الجامعة المستنصرية

كلية العلوم

قسم علوم الحياة

المرحلة الرابعة

فرع المايكرو

الفايروسات الطبية العملي

كافة المختبرات

يطلب من

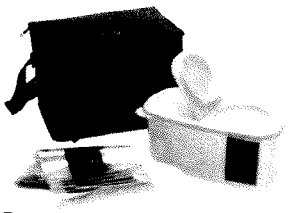
مكتبة حسنين

للطباعة والأستنساخ

07709265858

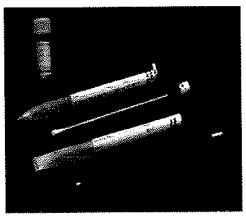
السعر. 1250

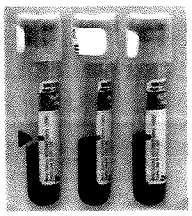
سعر الأستنساخ 6 بربع



Laboratory diagnosis of viral infections

Specimen collection and transport



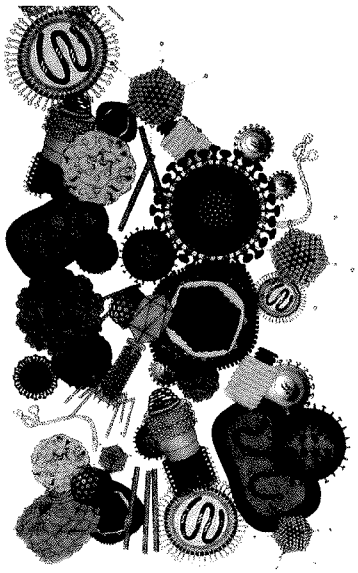


Steps in Pathogenesis of Viral Diseases

1. **Entry**
   - through skin
   - through mucosa (respiratory, gastrointestinal or genital)
   - through conjunctive
   - directly in the bloodstream by animal or insect bite or by needles

2. **Spread**
   - to bloodstream (first viremia)
   - to the cells of RES
   - to the bloodstream again (second viremia)
   - to the target organ

3. **Excretion (Shedding)**
   - from the same site as entry (skin, mucosa)
   - from site different than entry (by urine, mother's milk…)



Laboratory Diagnosis of Viral Infections

Most viral infections have typical clinical manifestations and not need to be confirmed by laboratory diagnosis.

Laboratory Diagnosis of Viral Infections

Laboratory diagnosis is necessary in cases:

- **When treatment and prognosis depend entirely on correct diagnosis**
  (teratogenic viruses, differential diagnosis of encephalitis, viral diseases with atypical clinical manifestationes)

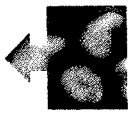
- **When there is an epidemic spread of viral disease**
  (influenza, HAV, variolla)

- **When viral disease can be treated with antiviral drugs**
  (HSV, CMV, HIV)

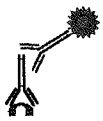
Laboratory Diagnosis of Viral Infections

Viral (direct) diagnosis

- isolation and identification of viruses in the systems of living cells
- by electron microscopy



- detection of viral antigenes



- detection of viral nucleic acids

Serological (indirect) diagnosis

- detection of anti-viral antibodies in serum

Laboratory Diagnosis of Viral Infections

Includes:

✓Taking speciment
✓Transport speciment
✓Speciment processing and inoculation in system of living cell
✓Virus identification

Important:
- Correctly interpretate the results, especially in serology!
- Normal viral microflora **doesn't** exist!

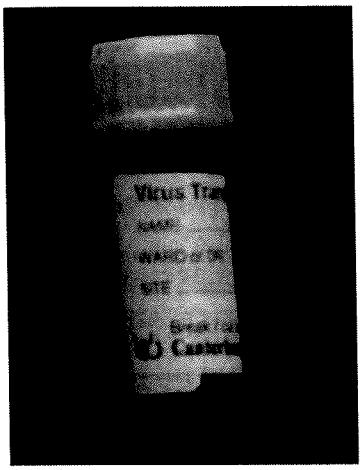
Rules of Specimen Collection

1. At the correct time
2. From the correct site
3. In the correct way
4. With active (urine, feces, sputum) or passive (swabs, blood, aspirates) participation of the patient
5. In the adequate volume (for all tests needed)
6. In the proper containers (sterile and chemically clean)
7. Correctly labeled (name, date, type of specimen) and with additional information (age, sex, clinical diagnosis, epidemiological data – vaccinations, recent trips, animal bites etc.)

VIRAL TRANSPORT MEDIUM (VTM)

VTM is used to:

- preserve viral infectivity within the specimen
- prevent specimen from drying
- stop the growth of bacteria and fungi



Effect of phisical and chemical factors on viruses

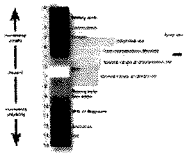
Inactivation of virus represents permanent loss of contamination.

-**Temperature**: - viruses are stable on low temperature (keeping)
- viruses with envelope are heat sensitive

-**Drying:** most of the viruses are sensitive

-**Irradiation (UV, X-ray...):**
inactivation of viruses

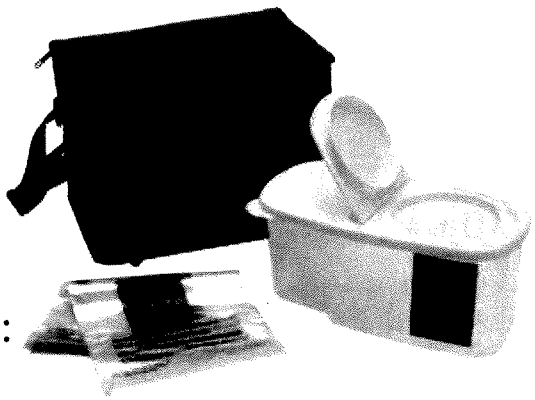
- **Chemical agents**:
organic solvent (chloroform) – inactivation of the viruses with envelope
oxido-reduct. agents (formaldehyde, chlorine, iodine) – inactivation



- **pH**: low pH - viruses without envelope are mostly stable

SPECIMEN TRANSPORT

During transport specimen should be:



→ protected from breaking

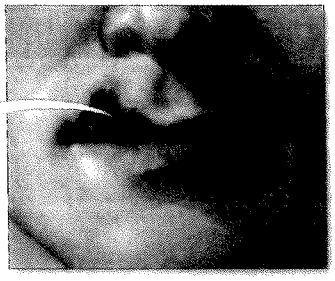
→ protected from light

→ At adequate temperature:

48 h at +4 °C (refrigerator, wet ice)
more than 48 h at -70 °C (dry ice)
must not be frozen at -20 °C!

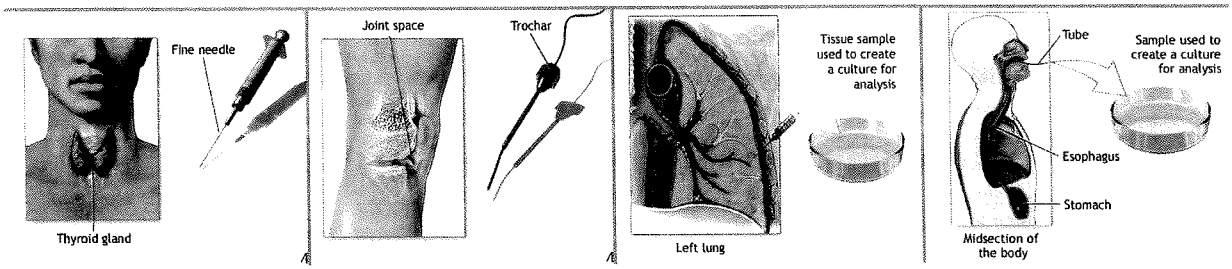
Specimen from mucosae lesion (oral and anogenital)

Swab from the lesion
with rotation
place in test-tube with VTM



Tissue samples from biopsy or autopsy

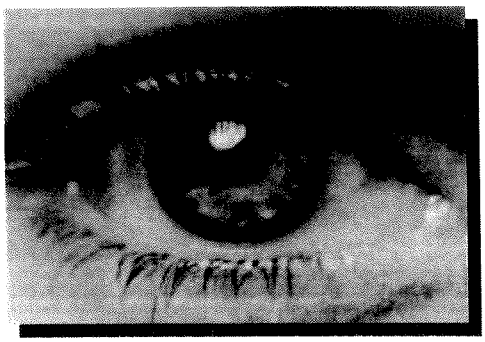
Place with VTM



Specimen from eye

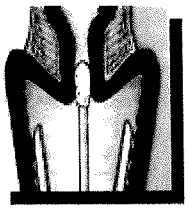
Swab from conjuctiva
imbue with sterile physiologic solution
take with rotation
inVTM

Swab from cornea/scraping
Ophthalmologist only!
inVTM

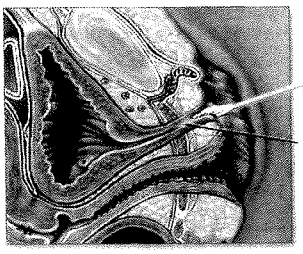


Specimen from genital tract

Endocervical swab
Thin swab – 1 cm in cervix
place in VTM



Urethral swab
The patient should not urinate 1 h before this swab.
Thin swab - 2-4 cm in uretra
place in VTM

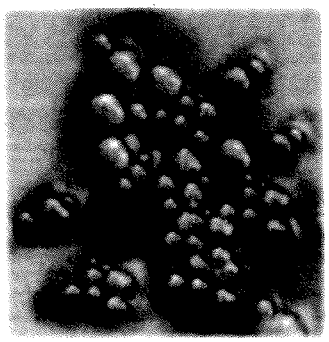


Sperm
Place in sterile container

Specimen from skin

Swab from the lesion
imbue with sterile physiologic solution
place in the test-tube in VTM

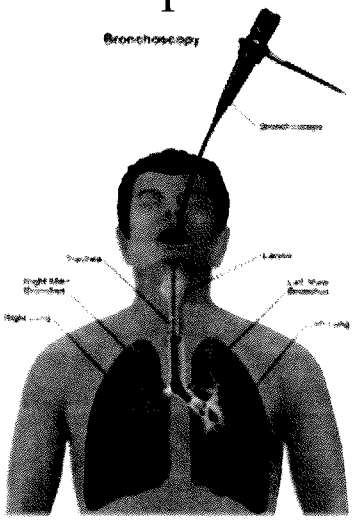
Vesicle swab
cleaning with sterile saline; piercing with sterile instrument
1 swab for the fluid
1 swab for the material from the bottom of the vesicle
both swabs in VTM



Vesicle aspirate
piercing with sterile needle
aspiration of fluid with syringe
washing the syringe with VTM

Specimen from lower respiratory tract

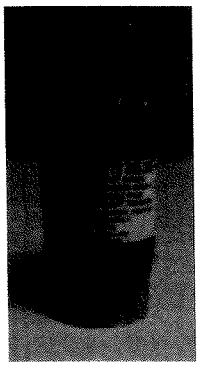
Bronchoalveolar lavage (BAL):
patient in anestesia, placement of bronchoscope
8-10 mL in sterile container
no VTM



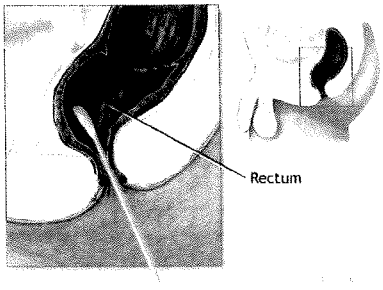
Sputum:
Not usefull for viral detection

Specimen from gastrointestinal tract

Feces
2-4 g of feces (sterile container)
place in VTM (8-10 ml)



Rectal swab
place in VTM

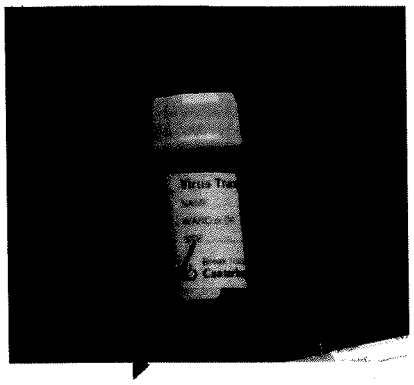


VIRAL TRANSPORT MEDIUM (VTM)

VTM contains:

- saline (adequate ion concentration)
- proteins (albumine or gelatine)
- buffer (adequate pH)
- antibiotics and fungicides

* MEM, Hank's solution, Stuart's



SPECIMEN COLLECTION

SWAB/SCRAPING VESICLE ASPIRATE SMALL TISSUE SAMPLES

*PLACE IN STERILE TUBE WITH **1-2 mL** VTM*

FLUIDS: URINE,CSF, BLOOD IN EDTA OR CITRATE, BRONCHOALVEOLAR LAVAGE, NASOPHARYNGEAL ASPIRATE

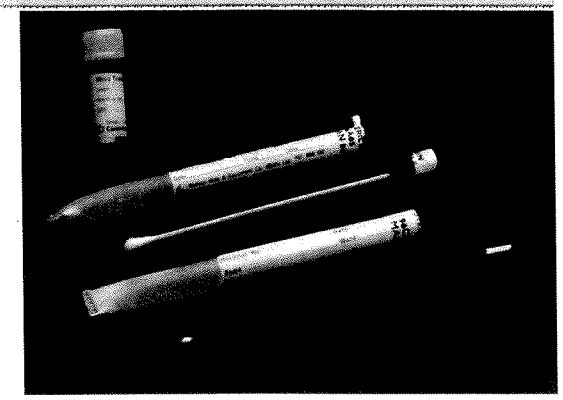
*PLACE INTO STERILE CONTAINER: VTM IS **NOT** ADDED*

SOLID SPECIMEN: STOOL, TISSUES

*PLACE INTO STERILE CONTAINER WITH **5-10 mL** VTM*

TRANSPORT TO LABORATORY
DURING TRANSPORT STORAGE AT **2-8 ℃**

SWABS

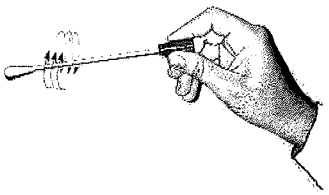


For diagnosis of viral infections, swabs should be:
- made of reyon

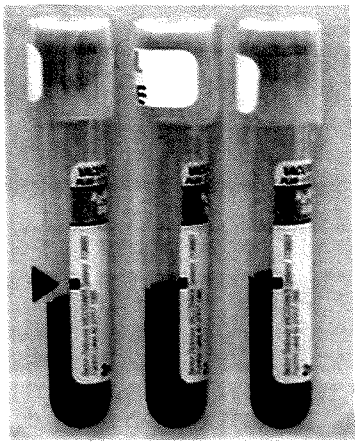
✓<u>Should not</u> be made of cotton or calcium alginate

Swab's shaft should be:
- made of plastics or metal

✓<u>Should not</u> be made of wood



COLLECTION OF SPECIMEN



<u>Blood</u>

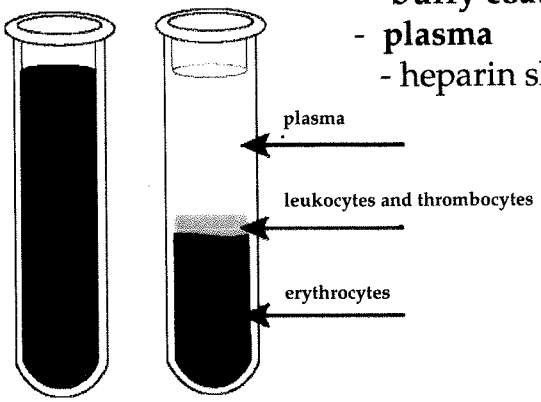
by venepuncture or through venal catheter
Blood is taken for viral or serology diagnosis

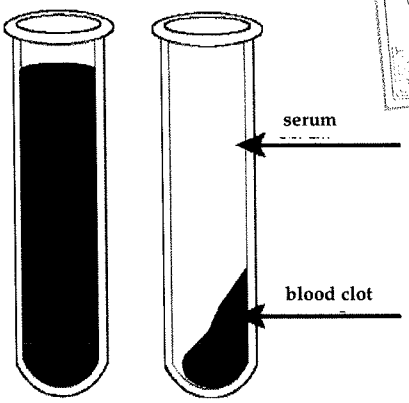
<u>Bone marrow</u>

by puncture - about 2 ml
(with EDTA, sodium-citrate or heparin)

Blood For viral diagnosis

- **whole blood** with EDTA, sodium-citrate or heparin
- **"buffy coat" (Leukocytes or Thrombocytes)** or
- **plasma**
  - heparin should not be used for PCR!





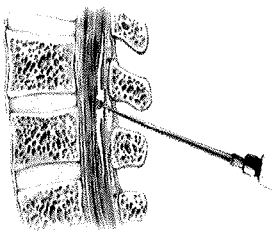
For serology - **serum**

Fluid Specimen

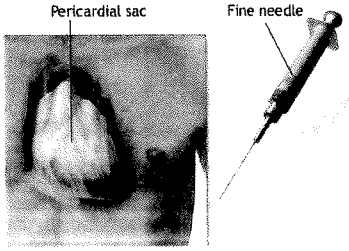
Saliva
1 swab from the bottom of the mouth
1 swab from the area around Stenson's ductus
place in tube with VTM

Urine
10-20 ml, middle stream (sterile container)

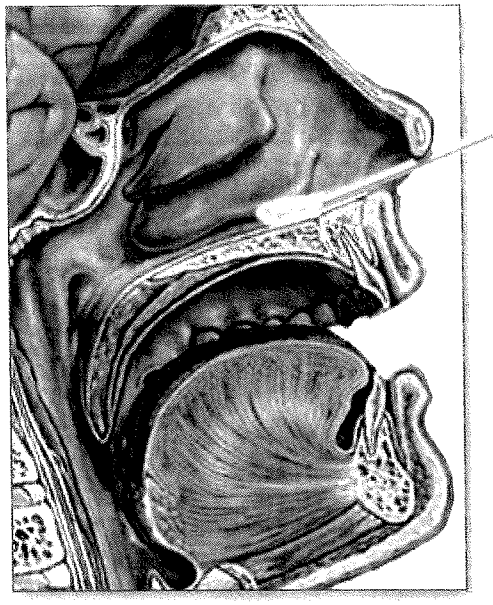
CSF
lumbal puncture (2-5 ml)



Pericardial fluid
pericardial puncture (2 ml)



Specimen from upper respiratory tract



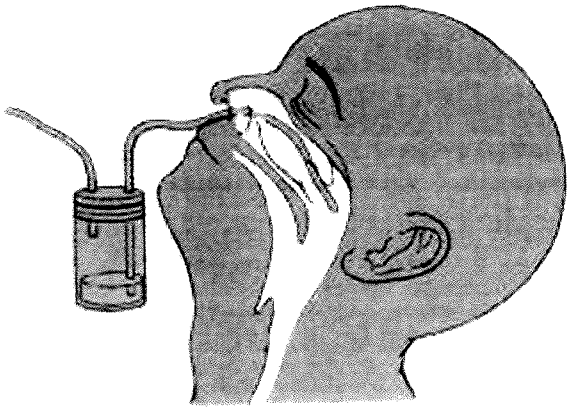
Nasopharingeal swab:

through nostrile to nasopharincs
place in VTM

Specimen from upper respiratory tract

Nasopharingeal wash:

pouring of saline by syringe through nostrile to nasopharings
aspirating of saline with respiratory secretions
place in VTM



Cell culture

Cell culture refers to the process by which cells are grown in a controlled artificial environment. Cells can be maintained in vitro outside of their original body by this process

Types of animal cell culture

Based on the number of cell division, cell culture can be classified as primary cell culture and cell lines. Cell lines can undergo finite or infinite cell divisions.

Animal cell culture

A/ Primary cell culture

This is the cell culture obtained straight from the cells of a host tissue. The cells dissociated from the parental tissue are grown on a suitable container and the culture thus obtained is called primary cell culture. Such culture comprises mostly heterogeneous cells and most of the cells divide only for a limited time. However, these cells are much similar to their parents,Depending on their origin, primary cells grow either as an adherent monolayer or in a suspension.

Adherent cells

These cells are anchorage and propagate as a monolayer. These cells need to be attached to a solid or semi-solid substrate for proliferation.. Fibroblasts and epithelial cells are of such types. When the bottom of the culture vessel is covered with a continuous layer of cells, usually one cell in thickness, these are known as monolayer cultures. Majority of continuous cell lines grow as monolayers. As being single layers, such cells can be transferred directly to a cover slip to examine under microscope.

Suspension cells

Suspension cells do not attach to the surface of the culture vessels. These cells are also called anchorage independent or non-adherent cells which can be grown floating in the culture medium. Hematopoietic stem **cells (derived from blood, spleen and bone marrow) and tumor cells can be grown in suspension. These cells grow much faster which do not require the frequent replacement of the medium and can be easily maintained. These are of homogeneous types and enzyme treatment is not required for the dissociation of cells; similarly these cultures have short lag period.**

dherent Cell Culture	Suspension Cell Culture
Appropriate for most cell types, including primary cultures	Appropriate for cells adapted to suspension culture and a few other cell lines that are nonadhesive (e.g., hematopoietic)
Requires periodic passaging, but allows easy visual inspection under inverted microscope	Easier to passage, but requires daily cell counts and viability determination to follow growth patterns; culture can be diluted to stimulate growth
Cells are dissociated enzymatically (e.g., TrypLE™ Express, trypsin) or mechanically	Does not require enzymatic or mechanical dissociation
Growth is limited by surface area, which may limit product yields	Growth is limited by concentration of cells in the medium, which allows easy scale-up
Requires tissue-culture treated vessel	Can be maintained in culture vessels that are not tissue-culture treated, but requires agitation (i.e., shaking or stirring) for adequate gas exchange
Used for cytology, harvesting	Used for bulk protein production,

products continuously, and many research applications	batch harvesting, and many research applications

Confluent culture and the necessity of sub-culture

After the cells are isolated from the tissue and proliferated under the appropriate conditions, they occupy all of the available substrate i.e. reach confluence. For a few days it can become too crowded for their container and this can be detrimental to their growth, generally leading to cell death if left for long time. The cells thus have to be subculture i.e. a portion of cells is transferred to a new vessel with fresh growth medium which provides more space and nutrients for continual growth of both portions of cells. Hence subculture keeps cells in healthy and in growing state.

A passage number refers specifically to how many times a cell line has been sub-cultured. In contrast with the population doubling level in that the specific number of cells involved is not relevant. It simply gives a general indication of how old the cells may be for various assays.

B/ Secondary cell culture and cell line

When a primary culture is sub-cultured, it is known as secondary culture or cell line or sub-clone. The process involves removing the growth media and disassociating the adhered cells (usually enzymatically).

Sub-culturing of primary cells to different divisions leads to the generation of cell lines. During the passage, cells with the highest growth capacity predominate, resulting in a degree of genotypic and phenotypic uniformity in the population. However, as they are sub-cultured serially, they become different from the original cell. On the basis of the life span of culture, the cell lines are categorized into two types:

❖ Finite cell lines

The cell lines which go through a limited number of cell division having a limited life span are known as finite cell lines. The cells passage several times and then lose their ability to proliferate, which is a genetically determined event known as senescence. Cell lines derived from primary cultures of normal cells are finite cell lines.

❖ Continuous cell lines

When a finite cell line undergoes transformation and acquires the ability to divide indefinitely, it becomes a continuous cell line. Such transformation/mutation can occur spontaneously or can be chemically or virally induced or from the establishment of cell cultures from malignant tissue. Cell cultures prepared in this way can be sub-cultured and grown indefinitely as permanent cell lines and are immortal.

These cells are less adherent, fast growing, less fastidious in their nutritional requirements, able to grow up to higher cell density and different in phenotypes from the original tissue. Such cells grow more in suspension. They also have a tendency to grow on top of each other in multilayers on culture-vessel surfaces.

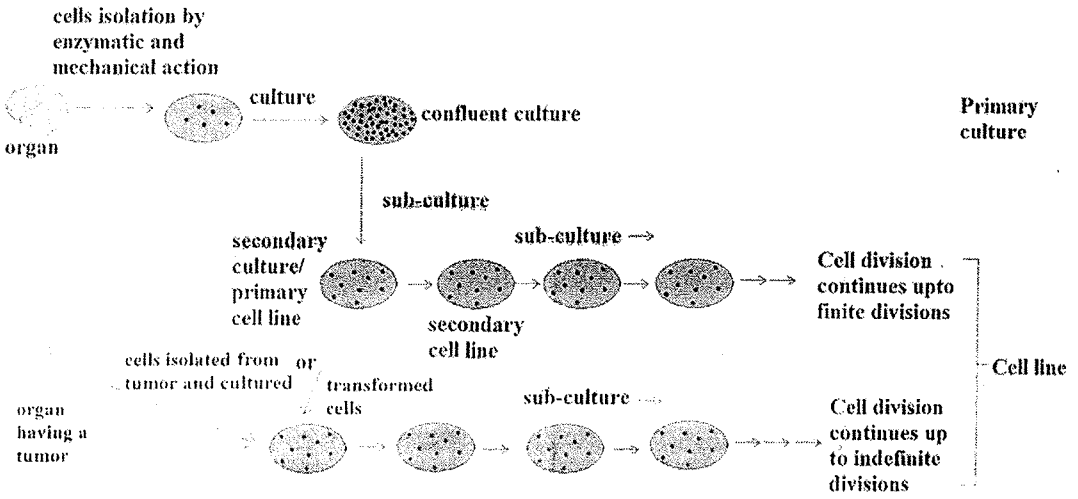


fig: animal cell culture

Methods

Growth Requirements

The culture media used for cell cultures are generally quite complex, and culture condition widely varies for each cell type. However, media generally include amino acids, vitamins, salts (maintain osmotic pressure), glucose, a bicarbonate buffer system (maintains a pH between 7.2 and 7.4), growth factors, hormones, $O_2$and CO_2. To obtain best growth, addition of a small amount of blood serum is usually necessary, and several antibiotics, like penicillin and streptomycin are added to prevent bacterial contamination.

Temperature varies on the type of host cell. Most mammalian cells are maintained at 37°C for optimal growth, while cells derived from cold-blooded animals tolerate a wider temperature range (i.e. 15°C to 26°C). Actively growing cells of log phage should be used which divide rapidly during culture.

Components of cell culture media[edit]

Component	Function
Carbon source (glucose/glutamine)	Source of energy
Amino acid	Building blocks of protein
Vitamins	Promote cell survival and growth
Balanced salt solution	An isotonic mixture of ions to maintain optimum osmotic pressure within the cells and provide essential metal ions to act as cofactors for enzymatic reactions, cell adhesion etc.
Phenol red dye	pH indicator. The color of phenol red changes from orange/red at pH 7-7.4 to yellow at acidic (lower) pH and purple at basic (higher) pH.

Bicarbonate /HEPES buffer	It is used to maintain a balanced pH in the media

Process to obtain primary cell culture

Primary cell cultures are prepared from fresh tissues. Pieces of tissues from the organ are removed aseptically; which are usually minced with a sharp sterile razor and dissociated by proteolytic enzymes (such as trypsin) that break apart the intercellular cement. The obtained cell suspension is then washed with a physiological buffer (to remove the proteolytic enzymes used). The cell suspension is spread out on the bottom of a flat surface, such as a bottle or a Petri dish. This thin layer of cells adhering to the glass or plastic dish is overlaid with a suitable culture medium and is incubated at a suitable temperature.

Aseptic techniques

Bacterial infections, like Mycoplasma and fungal infections commonly occur in cell culture creating a problem to identify and eliminate. Thus, all cell culture work is done in a sterile environment with proper aseptic techniques. Work should be done in laminar flow with constant unidirectional flow of HEPA filtered air over the work area. All the material, solutions and the whole atmosphere should be of contamination-free.

Cryopreservation

If a surplus of cells is available from sub-culturing, they should be treated with the appropriate protective agent (e.g., DMSO or glycerol) and stored at temperatures below –130°C until they are needed. This stores cell stocks and prevents original cell from being lost due to unexpected equipment failure or biological contaminations. When thawing the cells, the frozen tube of cells is warmed quickly in warm water, rinsed with medium and serum and then added into culture containers once suspended in the appropriate media

Applications of Cell Line

A. Vaccines Production

One of the most important uses of cell culture is in research and production of vaccines. The ability to grow large amounts of virus in cell culture eventually led to the creation of the polio vaccine, and cells are still used today on a large scale to produce vaccines for many other diseases, like rabies, chicken pox, hepatitis B, and measles. In early times, researchers had to use live animals to grow poliovirus, but due to the development of cell culture technique they were able to achieve much greater control over virus production and on a much larger scale which eventually develop vaccines and various treatments. However, continuous cell lines are not used in virus production for human vaccines as these are derived from malignant tissue or possess malignant characteristics.

B. Virus cultivation and study

Cell culture is widely used for the propagation of viruses as it is convenient, economic, easy to handle compared to other animals. It is easy to observe cytopathic effects and easy to select particular cells on which virus grow as well as to study the infectious cycle. Cell lines are convenient for virus research because cell material is continuously available. Continuous cell lines have been extremely useful in cultivating many viruses previously difficult or impossible to grow.

C. Cellular and molecular biology

Cell culture is one of the major tools used in cellular and molecular biology, providing excellent model systems for studying the normal physiology and biochemistry of cells (e.g., metabolic studies, aging), the effects of different toxic compounds on the cells, and mutagenesis and carcinogenesis. The major advantage of using cell culture for any of these applications is the consistency and reproducibility of results that can be obtained from using a batch of clonal cells.

D. In Cancer Research

Normal cells can be transformed into cancer cells by methods including radiation, chemicals and viruses. These cells can then be used to study cancer more closely and to test potential new treatments.

E. Gene therapy

Cells having a functional gene can be replaced to cells which are having non-functional gene, and for which the cell culture technique is used.

F. Immunological studies

Cell culture techniques are used to know the working of various immune cells, cytokines, lymphoid cells, and interaction between disease causing agent and host cell.

G. Others

Cell lines are also used in in-vitro fertilization (IVF) technology, recombinant protein and drug selection and improvement.

Preparation of primary Chicken embryo fibroblast

Following the method of (Hichner, 1980; Karel, *et al.,* 1998) to prepare chicken embryo fibroblast by:

(1) Nine-eleven days incubated eggs were chosen for the preparation of chick embryo fibroblasts. The eggs were candled and the air space marked. After disinfection with alcohol 70% the shell over the air space is removed.

(2) The contents of the egg were poured into a petridish and the embryo is taken out of the amnion sac.

(3) The chick embryos were discard from head, limbs, wings, and removed the viscera, washed the other part by PBS to discard from blood.

(4) The Minced tissue scratched to small fragments or pieces and transferred to sterile trypsinizing flask which contain sterile magnetic rod.

(5) The cells were dispersed by warm Trypsin (0.25% trypsin at 37 °C) and Leaving the trypsinizing flask on magnetic stirrer for 10 minutes.

(6) The cell suspension was filtrated through sterile gauze in to sterile beaker, 20 ml growth media was added to inhibition trypsin activity, the harvested cells Centrifuged at 1200 rpm for 15 min at 4 °C.

(6) Pelleted cells were re-suspended in RPMI growth medium at concentration 1ml of packed cells/200 ml of medium, cells were seeded in 96well plat for TCID50 and infected with ND virus in tissue culture flask for the gene expression .

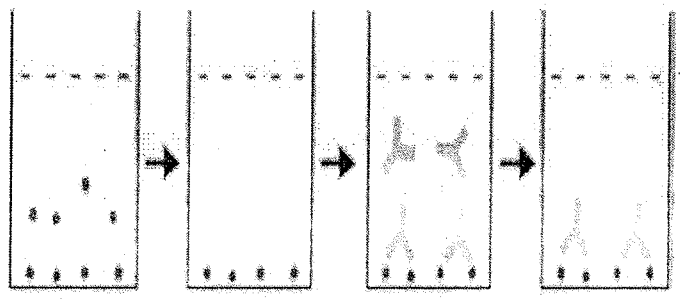
Shell vial assay

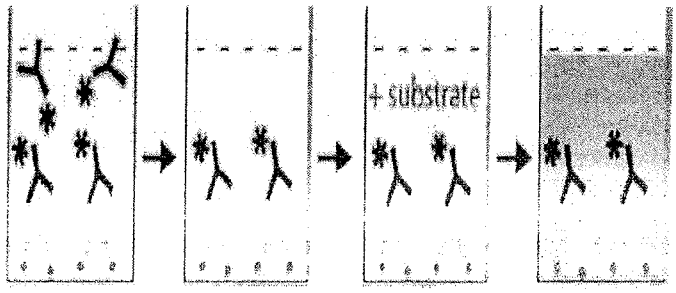
Viral culture is a laboratory test in which samples are placed with a cell type that the virus being tested for is able to infect. If the cells show changes, known as cytopathic effects, then the culture is positive. Human and monkey cells are used in both traditional viral culture and shell vial culture.
Human virus types that can be identified by viral culture include adenovirus, cytomegalovirus, enteroviruses, herpes simplex virus, influenza virus, parainfluenza virus, rhinovirus, respiratory syncytial virus, varicella zoster virus, measles and mumps.

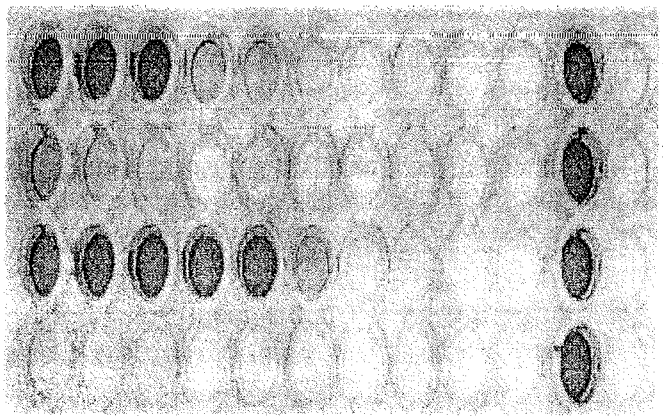
Mechanism of action:

Traditional viral culture has been generally superseded by shell vial culture, in which the sample is centrifuged on to a single layer of cells and viral growth is measured by antigen detection methods. This greatly reduces the time needed to detect slow growing viruses such as cytomegalovirus, for which the method was developed. In addition, the centrifugation step in shell vial culture enhances the sensitivity of this method because after centrifugation, the viral particles of the sample are in close proximity to the cells.
For these, the final identification method is generally by immunofluorescence, with exception of cytomegalovirus and rhinovirus, whose identification in a viral culture are determined by cytopathic effects.

Enzyme-linked Immunosorbent Assay







ELISA- Principle, Types and Applications

Enzyme-linked immunosorbent assay (ELISA) test is the most widely used type of immunoassay. ELISA is a rapid test used for detecting or quantifying antibody (Ab) against viruses, bacteria and other materials or antigen (Ag). ELISA is so named because the test technique involves the use of an enzyme system and immunosorbent.

ELISA test is being increasingly used in the detection of antigen (infectious agent) or antibody due to its simplicity and sensitivity. It is as sensitive as radioimmunoassay (RIA) and requires only microlitre quantities of test reagents. It has now been widely applied in detection of a variety of antibody and antigens such as hormones, toxins, and viruses.

Salient Features of ELISA Test

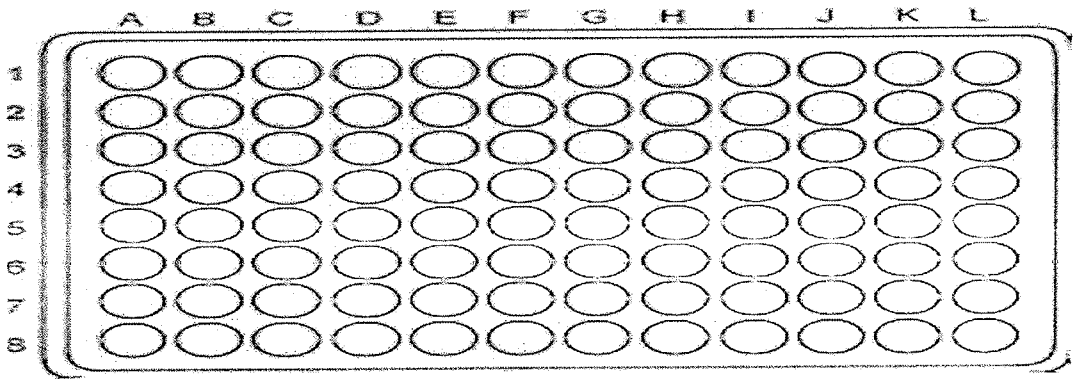
1. ELISA test has high sensitivity and specificity.
2. The result of quantitative ELISA tests can be read visually
3. A large number of tests can be done at one time.
   ELISAs are designed specifically for screening large numbers of specimens at a time, making them suitable for use in surveillance and centralized blood transfusion services
4. Reagents used for ELISA are stable and can be distributed in district and rural laboratories but as ELISAs require sophisticated equipment and skilled technicians to perform the tests, their use is limited to certain circumstances.

Materials needed in ELISA Testing

1. Pipettes, washer system, ELISA plate reader: Readers, washers and pipette are available as manual or automated system. One of the main factors affecting equipment selection is the number and types of test samples being run.
   1. **ELISA Readers:** Readers need to have appropriate filter (650 nm and 450 nm).
   2. **Pipette**: Are available as fixed as well as adjustable volume as well as single channel and multi-channel.
   3. **Washing system**: It can be manual system that washes one row or column at a time or semi automated systems that wash one strip or plate at a time or fully automated systems that can process multiple plates
2. **Reagents needed for the testing**– Concluded in the kit (coated plates, sample diluents, controls, wash concentrate, conjugate, substrate, stop solution)
   - **Coated plates:** The **96-well plates** are made of polystyrene and are coated with either inactivated antigen or antibody. The function of the plate has to hold the immobilized either antigen or antibody. Antigen or antibody present in the sample will bind to the plate. This coating acts as the binding site for the antibodies or antigens in the sample.
   - **Controls:** Negative and positive controls are provided in each kit. The controls help to normalize or standardize each plate. Controls are also used to validate the assay and to calculate sample results. Controls might be pre-diluted and ready to use. (Please refer to kit for specific instructions).
   - **Conjugates:** ELISA conjugates are enzyme labeled antibodies that react specifically to plate bound sample analytes. Unbound conjugates are washed away after incubation and before the addition of substrate.
   - **Wash Concentrate:** It acts as a buffered solution containing detergent to wash unbound material from the plate. (Not all test kits have wash concentrate; in that case distilled water can be used for washing; please refer to kit insert for specific instructions)
   - **Stop solution:** It stops the enzyme substrate reaction and color development.

Principle

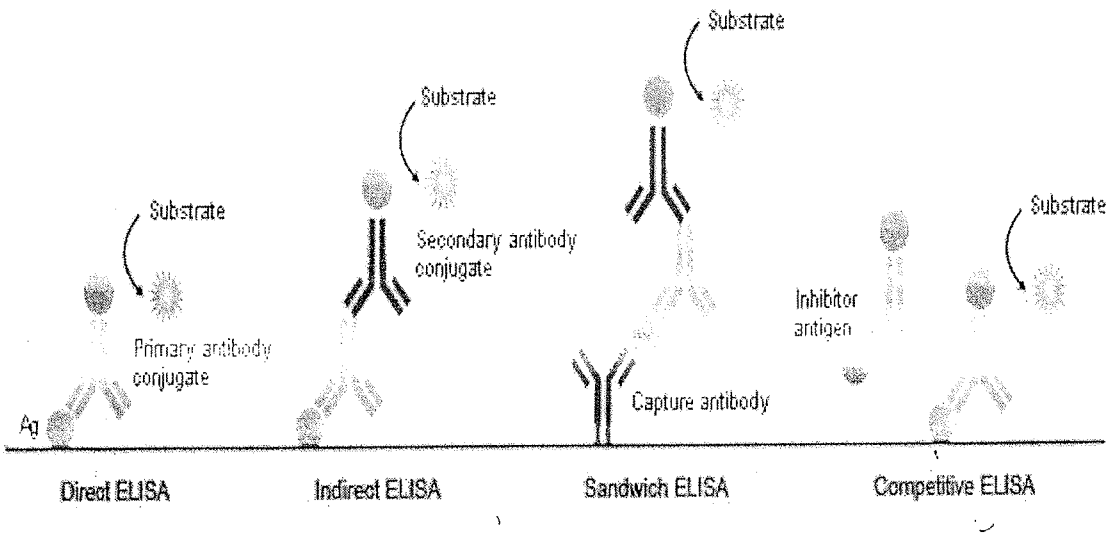
ELISAs are typically performed in 96-well polystyrene plates. The serum is incubated in a well, and each well contains a different serum. A positive control serum and a negative control serum would be included among the 96 samples being tested. Antibodies or antigens present in serum are captured by corresponding antigen or antibody coated on to the solid surface. After some time, the plate is washed to remove serum and unbound antibodies or antigens with a series of wash buffer. To detect the bound antibodies or antigens, a secondary antibodies that are attached to an enzyme such as peroxidase or alkaline phosphatase are added to each well. After an incubation period, the unbound secondary antibodies are washed off. When a suitable substrate is added, the enzyme reacts with it to produce a color. This color produced is measurable as a function or quantity of antigens or antibodies present in the given sample. The intensity of color/ optical density is measured at 450nm. The intensity of the color gives an indication of the amount of antigen or antibody.



Types of ELISA

Frequently there are 3 types of ELISA on the basis of binding structure between the Antibody and Antigen.

1. Direct ELISA
2. Indirect ELISA
3. Sandwich ELISA
4. Competitive ELISA



1. Direct ELISA

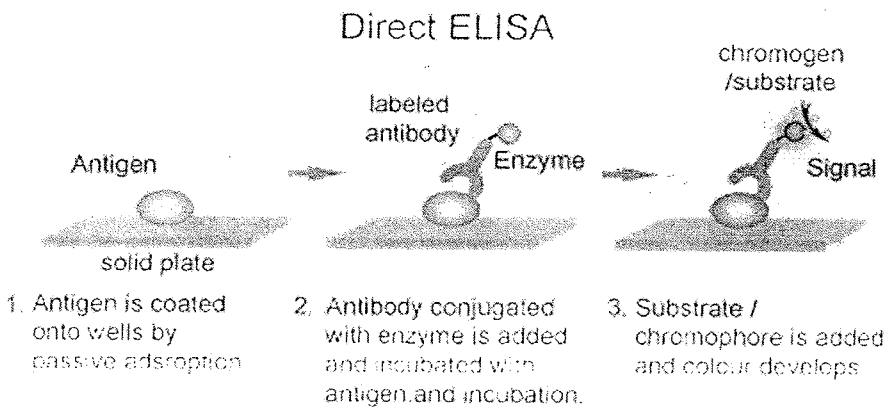
For direct detection, an antigen coated to a multi-well plate is detected by an antibody that has been directly conjugated to an enzyme. This detection method is a good option if there is no commercially available ELISA kits for your target protein.

Advantages

- Quick because only one antibody and fewer steps are used.
- Cross-reactivity of secondary antibody is eliminated.

Disadvantages

- Immunoreactivity of the primary antibody might be adversely affected by labeling with enzymes or tags.
- Labeling primary antibodies for each specific ELISA system is time-consuming and expensive.
- No flexibility in choice of primary antibody label from one experiment to another.
- Minimal signal amplification.



2. Indirect ELISA

Antibody can be detected or quantitatively determined by indirect ELISA. In this technique, antigen is coated on the microtiter well. Serum or some other sample containing primary antibody is added to the microtiter well and allowed to react with the coated antigen. Any free primary antibody is washed away and the bound antibody to the antigen is detected by adding an enzyme conjugated secondary antibody that binds to the primary antibody. Unbound secondary antibody is then washed away and a specific substrate for the enzyme is added. Enzyme hydrolyzes the substrate to form colored products. The amount of colored end product is measured by spectrophotometric plate readers that can measure the absorbance of all the wells of 96-well plate.

Procedure of Indirect ELISA

1. Coat the micro titer plate wells with antigen.
2. Block all unbound sites to prevent false positive results.
3. Add sample containing antibody (e.g. rabbit monoclonal antibody) to the wells and incubate the plate at 37°c.

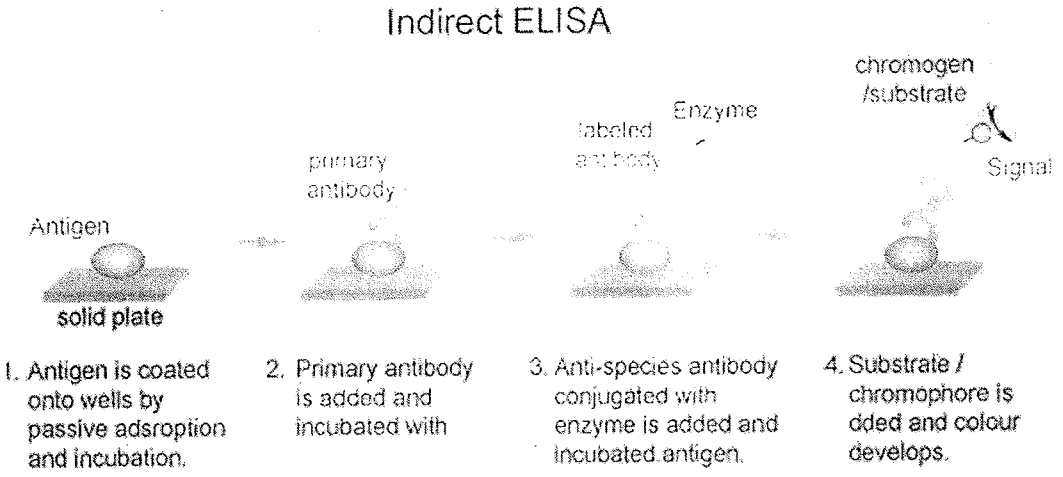
4. Wash the plate, so that unbound antibody is removed.
5. Add secondary antibody conjugated to an enzyme (e.g. anti- mouse IgG).
6. Wash the plate, so that unbound enzyme-linked antibodies are removed.
7. Add substrate which is converted by the enzyme to produce a colored product.
8. Reaction of a substrate with the enzyme to produce a colored product.

Advantages

- Increased sensitivity, since more than one labeled antibody is bound per primary antibody.
- A wide variety of labeled secondary antibodies are available commercially.
- Maximum immunoreactivity of the primary antibody is retained because it is not labeled.
- Versatile because many primary antibodies can be made in one species and the same labeled secondary antibody can be used for detection.
- Flexibility, since different primary detection antibodies can be used with a single labeled secondary antibody.
- Cost savings, since fewer labeled antibodies are required.
- Different visualization markers can be used with the same primary antibody.

Disadvantages

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



3. Sandwich ELISA

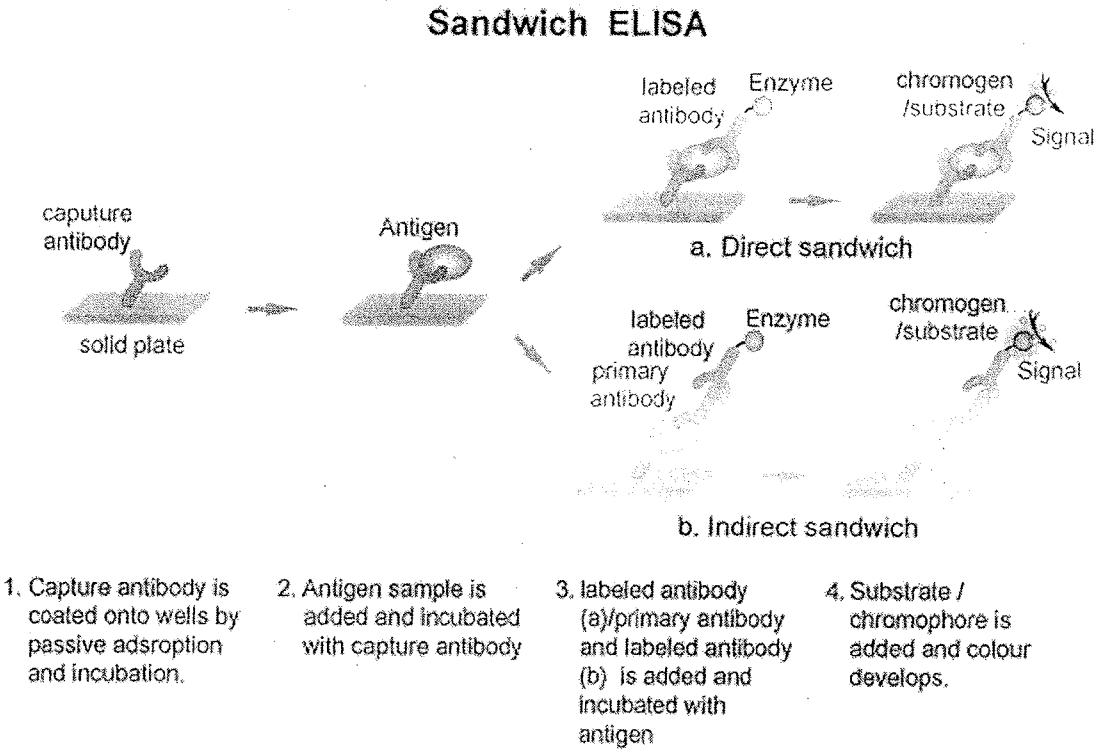
Antigen can be detected by sandwich ELISA. In this technique, antibody is coated on the microtiter well. A sample containing antigen is added to the well and allowed to react with the antibody attached to the well, forming antigen-antibody complex. After the well is washed, a second enzyme-linked antibody specific for a different epitope on the antigen is added and allowed to react with the bound antigen. Then after unbound secondary antibody is removed by washing. Finally substrate is added to the plate which is hydrolyzed by enzyme to form colored products.

Procedure of sandwich ELISA

1. Prepare a surface to which a known quantity of antibody is bound.
2. Add the antigen-containing sample to the plate and incubate the plate at 37°c.
3. Wash the plate, so that unbound antigen is removed.
4. Add the enzyme-linked antibodies which are also specific to the antigen and then incubate at 37°c.
5. Wash the plate, so that unbound enzyme-linked antibodies are removed.
6. Add substrate which is converted by the enzyme to produce a colored product.
7. Reaction of a substrate with the enzyme to produce a colored product.

Advantages

- High specificity, since two antibodies are used the antigen is specifically captured and detected.
- Suitable for complex samples, since the antigen does not require purification prior to measurement.
- Flexibility and sensitivity, since both direct and indirect detection methods can be used.



4. Competitive ELISA

This test is used to measure the concentration of an antigen in a sample.

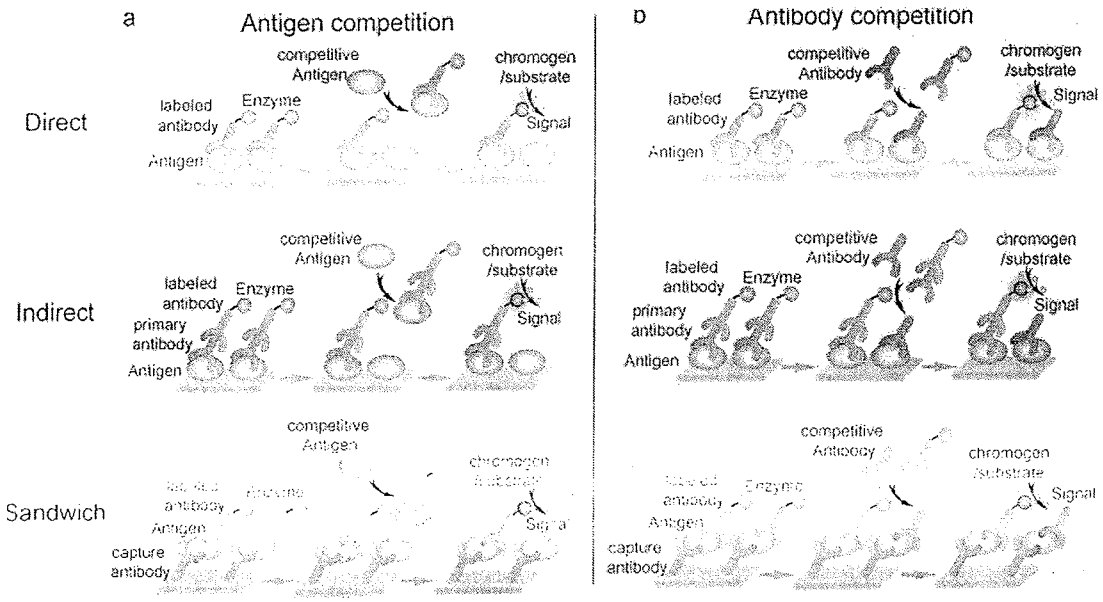
In this test, antibody is first incubated in solution with a sample containing antigen. The antigen-antibody mixture is then added to the microtitre well which is coated with antigen. The more the antigen present in the sample, the less free antibody will be available to bind to the antigen-coated well. After the well is washed, enzyme conjugated secondary antibody specific for isotype of the primary antibody is added to determine the amount of primary antibody bound to the well. The higher the concentration of antigen in the sample, the lower the absorbance.

Procedure

1. Antibody is incubated with sample containing antigen.
2. Antigen-antibody complex are added to the microtitre well which are pre-coated with the antigen.
3. Wash the plate to remove unbound antibody.
4. Enzyme linked secondary antibody which is specific to the primary antibody is added.
5. Wash the plate, so that unbound enzyme-linked antibodies are removed.
6. Add substrate which is converted by the enzyme into a fluorescent signal.

Advantages

- High specificity, since two antibodies are used.
- High sensitivity, since both direct and indirect detection methods can be used.
- Suitable for complex samples, since the antigen does not require purification prior to measurement.



Application of ELISA

1. Presence of antigen or the presence of antibody in a sample can be evaluated.
2. Determination of serum antibody concentrations in a virus test.
3. Used in food industry when detecting potential food allergens.
4. Applied in disease outbreaks- tracking the spread of disease e.g. HIV, bird flu, common, colds, cholera, STD etc.

Term Definition

Solid phase : Usually a microtiter plate well. Specially prepared ELISA plates are commercially available. These have an 8 ¡Á 12 well format and can be used with a wide variety of specialized equipment designed for rapid manipulation of samples including multichannel pipets.

Adsorption : The process of adding an antigen or antibody, diluted in buffer, so that it attaches passively to the solid phase on incubation. This is a simple way for immobilization of one of the reactants in the ELISA and one of the main reasons for its success.

Washing The simple flooding and emptying of the wells with a buffered solution to separate bound (reacted) from unbound (unreacted) reagents in the ELISA. Again, this is a key element to the successful exploitation of the ELISA.

Antigens : A protein or carbohydrate that when injected into animals elicits the production of antibodies. Such antibodies can react specifically with the antigen used and therefore can be used to detect that antigen.

Antibodies : Produced in response to antigenic stimuli. These are mainly protein in nature. In turn, antibodies are antigenic.

Antispecies antibodies : Produced when proteins (including antibodies) from one species are injected into another species. Thus, guinea pig serum injected into a rabbit elicits the production of rabbit anti¨Cguinea pig antibodies.

Enzyme : A substance that can react at low concentration as a catalyst to promote a specific reaction. Several specific enzymes are commonly used in ELISA with their specific substrates.

Enzyme conjugate : An enzyme that is attached irreversibly to a protein, usually an antibody. Thus, an example of antispecies enzyme conjugate is rabbit antiguinea linked to horseradish peroxidase.

Substrate : A chemical compound with which an enzyme reacts specifically. This reaction is used, in some way, to produce a signal that is read as a color reaction (directly as a color change of the substrate or indirectly by its effect on another chemical).

Chromophore : A chemical that alters color as a result of an enzyme interaction with substrate.

Stopping : The process of stopping the action of an enzyme on a substrate. It has the effect of stopping any further change in color in the ELISA.

Reading : Measurement of color produced in the ELISA. This is quantified using special spectrophotometers reading at specific wavelengths for the specific colors obtained with particular enzyme/chromophore systems. Tests can be assessed by eye.

Lab 2 4

HAEMAGGLUTINATION ASSAY – VIRAL QUANTITATION

Haemagglutination assay was developed by American virologist George Hirst in 1941-1942. The ability of certain viruses to bind with the red blood cells through their superficial glycoproteins and proteins had been utilised to quantitate these viruses and the assay is termed as haemagglutination assay. Red blood cells when suspended with adequate amount of such viral load is capable of forming lattices coating the container. But with insufficient viral load, red blood cells form sharp dots at the centre of the container indicating absence of agglutination.

Considering these facts, the unknown samples are diluted and analysed for the last dilution point, also called end point, capable of agglutinating the red cells completely. The amount of virus in the end point is termed as one hemagglutination unit (1 HAU). Considering the following protocol, 1 HAU is the amount of virus in 50 µl volume of sample required to agglutinate 50 µl of the 0.5% chicken RBC. Additionally, the reciprocal of the dilution of the virus at end point is said to be the haemagglutination titre (HA titre).

AIM

The main purpose of the assay is to estimate HA titre for the unknown viral sample. The basis of this assay is the ability of viral haemaglutinin to bind with the sialic acid present on the receptors of surface of the red blood cells causing haemagglutination

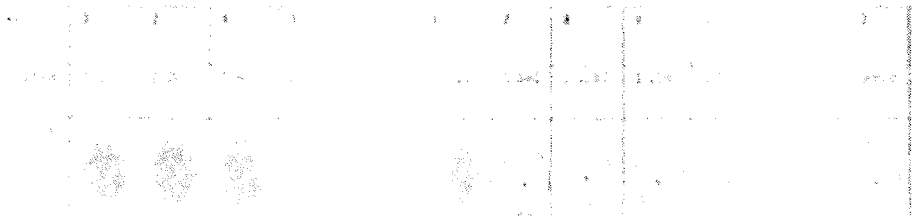
METHODS

- Label the first row of 96-well V-bottom plate as 1-12.
- Add 50 µl of the PBS buffer from well 2 till well 12.
- Add 100 µl of the pre-diluted virus in well 1.
- Perform serial dilution of the viral sample from well 1 to 11 using 50 µl pipette.
- Discard 50 µl of the diluted solution from well 11 to make the volume of the solution even in all the wells *(which is required for getting the right amount of virus in the well)*.
- Then, add 50 µl of properly mixed 0.5% chicken red blood cells to each well (1-12).

- Incubate for approximately 30 minutes at room temperature (*to ensure the occurrence of haemagglutination).*
- Lastly, note the end point of the sample and observe the pattern of haemagglutination to calculate HA titre.

SAMPLE RESULT

Table: Haemagglutination pattern of serially diluted influenza virus for determination of HA titre



1. Lattice formation upto well 6 shows the presence of one hemagglutination unit (1 HAU) viral load in the well.
2. HA Titre is the reciprocal of the highest dilution upto which haemagglutination was observed Here,
   HA titre = 320

3. Absence of hemagglutination from well 7 to well 11 – showing no or insufficient viral load to cause agglutination.
4. Well 12: Test control

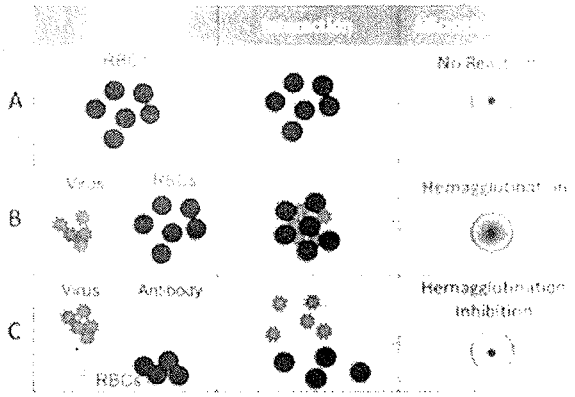
IMPORTANT NOTES

This haemagglutination assay is found to be effective, simple and easier to understand as compared to the other robust techniques, like nucleic acid amplification tests, used to assess the virus qualitatively as well as quantitatively. However, from this assay it is difficult to predict the subtypes of virus. Furthermore, if an unknown sample is to be analysed, this assay is not suitable for unravelling riddles of diagnostic approach because of the binding of red blood cells to various viruses or viral proteins. For these reasons, it is more likely to be a screening test rather than diagnostic assay. But the viral quantitation for a known type can be performed using this assay. Despite the simpler handling, high sensitivity, lower chances of error during performance, minimal skill requirement, relatively low cost and rapid assessment, this assay has very low specificity. Meanwhile, slight modification of the assay to haemagglutination inhibition assay, which detects specific antibodies against viral antigens, is much more specific that may be helpful in

determining the quality of the sample effectively. In addition to that, coating erythrocytes with specific antibodies can be beneficial in increasing the specificity of the assay. Thus, evidences support the modification of haemagglutination to be useful for ascertaining both quality and quantity of viruse

Hemagglutination Inhibition Test (HAI): Principle, procedure, result and interpretations

The nucleic acids of various viruses encode surface proteins that agglutinate the red blood cells (RBC) of a variety of species. For example; Influenza virus particles have an envelope protein called the hemagglutinin, or HA, which binds to erythrocytes , causing the formation of a lattice. This property is called hemagglutination. Reaction of viral hemagglutinins with red blood cells results in a lattice of agglutinated cells which settle irregularly in a tube or microtiter well. Unagglutinated cells settle in a compact button.



Hemagglutination and Hemagglutination Inhibition Test

Hemagglutination phenomenon is almost commonly used for diagnosis of infection produced by Orthomyxoviruses, paramyxoviruses, and the abroviruses-togaviruses (including rubella), flaviviruses, and bunyaviruses.

The presence of virus in infected cell cultures can be detected by hemagglutination; the identity of the virus or of antibodies in a patient's serum can be determined by specific inhibition of that hemagglutination. Although influenza viruses can be detected by hemadsorption test, typing of the isolate is done most efficiently by hemagglutination inhibition (HAI). Reagents and conditions for the test vary by virus.

Lab: 5

Determination of 50% endpoint titer

The procedure is performed to determine the infectious titer of any virus which can cause cytopathic effects (CPE) in tissue culture over a reasonable period of 5 to 20 days while cells in culture remain viable. This procedure is performed to quantify how much infectious virus is in a preparation. Not all virus types cause CPE in tissue culture, and the cell line and virus must be carefully matched in order to see a cytopathic effect.

The TCID50 is determined in replicate cultures of serial dilutions of the virus sample. The titer of the virus stock is expressed as the TCID50 which can be calculated using a statistical Excel program and is more accurate than a negative end-point

The TCID50 assay using tenfold serial dilutions of the virus sample, and plates those samples in quadruplicate in 48-well tissue culture dishes. This is a generic procedure, and the specific culture of the cell line such as the use of additives and the cell density and culture times may vary. Consult the managing virologist for appropriate cell lines, cell density, virus sample dilutions and incubation temperatures and times for the virus being tested.

Tissue Culture Infective Dose 50 (TCID50) test

Cells were seeded at 10000 cells/well in 96well microliter plates with a flat bottom, after 24hr. Confluent monolayer is achieved, growth media was discarded and virus suspension was serially tenfold dilution, then Cells were inoculated into 4 wells for each dilution (50µl per well), control cells were inoculated with serum free media.

The plate was covered with a sterile adhesive cover and incubated 2 hr. at room temperature to allow virus adsorption. After that, cells were washed with PBS and 200 µl serum free medium was added. the plate was covered again and incubated at 37°C, The inoculated plate was examined daily for three-five days and virus titer was calculated ,the titer determined as the dilution that cause 50% CPE in inoculated wells by used the equation bellow:

In this example of an end-point dilution assay, 10 monolayer cell cultures were infected with each virus dilution. After an incubation period, plates that displayed cytopathic effects were scored with a +. At high dilutions, none of the cell cultures are infected because no particles are present. At low dilutions, every cell culture is infected. Half of the cell cultures showed cytopathic effects at the 10^{-5} dilution. This is the *end point*: the dilution of virus at which 50% of the cell cultures are infected. This number can be calculated from the data and expressed as 50% infectious dose (ID_{50}) per milliliter. The virus stock in this example contains 10^{5} ID_{50} per ml.

Virus dilution	Cytopathic effect									
10^{-3}	+	+	+	+	+	+	+	+	+	+
10^{-4}	+	+	+	+	+	+	+	+	+	+
10^{-5}	+	+	–	+	+	+	+	+	+	+
10^{-6}	–	+	+	–	+	–	–	+	–	+
10^{-7}	–	–	–	–	–	–	+	–	–	–
10^{-8}	–	–	–	–	–	–	–	–	–	–

In real life, the 50% end point does not usually fall exactly on a dilution as shown in the example. Therefore statistical procedures are used to calculate the end point of the titration.

End-point dilution methods can also be used to determine the virulence of a virus in animals. The same approach is used: serial dilutions of viruses are made and inoculated into multiple test animals. Infection of the animal can be determined by death or clinical symptoms such as fever, weight loss, or paralysis. The results are expressed as 50% lethal dose (LD_{50}) per ml or 50% paralytic dose (PD) per ml when lethality or paralysis are used as end points. The following example illustrates the use of end point dilution to measure the lethality of poliovirus in mice. Eight mice were inoculated per virus dilution, and the end point was death. The statistical method of Reed and Muench was used to determine the 50% end point. In this method, the results are pooled, and the mortality at each dilution is calculated. The 50% end point, which falls between the fifth and sixth dilutions, is calculated to be 10 . Therefore the virus sample contains $10^{5.5}$ LD_{50} units.

Dilution	Alive	Dead	Total alive	Total dead	Mortality ratio	Mortality (%)
1 × 10	0	8	0	80	0/40	100
1 × 10	0	8	0	32	0/32	100
1 × 10	1	7	1	23	1/25	96
1 × 10	0	8	1	7	1/18	94
1 × 10	2	6	3	9	3/12	75
1 × 10	5	3	8	3	8/11	27

Reed, L.J., & Muench, H. (1938). A simple method of estimating fifty percent endpoints. Reed and Muench method

= [(mortality at dilution next above 50%)-50%] / [(mortality next above 50%)-(mortality next below 50%)].

Spearman-Karber method

$\log_{10}$ 50% end point dilution = $-(x_0 - d/2 + d \sum r_i/n_i)$

x_0 = $\log_{10}$ of the reciprocal of the highest dilution (lowest concentration) at which all animals are positive;

$d = \log_{10}$ of the dilution factor;

n_i = number of animals used in each individual dilution (after discounting accidental deaths);

r_i = number of positive animals (out of n_i).

Summation is started at dilution x_0.

Methods and Technology

What is gel electrophoresis?

Electrophoresis is a technique commonly used in the lab to separate charged molecules, like DNA, according to size.

- Gel electrophoresis is a technique commonly used in laboratories to separate charged molecules like DNA?, RNA? and proteins? according to their size.
- Charged molecules move through a gel when an electric current is passed across it.
- An electric current is applied across the gel so that one end of the gel has a positive charge and the other end has a negative charge.
- The movement of charged molecules is called migration. Molecules migrate towards the opposite charge. A molecule with a negative charge will therefore be pulled towards the positive end (opposites attract!).
- Smaller molecules migrate through the gel more quickly and therefore travel further than larger fragments that migrate more slowly and therefore will travel a shorter distance. As a result the molecules are separated by size.

Gel electrophoresis and DNA

- Electrophoresis enables you to distinguish DNA fragments of different lengths.
- DNA is negatively charged, therefore, when an electric current is applied to the gel, DNA will migrate towards the positively charged electrode.
- Shorter strands of DNA move more quickly through the gel than longer strands resulting in the fragments being arranged in order of size.
- The use of dyes, fluorescent? tags or radioactive? labels enables the DNA on the gel to be seen after they have been separated. They will appear as bands on the gel.

- A DNA marker with fragments of known lengths is usually run through the gel at the same time as the samples.
- By comparing the bands of the DNA samples with those from the DNA marker, you can work out the approximate length of the DNA fragments in the samples.

How is gel electrophoresis carried out?

Preparing the gel

- **Agarose gels? are typically used to visualise fragments of DNA. The concentration of agarose used to make the gel depends on the size of the DNA fragments you are working with.**
- **The higher the agarose concentration, the denser the matrix and vice versa. Smaller fragments of DNA are separated on higher concentrations of agarose whilst larger molecules require a lower concentration of agarose.**
- **To make a gel, agarose powder is mixed with an electrophoresis buffer and heated to a high temperature until all of the agarose powder has melted.**
- **The molten gel is then poured into a gel casting tray and a "comb" is placed at one end to make wells for the sample to be pipetted into.**
- **Once the gel has cooled and solidified (it will now be opaque rather than clear) the comb is removed.**
- **Many people now use pre-made gels.**
- **The gel is then placed into an electrophoresis tank and electrophoresis buffer is poured into the tank until the surface of the gel is covered. The buffer conducts the electric current. The type of buffer used depends on the approximate size of the DNA fragments in the sample.**

Preparing the DNA for electrophoresis

- A dye is added to the sample of DNA prior to electrophoresis to increase the viscosity of the sample which will prevent it from floating out of the wells and so that the migration of the sample through the gel can be seen.
- A DNA marker (also known as a size standard or a DNA ladder) is loaded into the first well of the gel. The fragments in the marker are of a known length so can be used to help approximate the size of the fragments in the samples.
- The prepared DNA samples are then pipetted into the remaining wells of the gel.
- When this is done the lid is placed on the electrophoresis tank making sure that the orientation of the gel and positive and negative electrodes is correct (we want the DNA to migrate across the gel to the positive end).

Separating the fragments

- The electrical current is then turned on so that the negatively charged DNA moves through the gel towards the positive side of the gel.
- Shorter lengths of DNA move faster than longer lengths so move further in the time the current is run.
- The distance the DNA has migrated in the gel can be judged visually by monitoring the migration of the loading buffer dye.
- The electrical current is left on long enough to ensure that the DNA fragments move far enough across the gel to separate them, but not so long that they run off the end of the gel.

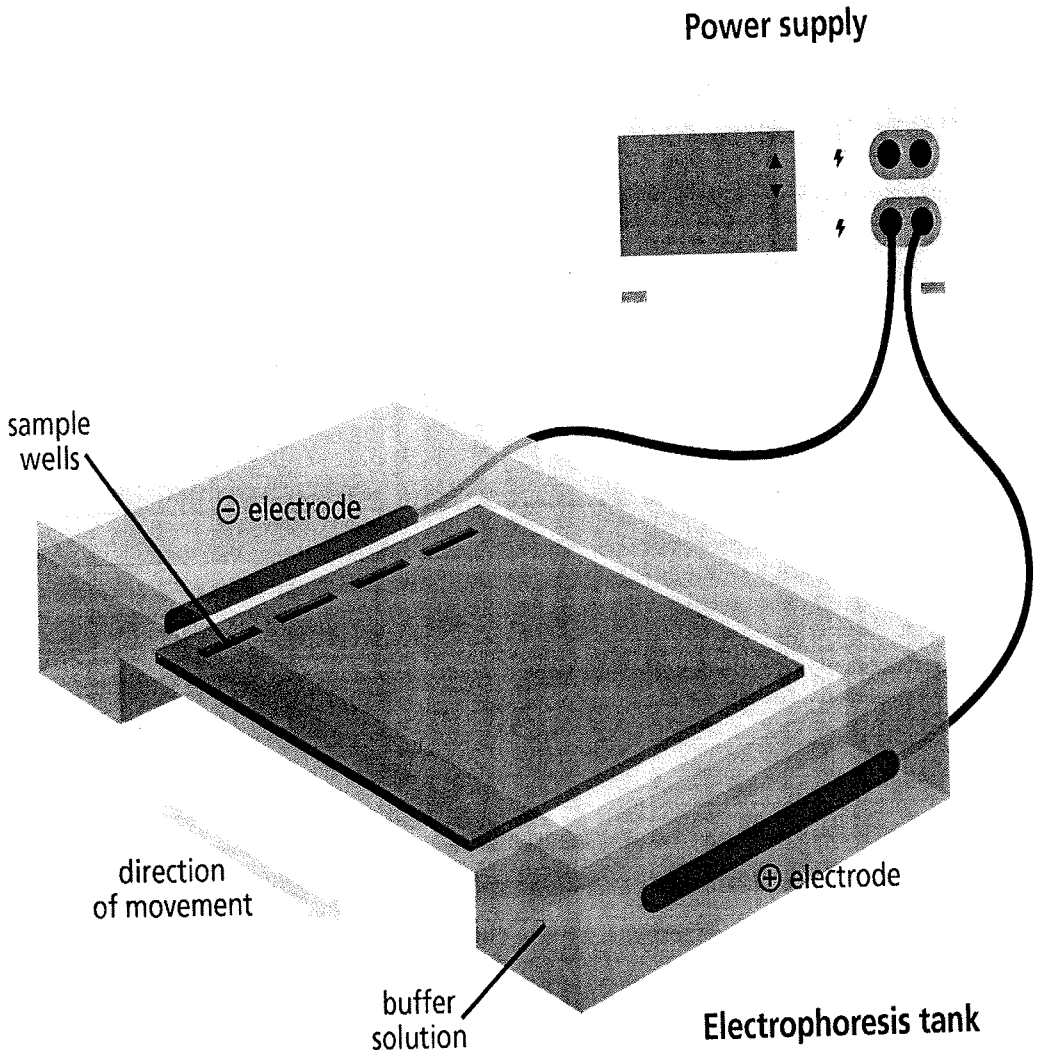


Illustration of DNA electrophoresis equipment used to separate DNA fragments by size. A gel sits within a tank of buffer. The DNA samples are placed in wells at one end of the gel and an electrical current passed across the gel. The negatively-charged DNA moves towards the postive electrode. Image credit: Genome Research Limited

Visualizing the results

- **Once the DNA has migrated far enough across the gel, the electrical current is switched off and the gel is removed from the electrophoresis tank.**
- **To visualize the DNA, the gel is stained with a fluorescent dye that binds to the DNA, and is placed on an ultraviolet transilluminator which will show up the stained DNA as bright bands.**
- **Alternatively the dye can be mixed with the gel before it is poured.**
- **If the gel has run correctly the banding pattern of the DNA marker/size standard will be visible.**
- **It is then possible to judge the size of the DNA in your sample by imagining a horizontal line running across from the bands of the DNA marker. You can then estimate the size of the DNA in the sample by matching them against the closest band in the marker.**

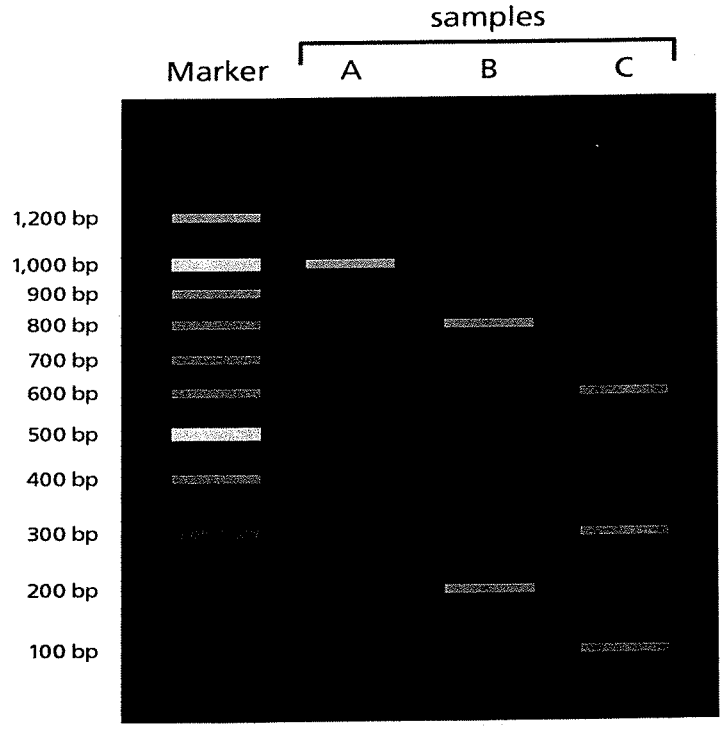


Illustration showing DNA bands separated on a gel. The length of the DNA fragments is compared to a marker containing fragments of known length. Image credit: Genome Research Limited

Lab--6- الفايروسات الطبية العملي

Electron Microscopy

The electron microscope is a type of microscope that uses a beam of electrons to create an image of the specimen. It is capable of much higher magnifications and has a greater resolving power than a light microscope, allowing it to see much smaller objects in finer detail.

Types of Electron Microscopes

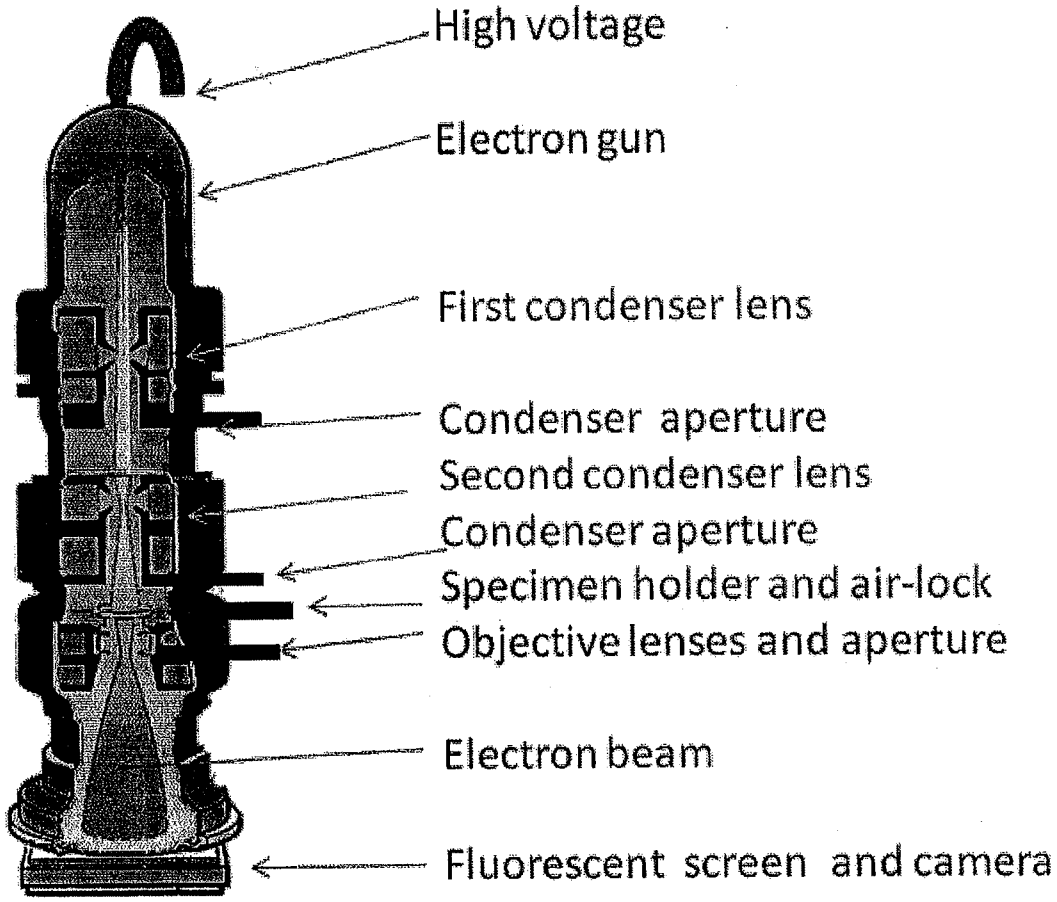
All electron microscopes use electromagnetic and/or electrostatic lenses to control the path of electrons. The electron beam passes through the centre of such solenoids on its way down the column of the electron microscope towards the sample. Electrons are very sensitive to magnetic fields.

The faster the electrons travel, the shorter their wavelength. The resolving power of a microscope is directly related to the wavelength of the irradiation used to form an image.

Although modern electron microscopes can magnify objects up to about two million times, they are still based upon the correlation between wavelength and resolution.. Researchers can use it to examine biological materials (such as microorganisms and cells), a variety of large molecules, medical biopsy samples.

Transmission Electron Microscope (TEM)

(TEM) involves a high voltage electron beam emitted by a cathode and formed by magnetic lenses. The electron beam that has been partially transmitted through the very thin (and so semitransparent for electrons) specimen carries information about the structure of the specimen. The spatial variation in this information (the "image") is then magnified by a series of magnetic lenses until it is recorded by hitting a fluorescent screen, photographic plate, or light sensitive sensor such as a CCD (charge-coupled device) camera. The image detected by the CCD may be displayed in real time on a monitor or computer. Transmission electron microscopes produce two-dimensional, black and white images.



Transmission Electron Microscope

Scanning Electron Microscope (SEM)

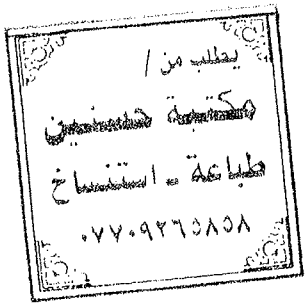
Unlike the TEM, where the electrons in the primary beam are transmitted through the sample, the Scanning Electron Microscope (SEM) produces images by detecting secondary electrons which are emitted from the surface due to excitation by the primary electron beam.

TEM resolution is about an order of magnitude better than the SEM resolution. Our TEM can easily resolve details of 0.2nm. Our two SEMs at are both relatively recent are high-resolution instruments capable of about 2 nm resolution on biological samples. Because the SEM image relies on electron interactions at the surface rather than transmission it is able to image bulk samples and has a much greater depth of view, and so can produce images that are a good representation of the 3D structure of the sample. .

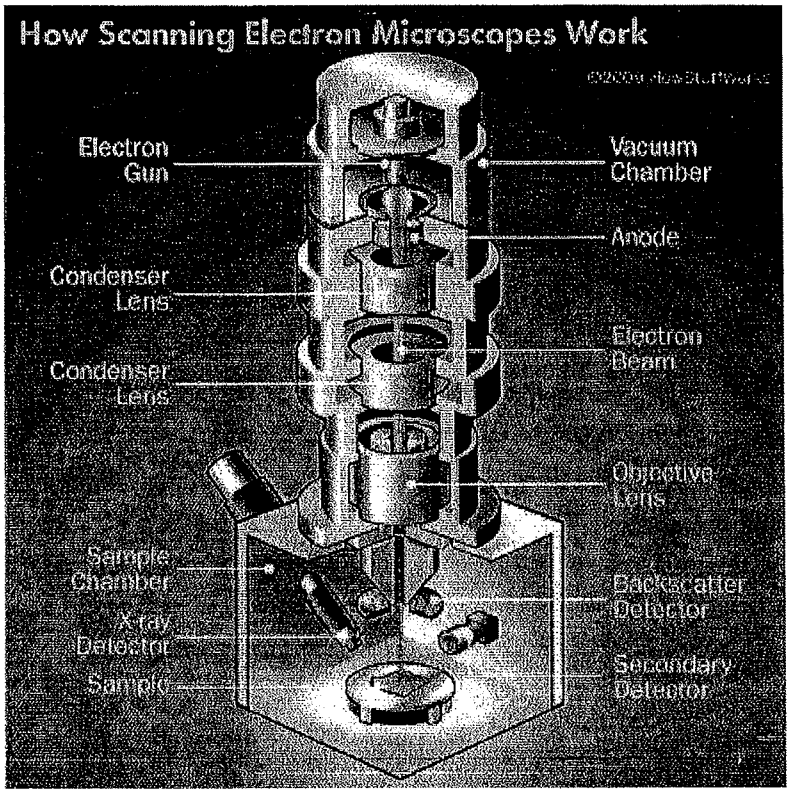
(11)

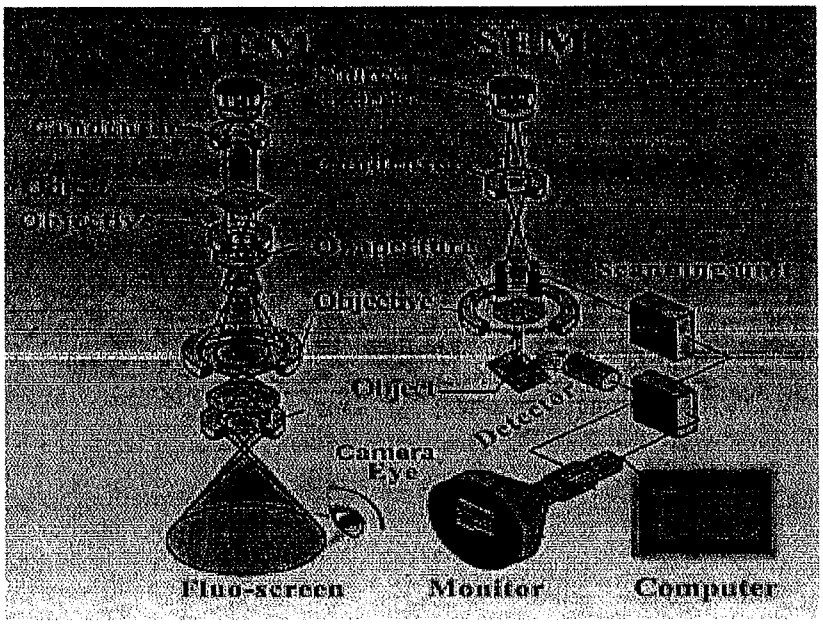
supplies, a cooling water supply circulation through the lenses and pumps. As they are very sensitive to vibration and external magnetic fields,

The samples have to be viewed in a vacuum, as the molecules that make up air would scatter the electrons. This means that the samples need to be specially prepared by sometimes lengthy and difficult techniques to withstand the environment inside an electron microscope.



(14)





Sample Preparation

Materials to be viewed in an electron microscope generally require processing to produce a suitable sample. This is mainly because the whole of the inside of an electron microscope is under high vacuum in order to enable the electron beam to travel in straight lines. The technique required varies depending on the specimen, the analysis required and the type of microscope:

Cryofixation - freezing a specimen rapidly, typically to liquid nitrogen temperatures or below, An entire field called cryo-electron microscopy has branched from this technique.

Fixation - a general term used to describe the process of preserving a sample at a moment in time and to prevent further deterioration so that it appears as close as possible to what it would be like in the living state, although it is now dead. In chemical fixation for electron microscopy, glutaraldehyde is often used to crosslink protein molecules and osmium tetroxide to preserve lipids.

Dehydration - removing water from the samples. The water is generally replaced with organic solvents such as ethanol or acetone as a stepping stone towards total drying for SEM specimens or infiltration with resin and subsequent embedding for TEM specimens.

Embedding - a resin (for electron microscopy) which can then be polymerised into a hardened block for subsequent sectioning.

Sectioning - the production of thin slices of the specimen so that they are semitransparent to electrons, typically around 90nm. These ultra-thin sections for electron microscopy are cut on an ultramicrotome with a glass or diamond knife.

Staining - uses heavy metals such as lead and uranium to scatter imaging electrons and thus give contrast between different structures, since many (especially biological) materials are nearly "transparent" to the electron beam.

Freeze-fracture and **freeze-etch** - a preparation method particularly useful for examining lipid membranes and their incorporated proteins in "face on" view. For the SEM, the sample is now ready for imaging. For the TEM, it can then be rotary-shadowed with evaporated platinum at low angle in a high vacuum evaporator. A second coat of carbon, The specimen is returned to room temperature and pressure.

Sputter Coating - an ultra-thin coating of electrically-conducting material, deposited by low vacuum coating of the sample. This is done to prevent charging of the specimen which would occur because of the accumulation of static electric fields due to the electron irradiation required during imaging. It also increases the amount of secondary electrons that can be detected from the surface of the sample in the SEM and therefore increases the signal to noise ratio. Such coatings include gold, gold/palladium, platinum, chromium etc.

Disadvantages of Electron Microscopy

Electron microscopes are very expensive to buy and maintain. They are dynamic rather than static in their operation: requiring extremely stable high voltage

Polymerase Chain Reaction

Introduction

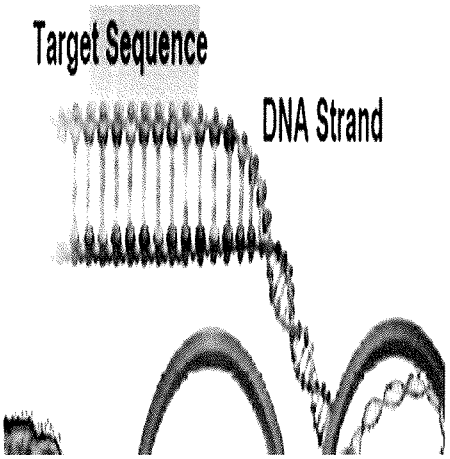
- PCR, polymerase chain reaction, is an in-vitro technique for amplification of a region of DNA whose sequence is known or which lies between two regions of known sequence
- Before PCR, DNA of interest could only be amplified by over-expression in cells and this with limited yield

- 1966, Thomas Brock discovers Thermus Aquaticus, a thermostable bacteria in the hot springs of Yellowstone National Park
- 1983, Kary Mullis postulated the concept of PCR (Nobel Prize in 1993)
- 1985, Saiki publishes the first application of PCR (beta-Globin)
- 1985, Cetus Corp. Scientists isolate Thermostable Taq Polymerase (from T.Aquaticus), which revolutionized PCR

Reaction Components

- DNA template
- Primers
- Enzyme
- dNTPs
- Mg^{2+}
- buffers

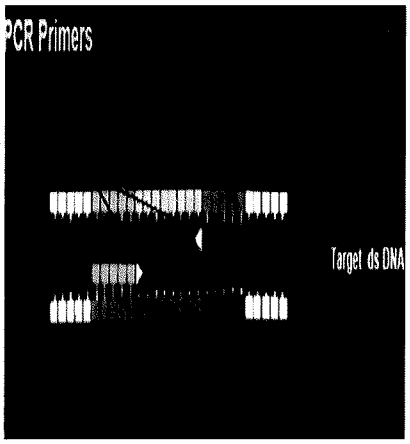
1- DNA template



- DNA containing region to be sequenced
- Size of target DNA to be amplified : up to 3 Kb

2- Primers

- 2 sets of primers
- Generally 20-30 nucleotides long
- Synthetically produced
- complimentary to the 3' ends of target DNA
- not complimentary to each other



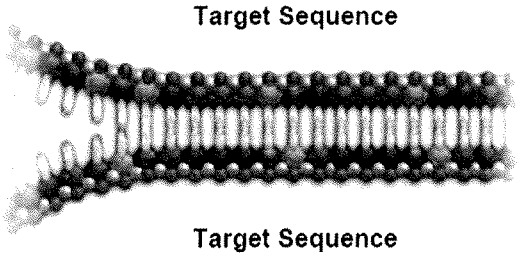
Primers (ctnd)

- Not containing inverted repeat sequences to avoid formation of internal structures
- 40-60% GC content preferred for better annealing
- Tm of primers can be calculated to determine annealing T^0

The PCR Cycle

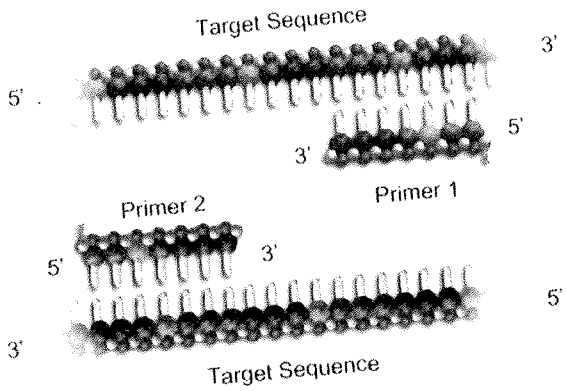
- Comprised of 3 steps:
  - Denaturation of DNA at 95^0C
  - Primer hybridization (annealing) at $40-50^0C$
  - DNA synthesis (Primer extension) at 72^0C

PCR Cycle - Step 1 - Denaturation Template DNA by Heat (95°C)



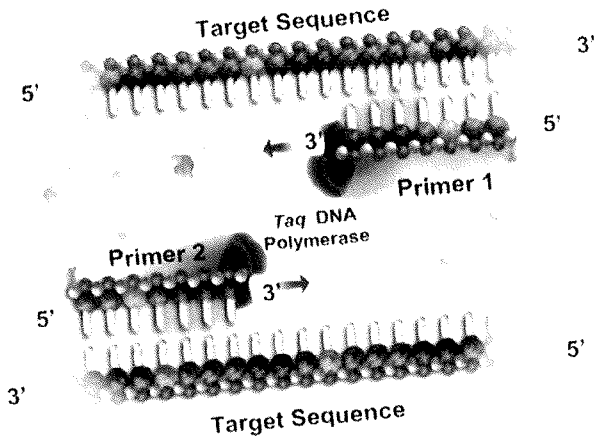
PCR Cycle - Step 2 –

Temperature is lowered (T_m) and primers anneal to target sequences



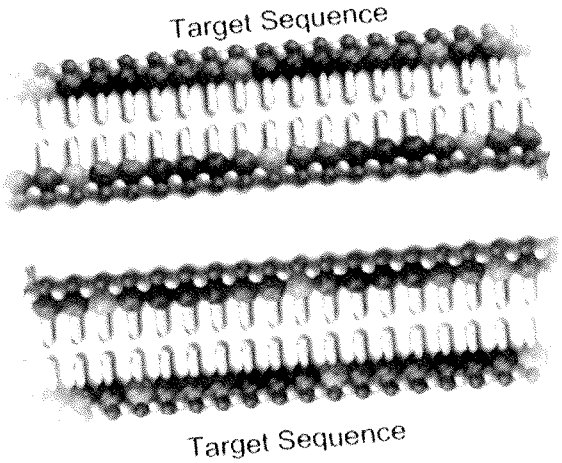
PCR Cycle - Step 3 -

At 72 °C *Taq* DNA polymerase catalyses primer extension as complementary nucleotides are incorporated

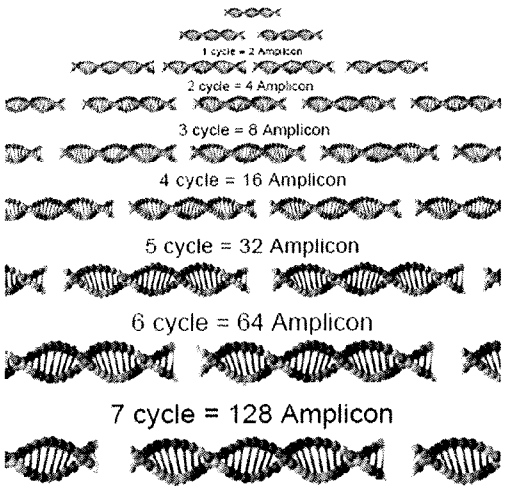


End of the 1st PCR Cycle –

Results in two copies of target sequence

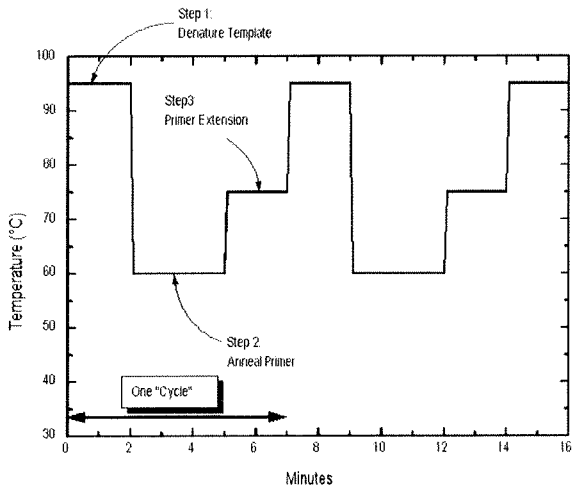


Target Amplification



No. of Cycles	No. Amplicon Copies of Target
1	2
2	4
3	8
4	16
5	32
6	64
20	1,048,576
30	1,073,741,824

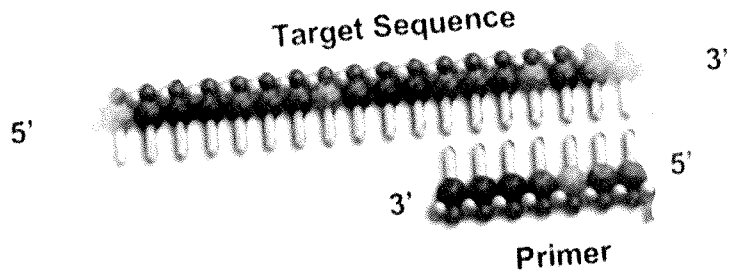
Standard thermocycle



RT-PCR

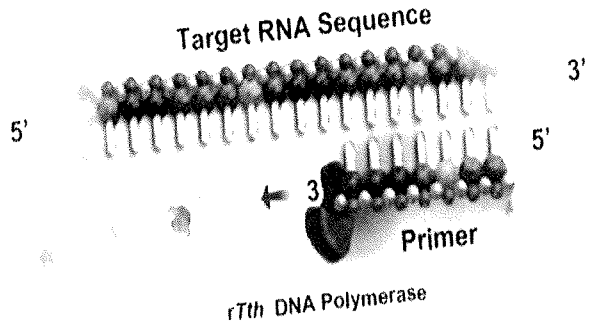
- Reverse Transcriptase PCR
- Uses RNA as the initial template
- RNA-directed DNA polymerase (rTh)
- Yields ds cDNA

Reverse Transcription - Step 1 –
Primer Anneals to Target RNA Sequence

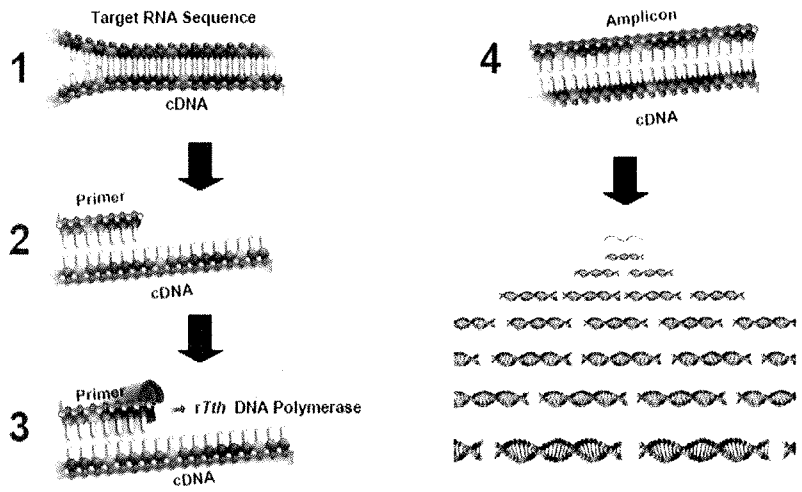


Reverse Transcription - Step 2 –

r*Tth* DNA Polymerase also has RT activity Catalyses Primer Extension by Incorporating Complementary Nucleotides



PCR Step 1 - Denaturation by Heat
PCR Step 2 - Annealing of Primer to cDNA
PCR Step 3 - r*Tth* DNA Polymerase Catalyses Primer Extension
End of 1st PCR Cycle - Yields a Double-Stranded DNA Copy (Amplicon) of the Target Sequence



Detection of amplification products

- Gel electrophoresis
- Sequencing of amplified fragment
- Southern blot
- etc...

Applications

- Genome mapping and gene function determination
- Biodiversity studies (e.g. evolution studies)
- Diagnostics (prenatal testing of genetic diseases, early detection of cancer, viral infections...)
- Detection of drug resistance genes
- Forensic (DNA fingerprinting)

Advantages

- Automated, fast, reliable (reproducible) results
- Contained :(less chances of contamination)
- High output
- Sensitive
- Broad uses
- Defined, easy to follow protocols