staph Aureus. The antigen-recognizing regions of the molecule are concentrated in the Fab regions. It is possible to treat antibodies with proteases to cleave the antibody near the hinge region and produce the monovalent Fab fragments which must be used if there is a need to avoid dimer formation.



Antigens

The proteins that can be recognized (bound) by the antibody are called antigens*. These antigens are most commonly polypeptides or carbohydrates, but they can also be lipids, nucleic acids, or even small molecules like neurotransmitters. A particular antibody molecule can only interact with a small region of an antigen and in the case of a polypeptide this is generally about 5-12 amino acids. This region can be continuous or it can be distributed in different regions of a primary structure that are brought together because of the secondary or tertiary structure of the antigen. The region of an antigen that is recognized by an antibody is called an epitope*. It is possible, although unlikely, that the same, or closely related epitopes, could be shared by the different antigens. In some cases, it is possible to make an antibody that is directed against the antigen binding site of another antibody (i.e., the antigen binding site is the epitope). This type of antibody is called an anti-idiotype antibody.* In this case, the antigen binding site of the anti-idiotype antibody can be similar in structure to the original antigen (they both recognise the same antibody.)

Adding an epitope:

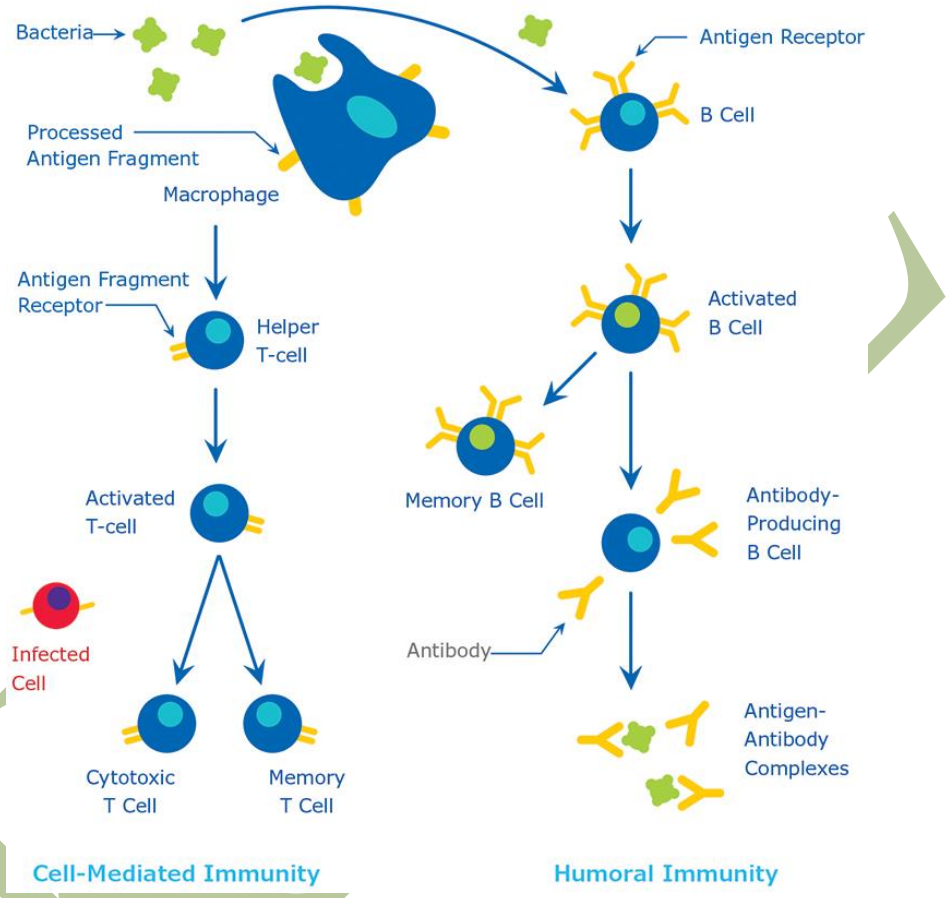
If a cDNA for a protein is available, it is possible to add a new coding sequence before expression, producing a fusion protein. Since only a few amino acids are required to form an epitope*, only a few amino acids need to be added to a protein to produce a strong antigen binding site. This forms an epitope-tag, and common versions of the epitope tag are FLAG-tag*, His-tag, and GST-tags.

Thinking about antibody antigen interactions.

It is important to be constantly aware of the way antibodies recognize antigens and the nature of the antibodies that are used. It is possible to use serum as a source of antibody but in this case the antibodies that are present are derived from many independent clones each of which produces a separate antibody. (Serum is the fluid that is produced when blood clots.) In this case, multiple antibodies may recognize multiple epitopes on a single protein which can be useful experimentally.

In other cases antibodies can be produced by a single clonal cell line (called monoclonal antibodies) and in this case every antibody molecule is identical to every other one and all recognize the same epitope. This is an important distinction to remember since using both sera and monoclonal antibodies have advantages and disadvantages. It is clearly easier to think about how a monoclonal antibody can be recognized, but it must be remembered that since any single antibody molecule recognizes only a single epitope, it is always possible that this epitope may be shared among very different proteins. So this must be considered in experimental design. This problem is partially avoided by the use of antisera which are by their nature polyclonal. An antisera that has been produced with a high titer to a particular antigen will have many antibodies recognizing many epitopes on that antigen. Because of this it is less likely that a cross-reacting epitope could be a problem. On the other hand, antisera include not only antibodies directed against a particular antigen of interest, but they may also include many other types of antibodies present in the serum (yes, they are always there). These antibodies may be present due to the immunological history of the animal, or even the presence of an autoimmune reaction. Experimentally it is essential that the experimentalist

considers the possibility that reacitivities present in antiserum are due not only to reaction to an antigen of interest but due to some other antigen. Thus, controls, including the use of the preimmune serum are always essential. Frequently, using both an antiserum and the monoclonal antibody for a specific experiment can help make a convincing case.



Making polyclonal antibodies (antiserum). Historically, serum was the first source of antibodies. In some cases, a serum may have a high concentration of an antigen of interest without any experimental manipulation. Good examples of this are the existence of human autoimmune antisera that are produced by patients with Lupus or multiple sclerosis or paraneoplastic disorders. When such a serum is available it provides an experimental tool that can be used to study the underlying immune response and to isolate and study the antigen or the cDNA for the antigen that is recognized by the antibody. A number of antisera have serendipitously recognized particular antigens of interest. For example, there is one antiserum

that specifically stains neuronal precursors in Drosophila embryo. More commonly, it is necessary to experimentally produce a high titer antisera.

The titer of an antisera refers to the amount of specific, functional antibody present in the serum. A 'high titer' serum has more antibody than a 'low titer' serum. Functionally, titer is generally indicated by the amount a serum can be diluted and still give a good signal in a particular assay (e.g., a serum that works at a dilution of 1/10,000 has a higher titer than one that works only at a dilution of 1/100). Thus, the titer depends on the assay used and it measures both the affinity and amount of an antibody directed against the antigen of interest. A high titer is valuable not only because it means the serum can be used in more experiments, but, more importantly, that the non-specific components present in any serum will be less of a problem (they can be diluted out).

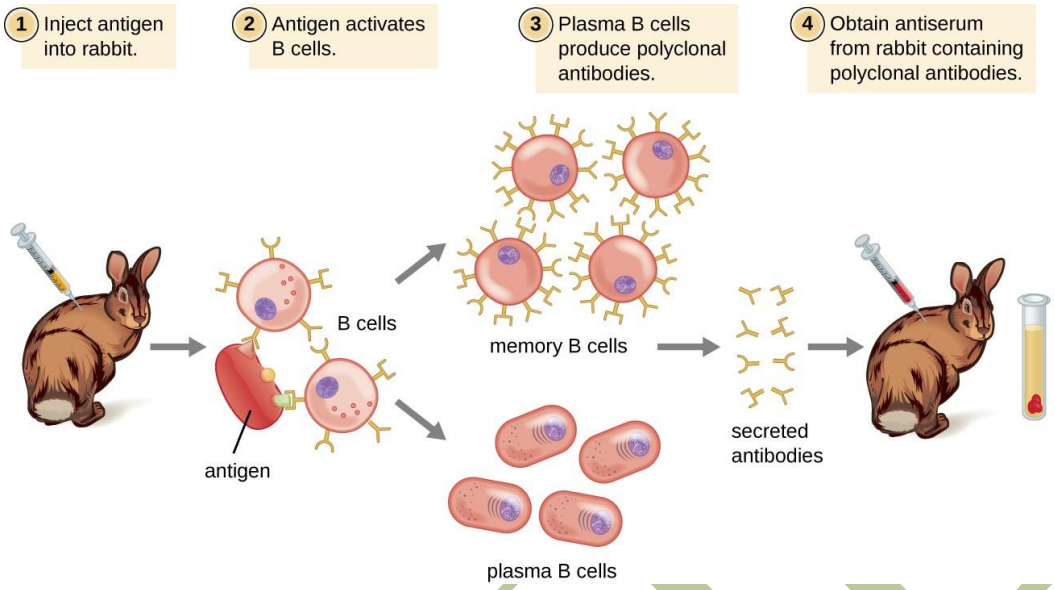
an antiserum is made by injecting an antigen into an animal, most commonly a rabbit or a chicken (sometimes to bypass the problems of tolerance) but also hamsters, rats, goats, and even cows. The quality of the antisera produced will be determined in part by the quality of the antigen selected. An antigen can be either a purified protein, or a relatively complicated mixture of several macromolecules. Antisera have been produced, for example, against preparations of secretory granules (which contain multiple proteins and other antigenic macromolecules). Such an antisera is obviously complicated, but it has proved useful to some investigators.

If the sequence of a protein is available, it is also possible to generate a synthetic peptide which can be covalently attached to a "carrier" protein and used as an antigen to make an anti-peptide antibody.* Typically, an antigen is mixed with an adjuvent mixture and injected into an animal. Adjuvents are frequently chosen in an attempt to increase the strength of the immune response, but there is much debate about which adjuvents are effective or even if they are effective at all. After an initial injection an animal undergoes a primary immune response which produces a relatively low titer antiserum and includes antigens of multiple classes including IgM and IgG. After a waiting period, the animal is injected again (boosted) to produce a secondary response. This procedure can be repeated multiple times, but each

time the injection procedure is repeated, the repertoire of antibodies produced by animal is somewhat different. Thus, every antiserum has a slightly different specificity and some scientists are apt to guard their pool of "good" antisera for fear that the fraction they have may be all that will ever be produced. The serum is produced by withdrawing blood from the animal. The blood is allowed to clot removing red blood cells, white blood cells, and some serum proteins. The remaining fractions is a heterogeneous mixture that includes many proteins including the antibodies of interest. In some cases the serum is further purified before use. It is possible to absorb an antisera with material in an attempt to increase the specificity of the antiserum. For example, an antiserum directed against brain synaptic vesicles might be absorbed with a microsome fraction from liver in an attempt to increase specificity. There are also straight forward procedures to purify the IgG fraction from serum so that potentially interfering substances in the serum can be removed. Finally, the antibodies that specifically recognize an antigen of interest can frequently be purified using an affinity column that contains the substance (peptide, carbohydrate, protein, etc.) that was used as an antigen initially. The quality of an antiserum of course must be judged on the basis of specific assays and these will be discussed below.

Producing Polyclonal Antibodies

Antibodies used for research and diagnostic purposes are often obtained by injecting a lab animal such as a rabbit or a goat with a specific **antigen**. Within a few weeks, the animal's immune system will produce high levels of antibodies specific for the antigen. These antibodies can be harvested in an **antiserum**, which is whole serum collected from an animal following exposure to an antigen. Because most antigens are complex structures with multiple epitopes, they result in the production of multiple antibodies in the lab animal. This so-called **polyclonal antibody** response is also typical of the response to infection by the human immune system. Antiserum drawn from an animal will thus contain antibodies from multiple clones of B cells, with each B cell responding to a specific epitope on the antigen.



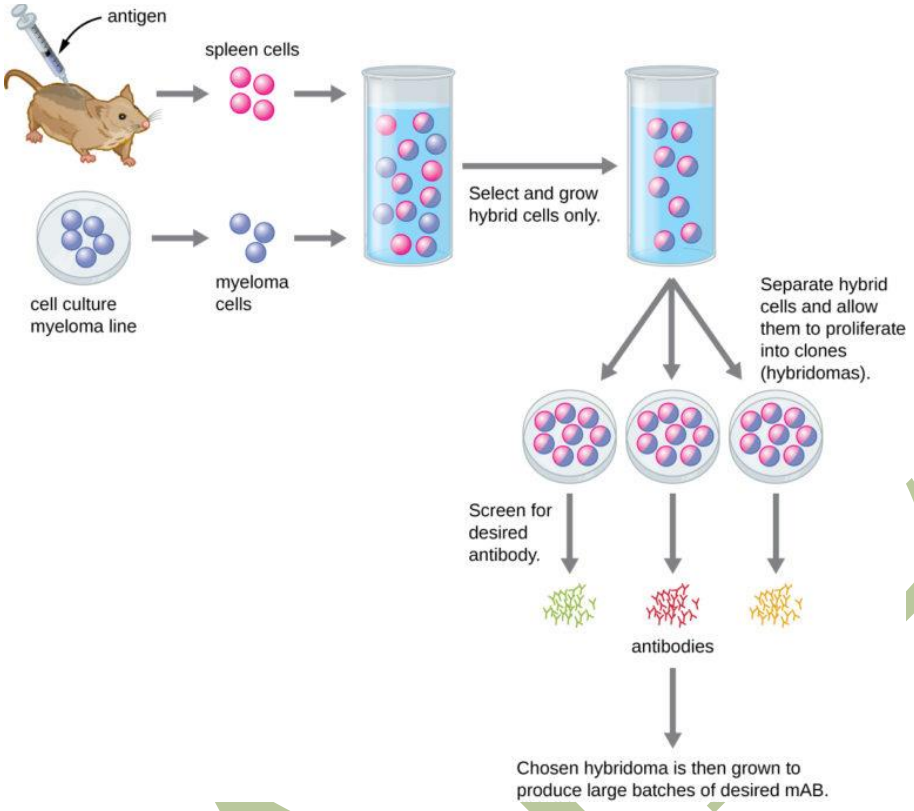
Lab animals are usually injected at least twice with antigen when being used to produce antiserum. The second injection will activate memory cells that make class **IgG** antibodies against the antigen. The memory cells also undergo **affinity maturation**, resulting in a pool of antibodies with higher average affinity. Affinity maturation occurs because of mutations in the immunoglobulin gene variable regions, resulting in B cells with slightly altered antigen-binding sites. On re-exposure to the antigen, those B cells capable of producing antibody with higher affinity antigen-binding sites will be stimulated to proliferate and produce more antibody than their lower-affinity peers. An **adjuvant**, which is a chemical that provokes a generalized activation of the immune system that stimulates greater antibody production, is often mixed with the antigen prior to injection.

Antiserum obtained from animals will not only contain antibodies against the antigen artificially introduced in the laboratory, but it will also contain antibodies to any other antigens to which the animal has been exposed during its lifetime. For this reason, antisera must first be "purified" to remove other antibodies before using the antibodies for research or diagnostic assays.

Producing Monoclonal Antibodies

Some types of assays require better antibody specificity and affinity than can be obtained using a polyclonal antiserum. To attain this high specificity, all of the antibodies must bind with high affinity to a single epitope. This high specificity can be provided by **monoclonal antibodies (mAbs)**.

Unlike polyclonal antibodies, which are produced in live animals, monoclonal antibodies are produced *in vitro* using tissue-culture techniques. mAbs are produced by immunizing an animal, often a mouse, multiple times with a specific antigen. B cells from the spleen of the immunized animal are then removed. Since normal B cells are unable to proliferate forever, they are fused with immortal, cancerous B cells called myeloma cells, to yield hybridoma cells. All of the cells are then placed in a selective medium that allows only the hybridomas to grow; unfused myeloma cells cannot grow, and any unfused B cells die off. The hybridomas, which are capable of growing continuously in culture while producing antibodies, are then screened for the desired mAb. Those producing the desired mAb are grown in tissue culture; the culture medium is harvested periodically and mAbs are purified from the medium. This is a very expensive and time-consuming process. It may take weeks of culturing and many liters of media to provide enough mAbs for an experiment or to treat a single patient. mAbs are expensive.



Uses of antibodies. Antibodies have a wide variety of uses.

1. They can be used to immuno-localize a particular antigen in a tissue (immunohistochemistry*). Tissue can be fixed and incubated with the antibodies of interest. These antibodies can then be localized using a 'secondary' antibody coupled to a gold particle or another enzyme that gives a chemical reaction like horse radish peroxidase or beta galactosidase. A secondary antibody is frequently made by generating an immune response to the Fc region of the primary antibody in another species. Thus, if the primary antibody is a mouse IgG, then the secondary could be a rabbit anti-mouse antibody which has been linked to beta galactosidase. Alternatively the antibody can be purified and then conjugated to another molecule to produce a fluorescent antibody.

2. Antibodies can be used to quantitate the presence of an antigen using either a radioimmunoassay* (RIA) or an enzyme-linked immunoabsorbent assay (ELISA)*. There are many variants of these approaches, but those are based on a similar idea. For example, if an

antigen can be bound to a solid support or surface, it can be detected by reacting it with a specific antibody and the antibody can be quantitated by reacting it with either a secondary antibody or by incorporating a label directly into the primary antibody. Alternatively, an antibody can be bound to a solid surface and the antigen added. A second antibody that recognizes a distinct epitope on the antigen can then be added and detected. This is frequently called a 'sandwich assay' and can frequently be used to avoid problems of high background or non-specific reactions. It was only an assay of this type, for example that was sensitive and reproducible enough to measure low concentrations of nerve growth factor present in the body.

3. Antibodies can have effects in vivo. These effects can be used in an experimental sense to determine the importance of a particular molecule in vivo. For example, antibodies to nerve growth factor demonstrated that nerve growth factors had to be present for the sympathetic and sensory nervous system to develop. Antibodies to particular hormones or cell-surface receptors can also have a therapeutic use. For example, antibodies to EGF receptor have effects on the development of some tumor types.

4. Since antibodies have a high affinity for a particular epitope, they can be used as affinity reagents in protein purification.

5. Because particular antibodies bind to proteins with high affinity they can also be used as a classic criteria for the importance of a particular enzyme or other macromolecule in a particular reaction. If an antibody can interfere with a reaction in a solution, that suggests that it may be binding specifically to a protein involved in that reaction. Of course, it must be remembered that some antibodies may be directed against a site on a protein that is not important for enzymatic activity and so may not have the classic "blocking" activity.

6. Antibodies can also be used in conjunction with gel-shift experiments to identify specific DNA-binding proteins. As discussed in the section on transcription analysis, DNA-binding proteins can be frequently assayed by their ability to bind with high affinity to a particular oligonucleotide. The mobility of an oligonucleotide associated with the protein is far different than the mobility of a free oligonucleotide giving a signal that is frequently called a gel shift.

If an antibody to a protein present in such a complex is available, the addition of the antibody to the binding assay can have either of two effects. If the antibody binds to a region of a protein not involved in DNA binding it can produce a complex that has even a slower mobility leading to another shift in mobility (a super-shift). Alternatively, if the antibody binds to a region of the protein involved in recognizing the DNA then it can disrupt the binding and eliminate the shift. In either case, this can serve as a criterion to identify DNA-binding protein.

7. It is also possible to use an antibody to detect a protein after fractionation by SDS-PAGE. This approach, which is called 'western blotting*' because it is analogous with northern blotting and Southern blotting, involves transferred a fractionationed protein to a nitrocellulose sheet. These proteins can then be exposed to a particular antibody and the antibody can recognize the protein bound to the blot. This allows the identification of a particular protein. This approach is particularly useful if the mobility of the protein changes during an experiment. For example, incorporation of a phosphate, or a carbohydrate or cleavage of the protein results in a change in mobility and this can be followed in straight forward manner by western analysis. With appropriate controls, this approach can be use to measure the abundance of a protein in response to experimental manipulations.

8. The combination of SDS gels and immunoprecipitations* can also be extremely effective. If a particular protein can be immunoprecipitated in a solution, both supernatant and precipitated fractions can be separated on an SDS gel and studied.

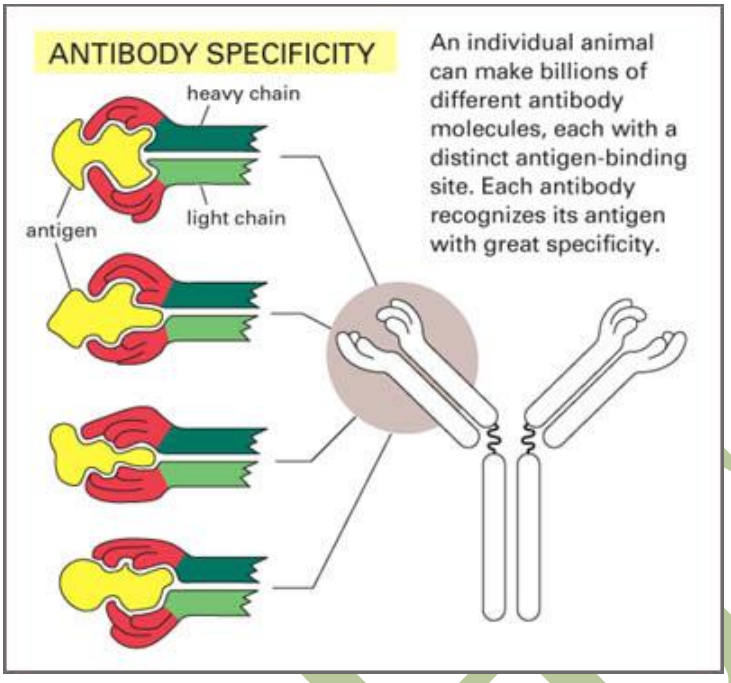
9. Antibodies, including antisera, can be used to screen expression libraries to isolate candidate genes that express a particular epitope.

10. If a fluorescent antibody is bound to a cells surface it can be used as a marker to quantitate the fraction of cells expressing that marker using flow cytometry*. If different antibodies / fluorescent dye combinations are used, the fraction of cells expressing several antigens can be determined.

11. Anti-idiotype antibodies, which are antibodies against the binding domain of another antibody, can sometimes be used to mimic the structure of the original antigen.

Antibody Specificity

Perhaps most importantly, it is important to consider the question of antibody specificity and reactivity. The quality of experimental work using an antibody cannot be any greater than the quality of the antibody that is used. Doing immunolocalization or immunoprecipitation with a poorly characterized antibody is more likely to add confusion than enlightenment to a field, but a well characterized antibody is invaluable. Once an antibody (serum or monoclonal) is produced, it may be characterized by a number of approaches. Does it precipitate an antigen of the correct size? Does it react with a single peptide by western analysis? Is the protein it recognizes present in the 'right' tissues (remembering that antigens are sometimes found in unexpected places)? If it is an anti-peptide antisera, is its reactivity competed by the peptide used to make it? (This is a good reason to make anti-peptide antiseras.) Is it localized where it might be expected by immunohistochemistry (tissue distribution or subcellular localization)? Does the antigen appear when the gene is expressed (either during development or with an expression vector)? Is the protein missing (or changed in its properties) in an animal with a mutation? All these are useful criteria, and it is incumbent on the experimenter to develop sufficient criteria that the experiments using an antibody are convincing.



Suitability of antibodies for different purposes.

It should be appreciated that not all antibodies will work equally well in all assays. An antibody for example may work beautifully in a western style analysis but fail in immunohistochemistry. Thinking about the nature of antibodies in the epitopes they recognize, may explain this initially puzzling phenomenon. If an antibody is directed against an epitope that is buried in the interior of the protein, it may not be accessible in immunohistochemistry. Likewise, if a reagent used to fix a tissue modifies a residue that is a key residue in a particular epitope an antibody may not recognize a protein in a fixed tissue. Thus, immunohistochemical localization may depend not only on the quality of the antibody but also on the type and extent of fixation. If an epitope is on the surface of the protein and is made up by amino acids that are widely separated in the primary structure of the protein, then the denaturation of the protein on an SDS gel may irreversibly separate and disrupt this epitope. If one is interested in immunoprecipitating a native protein, it is presumably essential to have an antibody that recognizes a surface epitope on that protein with reasonably high affinity. If the affinity of the antibody is too low then the complex may dissociate before the

particular insoluble fractions are separated. Despite these considerations, the use of antibodies is an important approach to studying cells and their proteins.

SYLLABUS

Diagnostic immunology: Concepts and methods in idiotypes. Anti idiotypes and molecular mimicry and receptors. Epitope design and applications. Immunodiagnostic tests. Immuno-florescence. Radioimmunoassay.

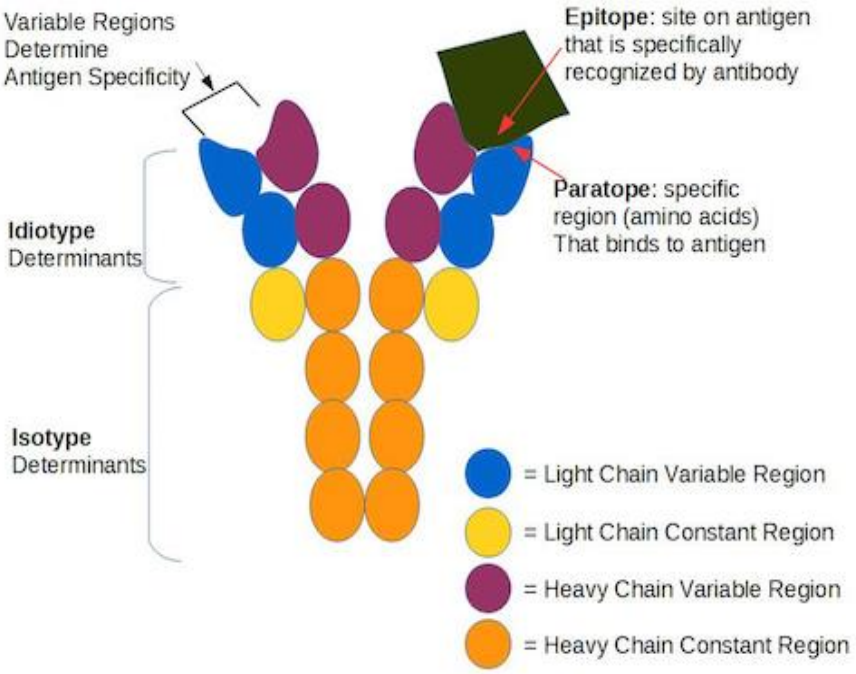
Concepts and methods in idiotypes and Anti idiotypes

I: Defining the idiotype

The immune system has the capacity to develop antibodies that can specifically bind to almost any biological or chemical structure imaginable. This remarkable repertoire of antibody specificity is defined by the variable region domains of the heavy (VH) and light (VL) chains of the heterodimeric antigenic determinant region, which generates an estimated 1-6 X 10^6 combinations in mice and humans through the rearrangement of VH and VL genes. Additional modifications to the VH and VL genes, through somatic mutation and junctional diversity, increase the estimated capacity to produce approximately 10^{11} to 10^{13} unique antibodies (Eichmann, 2008; Mak and Saunders, 2005). To put this in perspective: there are about 3 X 10^{11} stars in our galaxy; so when you look up at the stars consider you have approximately 10^{20} antibodies circulating in your bloodstream that have the potential to recognize a diversity of structures as vast as the galaxy.

Antibodies have bispecific functionality with the antigenic determinant defining the binding specificity to the antigen, and the heavy chain constant (CH) region defining its immunological functionality, through the interactions of the CH region with specific receptors or complement. Two identical heavy and light chain pairs combine (through disulfide bonds) to form a complete antibody structure, which contains two antigen binding domains and a single constant region, known as the FC composed of two CH regions (Figure 1).

Figure 1. Antibody Structure



The CH region defines the isotype, or class, of antibody (Figure 1): IgM, IgG, IgA, IgE, and IgD. Therefore the isotype of the antibody defines the role the antibody will play in a specific immunological response. The variable regions that define the antigen specificity is referred to as the idiotype (Figure 1), each unique combination of VH and VL generates a unique idiotype. Therefore, the specific antigen or target that is recognized by antibodies in the immune response are determined by the idiotype. A specific idiotype can be represented as multiple isotypes: i.e. an antibody that is specific for a unique antigen or epitope of an antigen (Figure 1), can be represented by multiple isotypes: IgM and IgG for example. Conversely, a single isotype can be represented with many different idiotypes— which would define an individual's antibody repertoire.

II: Idiotype network theory

Antibodies that recognize the unique structures of the VH and VL regions are referred to as anti-idiotypes (or anti-Ids). The principle of idiotypy, the determination of the the unique

structure of one antibody by another antibody, was characterized by Henry Kunkel's laboratory and Jacques Oudin's laboratory in the 1950's. Through the work of Kunkel, Oudin and others it was discovered that immunization of an animal with its own antibody produced anti-Ids, which was an indication that idiotypes and anti-idiotypes were present together in an immunological repertoire. Niels Jerne used these findings to develop his "network" theory, which described antibody idiotypes and anti-idiotypes generating a functional "network" that regulated immune responses and contributed to antibody diversity. While it was controversial at the time, and remains so today, it has guided the discovery of mechanisms of antibody development and regulation of the immune system for decades, leading to current models which favor a more cellular regulatory network model. The principles of the idiotype network theory have been used to explain the development and expansion of autoimmune responses, autoantibody development, and the development of animal models of autoimmunity.

One of the principle concepts of the idiotype network theory was the definition of anti-id antibodies (Figure 2). Experimentally anti-ids were demonstrated to interact with idiotypic regions in several ways:

i) binding to the variable region outside of the antigen binding site(Ab2α);

ii) binding to the antigen binding site($Ab2_{\beta}$), and

iii) binding near the antigen binding site ($Ab2_{\gamma}$). $Ab2_{\beta}$, and possibly $Ab2_{\gamma}$, would mimic the structure of the antigen as it is structurally complementary to the antigen binding site of the original antibody (Ab1). An anti-id to $Ab2_{\beta}$ would be structurally similar (if not identical) to Ab1.

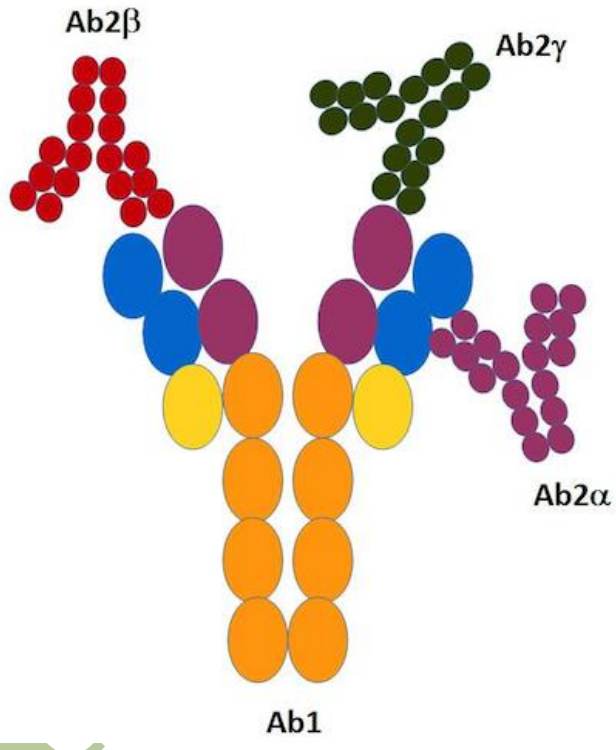
III. Anti-Idiotypes as therapeutics & diagnostics

As demonstrated by Jerne's idiotype network theory, antibodies themselves can be antigens and used to drive and/or regulate immune responses. It is this feature that allows anti-ids to be useful as analytical tools and potentially important as therapeutics (Kieber-Emmons et al., 2012). As antibodies have become important biologic therapeutics it is critical both for quality control of the antibodies as well as the evaluation of the pharmacokinetics (PK) of the

antibody therapeutic to have the ability to specifically detect the antibody drug. This is frequently accomplished by the development of an anti-id to the antibody drug, which then can be used in immunoassays for the characterization of the antibody drug. In addition as the $Ab2_{\beta}$, and possibly $Ab2_{\gamma}$, antigen determinant region would represent structures mimicking the antigen, these anti-ids have been used to induce immune responses for vaccinations as well as the treatment of cancer.

IV. Development of anti-idiotypes.

Figure 2. Anti-idiotype classes



Anti-ids are simply antibodies that bind to the unique structure of another antibody (idiotype). Therefore the development of anti-idiotypes follows the same paradigm as developing any monoclonal antibody targeted towards a protein antigen. The key is to understand what class (Figure 2) of anti-id antibody is necessary for the intended applications. Does it need to functionally block or mimic the target antigen ($Ab2_{\beta}$) or prevent the association of the antibody with its target ($Ab2_{\beta}$ or $Ab2_{\gamma}$), or does it simply need to

uniquely identify the antibody (Abα, $Ab2_{\beta}$, or $Ab2_{\gamma}$)? The key to developing the appropriate anti-idiotype is to have a screening strategy that will specifically determine the class of anti-id, generally defined by immunoassay.

Molecular Mimicry

The concept that molecular mimicry is an important factor in autoimmune disease was first published in 1985 and since that time substantial evidence has accumulated such that it has become the favoured mechanism for causing many autoimmune diseases including Multiple Sclerosis.

The concept is simple but entails a lot of understanding of the workings of the immune system. Basically molecular mimicry means that part of a molecule of a given protein closely resembles a part of another totally different protein. Proteins are made up of strings of amino acids and in molecular mimicry one series amino acids (eg~10) in one protein is very similar to a string of ten amino acids in another protein. Given that there are 20 different amino acids it is a rather rare occurrence to find such mimicking arrangements but many examples have been demonstrated.

The main types of proteins which came into play in autoimmune disease are:

1. self proteins which are part of the human body. An example of this would be myelin basic protein which is the most common protein in myelin;
2. proteins of infectious agents such as viruses and bacteria;
3. food proteins. For example over 400 different proteins occur in cow's milk and most have over 150 amino acids.

To understand how molecular mimicry works in the induction of autoimmunity one must understand the basic mechanisms of an immune response to a foreign invader in the body. The immune system recognizes a part of the protein portion of the invader. It does this with T cells which have receptors which bind to short segments (~10 amino acids) of a foreign protein. It is helped in this task by so called antigen presenting cells such as macrophages. A macrophage will engulf a foreign invader (eg a bacteria or food particle) and break it down

into fragments. A special molecule in the macrophage then carries a protein fragment(peptide) to the surface of the cell and "presents" it to the millions of circulating T cells. A T cell which has a matching receptor locks onto the presented protein fragment. The T cell then becomes activated and stimulates other portions of the immune system to begin an immune response against all proteins which contain a similar looking amino acid string. The details of what constitutes a similar looking string are beyond this summary but suffice to say it has been found that a variety of similar, yet somewhat different strings, can be recognized by the same T cell.

Thus, it is easy to understand how molecular mimicry can trigger an autoimmune reaction. If the protein fragment from a foreign invader which is presented to the T cell closely resembles part of a self protein then the activated immune system will not only attack all foreign invaders which have the same string of amino acids but will also attack a very similar string in a self protein. It has been shown that parts of proteins in various foods and infectious agents resemble parts of various self proteins. Sometimes a three-way mimicry occurs with a protein fragment from a food closely resembling that of an infectious agent which in turn closely resembles part of a self protein. In Celiac disease part of the gliadin molecule (found in various grains such as wheat and rye), part of adenovirus 12 and part of a gut protein all closely resemble each other, and the result of such mimicry is an immune attack on the gut when food containing gliadin protein is eaten. A similar three-way mimicry occurs between a cell wall protein in grains and legumes, part of the Epstein Barr virus and part of the collagen in joints. This leads to rheumatoid arthritis in genetically susceptible people. For type 1 diabetes parts of milk proteins and viral proteins mimic proteins in the insulin-producing beta cells of the pancreas.

Receptors

Receptors are proteins, usually cell surface receptors, which bind to ligands and cause responses in the immune system, including cytokine receptors, growth factor receptors and Fc receptor. Receptors can be found in various immune cells like B cells, T cells, NK cells, monocytes and stem cells. A molecule that binds to a receptor is called a ligand, and can be a

peptide (short-protein) or another small molecule such as a neurotransmitter, hormone, pharmaceutical-drug, toxin, or parts of the outside of a virus or microbe. When a ligand binds to its corresponding receptor, it activates or inhibits the receptor's associated-biochemical pathway.

Receptors can induce cell growth, division and death; control membrane channels or regulate cell binding. Receptors play an important role in signal transduction, immunetherapy and immune responses.

Growth Factor Receptors

Growth factors and their receptors play important roles in the regulation of cell division, development and differentiation. Growth factor receptors include wnt receptor, tie, neurotrophin receptor, ephrin receptor, insulin-like growth factor receptor (IGF receptor), epidermal growth factor receptor (EGF receptor), fibroblast growth factor receptor (FGF receptor), platelet-derived growth factor receptor (PDGF receptor) and vascular endothelial growth factor receptor (VEGF receptor), etc.

Cytokine Receptors

The patterns of expression of cytokine receptors are a product of differentiation and provide for changes in physiological regulation. These cytokine receptors enable cells to communicate with the extracellular environment by responding to signals generated in the vicinity or in other parts of the organism. Thus, the initial binding of cytokines to their receptors is a key event that occurs rapidly, at very low cytokine concentrations, is usually virtually irreversible, and leads to intracellular changes resulting in a biologic response. The biologic response can vary between cytokine receptors and from cell to cell but in general it involves gene expression, changes in the cell cycle, and release of mediators such as cytokines themselves.

B Cell Receptor

B cell, also called B lymphocyte, developed in bone marrow of most mammals, that circulates in the blood and lymph. One of the main functions of a B cell is antibody production, which aids in adaptive immunity, complement activation, and induction of allergic responses.

T Cell Receptor

The T cell antigen receptor (TCR) is the principle defining marker of all T cells. Also associated with the TCR is a complex of proteins known as CD3, which participate in the transduction of an intracellular signal following TCR binding to its cognate MHC/antigen complex. With the help of chemokine receptor CCR5 or CXCR4, HIV binds CD4 and penetrates its nucleic acid into the host cell. This cause CD4 T-cell destruction and immune system collapse.

Granulocyte Receptor

Granulocyte is a type of immune cell that has granules with enzymes released during infections, allergic reactions and asthma. A granulocyte is a type of white blood cell. Neutrophils, eosinophils, and basophils are three different forms of granulocytes. Granulocytes are crucial players in innate and adaptive immunity that express a large number of cell surface receptors for the recognition of pathogen invasion and the inflammatory environment. Those include G-protein-coupled chemokine receptors, Fc-receptors, various cytokine receptors, as well as innate immune receptors such as toll-like receptors and C-type lectins.

Epitope design and applications

An **epitope**, also known as **antigenic determinant**, is the part of an antigen that is recognized by the immune system, specifically by antibodies, B cells, or T cells. For example, the epitope is the specific piece of the antigen to which an antibody binds. The part of an antibody that binds to the epitope is called a paratope. Although epitopes are usually non-self proteins, sequences derived from the host that can be recognized (as in the case of autoimmune diseases) are also epitopes.

The epitopes of protein antigens are divided into two categories, conformational epitopes and linear epitopes, based on their structure and interaction with the paratope. Conformational and linear epitopes interact with the paratope based on the 3-D conformation adopted by the epitope, which is determined by the surface features of the involved epitope residues and the shape or tertiary structure of other segments of the antigen. A conformational epitope is formed by the 3-D conformation adopted by the interaction of discontiguous amino acid residues. In contrast, a linear epitope is formed by the 3-D conformation adopted by the interaction of contiguous amino acid residues. A linear epitope is not determined solely by the primary structure of the involved amino acids. Residues that flank such amino acid residues, as well as more distant amino acid residues of the antigen affect the ability of the primary structure residues to adopt the epitope's 3-D conformation.

Function

T cell epitopes

T cell epitopes are presented on the surface of an antigen-presenting cell, where they are bound to MHC molecules. In humans, professional antigen-presenting cells are specialized to present MHC class II peptides, whereas most nucleated somatic cells present MHC class I peptides. T cell epitopes presented by MHC class I molecules are typically peptides between 8 and 11 amino acids in length, whereas MHC class II molecules present longer peptides, 13-17 amino acids in length, and non-classical MHC molecules also present non-peptidic epitopes such as glycolipids.

Cross-activity

Epitopes are sometimes cross-reactive. This property is exploited by the immune system in regulation by anti-idiotypic antibodies. If an antibody binds to an antigen's epitope, the paratope could become the epitope for another antibody that will then bind to it. If this second antibody is of IgM class, its binding can upregulate the immune response; if the second antibody is of IgG class, its binding can downregulate the immune response.

Epitope mapping

Epitopes can be mapped using protein microarrays, and with the ELISPOT or ELISA techniques. Another technique involves high-throughput mutagenesis, an epitope mapping strategy developed to improve rapid mapping of conformational epitopes on structurally complex proteins.

MHC class I and II epitopes can be reliably predicted by computational means alone, although not all in-silico T cell epitope prediction algorithms are equivalent in their accuracy.

Epitope tags

Epitopes are often used in proteomics and the study of other gene products. Using recombinant DNA techniques genetic sequences coding for epitopes that are recognized by common antibodies can be fused to the gene. Following synthesis, the resulting epitope tag allows the antibody to find the protein or other gene product enabling lab techniques for localisation, purification, and further molecular characterization. Common epitopes used for this purpose are Myc-tag, HA-tag, FLAG-tag, GST-tag, 6xHis, V5-tag and OLLAS. Peptides can also be bound by proteins that form covalent bonds to the peptide, allowing irreversible immobilisation. These strategies have also been successfully applied to the development of "epitope-focused" vaccine design.

Neoantigenic determinant

A neoantigenic determinant is an epitope on a neoantigen, which is a newly formed antigen that has not been previously recognized by the immune system. Neoantigens are often associated with tumor antigens and are found in oncogenic cells. Neoantigens and, by extension, neoantigenic determinants can be formed when a protein undergoes further modification within a biochemical pathway such as glycosylation, phosphorylation or proteolysis. This, by altering the structure of the protein, can produce new epitopes that are called neoantigenic determinants as they give rise to new antigenic determinants. Recognition requires separate, specific antibodies.

Diagnostic Tests

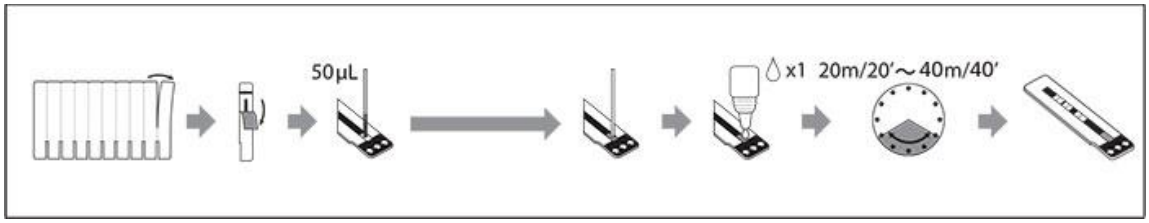
HIV Combo – Rapid Test

- *in vitro*
- Qualitative immunoassay
- 4th generation Rapid Test
- Detection of antibodies (Ab) to HIV-1 and HIV-2
- Detection of free HIV-1 p24 antigen (Ag)
- As it detects p24 antigens, it may identify HIV infection earlier than antibody-only tests

The multiplication of the HIV in the infected cells leads to cell rupture and thus the release of HIV virus particles, which are first detected in the form of HIV RNA and next in the form of HIV antigen. This is followed by production of specific antibodies to either HIV-1 or HIV-2. The HIV antigen may become undetectable at this time because of the formation of antibody-antigen complexes. Alere HIV Combo can only detect free p24 antigens.

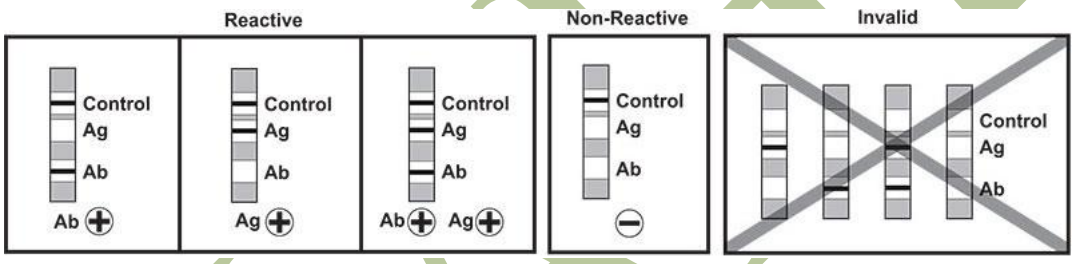
Test Procedure

- Rapid and easy-to-use
- Lateral flow assay
- 50 µL of sample applied to a sample pad
- 1 drop of Chaser Buffer applied to sample pad
- Sample diffuses along the strip
- Results for HIV-1 p24 antigen and HIV-1 and HIV-2 antibodies
- Control window
- Results read in designated windows
- The small strip format is suitable for use with whole blood, serum, or plasma samples
- Results in 20-40 minutes



Test Results

- Control and Ab bars – Antibody reactive
- Control and Ag bars – Antigen p24 reactive
- Control, Ab and Ag bars – Antibody and Antigen p24 reactive
- Only Control bar – Non-reactive
- No Control bar – Test invalid



How the Test Works

- A specimen is added to the sample pad.
- The specimen mixes with biotinylated anti-p24 antibodies and selenium colloid-conjugates coated with recombinant HIV-1, HIV-2 and HIV-1 group O antigens, synthetic HIV-2 peptide and anti p24 mouse monoclonal antibody.
- This mixture continues to migrate through the solid phase to the immobilized recombinant HIV-1/HIV-1 group O antigens and synthetic HIV-1/HIV-2 peptides at the Antibody (Ab) window, immobilized avidin at the Antigen (Ag) window.
- If antibodies to HIV-1 and/or HIV-2 are present in the specimen, the antibodies bind to the selenium colloid-conjugates coated with recombinant HIV-1, HIV-2 and HIV-1 group O antigens and synthetic HIV-2 peptide and to the immobilized recombinant

HIV-1/HIV-1 group O antigens and synthetic HIV-1/HIV-2 peptides, forming one red bar at the Ab window site.

- If antibodies to HIV-1 and HIV-2 are absent, the selenium colloid-conjugates flow past the Ab window and no red bar is formed at the Ab window site.
- If free HIV-1 p24 antigen is present in the specimen, the antigen binds to the biotinylated anti-p24 antibodies and the selenium colloid-conjugate coated with anti p24 mouse monoclonal antibody. This complex binds to the immobilized avidin forming a red bar at the Ag window site.
- If HIV-1 p24 antigen is not present, both the biotinylated anti-p24 antibodies and selenium colloid conjugate flow past the Ag window and no red bar is formed at the Ag window site.
- To ensure assay validity, a procedural control bar is incorporated in the assay device at the Control window.

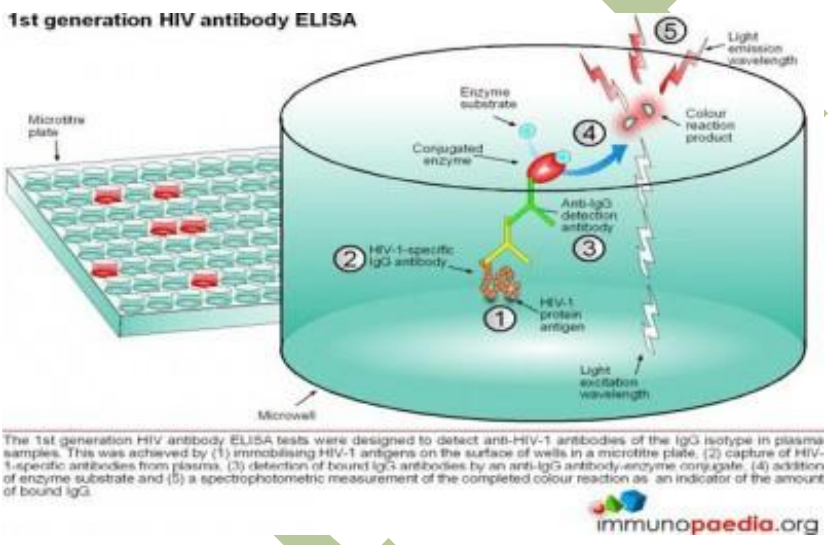
Test Limitations

- No test provides absolute assurance that a specimen does not contain low levels of HIV-1 p24 antigen and/or antibodies to HIV-1 and HIV-2 such as those present at a very early stage or late stage of HIV infection.
- Alere HIV Combo is designed to detect antibodies to HIV-1 and/or HIV-2 and free HIV-1 p24 antigen, in human serum, plasma and capillary and venous whole blood specimens. Other body fluids or pooled specimens should not be used.
- The intensity of the Ab and Ag bars does not necessarily correlate to the titer of antibody and antigen in the specimen.
- The absence of Ag bar may occur when all p24 antigen is bound by antibodies. When high levels of antibodies against the p24 antigen are present in the blood after seroconversion, the antibodies tend to bind to the antigens, forming immunocomplexes. Alere HIV Combo detects only non-immunocomplexed (free) antigens; it does not detect immunocomplexed (bound) antigens.
- HIV-infected persons taking antiretroviral medication have been shown to produce false negative results when tested by rapid diagnostic tests.

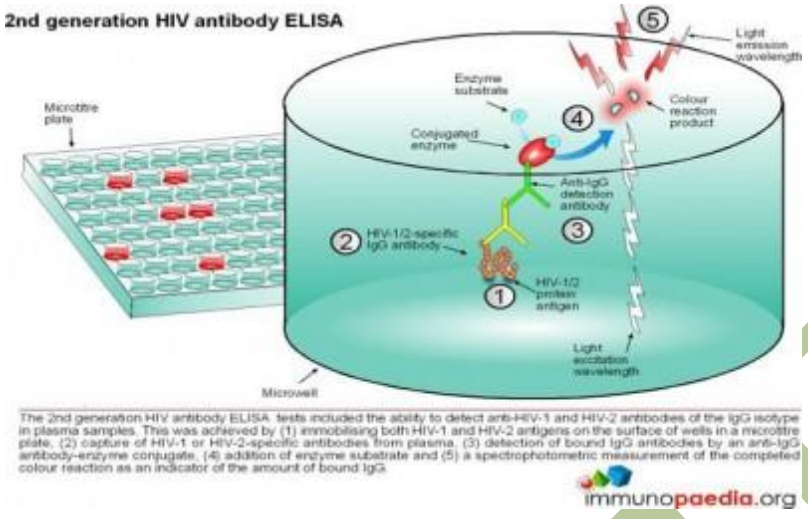
- Infants born to HIV-infected mothers may carry maternal antibodies and will test antibody positive until eighteen months of age, which may not necessarily indicate the true infection status of the new born. The use of HIV- 1 p24 antigen testing to exclude infection in neonates (up to around eighteen months) is not recommended by CDC, because of poor sensitivity, especially in the presence of HIV antibody. Definitive diagnosis of HIV infection in early infancy requires other assays, including HIV nucleic acid test or viral culture.

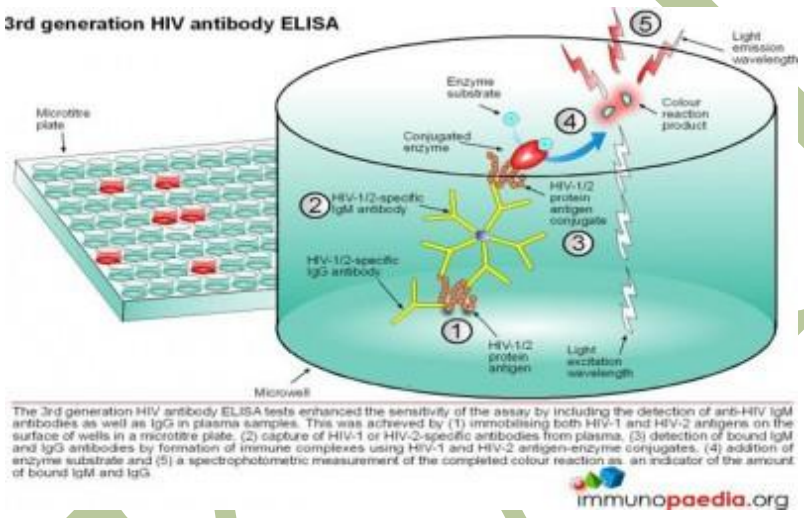
ELISA tests

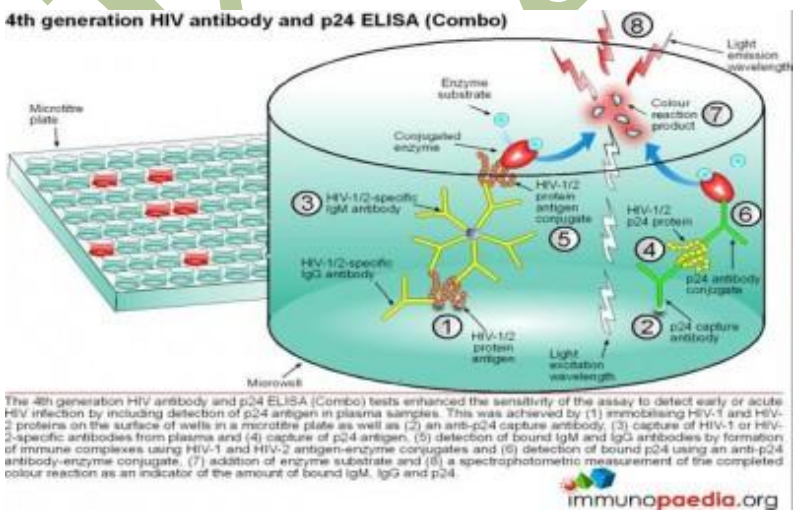
ELISA (enzyme –linked immunosorbent assay) is a serological assay in which a bound antigen or antibody is detected by a linked enzyme that converts a colourless substrate into a coloured product. There are 4 generations of HIV antibody ELISA tests. The progression of these assays has been depicted in our series of 4 graphics.



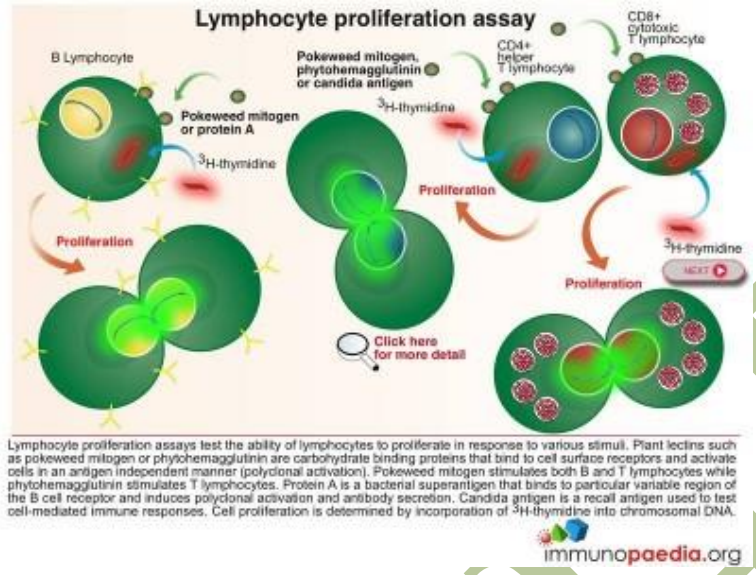
The 1st generation HIV antibody ELISA tests were designed to detect anti-HIV-1 antibodies of the IgG isotype in plasma samples. This was achieved by (1) immobilising HIV-1 antigens on the surface of wells in a microtitre plate, (2) capture of HIV-1-specific antibodies from plasma, (3) detection of bound IgG antibodies by an anti-IgG antibody-enzyme conjugate, (4) addition of enzyme substrate and (5) a spectrophotometric measurement of the completed colour reaction as an indicator of the amount of bound IgG.


2nd generation HIV antibody ELISA
Microtitre plate
Microwell
Enzyme substrate
Conjugated enzyme
Colour reaction product
Light emission wavelength
Anti-IgG detection antibody
HIV-1/2-specific IgG antibody
HIV-1/2 protein antigen
Light excitation wavelength
1
2
3
4
5
The 2nd generation HIV antibody ELISA tests included the ability to detect anti-HIV-1 and HIV-2 antibodies of the IgG isotype in plasma samples. This was achieved by (1) immobilising both HIV-1 and HIV-2 antigens on the surface of wells in a microtitre plate, (2) capture of HIV-1 or HIV-2-specific antibodies from plasma, (3) detection of bound IgG antibodies by an anti-IgG antibody-enzyme conjugate, (4) addition of enzyme substrate and (5) a spectrophotometric measurement of the completed colour reaction as an indicator of the amount of bound IgG.
immunopaedia.org


3rd generation HIV antibody ELISA
Microtitre plate
Microwell
Enzyme substrate
Conjugated enzyme
Colour reaction product
Light emission wavelength
HIV-1/2-specific IgM antibody
HIV-1/2-specific IgG antibody
HIV-1/2 protein antigen conjugate
HIV-1/2 protein antigen
Light excitation wavelength
1
2
3
4
5
The 3rd generation HIV antibody ELISA tests enhanced the sensitivity of the assay by including the detection of anti-HIV IgM antibodies as well as IgG in plasma samples. This was achieved by (1) immobilising both HIV-1 and HIV-2 antigens on the surface of wells in a microtitre plate, (2) capture of HIV-1 or HIV-2-specific antibodies from plasma, (3) detection of bound IgM and IgG antibodies by formation of immune complexes using HIV-1 and HIV-2 antigen-enzyme conjugates, (4) addition of enzyme substrate and (5) a spectrophotometric measurement of the completed colour reaction as an indicator of the amount of bound IgM and IgG.
immunopaedia.org


4th generation HIV antibody and p24 ELISA (Combo)
Microtitre plate
Microwell
Enzyme substrate
Conjugated enzyme
Colour reaction product
Light emission wavelength
HIV-1/2-specific IgM antibody
HIV-1/2-specific IgG antibody
HIV-1/2 protein antigen conjugate
HIV-1/2 p24 protein
p24 antibody conjugate
p24 capture antibody
HIV-1/2 protein antigen
Light excitation wavelength
1
2
3
4
5
6
7
8
The 4th generation HIV antibody and p24 ELISA (Combo) tests enhanced the sensitivity of the assay to detect early or acute HIV infection by including detection of p24 antigen in plasma samples. This was achieved by (1) immobilising HIV-1 and HIV-2 proteins on the surface of wells in a microtitre plate as well as (2) an anti-p24 capture antibody, (3) capture of HIV-1 or HIV-2-specific antibodies from plasma and (4) capture of p24 antigen, (5) detection of bound IgM and IgG antibodies by formation of immune complexes using HIV-1 and HIV-2 antigen-enzyme conjugates and (6) detection of bound p24 using an anti-p24 antibody-enzyme conjugate, (7) addition of enzyme substrate and (8) a spectrophotometric measurement of the completed colour reaction as an indicator of the amount of bound IgM, IgG and p24.
immunopaedia.org

Lymphocyte Proliferation Test



Lymphocyte proliferation assays test the ability of lymphocytes to proliferate in response to various stimuli. Plant lectins such as pokeweed mitogen or phytohemagglutinin are carbohydrate binding proteins that bind to cell surface receptors and activate cells in an antigen independent manner (polyclonal activation). Pokeweed mitogen stimulates both B and T lymphocytes while phytohemagglutinin stimulates T lymphocytes. Protein A is a bacterial superantigen that binds to particular variable region of the B cell receptor and induces polyclonal activation and antibody secretion. Candida antigen is a recall antigen used to test cell-mediated immune responses. Cell proliferation is determined by incorporation of ^{3}H-thymidine into chromosomal DNA.

The Lymphocyte Proliferation Assay (LPA) is a test used to measure the ability of lymphocytes to proliferate in response to various stimuli such as candida, pokeweed and phytohaemagglutinin. These plant lectins are carbohydrate binding proteins that bind to cell surface receptors and activate cells in an antigen independent manner. Most people will respond to at least one of several common microbial antigens. LPA can be used to measure improvements in immunological function following antiretroviral therapy and to detect the presence of immune responses against specific opportunistic pathogens.

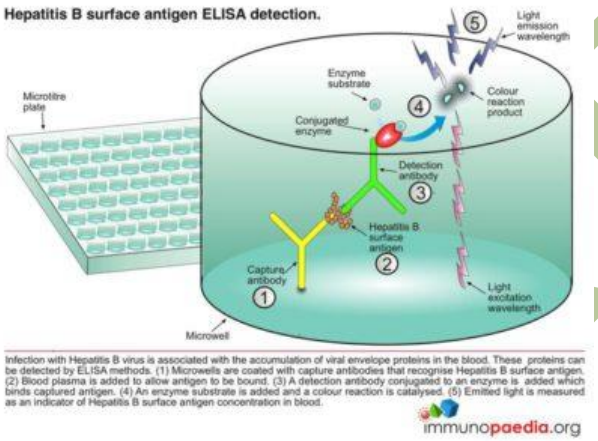
The 3H-thymidine incorporation assay is used to determine the extent of cell division in response to a proliferation signal. 3H-thymidine which is a radioactive nucleoside and precursor of thymine found in DNA becomes incorporated in DNA strands of proliferating cells and can be measured to determine extent of cell proliferation.

PCR test

The polymerase chain reaction (PCR) is a technique used for a wide range of tasks including detection and diagnosis of infectious disease. The technique exponentially amplifies a fragment of DNA or RNA by in vitro enzymatic replication and because PCR amplifies the regions of DNA or RNA that it targets, PCR can be used to analyze extremely small amounts

of sample. Viral DNA or RNA can therefore be detected by PCR. The primers used need to be specific to the targeted sequences in the DNA or RNA of a virus, and the PCR can be used for diagnostic analyses or DNA or RNA sequencing of the viral genome. The high sensitivity of PCR permits virus detection soon after infection and even before the onset of disease, allowing for a significant lead in treatment. A patient's viral load can also be quantified by PCR-based DNA or RNA quantitation techniques.

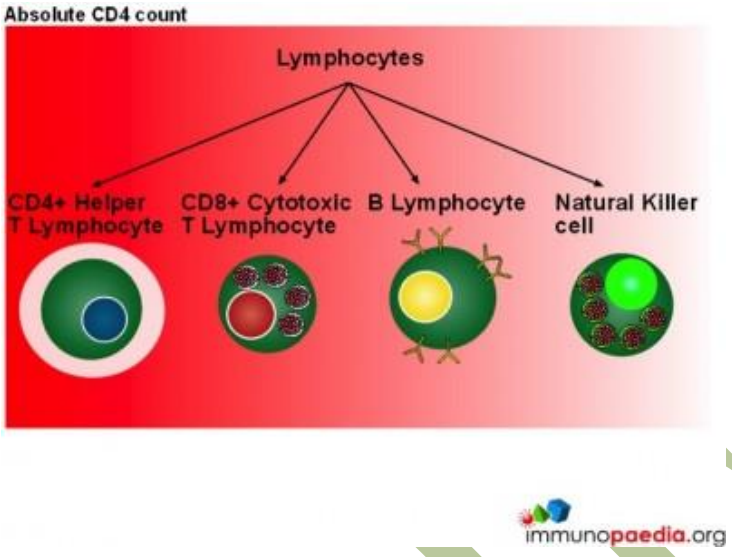
Hepatitis B Surface Antigen



Hepatitis B surface antigen (HBsAg) is a protein antigen produced by hepatitis B virus (HBV). This antigen is the earliest indicator of acute hepatitis B infection and is able to identify infected people before they are symptomatic. HBsAg becomes undetectable in the blood during the recovery period. Children and immunocompromised patients such as patients with HIV infection who become chronically infected may remain HBsAg positive.

To detect this antigen we perform an Enzyme-Linked Immunosorbent Assay test (HIV ELISA).

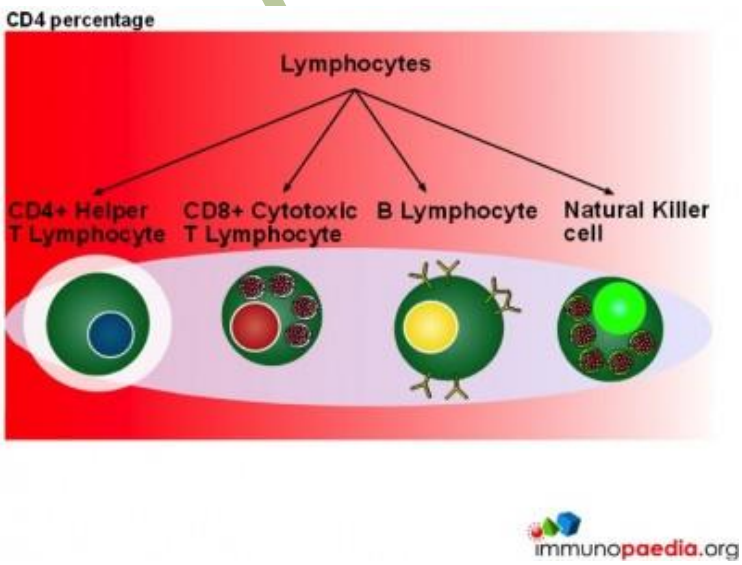
CD4 Count



In this series of graphics the three basic cell types found in blood (leukocytes, erythrocytes and platelets) have been depicted. The graphics further explain the leukocyte cell lines and include the granulocytes, monocytes and lymphocytes and the different cells which make up these groups of cells.

When measuring disease states it is an absolute CD4 count which is used to monitor patients.

Although CD4 cell counts are used to measure HIV disease states absolute CD4 counts are not a reliable marker when determining disease states in children.

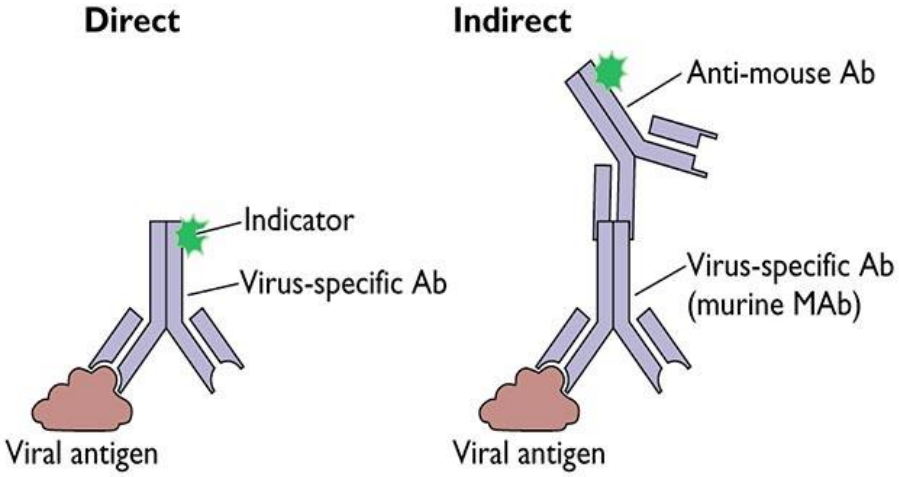


This is because lymphocytes are present in higher numbers in children thus making the count less accurate. The WHO has therefore recommended that all children under the age of 5 should be evaluated with a CD4 percentage when assessing disease status and decision making for initiation of treatment.

Immunofluorescence

Immunofluorescence is an assay which is used primarily on biological samples and is classically defined as a procedure to detect antigens in cellular contexts using antibodies. The specificity of antibodies to their antigen is the base for immunofluorescence.

The property of certain dyes absorbing light rays at one particular wavelength (ultraviolet light) and emitting them at a different wavelength (visible light) is known as **fluorescence.** In immunofluorescence test, fluorescent dye which illuminates in UV light are used to detect/show the specific combination of an antigen and antibody. The dye usually used is **fluorescein isothiocynate**, which gives yellow-green fluorescence. Immunofluorescence tests are also termed as **fluorescent antibody test (FAT).**



Fluorescent dyes, such as fluorescein isothiocyanate and lissamine rhodamine, can be tagged with antibody molecules. They emit blue-green and orange-red fluorescence, respectively under ultraviolet (UV) rays in the fluorescence microscope. This forms the basis of the

immunological test. Immunofluorescence tests have wide applications in research and diagnostics. These tests are broadly of two types:

1. Direct immunofluorescence test
2. Indirect immunofluorescence test

Direct immunofluorescence test is used to detect unknown antigen in a cell or tissue by employing a known labeled antibody that interacts directly with unknown antigen. If antigen is present, it reacts with labeled antibody and the antibody coated antigen is observed under UV light of the fluorescence. It involves use of labeled antiviral antibody.

Method

The specimen is placed on slide; fluorescent labeled antibody is then added to it and allowed for some time for Antigen-Antibody reaction. The preparation is then washed which will allow the removal of other components except the complex of antigen and fluorescent labeled antibody. On microscopy (Fluorescence Microscopy), Antigen- Antibody complex are observed fluorescing due to the dye attached to antibody.

The need for preparation of separate labeled antibody for each pathogen is the major disadvantage of the direct immunofluorescence test.

Indirect immunofluorescence test

Indirect fluorescence is a double antibody technique. The unlabeled antibodies which have bound to the antigens are visualized by a fluorescent antiglobulin reagent directed at the unlabeled antibodies. The indirect immunofluorescence test is used for detection of specific antibodies in the serum and other body fluids for sero-diagnosis of many infectious diseases.

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Indirect immunofluorescence is a two-stage process.

First stage: A known antigen is fixed on a slide. Then the patient's serum to be tested is applied to the slide, followed by careful washing. If the patient's serum contains antibody against the antigen, it will combine with antigen on the slide.

Second stage: The combination of antibody with antigen can be detected by addition of a fluorescent dye-labeled antibody to human IgG, which is examined by a fluorescence microscope.

The first step in the indirect immunofluorescence test is the incubation of a fixed antigen (e.g., in a cell or tissue) with unlabeled antibody, which becomes associated with the antigen. After careful washing, a fluorescent antibody (e.g. fluorescent labeled anti-IgG) is added to the smear. This second antibody will become associated to the first, and the antigen–antibody complex can be visualized on the fluorescence microscope.

The indirect method has the advantage of using a single labeled antiglobulin (antibody to IgG) as a "universal reagent" to detect many different specific antigen–antibody reactions. The test is often more sensitive than the direct immunofluorescence test.

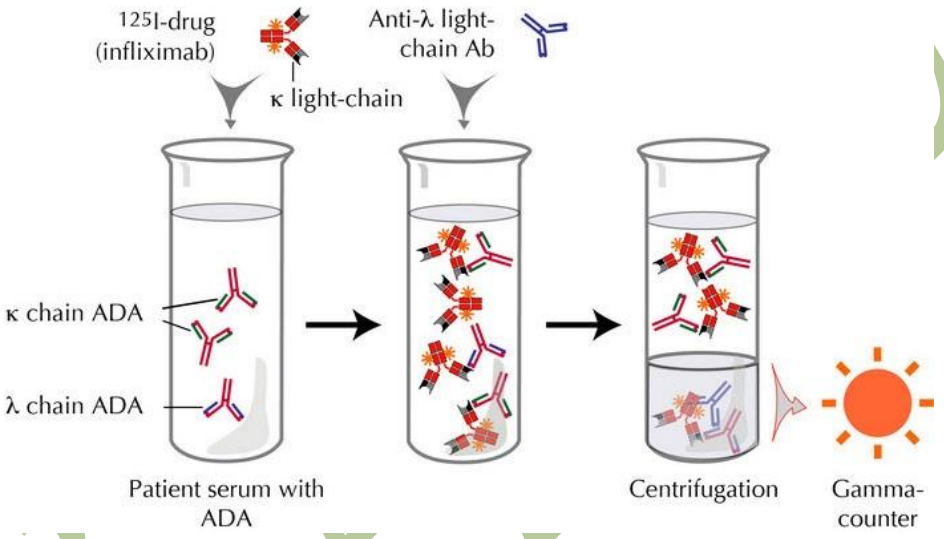
Indirect immunofluorescence test is used widely to:

1. Detect specific antibodies for serodiagnosis of syphilis, leptospirosis, amoebiasis, toxoplasmosis, and many other infectious diseases;
2. Identify the class of a given antibody by using fluorescent antibodies specific for different immunoglobulin isotypes;
3. Identify and enumerate lymphocyte subpopulations by employing monoclonal antibodies and cytofluorographs; and
4. Detect autoantibodies, such as antinuclear antibodies in autoimmune diseases.

Immunofluorescence may also be used to analyze the distribution of proteins, glycans, and small biological and non-biological molecules. Immunofluorescence has been widely used in biological research and medical research.

Radioimmunoassay

When radioisotopes instead of enzymes are used as labels to be conjugated with antigens or antibodies, the technique of detection of the antigen-antibody complex is called radioimmunoassay (RIA). Radioimmunoassay (RIA) is an *in vitro* assay that measures the presence of an antigen with very high sensitivity. RIA was first described in 1960 for the measurement of endogenous plasma insulin by **Solomon Berson and Rosalyn Yalow** of the Veterans Administration Hospital in New York.



The classical RIA methods are based on the principle of competitive binding. In this method, an unlabeled antigen competes with a radiolabeled antigen for binding to an antibody with the appropriate specificity. Thus, when mixtures of radiolabeled and unlabeled antigen are incubated with the corresponding antibody, the amount of free (not bound to antibody) radiolabeled antigen is directly proportional to the quantity of unlabeled antigen in the mixture.

Principle of Radioimmunoassay

It involves a combination of three principles.

1. An immune reaction i.e. antigen, antibody binding.
2. A competitive binding or competitive displacement reaction. (It gives specificity)

3. Measurement of radio emission. (It gives sensitivity)

Immune Reaction:

When a foreign biological substance enters into the body bloodstream through a non-oral route, the body recognizes the specific chemistry on the surface of foreign substance as antigen and produces specific antibodies against the antigen so as nullify the effects and keep the body safe. The antibodies are produced by the body's immune system so, it is an immune reaction. Here the antibodies or antigens bind move due to chemical influence. This is different from principle of electrophoresis where proteins are separated due to charge.

Competitive binding or competitive displacement reaction:

This is a phenomenon wherein when there are two antigens that can bind to the same antibody, the antigen with more concentration binds extensively with the limited antibody displacing others. So here in the experiment, a radiolabelled antigen is allowed to bind to high-affinity antibody. Then when the patient serum is added unlabeled antigens in it start binding to the antibody displacing the labeled antigen.

Measurement of radio emission:

Once the incubation is over, then washings are done to remove any unbound antigens. Then radio emission of the antigen-antibody complex is taken, the gamma rays from radiolabeled antigen are measured.

The target antigen is labeled radioactively and bound to its specific antibodies (a limited and known amount of the specific antibody has to be added). A sample, for e.g. blood-serum, is added in order to initiate a competitive reaction of the labeled antigens from the preparation, and the unlabeled antigens from the serum-sample, with the specific antibodies. The competition for the antibodies will release a certain amount of labeled antigen. This amount is proportional to the ratio of labeled to an unlabeled antigen. A binding curve can then be generated which allows the amount of antigen in the patient's serum to be derived. That means as the concentration of unlabeled antigen is increased, more of it binds to the antibody,

displacing the labeled variant. The bound antigens are then separated from the unbound ones, and the radioactivity of the free antigens remaining in the supernatant is measured.

Antigen-antibody complexes are precipitated either by crosslinking with a second antibody or by means of the addition of reagents that promote the precipitation of antigen-antibody complexes. Counting radioactivity in the precipitates allows the determination of the amount of radiolabeled antigen precipitated with the antibody. A standard curve is constructed by plotting the percentage of antibody-bound radiolabeled antigen against known concentrations of a standardized unlabeled antigen, and the concentrations of antigen in patient samples are extrapolated from that curve.

Uses of Radioimmunoassay

1. The test can be used to determine very small quantities (e.g. nanogram) of antigens and antibodies in the serum.
2. The test is used for quantitation of hormones, drugs, HBsAg, and other viral antigens.
3. Analyze nanomolar and picomolar concentrations of hormones in biological fluids.

The limitations of the RIA include:

1. The cost of equipment and reagents
2. Short shelf-life of radiolabeled compounds
3. The problems associated with the disposal of radioactive waste.

SYLLABUS

GC, HPLC, Electron microscopy, flow cytometry and cell sorting. Transgenic animals.

Gas Chromatograph (GC)

Instrumentation for Gas chromatography has continually evolved since the inception of the technique in 1951 and the introduction of the first commercial systems in 1954.

Most modern commercial GC systems operate in the following way (Figure):

An inert carrier gas, such as helium, is supplied from gas cylinders to the GC where the pressure is regulated using manual or electronic (pneumatic) pressure controls

The regulated carrier gas is supplied to the inlet and subsequently flows through the column and into the detector

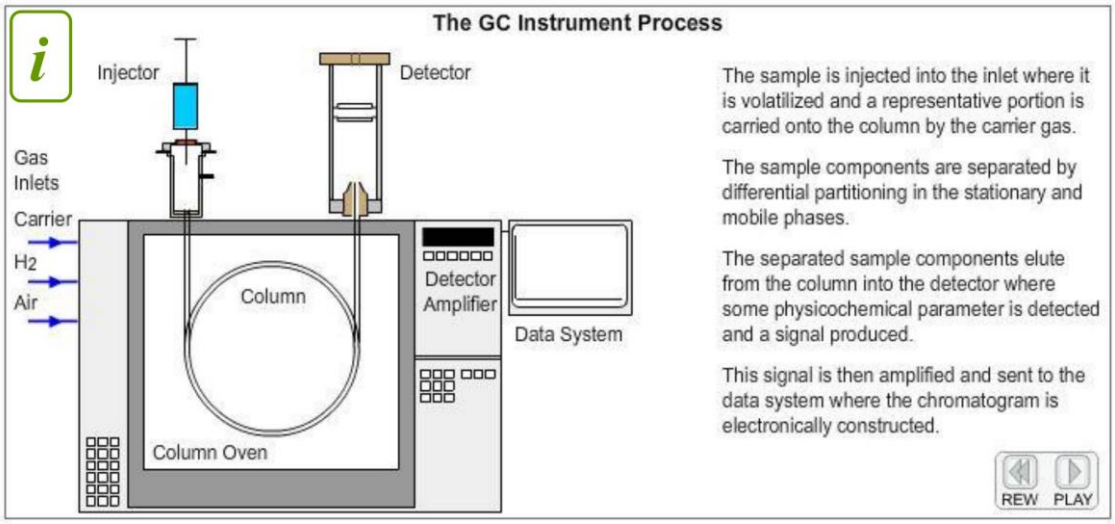
The sample is injected into the (usually) heated injection port where it is volatilized and carried into the column by the carrier gas

The sample is separated inside the column - usually a long silica based column with small internal diameter. The sample separates by differential partition of the analytes between the mobile and stationary phases, based on relative vapor pressure and solubility in the immobilized liquid stationary phase

On elution from the column, the carrier gas and analytes pass into a detector, which responds to some physicochemical property of the analyte and generates an electronic signal measuring the amount of analyte present

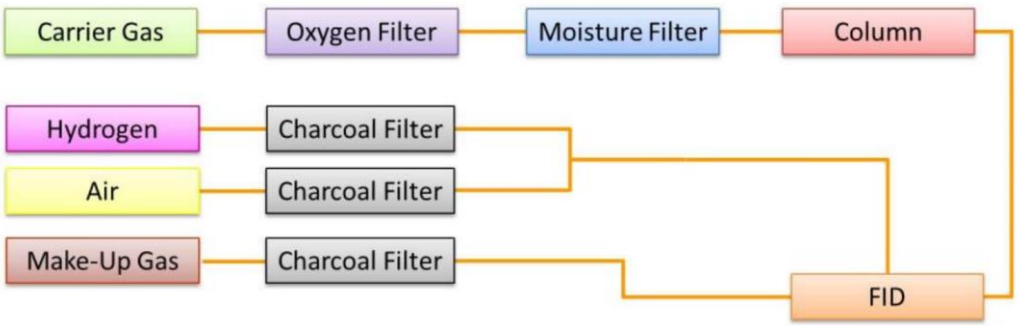
The data system then produces an integrated chromatogram

Gas chromatography uses ovens that are temperature programmable. The temperature of the GC oven typically ranges from 5 °C to 400 °C but can go as low as -25 °C with cryogenic cooling.



Gas Inlets:

Gas is fed from cylinders through supply piping to the instrument. It is usual to filter gases to ensure high gas purity and the gas supply may be regulated at the bench to ensure an appropriate supply pressure.



Required gases might include:

Carrier - (H 2, He, N 2)

Make-up gas - (H 2, He, N 2)

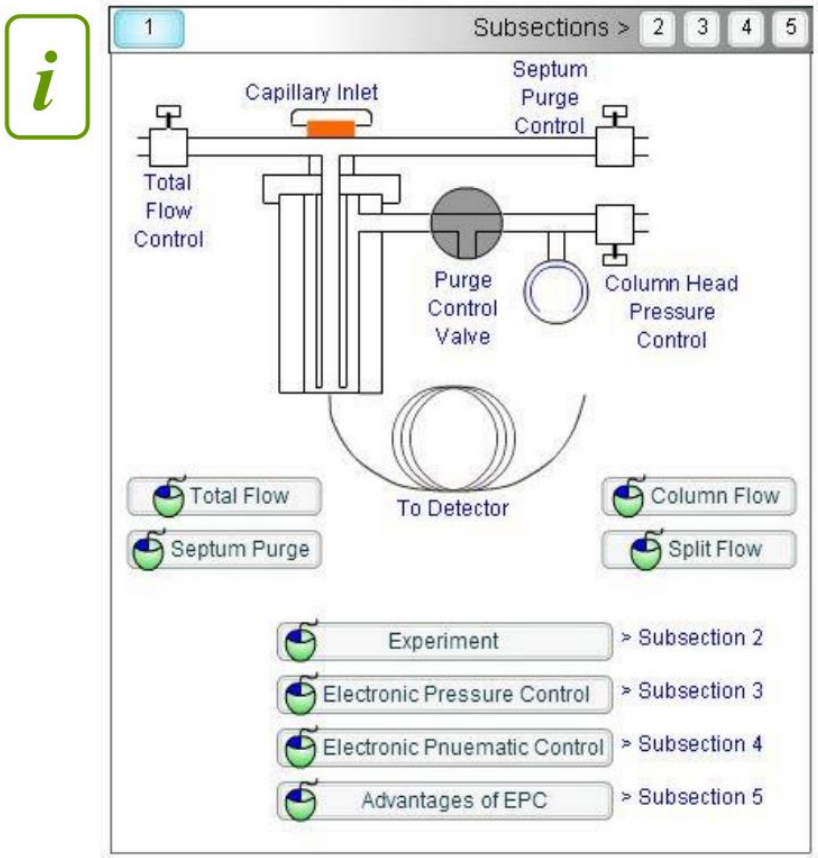
Detector Fuel Gas - (H 2 & Air, Ar or Ar & CH 4, N 2) depending on the detector type

Pneumatic controls:

The gas supply is regulated to the correct pressure (or flow) and then fed to the required part of the instrument. Control is usually required to regulate the gas coming into the instrument and then to supply the various parts of the instrument. A GC fitted with a Split/Splitless inlet, capillary GC column and Flame Ionization detector may have the following different gas specifications:

Carrier gas supply pressure, column inlet pressure (column carrier gas flow), inlet split flow, inlet septum purge flow, detector air flow, detector hydrogen flow, detector make-up gas flow.

Modern GC instruments have Electronic Pneumatic pressure controllers – older instruments may have manual pressure control *via* regulators

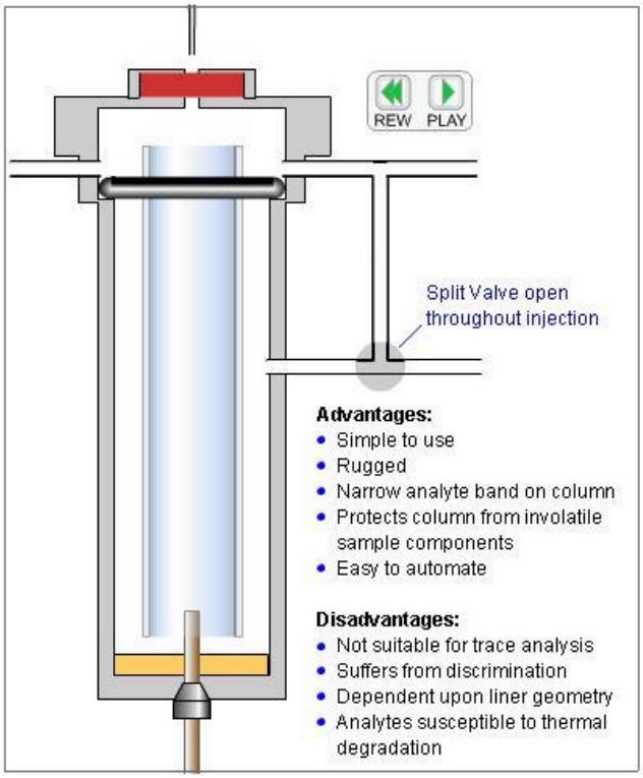


Injector:

Here the sample is volatilized and the resulting gas entrained into the carrier stream entering the GC column.

- Many inlet types exist including:
- Split / Splitless
- Programmed Thermal Vaporizing (PTV)
- Cool-on-column (COC) etc.

The COC injector introduces the sample into the column as a liquid to avoid thermal decomposition or improve quantitative accuracy.



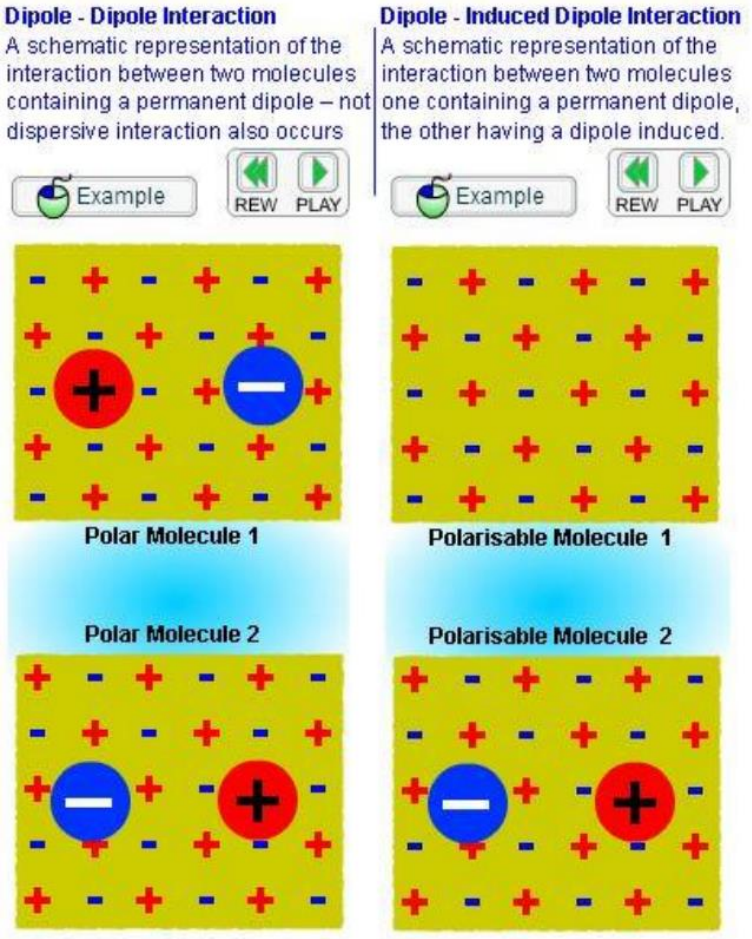
Column:

In GC, retention of analyte molecules occurs due to stronger interactions with the stationary phase than the mobile phase. This is unique in GC and, therefore, interactions between the

stationary phase and analyte are of great importance. The interaction types can be divided into three broad categories:

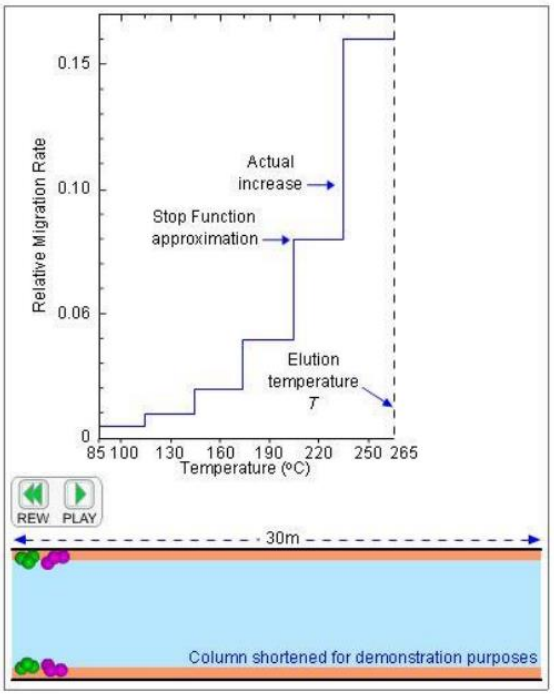
- Dispersive
- Dipole
- Hydrogen bonding

The sample is separated into its constituent components in the column. Columns vary in length and internal diameter depending on the application type and can be either packed or capillary. Packed columns (typical dimension 1.5 m x 4 mm) are packed with a solid support coated with immobilized liquid stationary phase material (GLC). Capillary columns (typical dimension 30 m x 0.32 mm x 0.1 mm film thickness) are long hollow silica tubes with the inside wall of the column coated with immobilized liquid stationary phase material of various film thickness. Many different stationary phase chemistries are available to suit a host of applications. Columns may also contain solid stationary phase particles (GSC) for particular application types.



Column Oven:

Temperature in GC is controlled via a heated oven. The oven heats rapidly to give excellent thermal control. The oven is cooled using a fan and vent arrangement usually at the rear of the oven. A hanger or cage is usually included to support the GC column and to prevent it touching the oven walls as this can damage the column. The injector and detector connections are also contained in the GC oven. For Isothermal operation, the GC is held at a steady temperature during the analysis. In temperature programmed GC (pTGC) the oven temperature is increased according to the temperature program during the analysis.

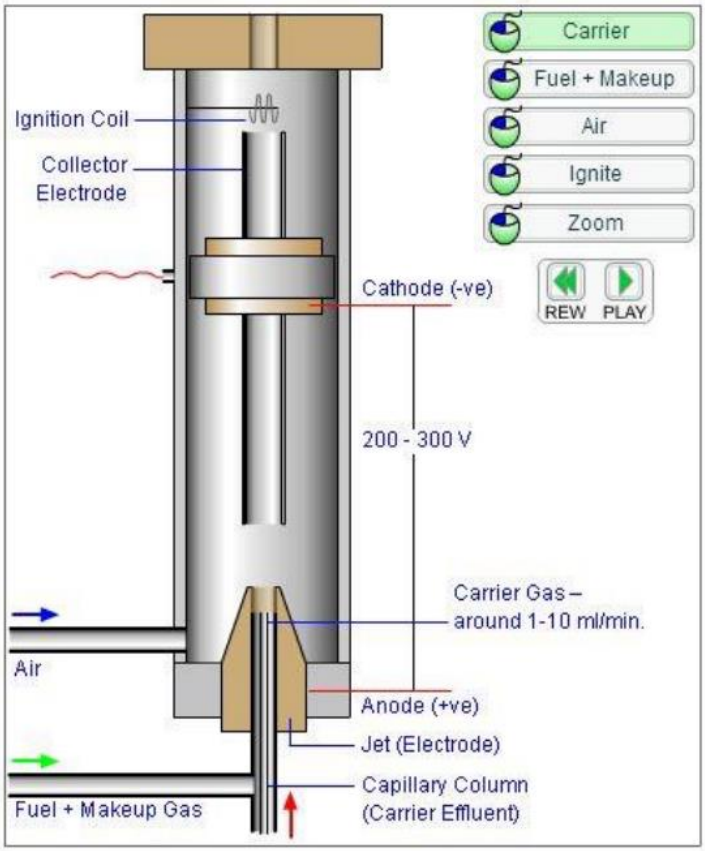


Detector:

The detector responds to a physicochemical property of the analyte, amplifies this response and generates an electronic signal for the data system to produce a chromatogram. Many different detector types exist and the choice is based mainly on application, analyte chemistry and required sensitivity – also on whether quantitative or qualitative data is required.

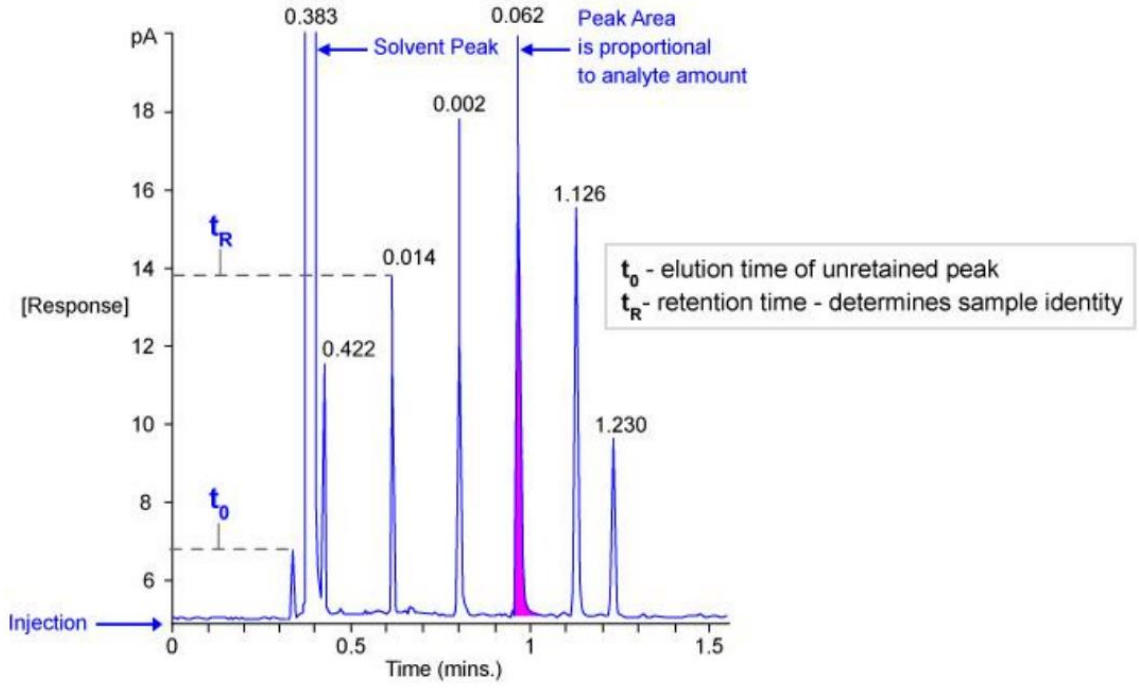
Detector choices include:

- Flame Ionization (FID)
- Electron Capture (ECD)
- Flame Photometric (FPD)
- Nitrogen Phosphorous (NPD)
- Thermal Conductivity (TCD)
- and Mass Spectrometer (MS)



Data System:

The data system receives the analogue signal from the detector and digitizes it to form the record of the chromatographic separation known as the 'Chromatogram'. The data system can also be used to perform various quantitative and qualitative operations on the chromatogram – assisting with sample identification and quantitation.



GC Advantages and Disadvantages

Gas chromatography has several important advantages which are listed opposite. GC techniques produce fast analyses because of the highly efficient nature of the separations achieved – this will be studied further in the Band Broadening Section. It can be argued that modern GC produces the fastest separations of all chromatographic techniques. A column has been produced to separate 970 components within a reasonable analysis time - proving that very complex separations may be carried out using GC.

By using a combination of oven temperature and stationary phase chemistry (polarity) very difficult separations may also be carried out – including separations of chiral and other positional isomers. GC is excellent for quantitative analysis with a range of sensitive and linear detectors to choose from.

GC is however limited to the analysis of volatile samples. Some highly polar analytes can be derivatized to impart a degree of volatility, but this process can be difficult and may incur quantitative errors.

A practical upper temperature limit for conventional GC columns is around 350-380 °C. Analyte boiling points rarely exceed 400 °C in GC analysis and the upper Molecular Weight is usually around 500 Da.

Advantages

- Fast analysis
- High efficiency – leading to high resolution
- Sensitive detectors (ppb)
- Non-destructive – enabling coupling to Mass Spectrometers (MS) - *an instrument that measures the masses of individual molecules that have been converted into ions, i.e. molecules that have been electrically charged*
- High quantitative accuracy (<1% RSD typical)
- Requires small samples (<1 mL)
- Rugged and reliable techniques
- Well established with extensive literature and applications

Disadvantages

- Limited to volatile samples
- Not suitable for samples that degrade at elevated temperatures (thermally labile)
- Not suited to preparative chromatography
- Requires MS detector for analyte structural elucidation (characterization)
- Most non-MS detectors are destructive.

High Performance Liquid Chromatography (HPLC) : Principle, Types, Instrumentation and Applications

Chromatography is a technique to separate mixtures of substances into their components on the basis of their molecular structure and molecular composition.

This involves a stationary phase (a solid, or a liquid supported on a solid) and a mobile phase (a liquid or a gas). The mobile phase flows through the stationary phase and carries the

components of the mixture with it. Sample components that display stronger interactions with the stationary phase will move more slowly through the column than components with weaker interactions.

This difference in rates cause the separation of variuos components. Chromatographic separations can be carried out using a variety of stationary phases, including immobilized silica on glass plates (thin-layer chromatography), volatile gases (gas chromatography), paper (paper chromatography) and liquids (liquid chromatography).

High perfomance Liquid Chromatography

High performance liquid chromatography (HPLC) is basically a highly improved form of column liquid chromatography.

Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres. That makes it much faster.

All chromatographic separations, including HPLC operate under the same basic principle; separation of a sample into its constituent parts because of the difference in the relative affinities of different molecules for the mobile phase and the stationary phase used in the separation.

Types of HPLC

There are following variants of HPLC, depending upon the phase system (stationary) in the process :

1. Normal Phase HPLC

This method separates analytes on the basis of polarity. NP-HPLC uses polar stationary phase and non-polar mobile phase. Therefore, the stationary phase is usually silica and typical mobile phases are hexane, methylene chloride, chloroform, diethyl ether, and mixtures of these.

Polar samples are thus retained on the polar surface of the column packing longer than less polar materials.

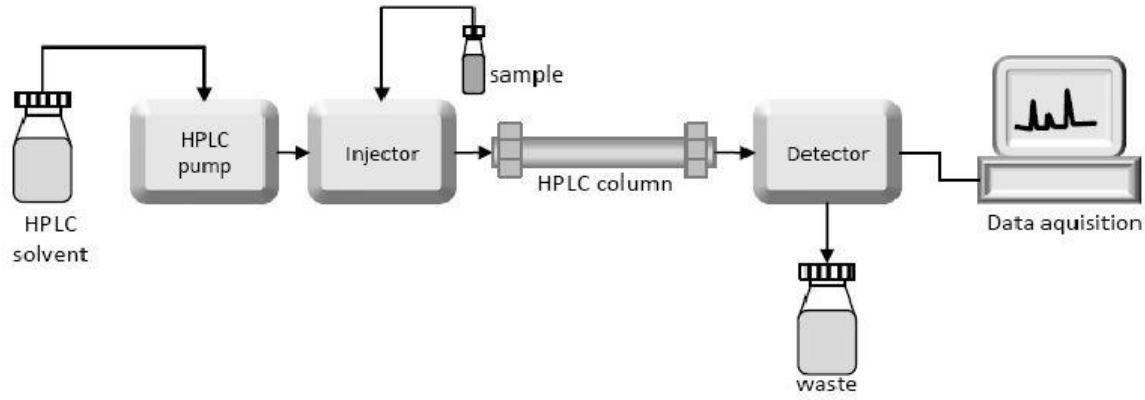
2. Reverse Phase HPLC

The stationary phase is nonpolar (hydrophobic) in nature, while the mobile phase is a polar liquid, such as mixtures of water and methanol or acetonitrile. It works on the principle of hydrophobic interactions hence the more nonpolar the material is, the longer it will be retained.

3. Size-exclusion HPLC

The column is filled with material having precisely controlled pore sizes, and the particles are separated according to its their molecular size. Larger molecules are rapidly washed through the column; smaller molecules penetrate inside the porous of the packing particles and elute later.

Instrumentation of HPLC



As shown in the schematic diagram in Figure above, HPLC instrumentation includes a pump, injector, column, detector and integrator or acquisition and display system. The heart of the system is the column where separation occurs.

1. Solvent Resorvoir

Mobile phase contents are contained in a glass resorvoir. The mobile phase, or solvent, in HPLC is usually a mixture of polar and non-polar liquid components whose respective concentrations are varied depending on the composition of the sample.

2. Pump

A pump aspirates the mobile phase from the solvent resorvoir and forces it through the system's column and detecter. Depending on a number of factors including column dimensions, particle size of the stationary phase, the flow rate and composition of the mobile phase, operating pressures of up to 42000 kPa (about 6000 psi) can be generated.

3. Sample Injector

The injector can be a single injection or an automated injection system. An injector for an HPLC system should provide injection of the liquid sample within the range of 0.1-100 mL of volume with high reproducibility and under high pressure (up to 4000 psi).

4. Columns

Columns are usually made of polished stainless steel, are between 50 and 300 mm long and have an internal diameter of between 2 and 5 mm. They are commonly filled with a stationary phase with a particle size of 3–10 µm.

Columns with internal diameters of less than 2 mm are often referred to as microbore columns. Ideally the temperature of the mobile phase and the column should be kept constant during an analysis.

5. Detector

The HPLC detector, located at the end of the column detect the analytes as they elute from the chromatographic column. Commonly used detectors are UV-spectroscopy, fluorescence, mass-spectrometric and electrochemical detectors.

6. Data Collection Devices

Signals from the detector may be collected on chart recorders or electronic integrators that vary in complexity and in their ability to process, store and reprocess chromatographic data. The computer integrates the response of the detector to each component and places it into a chromatograph that is easy to read and interpret.

Applications of HPLC

The information that can be obtained by HPLC includes resolution, identification and quantification of a compound. It also aids in chemical separation and purification. The other applications of HPLC include :

Pharmaceutical Applications

1. To control drug stability.
2. Tablet dissolution study of pharmaceutical dosages form.
3. Pharmaceutical quality control.

Environmental Applications

1. Detection of phenolic compounds in drinking water.
2. Bio-monitoring of pollutants.

Applications in Forensics

1. Quantification of drugs in biological samples.
2. Identification of steroids in blood, urine etc.
3. Forensic analysis of textile dyes.
4. Determination of cocaine and other drugs of abuse in blood, urine etc.

Food and Flavour

1. Measurement of Quality of soft drinks and water.
2. Sugar analysis in fruit juices.
3. Analysis of polycyclic compounds in vegetables.
4. Preservative analysis.

Applications in Clinical Tests

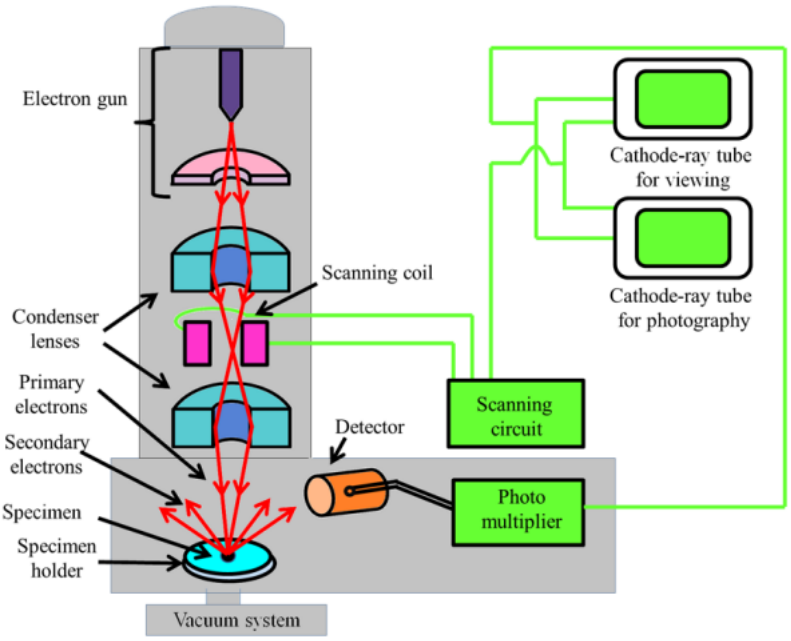
1. Urine analysis, antibiotics analysis in blood.
2. Analysis of bilirubin, biliverdin in hepatic disorders.
3. Detection of endogenous Neuropeptides in extracellular fluid of brain etc.

Electron Microscopy

There are two basic models of the electron microscopes: *Scanning electron microscopes* (SEM) and *transmission electron microscopes* (TEM). In a SEM, the secondary electrons produced by the specimen are detected to generate an image that contains topological features of the specimen. The image in a TEM, on the other hand, is generated by the electrons that have transmitted through a thin specimen. Let us see how these two microscopes work and what kind of information they can provide:

Scanning electron microscope

Figure shows a simplified schematic diagram of a SEM. The electrons produced by the electron gun are guided and focused by the magnetic lenses on the specimen.



The focused beam of electrons is then scanned across the surface in a raster fashion (Figure 18.2). This scanning is achieved by moving the electron beam across the specimen surface by using deflection/scanning coils. The number of secondary electrons produced by the specimen at each scanned point are plotted to give a two dimensional image.

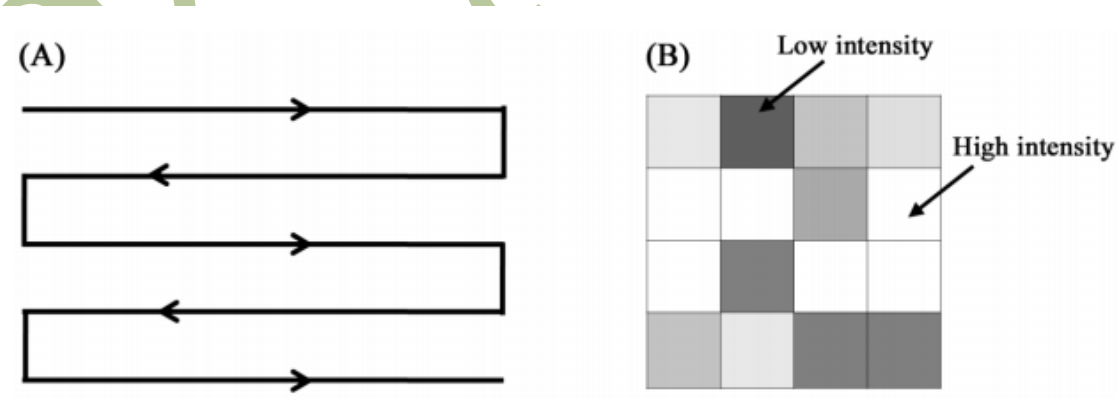


Figure 18.2 A diagrammatic representation of the raster scanning (A) and the intensity plot for the scanned area (B).

In principle, any of the signals generated at the specimen surface can be detected. Most electron microscopes have the detectors for the secondary electrons and the backscattered electrons. Figure 18.3 shows the interaction volume within the specimen showing the regions of secondary electrons (energy < 50 eV) and backscattered electrons.

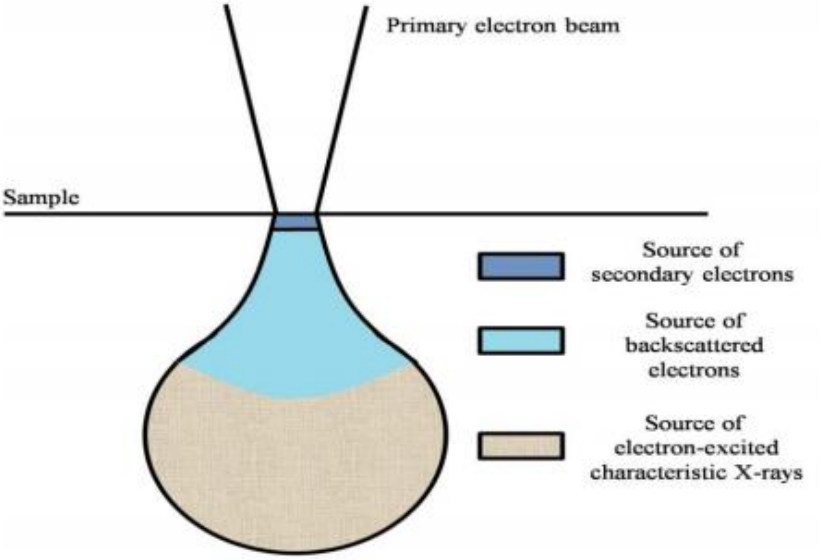


Figure 18.3 Specimen-electron interaction volume within the specimen. Notice the different regions where secondary electrons and backscattered electrons come from.

A secondary electron detector is biased with positive potential to attract the low energy secondary electrons. Detector for backscattered electrons is not biased; the high energy backscattered electrons strike the unbiased detector. As backscattered electrons come from a significant depth within the sample (Figure 18.3), they do not provide much information about the specimen topology. However, backscattered electrons can provide useful information about the composition of the sample; materials with higher atomic number produce brighter images.

Sample preparation for SEM: A specimen to be analyzed by electron microscopy has to be dry which most biological samples are not. As dehydration might lead to structural changes, the specimens are first fixed to preserve their structural features. Fixation is the first step and

can be achieved using chemical methods such as fixation with glutaraldehyde or physical methods such as cryofixation in liquid nitrogen. The fixed specimens are then dehydrated usually by exposing them to an increasing gradient of ethanol (up to 100%). The specimens are then dried using critical point method. The dried specimens are then coated with a conducting material usually gold to make the surface conducting and cause it emit more secondary electrons. A SEM image of human erythrocytes coated with gold is shown in figure 18.4.

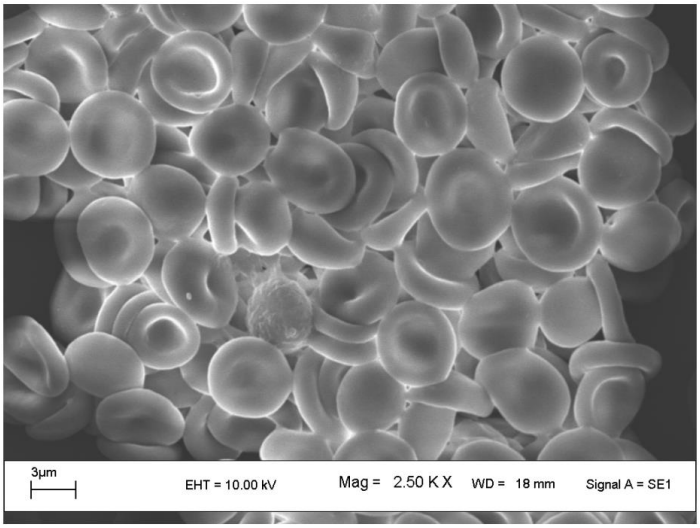


Figure 18.4 A scanning electron micrograph of human erythrocytes.

Transmission electron microscope

The first electron microscope was developed by Knoll and Ruska in 1930s. It was a transmission electron microscope; the electrons were focused on a thin specimen and the electrons transmitted through the specimen were detected. Figure 18.5 shows a simplified optical diagram comparing a light microscope with a transmission electron microscope.

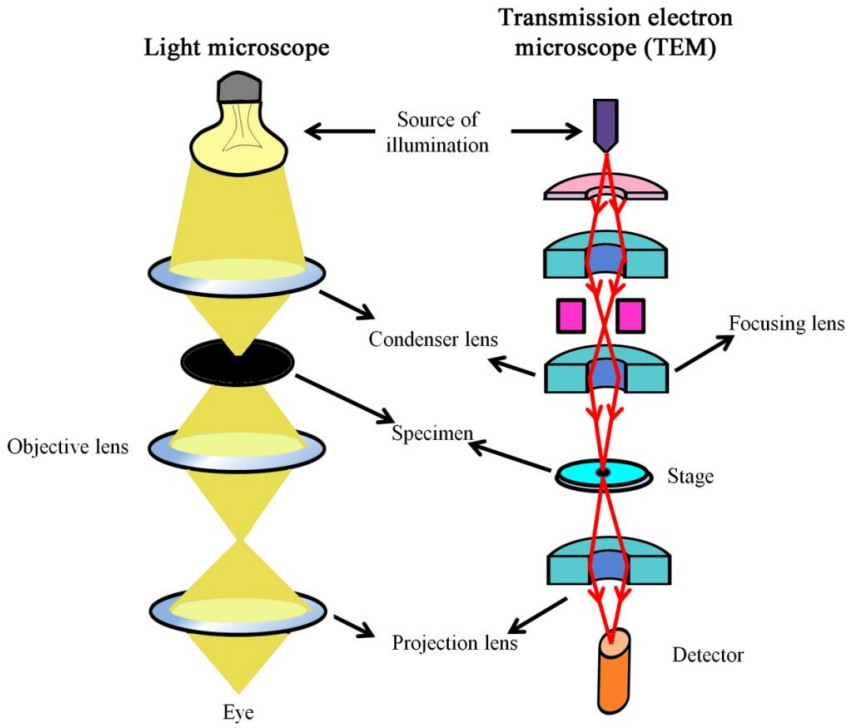


Figure 18.5 A simplified comparison of optics in a light microscope with that in a TEM.

Transmission electron microscopes usually have thermionic emission guns and electrons are accelerated anywhere between 40 – 200 kV potential. However, TEM with >1000 kV acceleration potentials have been developed for obtaining higher resolutions. Owing to their brightness and very fine electron beams, field emission guns are becoming more popular as the electron guns.

Sample preparation for TEM: The very first requirement of TEM is that the specimens have to be very thin. As for SEM, the specimens to be used for TEM also need to be fixed and dried. Preparation of specimens for TEM can be a fairly tedious process: The samples are usually fixed using a combination of glutaraldehyde and paraformaldehyde. A secondary staining can be done with OsO4 (Osmium tetroxide). OsO4 fixes the unsaturated lipids and being a heavy metal acts as an electron stain too. The samples are then dehydrated exactly as done for SEM analysis. The dried samples are then sectioned to obtain ultrathin (<100 nm thickness) sections. This is typically achieved by embedding the sample in a plastic mold and

cutting the sections. Epoxy and acrylic resins are also used for embedding the samples for sectioning. The sections are then stained with a heavy metal stain such as uranyl acetate and phosphotungstic acid. The stained sample is then deposited on a carbon coated grid and analyzed by TEM. Figure 18.6 shows a TEM image recorded for a peptide that self-assembled into spherical structures.

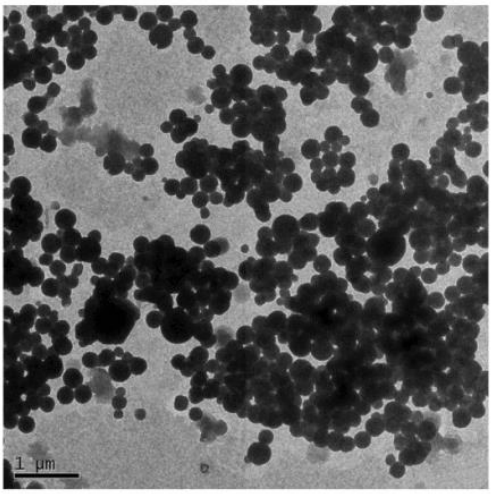


Figure 18.6 A transmission electron micrograph of a self-assembled peptide.

Scanning transmission electron microscopy: A scanning transmission electron microscope or STEM is a transmission electron microscope that works in the scanning mode like a SEM. An electron beam is focused to a small spot and scanned across the specimen exactly as done in SEM. A STEM allows detecting the transmitted as well as secondary and backscattered electrons. This mode of electron microscope provides spatially resolved information about the specimen.

All other types of electron microscopes are the modifications of SEM or TEM.

Flow cytometry principle - How the fluorescence activated cell sorting (FACS) work ?

Flow cytometry is a technique to identify and isolate cells from a mixture of other cells using fluorescence activity. Flow cytometry was developed by Fulwyler in 1965. Till today it is used for research in cell biology. In that technique cell sorting and cell counting was done by using laser light technology.

There are different steps involved in a process of flow cytometer; First step is Flow of cell in that liquid containing cells i.e. liquid stream is passing single file through light beam of laser light for sensing. Second step is that measuring system, which commonly used for measurement of conductivity, Optical system containing Mercury and Xenon lamp resulting in light signal. The third step is to detection of light scattering, in that step light signal are converted analogue to digital signal with the help of Analogue to digital conversion system. It will detect Forward scatter light (FSC) and Side scatter light as well as fluorescence signal from light in to electrical signal that can be processed by computer. The fourth step is that analysis of signal by computer, in that collecting of data from sample using cytometer this collecting of data is termed as Acquisition. This acquisition is carried out by computer connected to the flow cytometer software. This software handle the digital interface with cytometer, it is able to adjusting the parameter required for the voltage compensation. It is also monitor initial sample analysis.

Fluorescence labelled antibodies was developed for clinical research. In modern instrument contain multiple laser and fluorescence detector, currently in industrial instrument ten laser and 18 fluorescence detector. More number of detector and laser allow for multiple antibody labelling and identify a target population by their marker. In certain instrument can even take digital image of individual cell for the analysis.

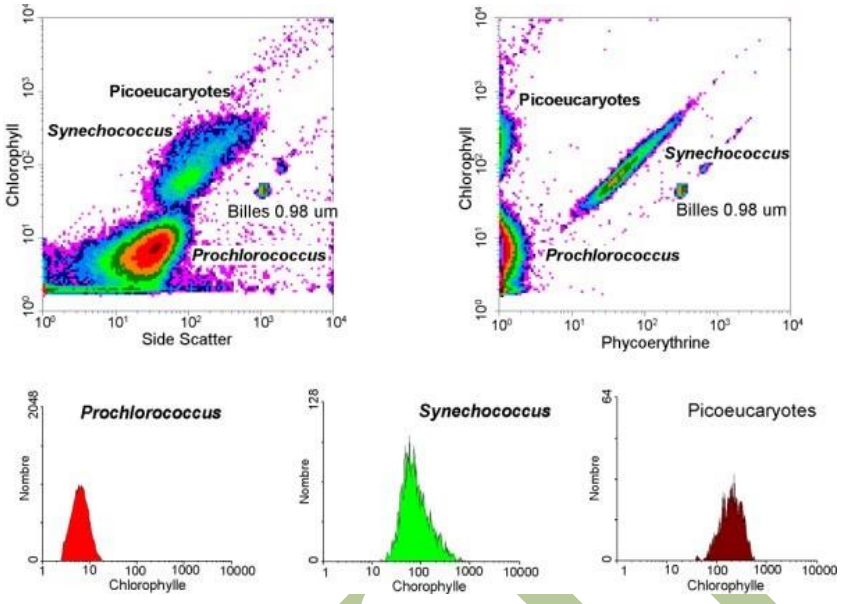


Fig- Analysis of a marine sample of photosynthetic picoplankton by flow cytometry showing three different populations (Prochlorococcus, Synechococcus, and picoeukaryotes)- from Wekipedia

The data is to be analysed by data generated by computer either in histogram or dot plot. Computer analysis give automated population identification, this automated identification could potentially help finding of rare hidden population.

Fluorescence-activated cell sorting (FACS) is a specialized type of flow cytometry. It provides a method for sorting a heterogeneous mixture of biological cells into two or more containers, one cell at a time, based upon the specific light scattering and fluorescent characteristics of each cell. It is a useful scientific instrument as it provides fast, objective and quantitative recording of fluorescent signals from individual cells as well as physical separation of cells of particular interest. The technique was expanded by Len Herzenberg, who was responsible for coining the term FACS.

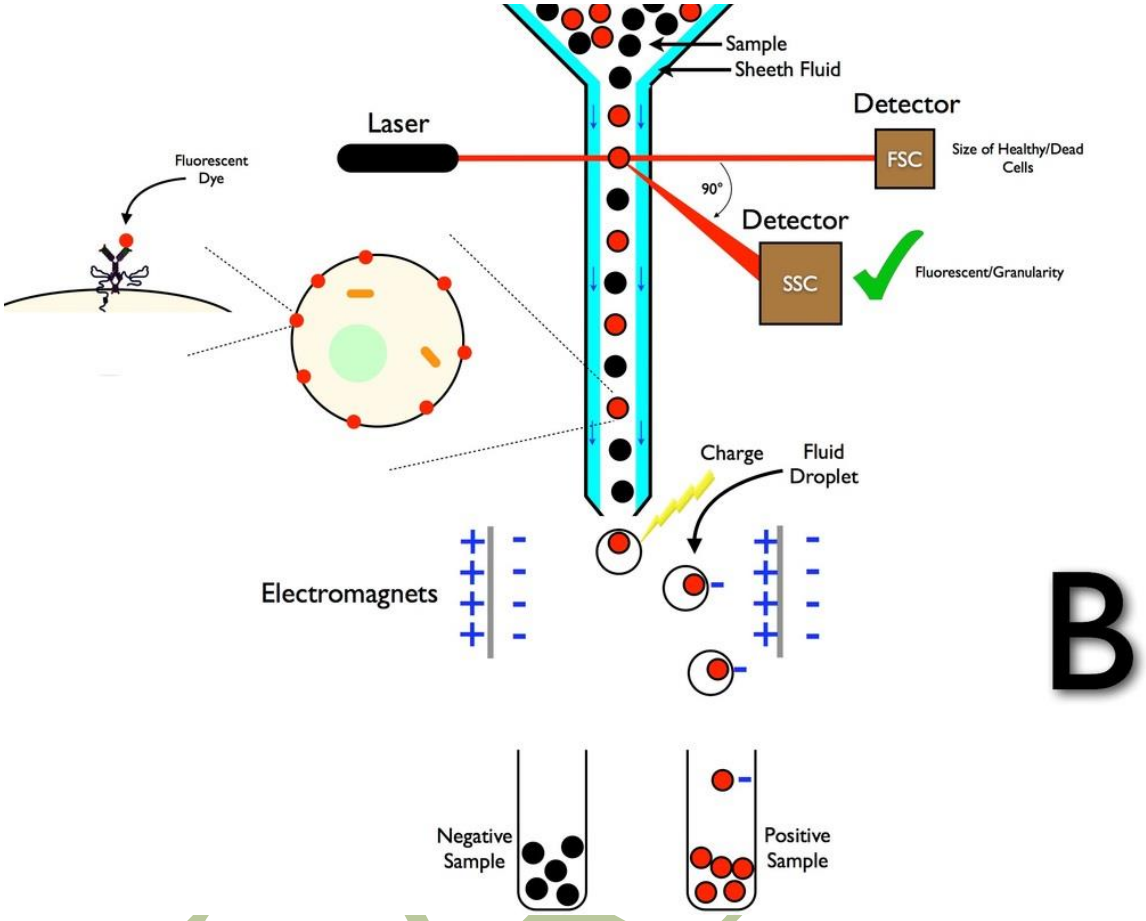


Fig- Fluorescence-Activated Cell Sorting (FACS) principle - image taken from Wikipedia

The cell suspension is entrained in the center of a narrow, rapidly flowing stream of liquid. The flow is arranged so that there is a large separation between cells relative to their diameter. A vibrating mechanism causes the stream of cells to break into individual droplets. The system is adjusted so that there is a low probability of more than one cell per droplet. Just before the stream breaks into droplets, the flow passes through a fluorescence measuring station where the fluorescent character of interest of each cell is measured. An electrical charging ring is placed just at the point where the stream breaks into droplets. A charge is placed on the ring based on the immediately prior fluorescence intensity measurement, and the opposite charge is trapped on the droplet as it breaks from the stream. The charged droplets then fall through an electrostatic deflection system that diverts droplets into containers based upon their charge. In some systems, the charge is applied directly to the

stream, and the droplet breaking off retains charge of the same sign as the stream. The stream is then returned to neutral after the droplet breaks off.

The technology has applications in a number of fields, including molecular biology, pathology, immunology, plant biology and marine biology It has broad application in medicine (especially in transplantation, hematology, tumor immunology and chemotherapy, prenatal diagnosis, genetics and sperm sorting for sex preselection). Also, it is extensively used in research for the detection of DNA damage, caspase cleavage and apoptosis In marine biology, the autofluorescent properties of photosynthetic plankton can be exploited by flow cytometry in order to characterise abundance and community structure. In protein engineering, flow cytometry is used in conjunction with yeast display and bacterial display to identify cell surface-displayed protein variants with desired properties

Summary of flow cytometry and FACS

Fluorescence activated cell sorting it is part of Flow cytometry. This technique used for the counting, sorting of cell and protein engineering, Based upon their properties of Bio molecules.

- In that techniques laser is going to monitor cell i.e. cells either have high cell weight or light weight.
- Cells containing sample is passed from laser light through niddle like column so as to perfectly get forward scatter value and side scatter value of laser light pattern, These FSC and SSC Detector gathering the data of laser light intensity and it will show the value on computer as graph i.e. graph is plotted FSC Vs SSC and in that at the bottom side strong electromagnet is present this working as a cell sorter
- Electromagnet is going to separate positively and negatively charged cell
- On the basis of graph value of FSC and SSC we can estimate how much amount of cell are healthy and how much are apoptic i.e. the cells are going to shrink and due to that cell shows low FSC and SSC value on the graph.
- It is majorly used in cancer cell, and animal cell culture

- It also contain analogue to digital conversion system which converts fluorescence light in to electrical signal for computer analysis.
- It is also applicable in immunology experiments and plant pathology i.e. disease
- In FACS size of healthy and dead cell is monitored by FSC light and Granularity of cell is monitored by SSC light. It shows the value by histogram and dot plot.
- Cells contains various organelle if healthy cells are present in higher concentration it will indicated by FSC value.

Transgenic Animals

The term transgenic animal refers to an animal in which there has been a deliberate modification of the genome - the material responsible for inherited characteristics - in contrast to spontaneous mutation (FELASA September 1992, revised February 1995). Foreign DNA is introduced into the animal, using recombinant DNA technology, and then must be transmitted through the germ line so that every cell, including germ cells, of the animal contain the same modified genetic material.

1. Historical background

Prior to the development of molecular genetics, the only way of studying the regulation and function of mammalian genes was through the observation of inherited characteristics or spontaneous mutations. Long before Mendel and any molecular genetic knowledge, selective breeding was a common practice among farmers for the enhancement of chosen traits, e.g., increased milk production.

During the 1970s, the first chimeric mice were produced (Brinster, 1974). The cells of two different embryos of different strains were combined together at an early stage of development (eight cells) to form a single embryo that subsequently developed into a chimeric adult, exhibiting characteristics of each strain.

The mutual contributions of developmental biology and genetic engineering permitted rapid development of the techniques for the creation of transgenic animals. DNA microinjection,

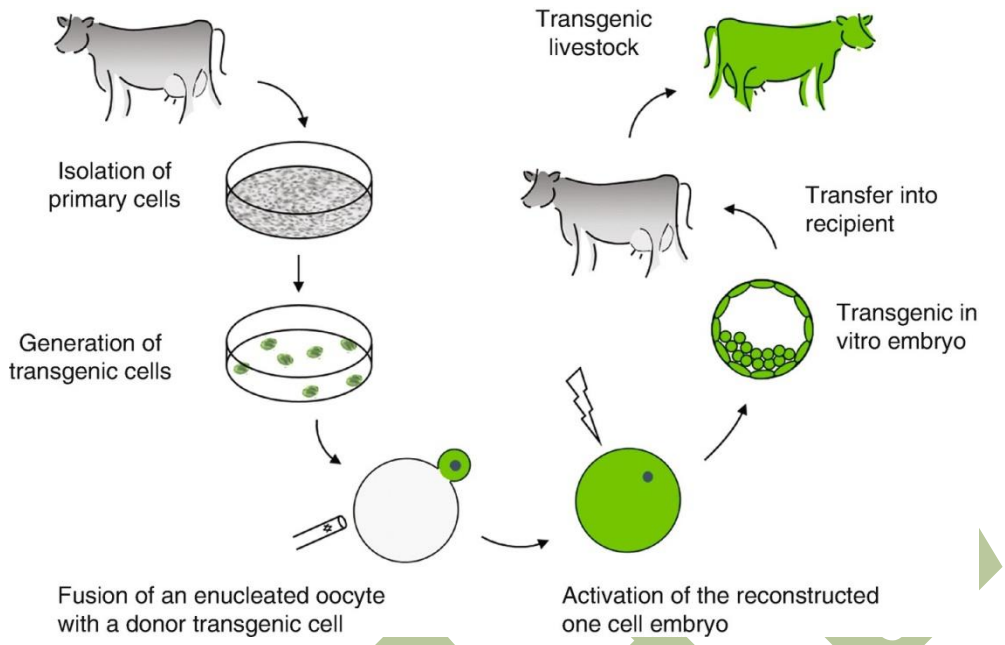
the first technique to prove successful in mammals, was first applied to mice (Gordon and Ruddle, 1981) and then to various other species such as rats, rabbits, sheep, pigs, birds, and fish. Two other main techniques were then developed: those of retrovirus-mediated transgenesis (Jaenisch, 1976) and embryonic stem (ES) cell-mediated gene transfer (Gossler et al., 1986).

Since 1981, when the term transgenic was first used by J.W. Gordon and F.H. Ruddle (1981), there has been rapid development in the use of genetically engineered animals as investigators have found an increasing number of applications for the technology.

2. Methods of creation of transgenic animals

For practical reasons, i.e., their small size and low cost of housing in comparison to that for larger vertebrates, their short generation time, and their fairly well defined genetics, mice have become the main species used in the field of transgenics.

The three principal methods used for the creation of transgenic animals are DNA microinjection, embryonic stem cell-mediated gene transfer and retrovirus-mediated gene transfer.



a) DNA microinjection.

This method involves the direct microinjection of a chosen gene construct (a single gene or a combination of genes) from another member of the same species or from a different species, into the pronucleus of a fertilized ovum. It is one of the first methods that proved to be effective in mammals (Gordon and Ruddle, 1981). The introduced DNA may lead to the over- or under-expression of certain genes or to the expression of genes entirely new to the animal species. The insertion of DNA is, however, a random process, and there is a high probability that the introduced gene will not insert itself into a site on the host DNA that will permit its expression. The manipulated fertilized ovum is transferred into the oviduct of a recipient female, or foster mother that has been induced to act as a recipient by mating with a vasectomized male.

A major advantage of this method is its applicability to a wide variety of species.

b) Embryonic stem cell-mediated gene transfer.

This method involves prior insertion of the desired DNA sequence by homologous recombination into an in vitro culture of embryonic stem (ES) cells. Stem cells are undifferentiated cells that have the potential to differentiate into any type of cell (somatic and germ cells) and therefore to give rise to a complete organism. These cells are then incorporated into an embryo at the blastocyst stage of development. The result is a chimeric animal. ES cell-mediated gene transfer is the method of choice for gene inactivation, the so-called knock-out method.

This technique is of particular importance for the study of the genetic control of developmental processes. This technique works particularly well in mice. It has the advantage of allowing precise targeting of defined mutations in the gene via homologous recombination.

c) Retrovirus-mediated gene transfer.

To increase the probability of expression, gene transfer is mediated by means of a carrier or vector, generally a virus or a plasmid. Retroviruses are commonly used as vectors to transfer genetic material into the cell, taking advantage of their ability to infect host cells in this way. Offspring derived from this method are chimeric, i.e., not all cells carry the retrovirus. Transmission of the transgene is possible only if the retrovirus integrates into some of the germ cells.

For any of these techniques the success rate in terms of live birth of animals containing the transgene is extremely low. Providing that the genetic manipulation does not lead to abortion, the result is a first generation (F1) of animals that need to be tested for the expression of the transgene. Depending on the technique used, the F1 generation may result in chimeras. When the transgene has integrated into the germ cells, the so-called germ line chimeras are then inbred for 10 to 20 generations until homozygous transgenic animals are obtained and the transgene is present in every cell. At this stage embryos carrying the transgene can be frozen and stored for subsequent implantation.

Transgenic Animals as Biotechnology

UNIT-V

Transgenic animals are just one in a series of developments in the area of biotechnology. Biotechnology has transformed the way in which we understand processes such as engineering and manufacturing. These terms now include the use of living organisms or their parts to make or modify products, to change the characteristics of plants or animals, or to develop micro-organisms for specific uses. The novel uses of biological techniques such as recombinant DNA techniques, cell fusion techniques, mono and polyclonal antibody technology and biological processes for commercial production have altered traditional distinctions and methods (US Congress, Office of Technology Assessment, 1989). Genetic manipulations at the level of DNA have also changed long held views as to what is considered to be animal, plant and human. In turn, these changes have made it more difficult to evaluate the ways in which animals are used and have obscured distinctions between pure and applied research.

Consideration of the acceptability of creating specific transgenic animal strains or genetic manipulation involving interchanging DNA between species and kingdoms could be a simple animal care issue or a societal decision. The following is an attempt to show what the ability to create transgenic animals or engage in other forms of DNA manipulation means in terms of traditional ACC functions, not forgetting that this impacts on wider considerations of human responsibility for the welfare of other life forms.

The creation of transgenic animals is resulting in a shift from the use of higher order species to lower order species, and is also affecting the numbers of animals used. This shift in the patterns of animal use is being monitored by the CCAC through the use of the Animal Use Data Form.

An example of the replacement of higher species by lower species is the possibility to develop disease models in mice rather than using dogs or non-human primates.

In the long term, a reduction in the number of animals used, for example to study human diseases, is possible due to a greater specificity of the transgenic models developed. On the other hand, the success of the method has led to using its potential for investigating a wider

range of diseases and conditions. The actual use of some species may be increased, in addition to the numbers of animals which are sacrificed as donors during the creation process. The potential of the technology has also made it possible to consider employing cattle, swine, sheep and goats as processing units to manufacture proteins or as organ donors.

The complex interactive processes of living mammals are not reproducible in vitro. However, transgenic animals provide a means of evaluating genetic modifications in terms of anatomical and physiological changes in a complex system. Transgenic models are more precise in comparison to traditional animal models, for example the oncomouse with its increased susceptibility to tumor development enables results for carcinogenicity studies to be obtained within a shorter time-frame, thus reducing the course of tumor development in experimentally affected animals. However, models are not strict equivalents, so as with any other system care must be taken in drawing conclusions from the data.

A representative, but non-inclusive, list of purposes for which transgenic animals have been used indicates the wide ranging application of this biotechnology:

- in medical research, transgenic animals are used to identify the functions of specific factors in complex homeostatic systems through over- or under-expression of a modified gene (the inserted transgene);
- in toxicology: as responsive test animals (detection of toxicants);
- in mammalian developmental genetics;
- in molecular biology, the analysis of the regulation of gene expression makes use of the evaluation of a specific genetic change at the level of the whole animal;
- in the pharmaceutical industry, targeted production of pharmaceutical proteins, drug production and product efficacy testing;
- in biotechnology: as producers of specific proteins;
- genetically engineered hormones to increase milk yield, meat production; genetic engineering of livestock and in aquaculture affecting modification of animal physiology and/or anatomy; cloning procedures to reproduce specific blood lines; and

- developing animals specially created for use in xenografting.

Important general considerations include the extent to which experience acquired in the laboratory with regard to husbandry should influence industry standards for keeping animals created specifically as living machines for the production of proteins, antibodies, etc. What words are appropriate to describe and evaluate the condition of animals now used as production units? The successful cloning of Dolly underlines the fact that innovative developments in animal science are part of the mainstream of biotechnology. In addition, the use of xenografts, at least at the public health level makes animal and human welfare inseparable.