Practical Virology

Biosafety

Principles of Biosafety

The action and/or process used to safely manage biohazardous materials in the laboratory environment is called "**containment**". The purpose of containment is to eliminate an infectious organism or toxin, thereby reducing for exposure to laboratory workers or persons outside the laboratory and release to the environment. The types of containment are:

1- Primary containment:

The primary means of physical containment and it is provided by good laboratory techniques and the use of personal protective equipment (PPE) and biosafety cabinets (BSCs) within the laboratory. Also, vaccination of laboratory workers may increase the level of personal protection.

2- Secondary containment (Secondary barrier):

The facility design and construction of a biological laboratory provide barrier protection to the laboratory workers from the infectious agents and toxins. It also provides barrier to protect people, animals and the environment outside of the laboratory from infectious agents or toxins that may be accidentally released from the laboratory.

Biosafety requirements

The term "biosafety" is describing the implementation of specific practices, safety equipment and specially designed laboratories to create a safe environment for the laboratory workers, people and animals from the infectious agents and toxins, within and outside the laboratory.

Biosafety containments are divided into four levels (BSL-1, BSL-2, BSL-3, and BSL-4) in ascending order of containment based on the degree of the health-related risk associated with the work being conducted, for example BSL-4 is the highest biosafety level. While, the infective microorganisms are divided into four categories (Risk groups 1-4) according to the risk posed to individuals and communities.

Table 1: Relation of risk groups to biosafety levels

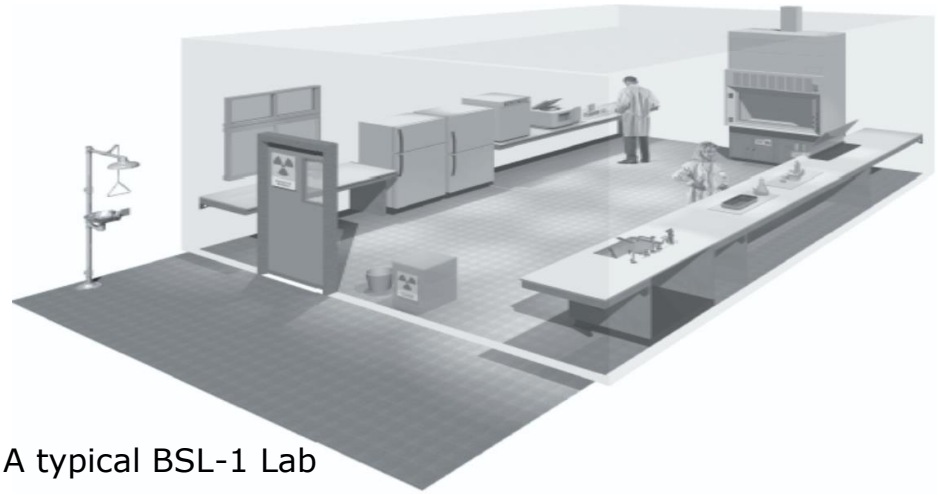
Risk group	Biosafety level	Laboratory type	Lab practices & Safety equipment	Lab facility construction
1. No or low individual and community risk, Ex: Adeno-associated virus (AAV)	Basic–in Biosafety Level 1	Basic teaching & research	Good microbiological techniques (GMT) + minimal requirements of PPE	Open bench, sink & doors to separate working place with other facility
2. Moderate individual risk, low community risk, Ex: Semiliki Forest virus (SFV)	Basic–in Biosafety Level 2	Primary health services; diagnostic Services & research	GMT + Biological Safety Cabinet II (BSCII), PPE as needed & biohazard sign	BSL-1 + self-closing door access & availability of a mechanism for decontamination.
3. High individual risk, low community risk, Ex: Japanese encephalitis virus (JEV)	Containment–in Biosafety Level 3	Special diagnostic Services & research	GMT+ requires significant training, BSC III, PPE required, controlled access & may the laboratory workers need to be immunized	BSL-2 + self-closing double door access, single-pass negative directional airflow, High Efficiency Particulate Air Filters(HEPA) filtered air exhaust & double-ended autoclave (through the wall)
4. High individual and community risk, Ex: Ebola virus	Maximum containment–in Biosafety Level 4	Dangerous pathogen units	BSL-3 + BSC III, access carefully controlled, positive pressure suit, shower upon exiting & decontaminate all materials before exiting.	BSL-3 + Lab in a separate building or a restricted zone in the building, supply and exhaust air detector & decontamination systems.

BSL-1

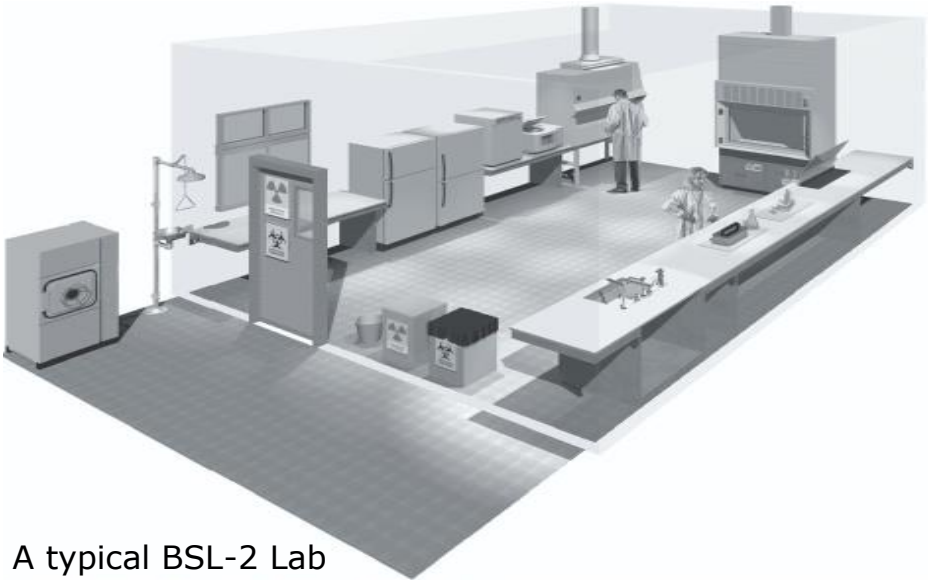
BSL-2

BSL-3

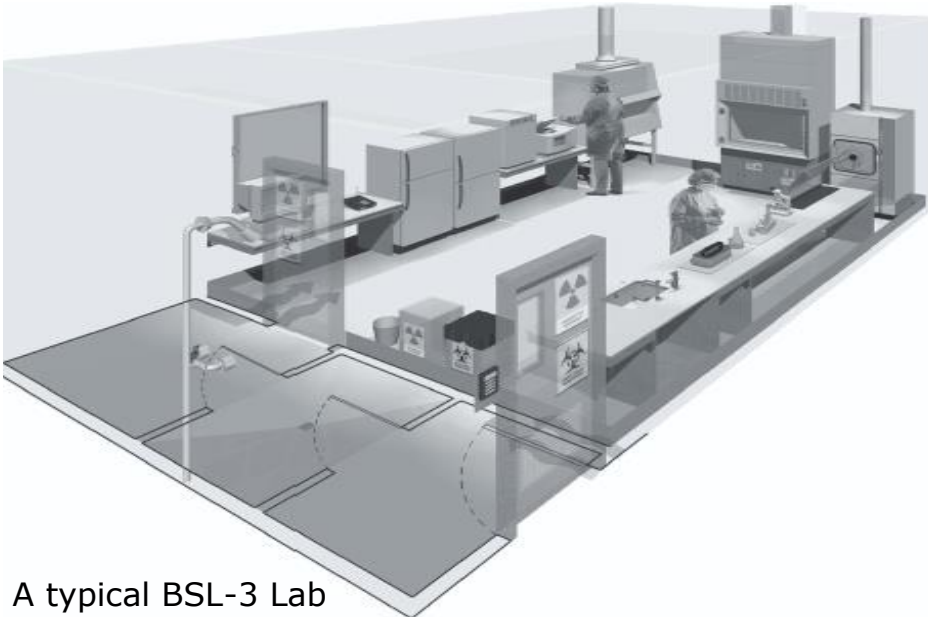
BSL-4



A typical BSL-1 Lab



A typical BSL-2 Lab

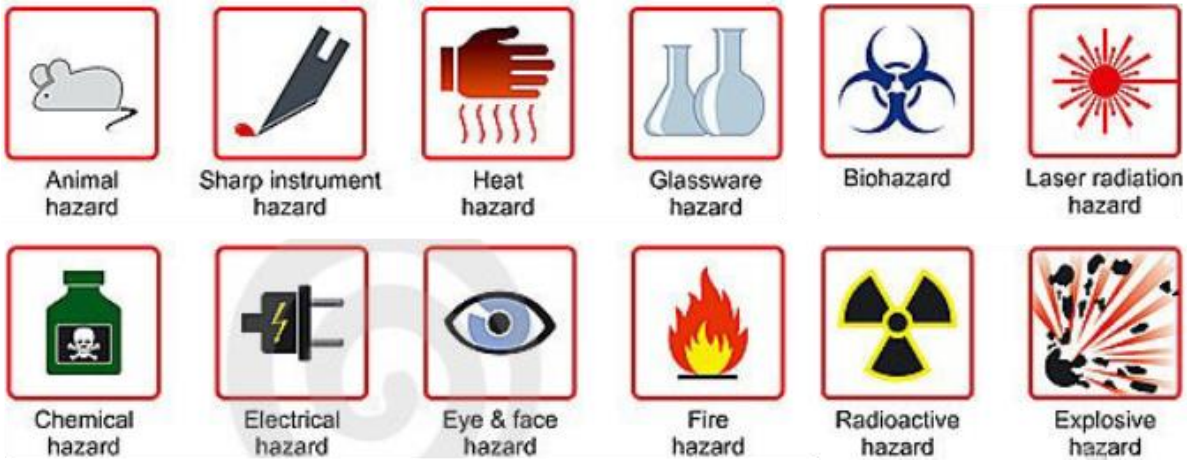


A typical BSL-3 Lab

The correct procedure to wear Personal Protective Equipment (PPE) steps are:

1- Wash hands. 2-Put on boots. 3-First pair of gloves.
4-Gown. 5-Plastic aperon. 6- Second pair of gloves (make sure gloves cover cuff of gown sleeves). 7-N95 particulate respirator.
8-Hair cover. 9-Goggles or face shield.

Lab safety symbols: They alert about possible dangers in the lab.



Triple packaging system:

Primary Container

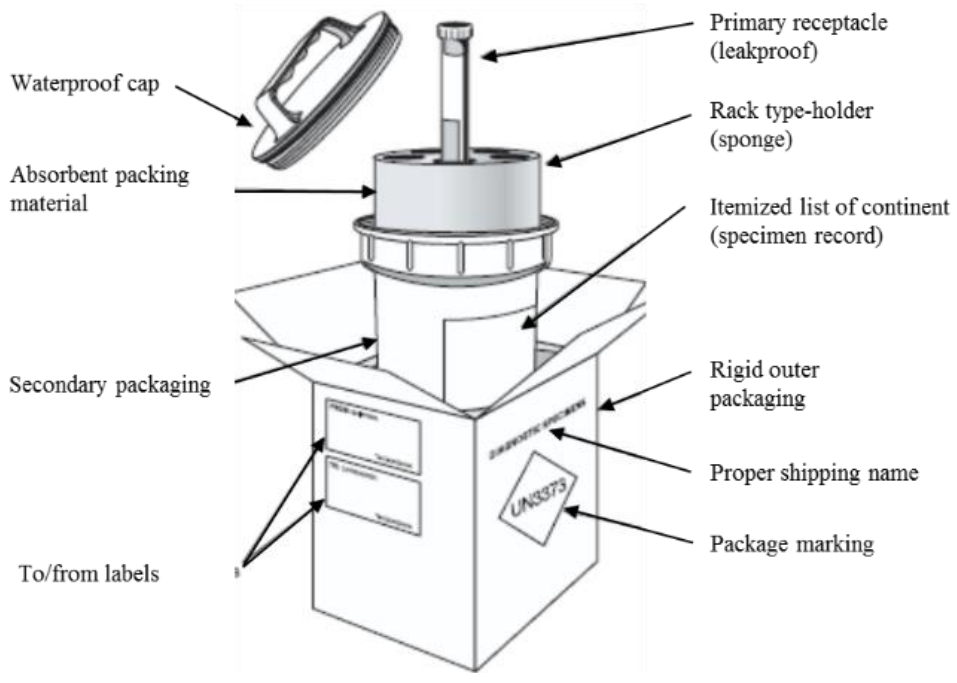
- ★ Leak proof, labeled, capped, and sealed with parafilm
- ★ Wrap specimen container with absorbent material or enough tissue/paper to prevent breakage and absorb contents in case of leakage.

Secondary container

- ★ Must be watertight.
- ★ If transporting with wet ice, surround secondary container with ice in sealed plastic bags (to prevent leakage,).
- ★ Place an itemized list of contents in a sealed plastic bag between the secondary container and the outer packaging.

Outer container

- ★ Protects the secondary packaging from physical damage while in transit.
- ★ Specimen data forms, letters and other types of information that identify or describe the specimen and any other documentation required, must also be provided
- ★ The outer package should display the sender, the laboratory name with complete address and telephone numbers for the sender and the recipient, the appropriate biohazard labels, and storage temperature requirements.



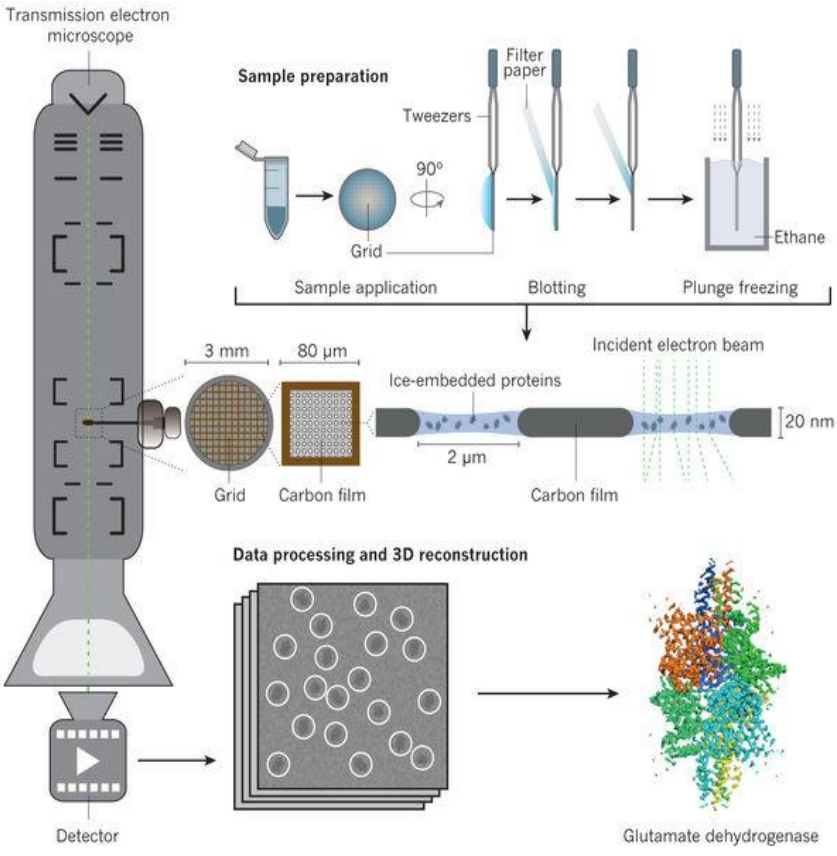
Measuring Sizes of Viruses

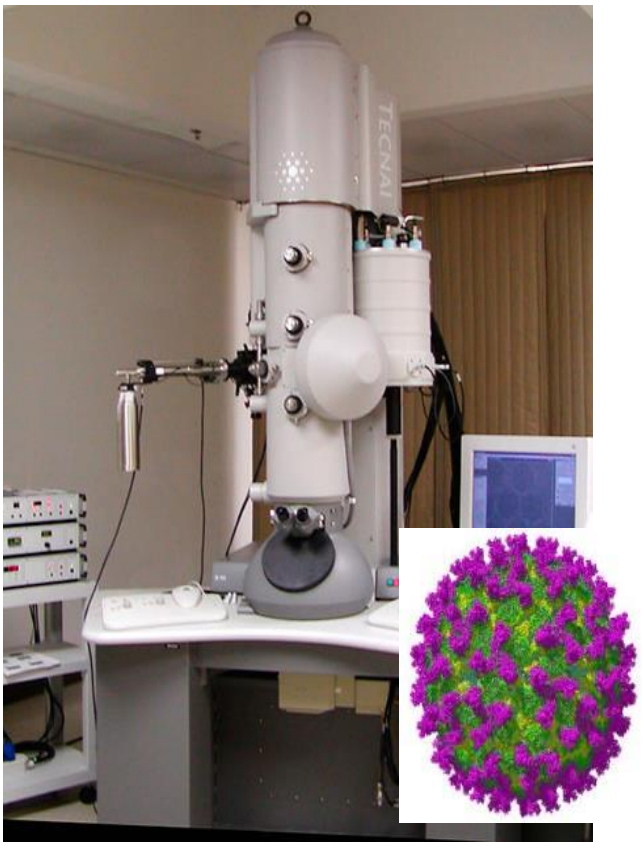
Very small size and ability to pass through bacterial filters are classic attributes of viruses. The following methods are used to determine the size of the viruses:

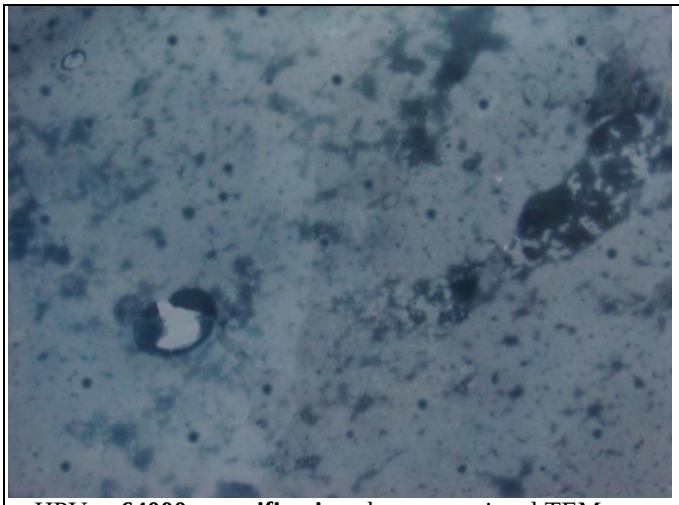
1- Direct observation by using electron microscope.

EM is the most widely used method for estimating virus particle size because viruses can be visualized in preparations from tissue extracts and in ultrathin sections of infected cells.

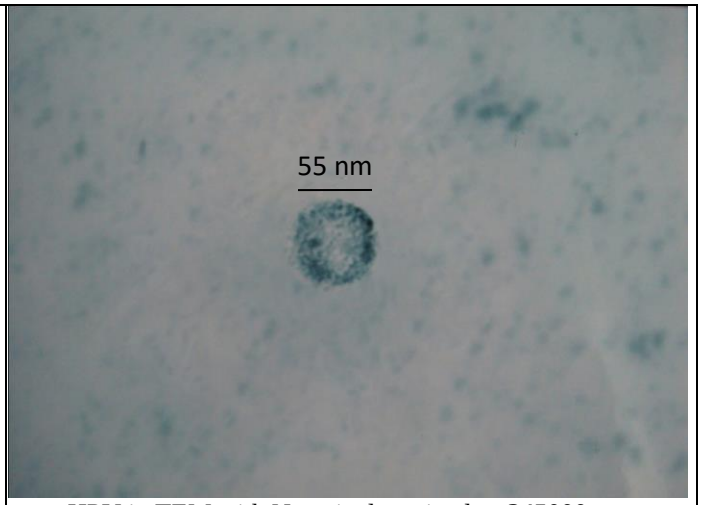
The EM uses electrons and electromagnetic lenses, therefore can be visualized that objects much smaller than the wavelength of visible or ultraviolet light that used in light microscope.







HPV at **64000 magnifications** by conventional TEM



HPV in TEM with Negatively stained **at 245000** magnifications

The key difference between Electron Microscope (EM) and Transmission Electron Microscope TEM is that EM creates an image by detecting reflected electrons, whereas TEM creates an image by detecting transmitted electrons. EM analyzes the surface of a sample while TEM analyses the internal structure. Since some biomoleculesare not compatible with the high-vacuum conditions and intense electron beams used in traditional TEM, The water that surrounds the molecules evaporates, and the high energy electrons burn and destroy the molecules. Cryo-EM uses frozen samples, gentler electron beams and sophisticated image processing to overcome these problems.

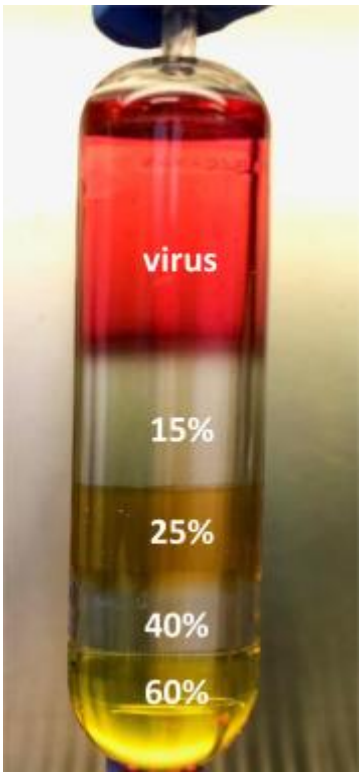
2- Filtration through membranes of graded porosity.

In this method, viruses are made to pass through a series of membranes of known pore size, the approximate size of any virus can be measured by determining which membrane allows the virus to pass through and which membrane holds it back. The size of the limiting APD (average pore diameter, ADP=P1+P2/2) multiplied by 0.64 yields the diameter of the virus.

$$\text{ADP}=\frac{\text{P1}+\text{P2}}{2} * 0.64$$

3- Sedimentation by ultracentrifugation in density gradient solution

When suspended viral particle in a liquid (**sucrose or CSCL**), then settled by using ultracentrifuge, force more than 100,000 xg (r.p.m) the particles are driven to the bottom of the tube. The relationship between the size and shape of a particle and its rate of sedimentation permits determination of particle size.



4- Comparative measurements.

The approximate estimate size of virus particle can be obtained by compared it's with known bacterial size or protein molecules. For example: *Staphylococcus* has a diameter of about 1000 nm. Serum albumin has a diameter of about 5 nm.

Reaction to Physical & Chemical Agents

Heat & Cold:

There is great variability in the heat stability of different viruses. Icosahedral viruses tend to be stable, losing little infectivity after several hours at 37 °C.

Enveloped viruses are much more heat-labile, rapidly dropping in titer at 37 °C. Enveloped viruses tend to lose infectivity after prolonged storage even at -90 °C and are particularly sensitive to repeated freezing and thawing. Viral infectivity is generally destroyed by heating at 50–60 °C for 30 minutes, though there are some notable exceptions (e.g., hepatitis B virus, polyomaviruses).

Viruses can be preserved by storage at subfreezing temperatures, and some may withstand lyophilization and can thus be preserved in the dry state at 4 °C or even at room temperature. Viruses that withstand lyophilization are more heat-resistant when heated in the dry state.

Stabilization of Viruses by Salts

Many viruses can be stabilized by salts in concentrations of 1 mol/L; ie, the viruses are not inactivated even by heating at 50 °C for 1 hour. The mechanism by which the salts stabilize viral preparations is not known. Viruses are preferentially stabilized by certain salts. $MgCl_2$, 1 mol/L, stabilizes picornaviruses and reoviruses; $MgSO_4$, 1 mol/L, stabilizes orthomyxoviruses and paramyxoviruses; and Na_2SO_4, 1 mol/L, stabilizes herpesviruses.

pH

Viruses are usually stable between pH values of 5.0 and 9.0. Some viruses (e.g., enteroviruses) are resistant to acidic conditions. All viruses are destroyed by alkaline conditions. In hemagglutination reactions, variations of less than one pH unit may influence the result.

Radiation

Ultraviolet, x-ray, and high-energy particles inactivate viruses. The dose varies for different viruses. Infectivity is the most radiosensitive property because replication

requires expression of the entire genetic contents. Irradiated particles that are unable to replicate may still be able to express some specific functions in host cells.

Photodynamic Inactivation

Viruses are penetrable to a varying degree by vital dyes such as toluidine blue, neutral red, and proflavine. These dyes bind to the viral nucleic acid, and the virus then becomes susceptible to inactivation by visible light.

Ether Susceptibility

Ether susceptibility can be used to distinguish viruses that possess an envelope from those that do not.

Detergents

Nonionic detergents e.g., Nonidet P40 and Triton X-100 solubilize lipid constituents of viral membranes. The viral proteins in the envelope are released (undenatured). Anionic detergents, e.g.: sodium dodecyl sulfate (SDS), also solubilize viral envelopes; in addition, they disrupt capsids into separated polypeptides.

Formaldehyde

Formaldehyde destroys viral infectivity by reacting with nucleic acid. Viruses with single-stranded genomes are inactivated much more readily than those with double-stranded genomes.

Antibiotics & Other Antibacterial Agents

Antibacterial antibiotics and sulfonamides have no effect on viruses.

Quaternary ammonium compounds, in general, are not effective against viruses.

Organic iodine compounds are also ineffective.

Larger concentrations of **chlorine** (5-10%) are required to destroy viruses than to kill bacteria.

Alcohols, such as isopropanol and ethanol, are relatively ineffective against certain viruses, especially picornaviruses.

Common Methods of Inactivating Viruses for Various Purposes

Viruses may be inactivated for various reasons: to sterilize laboratory supplies and equipment, disinfect surfaces or skin, make drinking water safe, and produce inactivated virus vaccines. Different methods and chemicals are used for these purposes.

Sterilization may be accomplished by steam under pressure (Autocleave), Oven (dry heat), ethylene oxide, and gamma irradiation. Surface disinfectants include sodium hypochlorite, glutaraldehyde, formaldehyde, and peracetic acid. Skin disinfectants include chlorhexidine, 70% ethanol, and iodophores.

Viral Diagnostic Tests

Viruses Cultivation (Propagation)

Viruses are **obligate intracellular** parasites that require living cells in order to replicate. There are three systems for their cultivation:

1- Lab. Experimental animals.
2- Hen's fertile eggs.
3- Cell culture technique.

Embryonated eggs and laboratory **animals** are very useful for the isolation of certain viruses, but **cell cultures** are the sole system for virus isolation in most laboratories.

1- Animal inoculation: the type of animal as well as the route of inoculation depends upon the type of the virus. Laboratory animals used as mice, G. Pigs and Rabbits. For example:

- Encephalitis viruses: cultivated in the white suckling mouse (1-3 days old) intracerebral .
- Herpes simplex virus: cultivated in **rabbit cornea ⟶ positive result appears as vesicles**
- Rabies virus: cultivated in **white mouse (baby/adult) intracerebral ⟶ 1 – 3 weeks ⟶ encephalitis**
- Dengue virus: cultivated in **baby white mouse (1 – 3 days) intracerebral/ subcutaneously ⟶ 3 – 7 days ⟶ tremor, paralysis**
- Polio virus: cultivated in **monkey intracutaneously, IM, intraneural, intraspinal ⟶ paralysis**

2- Embryonated Hen's Egg inoculation:

In most cases the advent of tissue culture techniques have superseded the need to isolate viruses in embryonated hen eggs. However, for some viruses, e.g., influenza and avian species, the embryonated egg is still often used for virus isolation. As will be determined later, the embryonated egg is also used for infectivity (pock) assays. The embryonated egg provides an ideal 'receptacle' in which to grow

viruses, as it is sterile and has a range of tissue types and cavity fluids which both support the replication and allow the concentration of infectious virus.

Many advantages are offered by the use of fertile eggs:

1- Cheap and easily maintained (require less space, no feeding and cleaning of cages).

2- Easily manipulated.

3- Easily identified and labeled.

4- Generally free from natural factors of defense.

5- Still used to produce many vaccines (Flu Vaccine)

Anatomy of embryonated eggs:

The embryonated eggs has several cavities which virus may be inoculated and cultivated, the site and method of inoculation chosen depend on the purpose of the procedure. The most important structures are:

1-Air sac: at the blunt end of the egg.

2-Chorioallantoic membrane (CAM): lies directly under egg membrane, serve as respiratory organ embryo. This route used for isolation of Herpes and Pox viruses.

3- Chorioallantoic sac (allantoic cavity): virus can be propagated in much larger quantities, used for vaccine production, the embryo used at 9-11 days old used for isolation of influenza viruses.

4-Amniotic cavity: the space surrounding the embryo, contain 1-2 ml of fluid. The embryo used at 10-14 days old. Used for influenza, A, B & C and mumps-virus.

5-Embryo: (Intraembryonal) used at 8 – 10 days embryo for Japanese B encephalitis virus.

6-Yolk sac: The embryos used at 5-8 days old. This route used for isolation of some toga and herpes viruses.

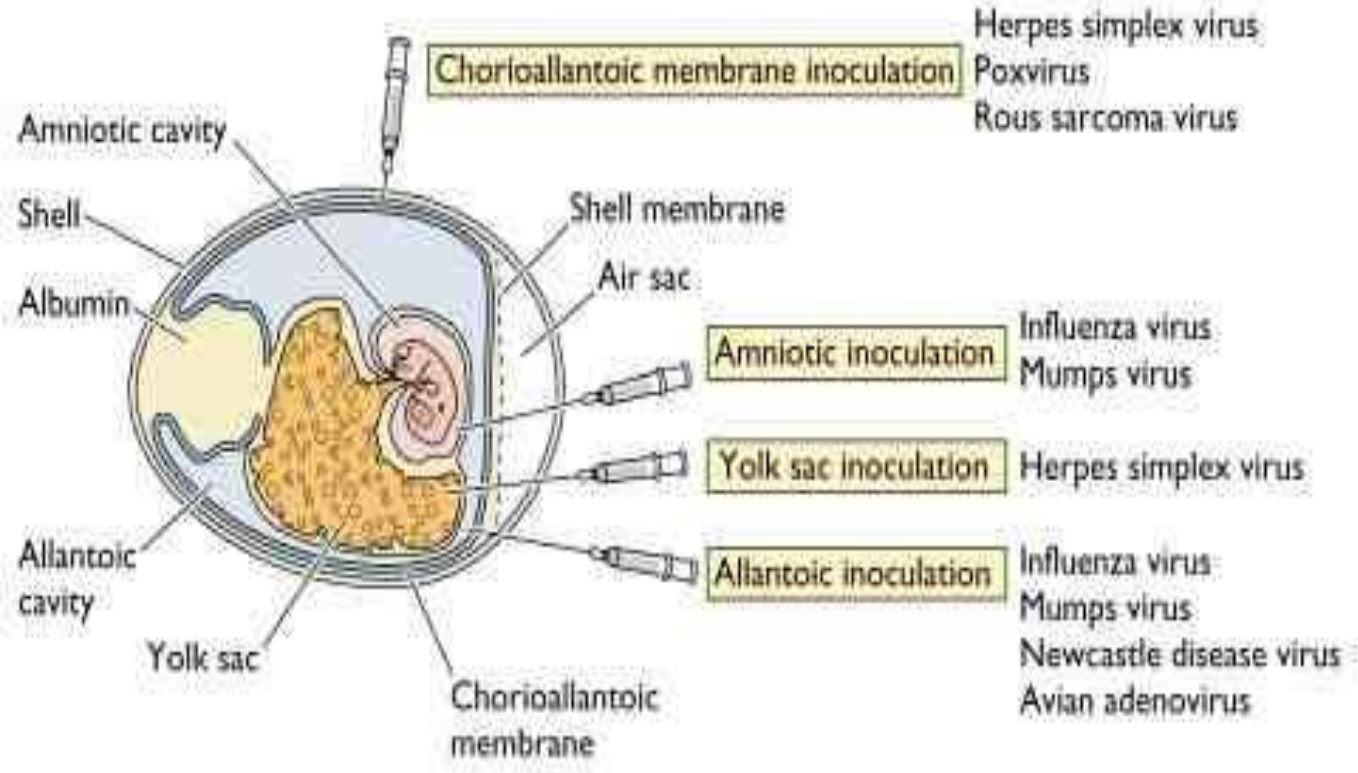


Figure 1: Routes of egg inoculation

Preparation of eggs for Viral Inoculation:

1- Incubation:

Fertile hens' eggs are acquired from a suitable hatchery and should be cleaned with 70% alcohol but not washed with water because the mechanically contaminants may be push inside through egg shell micropores and washing humidity facilitate the growth of contaminants. The eggs incubated at 37 degree Centigrate in an atmosphere of about 62% humidity with a forced (usually fan driven) air circulation. This prevents drying out of the egg and allows for good air exchange in the developing embryo. More elaborate incubators also have a mechanism for

gently rocking the egg at frequent time intervals. The embryo and developing membranes and cavities go through a variety of anatomical changes up to hatching.

2- Procedure:

The procedure of egg inoculation can be divided up into a series of steps.

1-Eggs are **'candled'** to check for viability and to determine the positions of the embryo, membranes and blood vessels. Dead eggs will have little or no vasculature and have a characteristic translucent appearance. Darkly stained eggs are usually heavily contaminated. Candling is carried out in a darkened room using a light box which has one small egg-shaped hole surrounded by a piece of foam on which the egg is placed. Rotating the egg immediately reveals its anatomical make-up.

2- Eggs are disinfected with alcohol and marked on the shell in preparation for the drilling of holes, care being taken to avoid areas rich in blood vessels.

3- The virus is injected via the appropriate route, and the hole is covered with tape, glue or wax.

4- Contaminated eggs (which appear 24 h post inoculation) are discarded such eggs can often be detected by their smell!

5-Eggs are chilled and harvested 2-5 days post infection.

Chorioallantoic membrane Inoculation:

This membrane is usually selected as an inoculation site for the quantity of virus, viral morphology and identification. The procedure outlined below is used routinely in our laboratory for demonstrations of the herpes simplex virus pock assay:

1. Candle 9-11 day old embryonated hen eggs to determine viability (described earlier).

2. Mark the air sac and an area for inoculation over the centre of the chorioallantoic membrane free from blood vessels.

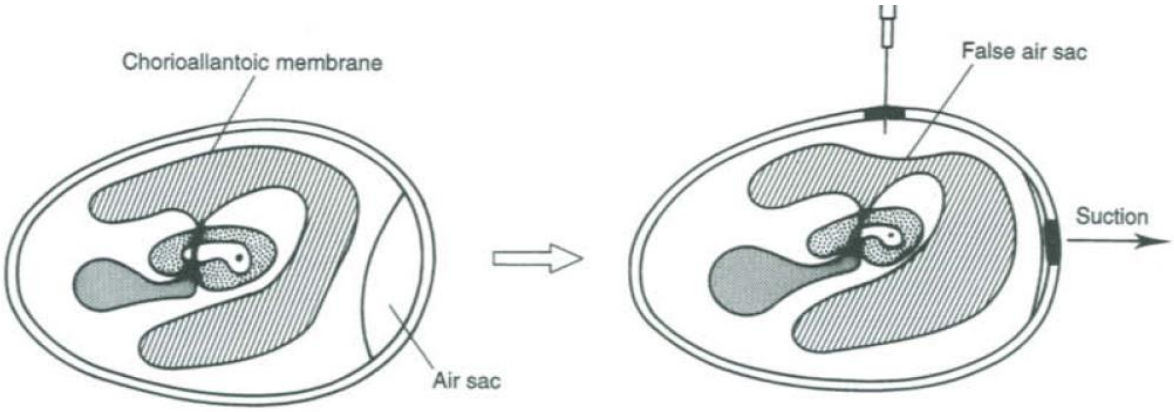


Figure 2: Creation of false air sac in embryonated egg.

3. Swab both areas of the egg with alcohol and drill a 3-4 mm groove in the shell at centre of the air sac and also over the CAM (position already marked). Avoid damage to the shell membrane.

4. Using a sterile mounting needle puncture the shell membrane over the air sac.

5. Place a small drop of sterile saline on the exposed shell membrane over the CAM.

6. Place the tip of a mounting needle or hypodermic syringe, through the drop of saline, on the exposed shell membrane, vertically to the long axis of the egg, and press gently but firmly to split the fibers of the shell membrane. Avoid puncturing the CAM which lies directly beneath.

7. Using a rubber teat apply suction to the air sac hole. The chorioallantoic membrane should detach and drop away from the shell membrane, the drop of saline acting as a 'wet wedge'.

8. The contents of the egg become transposed and an artificial air sac forms beneath the hole over the CAM. Check this by candling.

9. Using a 1 ml syringe filled with an appropriate virus dilution, inoculate the eggs (by inserting the needle into the artificial air sac through the hole over CAM to depth of about 4-5 mm) with 0.1 ml of virus suspension. Inoculate two eggs with each dilution of the virus.

10. Rock the eggs gently to distribute the inoculums.

11. Seal the holes in the shell with adhesive tape or wax.

12. Incubate the eggs at 37Ċ for two days in a humidified environment with the false air sac uppermost.

13. Chill eggs at 4 Ċ overnight prior to harvesting.

14. Place each egg in a petri dish with the inoculated area uppermost.

15. Cut the egg into two halves around the long axis using sterile scissors.

16. The chorioallantoic membrane should remain in the top half of the shell and can be removed with forceps. Discard the rest of the egg contents.

17. Place the harvested membrane in a fresh petri dish containing saline.

18. Wash the membrane free of yolk, shell, etc., and transfer to a further petri dish containing 10% normal saline.

19. Examine the membranes for presence of pocks. This is easier if they are placed over a black background. Count the pocks and calculate pock forming units per milliliter of original virus suspension.

Viruses Cultivation (Propagation)

3- Cell culture technique

This system of cultivation have several advantages over the animal and embryonated hen's egg inoculation because it is easier, more economical, the cells have no or seldom to have antibodies or other inhibitors to interfere with the growth of the virus and the effect of the virus on cell cultures can often be detected in a very short time. It is the most common method to grow viruses today.

There are three basic types of cell culture:

1- **Primary Cells**: made by dispersing cells from freshly removed host tissues, e.g., Monkey Kidney and chick embryo.

- *Advantages:*
  1) they are thought to represent the best experimental models for in vivo situations.
  2) they may express characteristics which are not seen in cultured cells.
  3) broader range of viral susceptibility.
- *Disadvantage:*
  1) Latent viruses may be present especially in primary monkey kidney cells.
  2) Unable to grow for more than a few passage (once or twice).
  3) Some are very expensive and difficult to obtain a reliable supply.

2- **Secondary Cells**: (diploid cell lines) may be passed up to 50 passages but retain their normal chromosome pattern, e.g. Human embryonic kidney (HEK), skin fibroblasts and MRC5 (human lung fibroblast).

- *Advantage:* These cells are widely used to study viruses *in vitro* and for vaccine production.
- *Disadvantage:* they do not continue to divide indefinitely and eventually, their physical characteristics may change, after which the cells will eventually senesce and die.

3- **Continuous cells**: These are immortalized cells; capable of passage indefinitely, altered & irregular numbers of chromosomes, e.g., Vero, Hep2 and tumor cell lines like HeLa cells.

- *Advantage:* May be the most easy to handle.

- *Disadvantage:* The range of viruses supported are limited.

Problems with cell culture:

- No one cell culture type can support the growth of all medically relevant viruses. Hence, virology laboratories must maintain several different cell culture types. The minimum requirements are HeLa and/or RD cells used for the isolation of enteroviruses, Baby Hamster Kidney 21 (BHK 21) cell lines used for the isolation of herpes simplex viruses, Madin-Darby Canine kidney cells (MDCK) and embryonated eggs for isolation of influenza viruses, Human Epithelial type 2 cells (Hep2) for the isolation of Respiratory Syncytial virus (RSV). Viro cells used for cultivation of measles and rubella viruses. A recent modification of the traditional cell culture involves the use of genetically engineered cell lines such as the L20B, which is used for the isolation of polioviruses.
- Long period (up to 4 weeks) required for result.
- Often very poor sensitivity, sensitivity depends to a large extent on the condition of the specimen.
- Susceptible to bacterial contamination.
- Susceptible to toxic substances which may be present in the specimen.
- Many viruses will not grow in cell culture, e.g. Hepatitis B, diarrheal viruses, parvovirus, papillomavirus.

Materials required for tissue culture:

1- **Cells**: e.g., MDCK cells.
2- **Tissue culture flasks or tubes, canted neck** (e.g., T-25, T-75, etc.).
3- **Complete Media** (may be *growth* , *maintenance media and freezing media*): e.g., Dulbecco's Modified Eagle Medium (**D-MEM**) and **RPMI Medium 1640**. These consist of medium plus:

a- **Antibiotics**: e.g., Penicillin or Vancomycin and Streptomycin or Gentamicin, stock solution (10,000 IU/ml penicillin G; 10,000 µg/ml streptomycin sulfate). Add 1ml concentrate to each 100ml cell culture fluid.

b- **Serum**: e.g., fetal bovine serum (FBS), act as protein source not been completely settled as yet. Used **10-20%** in growth media, **2.5%** in maintenance media and **20%** in freezing media. Various reasons have been suggested:

- The serum involved in the adhering of cells to the glass surface.
- The serum acts a carrier for one or more trace materials such as amino acids, vitamins, etc…..
- Serum may act as a detoxifying agent.

c- **L-glutamine** (1%): it has short life time so it adds to media immediately.

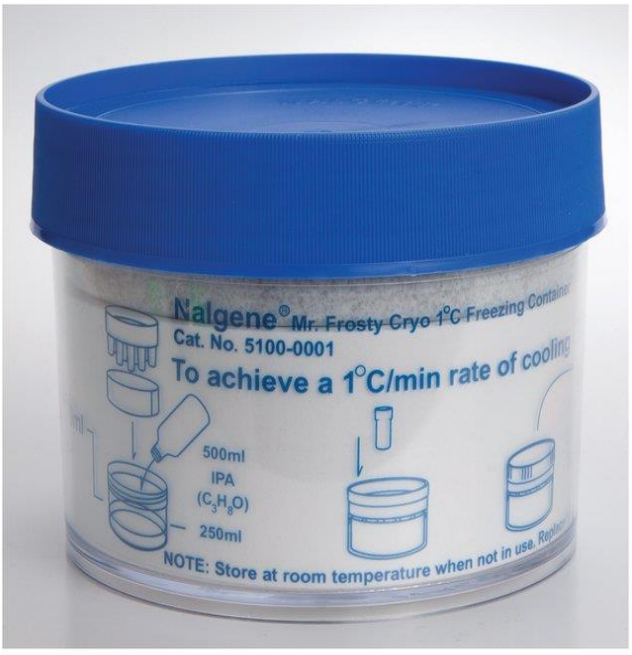
d- **HEPES** or sodium bicarbonate buffer (1%).

4- Trypsin: is used for dispersing & harvesting tissue cells from organs or cells sheets grown in vitro. It may be obtained in powder or crystalline form, powdered trypsin has been found to give excellent results.

a. Prepare 0.25% solution of trypsin in (Hanks Balanced salt solution BSS) containing phenol red.
b. Allow trypsin to dissolve in refrigerator overnight & then sterilize by Millipore (0.22 Mm) filtration.
c. Adjust to PH=7.4 & store in small quantities at -20 C°.

Do not expose the trypsin solution to temperatures above 37 C° when thawing several times, precipitate may form & the solution loses its effectiveness.

5- Dimethyl Sulphoxide (DMSO): is used with **freezing media** for cell or virus preservation. The most common freezing medium is **20% FBS+10% DMSO**. After centrifugation, resuspend the cell pellet in 1mL of freezing medium/cryovial 1 C°/min freezing container **(Mr. Frosty)** with isopropanol gradually process by placed at -80 C° for at least 5 hours, then transfer to liquid nitrogen storage container (-196°c) and record storage details.



Preparation of Primary Tissue Culture:

Chick embryo fibroblast cell culture: Most tissue whatever the source can be processed similar, but there are number of modification used in the preparation for ex: trypsinization may be carried out refrigerator temperature R.T. 4° C or at 37 °C.

In preparation of chick embryo fibroblast culture (cell culture), all instruments, glassware, solution & media should be sterile.

Procedure:

1. Select 9-11 embryonated egg (with a live embryo).
2. Swab the egg with 70 % alcohol & place with blunt end uppermost in small beaker.
3. Crack the top of shell & peel off to the edge of the air sac with sterile forceps as shown in figure 1.
4. Peel off the white shell membrane to reveal the (CAM) below with its blood vessells.
5. Transfer embryo to a 9 cm petridish containing Dulbecco's phosphate-buffered saline (**D-PBS**) or EmbryoMax Dulbecco's Phosphate Buffered Saline with Ca^{++} & Mg^{++} **(D-BSS**).

Note: take care not to apply any pressure extract it very slowly from egg.

6. Remove the head, feet & wings by cutting them with sterile scissors then cut the torsos to very small fragments.
7. Put the fragments in sterile flask & wash the tissue three times with PBS to remove blood as shown in figure 2.
8. Transfer tissue to trypsinization flask containing prewarmed to 37 C° (0.25%) trypsin & stir on magnetic stirrer, for 30 min. at 35 C°.
9. The suspension is then filtered through 6 layers of sterile gauze & collect the suspension in sterile container, contain 1ml FBS.
10. Centrifuge at 1000 rpm for 10 min.
11. Wash the cells with PBS or Hanks solution & recentrifuge at 1000 rpm for 10 min. then discard the supernatant.

12. Re-suspend the packed cells in sufficient growth medium at concentration of $1X10^6$ cell/ml (counting of cells done with haemocytometer).
13. The final suspensions are distributing into T-75 flasks, incubate at 37 C° and follow up until monolayer formation.
14. The preparation is incubated at 37 C° over night before use.

Cells counting with Trypan blue (vital stain):

To determine the number of viable cells per milliliter in the original suspension, using the following formula:

[(No. of cells counted) ÷ (no. of squares counted)] x [counting chamber conversion factor] x [dilution factor] = cells per ml.

Example: 1 part of dye added to a part of cell suspension = 1:2 dilution, therefore the dilution factor = 2. The conversion factor of the hemocytometer = 10,000. Therefore, 100 (cells) ÷ 4 (large squares) x 10,000 (conversion) x 2 (dilution)

$= 5 \times 10^5$ cells/ml

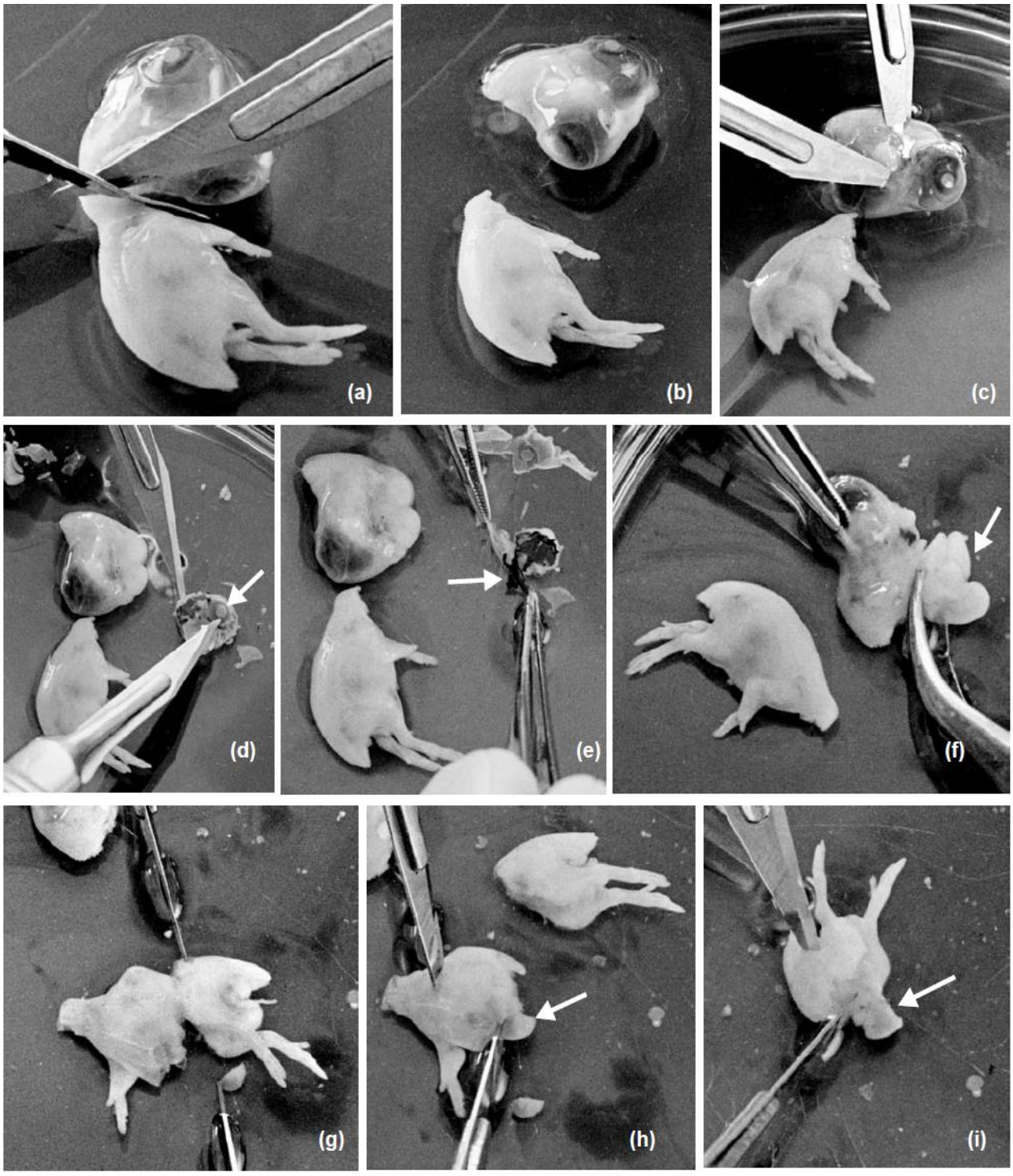
The volume of the initial cell suspension needed to seed subsequent cultures is determined by the standard volume/concentration equation:

Initial Concentration x Volume of Initial Concentrate = Final Concentration x Final Volume. For example: To determine what volume of a cell suspension containing 5×10^5 cells/ml should be diluted to 1 ml to yield 1×10^4 cells/ml

5×10^5 cells/ml × (X ml) = $1x\ 10^4$ cells/m1× 1 ml

Xml = $1x\ 10^4$ cells/m1× 1 ml ÷ 5×10^5 cells/ml

X = 0.02 ml. Therefore: 0.02 ml of the original suspension diluted with 0.98 ml of growth medium will yield 1 ml of suspension at 1×10^4 cells/ml.


(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)

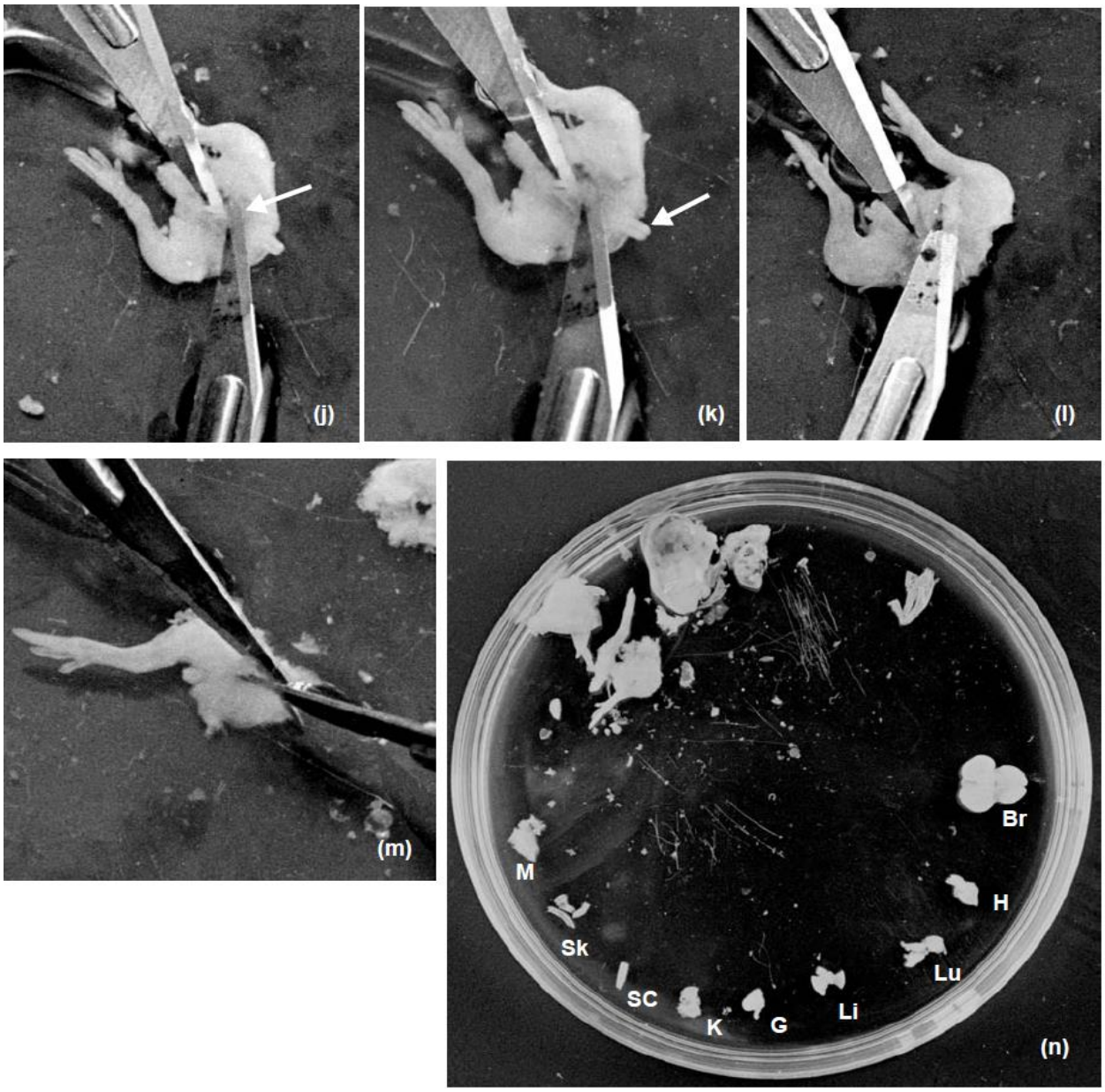


Figure 1: Dissection of a Chick Embryo. (a), (b) Removing the head. (c) Removing the eye. (d) Dissecting out the lens. (e) Peeling off the retina. (f) Scooping out the brain. (g) Halving the trunk. (h) Tearing out the heart and lungs from the anterior half. (i) Teasing out the liver and gut from the posterior half. (j) Inserting the tip of the scalpel between the left kidney and the dorsal body wall. (k) Squeezing out the spinal cord. (l) Peeling the skin off the back of the trunk and hind leg. (m) Slicing muscle from the thigh. (n) Organ rudiments arranged around the periphery of the dish. From the right, clockwise, we have the following organs: brain, heart, lungs, liver, gizzard, kidneys, spinal cord, skin, and muscle.

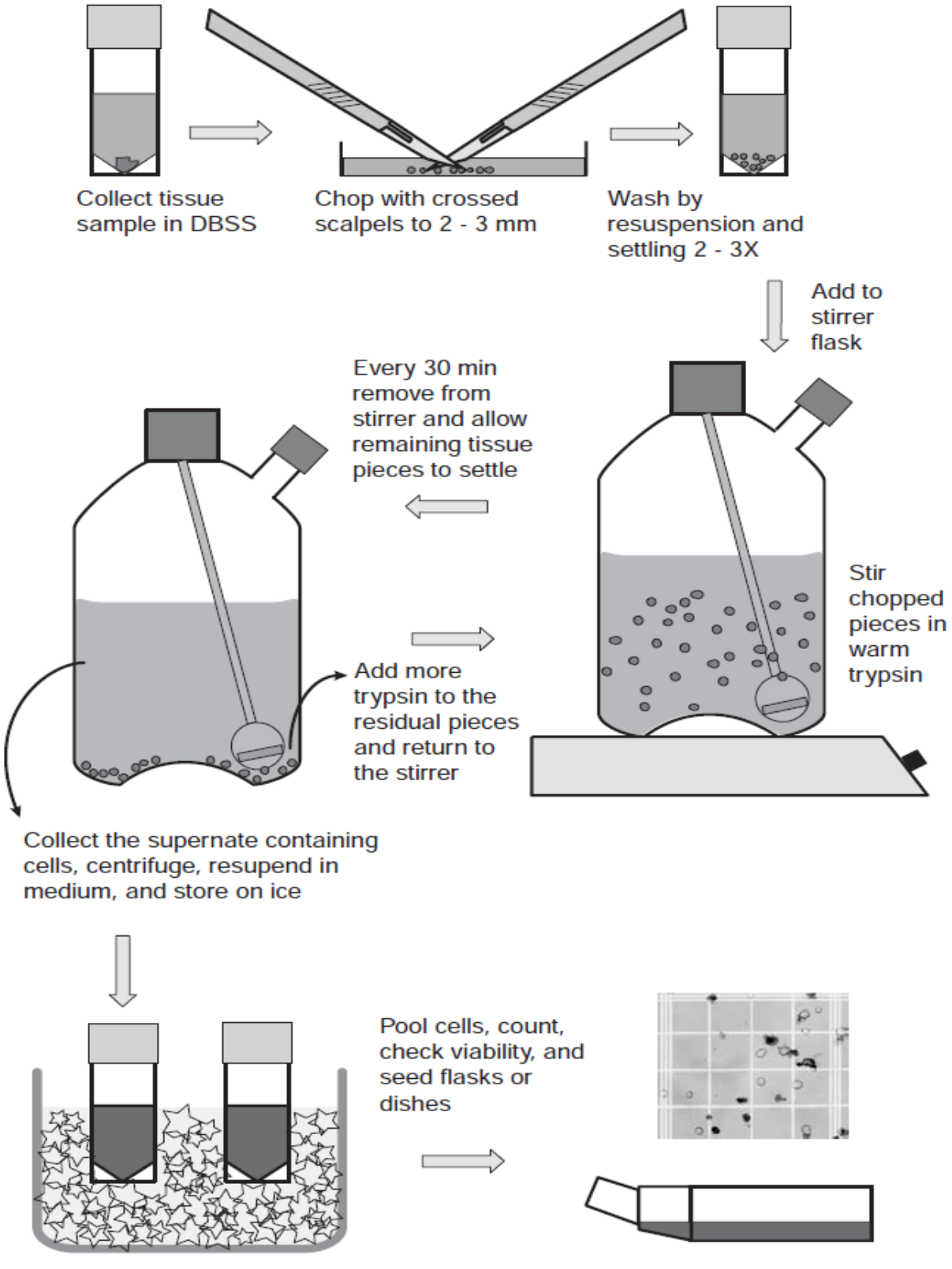


Figure 2: Schematic representation of the preparation of primary tissue culture

Subculture and storage of adherent and suspension Cells:

Prepare to subculture the cells when the culture has reached around 80% confluence in T-75 flask. The following protocol is for adherent cells; for suspension cells, start at step **8**. All procedure involving cell lines should be performed in a Class II Biosafety Cabinet.

1. Pre-warm the complete growth media, PBS and (1X) trypsin-EDTA at room-temperature or 37°C water bath before use (Prewarming).
2. Carefully aspirate the culture media from T-75 flask without disturbing the cell monolayer.
3. Wash cells by adding 5 ml of PBS, Swirl and then remove the PBS.
4. Add 5 ml of pre-warmed Trypsin-EDTA to the flask, gently rock the flask.
5. Incubate for 5-10 min in the incubator until the cells start to round up and detach from each other. Observe the cells under a microscope to confirm they are rounding up.
6. Add an equal volume of the complete media into the flask to inactivate the Trypsin-EDTA.
7. Transfer **the culture suspension** to a sterile centrifuge tube.

A- Subculture protocol:

8. Centrifuge the cell suspension at 1500 rpm for 3 minutes.
9. Aspirate the supernatant after checking all cells are pulled down into the pellet. Re-suspend the cell pellet in **10 ml** pre-warmed fresh complete media.
10. Perform a cell count and viability determination. For maintenance spilt cells 1:10 or 1:5 (this may vary depending on how fast the cells are growing). For a **10 ml** cell suspension, this means **1ml (1:10)** and **2 ml** (1:5) per flask. Add extra medium up to a total volume 10 ml.
11. Place the newly seeded culture vessel in a 37℃, 5% CO_2 incubator. Incubate for at least 48 –72 hr before processing the cells for downstream experiments.

B- Storage protocol:

1- Pellet cells at 1500 rpm for 3mins. Discard media into waste bottles.
2- Resuspend cell pellet in 1ml of freezing medium (**20% FBS complete media + 10% DMSO**) and place immediately in a pre-labled cryo vial. Labeled with cell name, passage number, date and initials of the person freezing the cells.

3- Put the cryo vial in Mister Frosty (cell freezing container). Immediately put the Mr. Frosty in a -80°C freezer.

4- The following day, transfer the cells to the appropriate rack in the liquid nitrogen storage container.

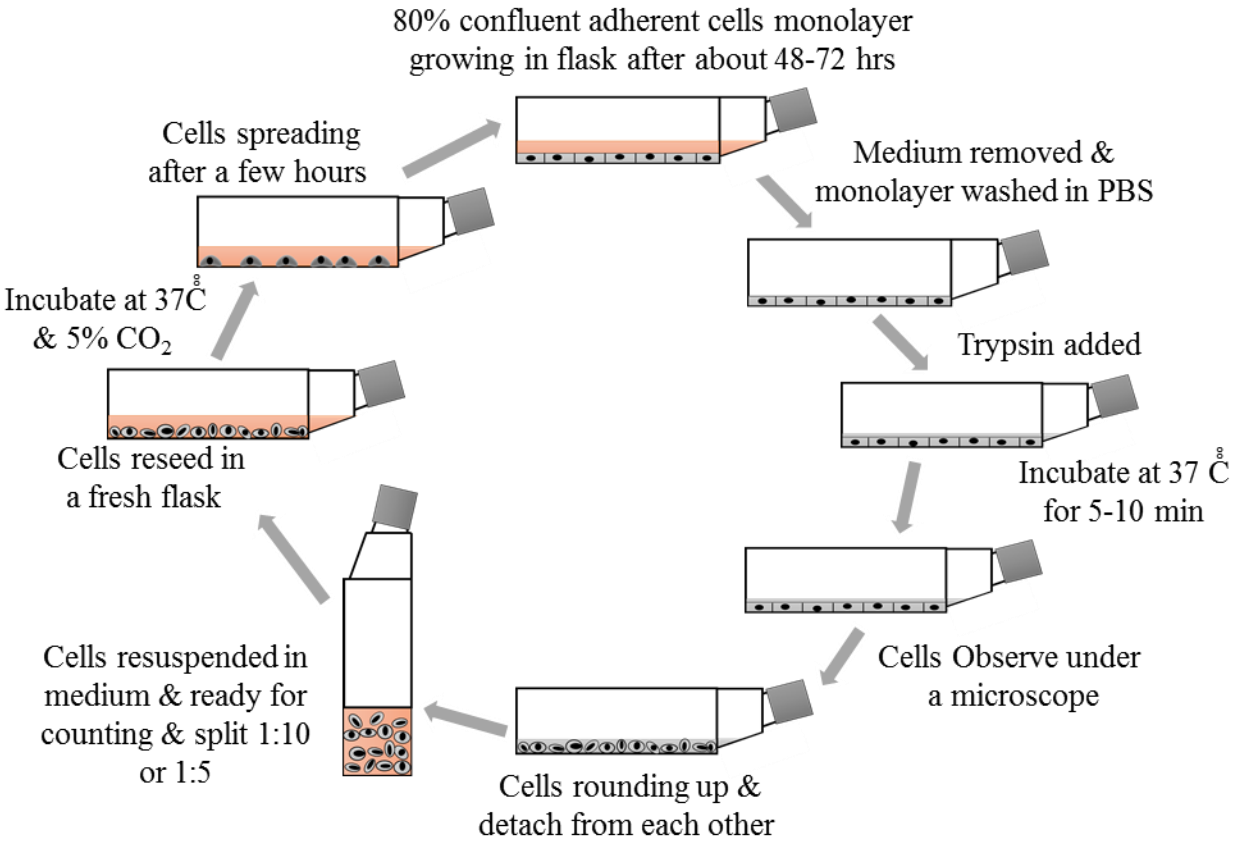


Figure: Schematic representation of subculture of adherent and suspension cells

Inoculation MDCK cells with clinical samples to isolate Influenza virus:

1. Remove D-MEM from flask containing MDCK cells.
2. Inoculate 200 µl of each specimen into a T-25 flask using sterile pipettes.
3. Allow inoculum to adsorb for 30 minutes at 37Cº. Rock the flask to distribute the virus evenly.

4. Add 6ml of complete media (D-MEM) containing 2 µg/ml of L-1-Tosylamido-2-Phenyl ethyl chloromrthyl keton (**TPCK) trypsin** without serum to T-25 flasks.

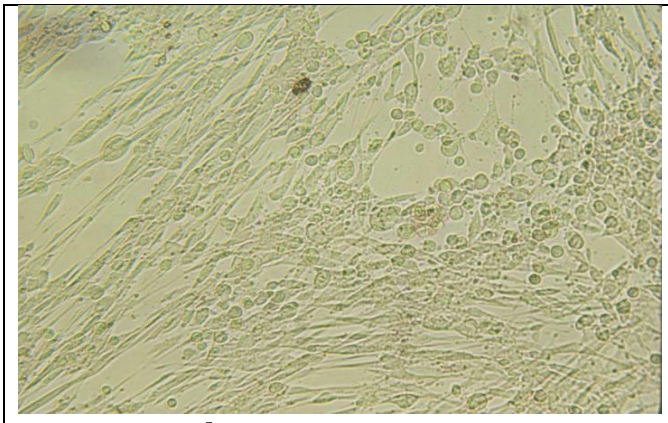
5. Observe daily for cytopathogenic effect (CPE).

Recognition of Virus Growth in Cell Cultures:

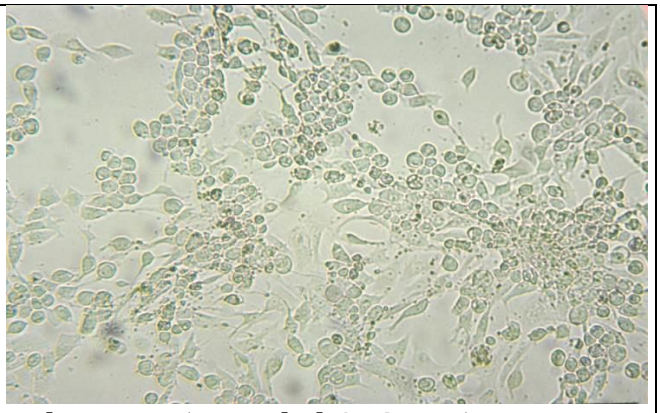
1-Viral Cytopathological Effects (CPE):

The CPE is visualizing a morphological changes in the cells when the virus growth in cell culture leads to cell death. CPE is characterized by:

- **The cell is rounded** with darkened margins and pyknotic nuclei e.g., polio, Echovirus, coxsackie virus A&B, herpes and varicella virus. Some cells progress to **aggregation** or **degeneration**.

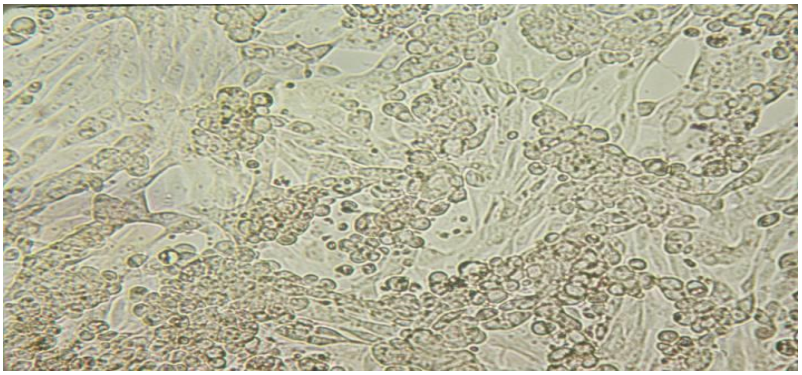


Herpes simplex Virus

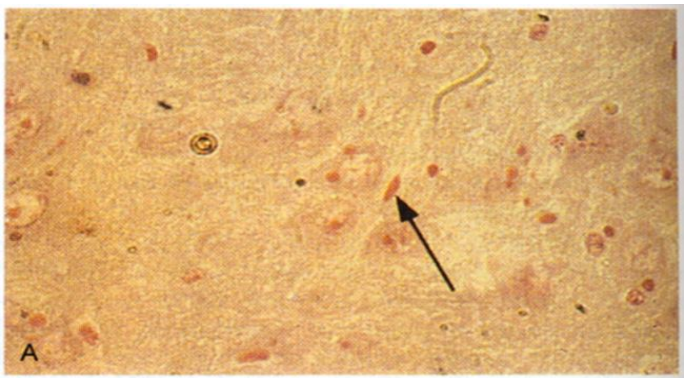


Poliovirus (Rounded & Grape)

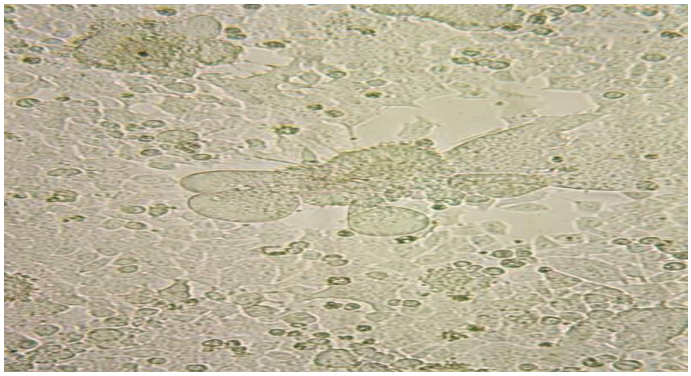
- **Grape**: is like clustering of rounded cells to form a pock e.g., Adenovirus.



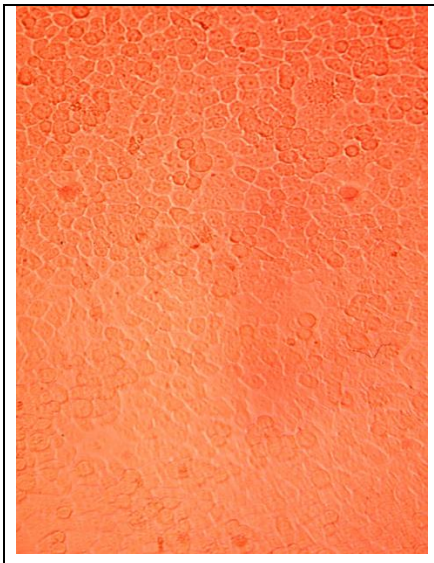
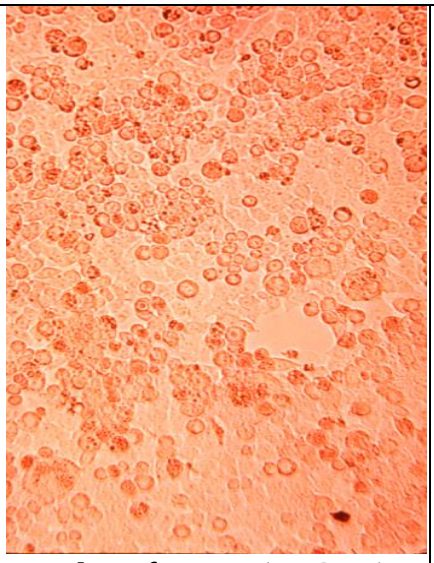
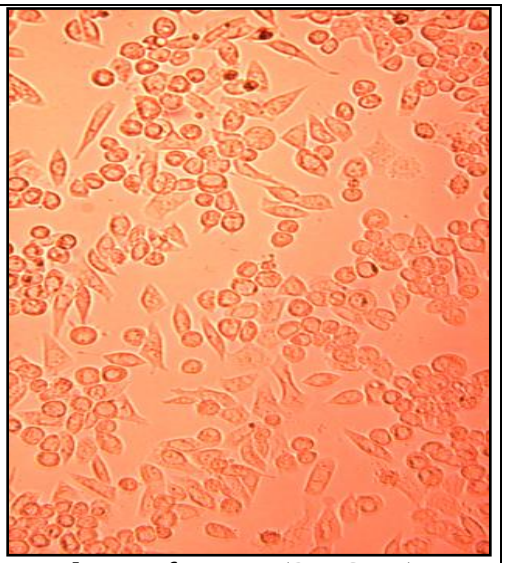
- **Inclusion bodies** in the nucleus or cytoplasm, margination of chromatin e.g., rabies virus.



- **Syncytia**: multinucleated giant cells caused by virus-induced cell-cell fusion that from slowly virus like measles, mumps and respiratory synsetial virus RSV.

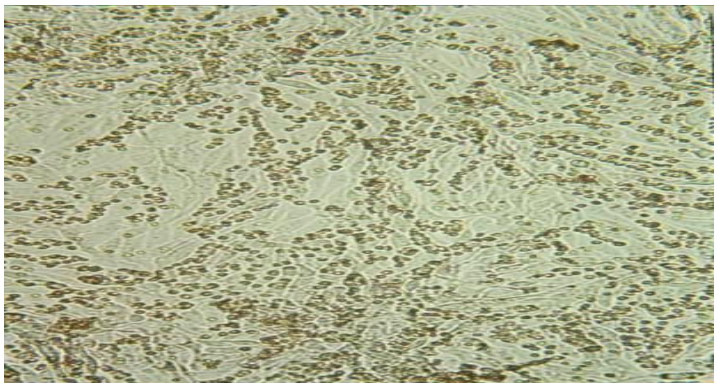


Note: Harvest by day 6 of cell culture incubation, if **3+ or 4+** CPE is observed by collecting supernatant fluid and adding stabilizer such as glycerol gelatin with bovine serum albumin to a final concentration of 20%. Whereas **1+** CPE during first week is considered as **false positive** or **low virus titer**, thus should perform passage 2 (P 2) to confirm result after freezing-thawing. Disappear of CPE in P 2 is meaning false positive for virus infection.

		
Uninfected	early infection (2+CPE)	late infection (3+ CPE)

2- Cell surface changes:

- Viral antigen expression (fluorescent specific antibodies).
- **Hemadsorption test** (hemagglutinin expression): is used to detect the presence of viruses that bind to red blood cells. It occurs when the red blood cells in an infected cell attach to the virus. This assay can be useful for detecting viruses that produce few or no CPE like measles virus.



Quality control:

Over a number of passages (15-20) MDCK cells might lose their susceptibility to respiratory viruses. For this reason, the laboratory should keep a stock of cells at a low passage level (baby cell) frozen in liquid nitrogen with 10% DMSO and 20% FBS. At intervals, the sensitivity of the MDCK cell line must be assessed using positive control virus of known titer tissue culture infective dose 50 (TCID50). If sensitivity of the isolation system is to be changed, a new vial of cells must be thawed out.

Quantitative assay of viruses (Viral load)

For most virology experiments, it is essential to know the concentration of the total or infectious virus particles. Two major types of quantitative assays for viruses exist, **biological & physical** methods.

Biological methods:

1- End point (Tissue Culture Infective Dose $_{50}$=TCID$_{50}$):

The $TCID_{50}$ is defined as that dilution of a virus required to infect 50% of a given batch of inoculated cell cultures. The assay relies on the presence and detection of cytocidal virus particles (i.e., those capable of causing CPE).

Procedure:

1. Seed cells into 96-well plates at a density of cells which will be confluent on the day of virus assay.

2. Make serial dilutions of virus suspension using **DMEM + 2.5% FCS** as diluant.

3. Remove tissue culture growth medium from healthy confluent monolayer and replace with **0.1 ml** dilution of virus. Set up at least 5 wells per virus dilution.

4. Also include at least 5 control wells which contain diluent alone (no virus).

5. Incubate at 37C°, 5% CO_2 and monitor the development of CPE. Record CPE after a designated time, having observed the cell control wells first.

6. CPE is usually graded on a 0-4 system: 0 (no CPE), 1 (less than 50% of cells showing CPE), 2 (about 50% of cells showing CPE), 3 (about 75% of cells showing CPE) and 4 (the monolayer is totally destroyed or shows 100% CPE).

7. Calculate the $TCID_{50}$ counting all the wells with 1-4 CPE as being positive according to the **Reed and Muench method** for estimating $TCID_{50}$.

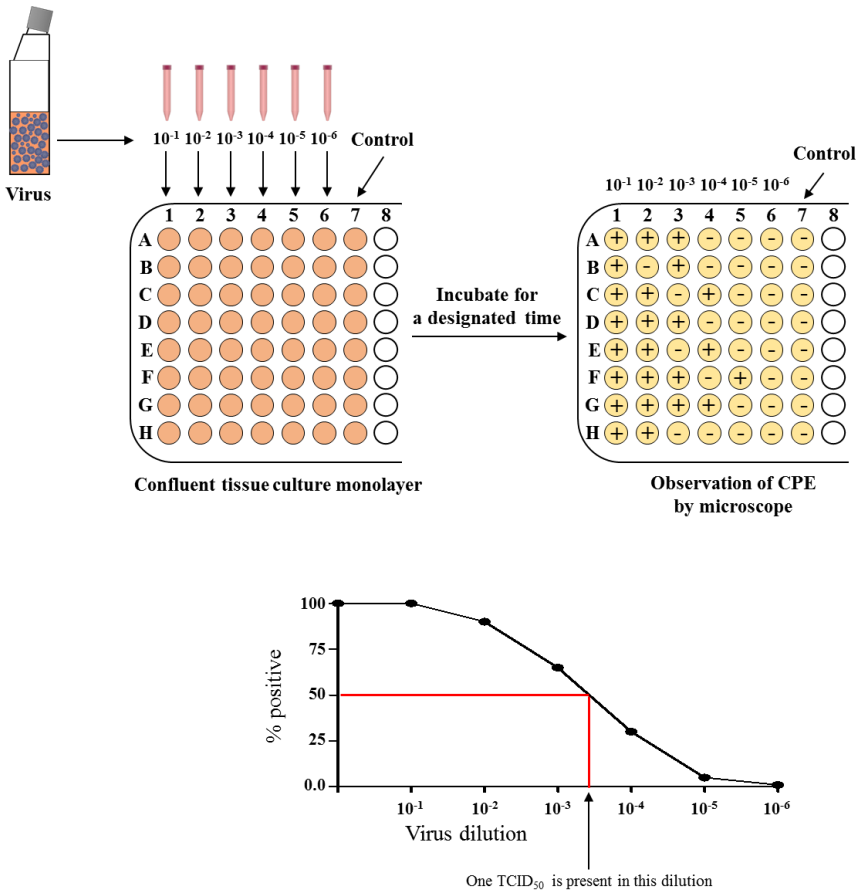


Figure: Schematic representation of the $TCID_{50}$ assay

Virus dilution	Infections per number inoculated	Observed values		Cumulative values[*1]		Infection ratio[*2]	% infection[*3]
		Positive	Negative	Positive	Negative		
10^{-1}	8/8	8	0	24	0	24/24	100
10^{-2}	7/8	7	1	16	1	16/17	94
10^{-3}	**5/8**	**5**	**3**	**9**	**4**	**9/13**	**69**
10^{-4}	3/8	3	5	4	9	4/13	31
10^{-5}	1/8	1	7	1	16	1/17	6
10^{-6}	0/8	0	8	0	24	0/24	0

[*1] The accumulative vales are derived by adding up the observed values in the direction of the arrows.

[*2] The infection ratio is the number of positive for the cumulative value out of the total for the cumulative value.

[*3] The % infection is the infection ratio converted to a percentage.

It had already been determined that the dilution of virus that contains one $TCID_{50}$ lies between 10^{-3} and 10^{-4}, so the end point can be expressed as $10^{-(3+x)}$, where x is the value to be estimated.

$$X = \log_{10} \text{dilution factor} \left[\frac{\text{\% infection at next dilution above 50\% - 50}}{\text{\% infection at next dilution above 50\% - \% infection at next dilution below 50\%}} \right]$$

$$X = 1 \left[\frac{69-50}{69-31} \right] = 0.5$$

End point = $10^{-(3+0.5)} = 10^{-3.5}$

i.e. 1ml of a $10^{-3.5}$ dilution contains one $TCID_{50}$ of virus.

i.e. 1ml of a **1/3200** dilution contains one $TCID_{50}$ of virus. (3.2 is the antilogarithm of 0.5).

The concentration of virus in the undiluted suspension is 3.2×10^3 $TCID_{50}$/ml.

Also, other applications of the end point are used in **cell Sensitivity** to virus and for **the Neutralization antibody test**.

2- Plaque assay:

The plaque assay is an infectivity assay that quantifies the number of infectious units in a given virus suspension. Plaques are localized discrete foci of infection denoted by zones of cell lysis or CPE within a monolayer of other-wise healthy tissue culture cells. Each plaque originates from a single infectious virion thus allowing a very precise calculation of the virus titer. For statistical reasons 20-100 plaques per monolayer are ideal to count, although the actual number is often dependent on the size of the plaque and the size of the vessel used for the assay. The infectivity titer is expressed as the number of plaque forming units per ml (pfu/ml).

Monolayer plaque assay procedure:

1. Day 1: Seed cells (BHK, Vero or any sensitive cells to virus infection) into 6-well plates.
2. Day 2: On the day of assay observe the cell monolayers, which must be healthy and 90-100% confluent.
3. Set up a series of 10 fold dilutions for virus using **DMEM + 2.5% FCS** as diluent. Mix well between each dilution and keep on ice.
4. Discard the medium from the previously seeded 6 well plate (s).

5. Carefully add 0.1 ml virus dilution to each of duplicate tissue culture monolayers and gently rock the dish to achieve an even distribution of virus.
6. Allow the virus to adsorb for 30 mins at 37°C in a 5% CO_2 incubator with rocking at intervals (10-15 min) to avoid the cells drying up.
7. During the last five minutes of virus adsorption, prepare an overlay medium using **2% Low Melting Point Agarose (LMP)** + **2X DMEM/5%FCS**.
*The LMP is prepared in advance and sterilize by autoclaving. Melt carefully in the microwave, and place the bottle in the 37°C water bath. Also warm up the **2X DMEM/5%FCS** to 37°C. **Mix equal volumes** of **2% LMP + 2xDMEM + 5 %FCS**.
8. Carefully, remove un adsorbed virus (virus inoculum).
9. Add 4 ml of overlay to the cells and allow to solidify at room temp, then transfer to tissue culture incubator.
10. After 48-72 hr., fix plates in Formalin (10%) for at least 2 hr.
11. In fume hood, remove the formalin and place in formalin waste container. Using a spatula carefully flick of the agarose layer.
12. Add 2 ml of crystal violet solution (10%) for 10 min. Wash the plates carefully under the tap with running water.
13. Invert and drain the plates and count the plaques and determine the virus titer.
e.g.: If the average plaque count from the two wells at 10^{-7} dilution was 50, then the titer is calculated as follows:
No. of plaques x reciprocal of dilution factor x reciprocal of volume inoculum in ml= pfu/ml
$50 \times 10^7 \times 10 = 5 \times 10^9$ pfu/ml

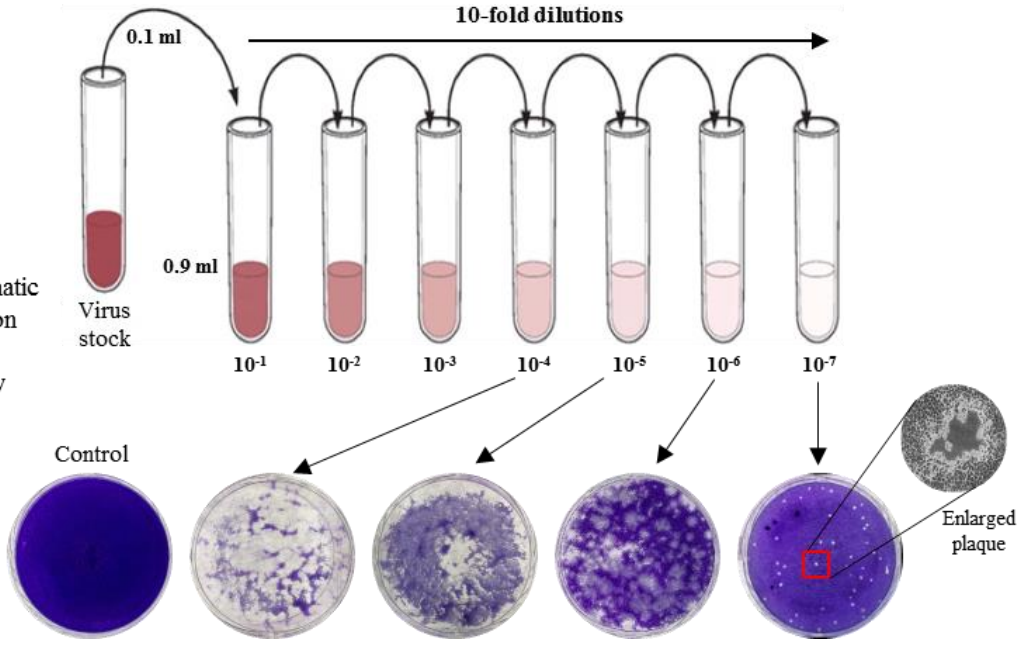
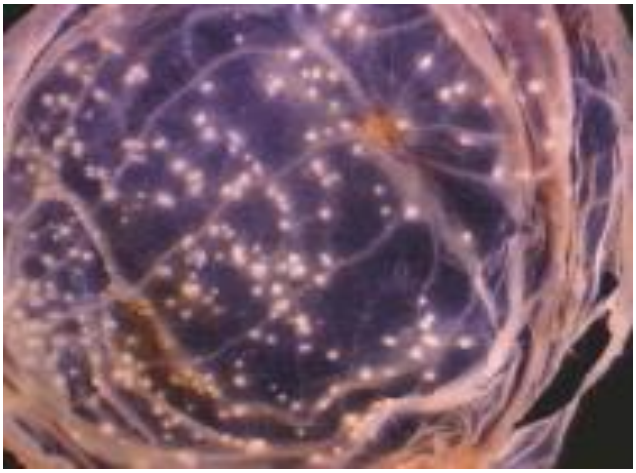


Figure: Schematic representation of the viral plaqe assay

3- Pock assays:



A pock is essentially an area of inflammatory response which results from the virus invasion of an epithelial cell on the chorioallantoic membrane (CAM) of a fertile hen's egg. Like the plaque assay the method is quantitative and each pock results from the infection caused by an infectious unit of virus (pock forming unit).

Pock assay procedure:

Retain to lab.3 from 13-18 points. The infectivity titer is obtained in the following way:

Pock number x reciprocal of dilution x reciprocal of volume in ml

e.g. if there is a mean number of 50 pocks from eggs inoculated with 0.1 ml of a 10^{-6} dilution there are:

$50 \times 10^{6} \times 10 = 5 \times 10^{8}$ pkfu/ml

Quantitative assay of viruses (Viral load)

Physical, Biochemical methods:

1- Direct Particle count.
2- Heamagglutination.
3- Immunological tests for proteins.
4- Assay of nucleic acid (Southern blot, Real-Time PCR).
5- Enzymatic (Reverse transcripts for retroviruses).

℘ Direct particle count:

The concentration of virus particles in a sample of purified virus can be counted directly using an electron microscope. Briefly, a purified preparation of virus is mixed with a known concentration of microscopic marker particles (e.g., latex beads), which can be easily distinguished from virus particles in the **electron microscope.**

Problems with Electron Microscopy

- Expensive equipment and maintenance.
- Require experienced observer.
- Sensitivity often low about 10^6 particles/ml.

℘ Principle of viral hemagglutination:

Many viruses have hemagglutinin (hemagglutinate RBCs) that are able to adhere to the surface of erythrocytes by the reaction of agglutinin protein (this specific protein project from envelop of orthomyxoviruses such as: (Influenza A, B and C), mumps, Newcastle, rubella and respiratory syncytial virus) and receptor sites on RBCs membrane (mostly glycoprotein) this reaction occurs as visible agglutination.

Screen test for hemagglutination:

The screen test is very simple and gives indication of the presence and viral replication in clinical samples and experimental tests.

1. Add 50µl amniotic or allantoic fluid (Embryonic fluid diluted 1:10) to test tubes 1&2.

2. Tube no.1; add 50µl of 0.5% RBCs suspensions (guinea pig RBCs or human group RBCs).

3. Tube no.2; add 50µl of 0.5% chick embryo RBCs suspensions.

Set up control tubes 3&4 by mixing 0.5µl of each tested RBCs with 50µl normal saline.

Mix all tubes and incubate in R.T for 1hour.

Examine tubes for agglutination which is indicated by a uniform film of agglutination covering the bottom of the tubes, control tubes RBCs is settle down to form compact bottom.

Note: All blood specimens that used to prepare RBCs must be washed 3 times with saline, preparation of RBCs suspension wanted from immunology Lab.

Titration of virus by hemagglutination (HA):

After the virus can be identified as a type, it is necessary to establish the concentration of the viral agent in term of hemagglutination unit (HAU) in embryonic fluids.

Procedure:

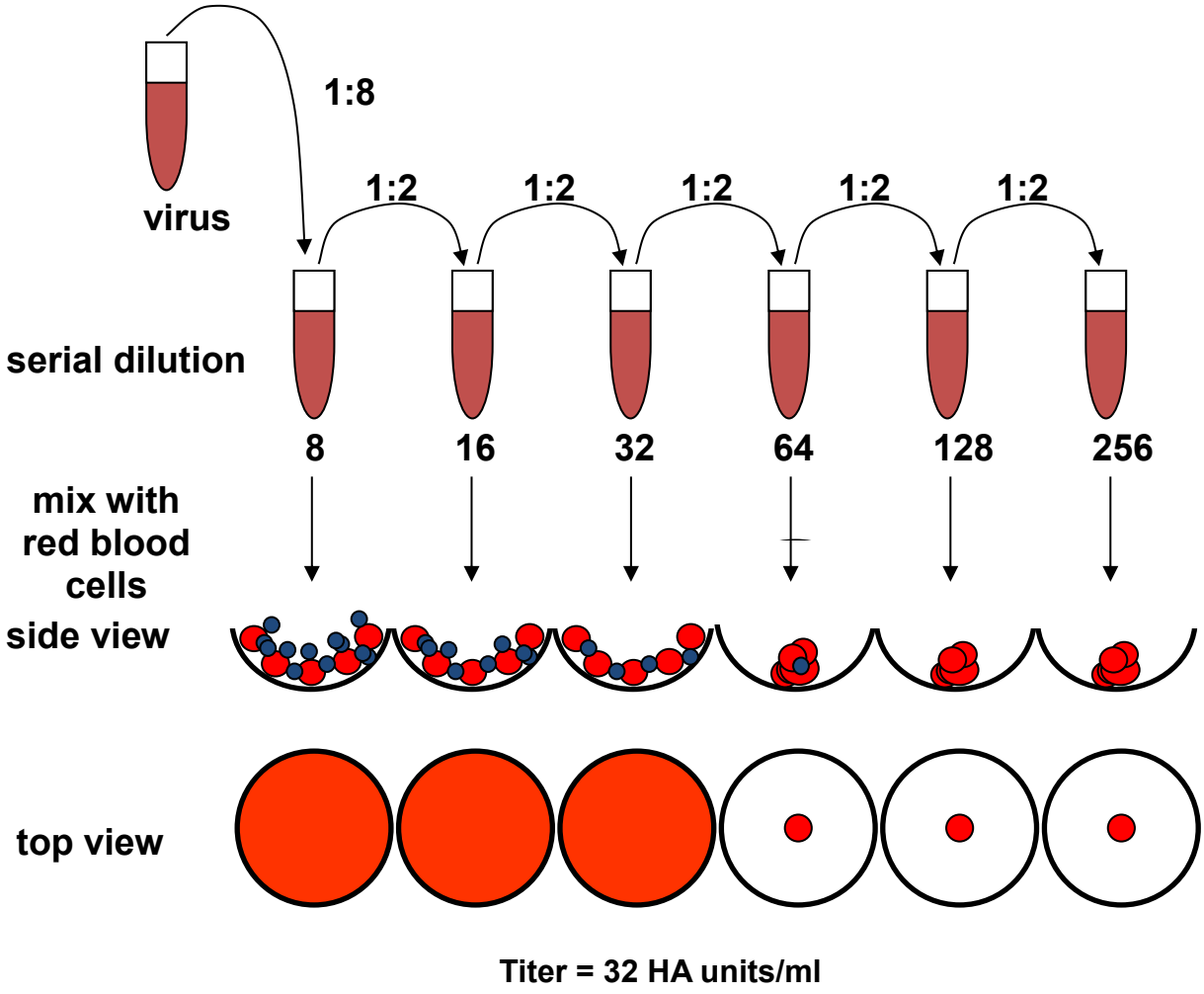
1-Prepare serial 2-fold dilution of allantoic fluid in saline (1:2)-(1:1024).

2-Add 50µl of 1% RBCs suspension of the species that were agglutinated in the screening test to 50µl of each dilution of allantoic fluid. Mix and incubate at R.T. (25Ċ°) for 1hour.

3-Examine for hemagglutination, the concentration of the viral agent with the highest dilution of embryonic fluid (allantoic fluid) at which complete visible agglutinate occur in the end point and 50µl is dilution contains 1HAU/50µl.

Note: To obtain 4HAU/50µl must be diluents the original allantoic fluid according to the following formula:

$$\text{Dilution of fluid containing 4HAU/50µl} = \frac{\text{Reciprocal dilution containing 1 HAU/50 µl}}{4}$$



Titer = 32 HA units/ml

Serological Tests

Serological diagnosis is an indirect test in which the level (titer) of antibody in the patient's serum was determined for specific virus. The role of serology can be for the (1)diagnosis of a cute or current infection or for the (2)determination of immune status to specific viruses. Serological diagnosis is important for viral agents that (3)do not replicate well in cell culture or for which there are as yet (4)no good reagents for direct detection of virus are routinely diagnosed using measurement of antibody. Routine testing for Arboviruses, Epstein-Barr virus (EBV), hepatitis B virus (HBV), hepatitis C virus (HCV), human immunodeficiency virus (HIV), human T lymphotropic viruses (HTLV) and in some circumstances cytomegalovirus (CMV), herpes simplex virus (HSV), varicella-zoster virus (VZV), measles and rubella is performed by serologic techniques. Examples for serological tests: ELISA, Immune EM (IEM), Heamagglutination Inhibition (HI) and Neutralization Test (NT).

℘ Hemagglutination Inhibition Test (HI):

The type of viral isolate from embryonic fluid or tissue culture can be identified by HI test.

Procedure:

1- Mix 50µl of allantoic fluid containing (4HAU/50µl) with 50µl (1:10) dilution of hyper immune type specific antisera (inactivate the complement in antiserum at 56Ċ water bath for 30min prior to use).
2- Set up the tube of antiserum for each virus**.**
3- Add 50µl of 1% RBCs suspension for each tube**.**
4- Set up control**:** Virus control containing 50µl allantioc fluid and 50µl 1% RBCs suspension.
5- RBCs control containing a 50µl RBCs suspension and 50µl normal saline.

Incubate all tubes at R.T. for 1hour. Identify of the virus is indicated by the type of antiserum that inhabits the hemagglutination reaction**.**

℘ Neutralization test (NT):

Virus-neutralizing antibodies are measured by adding serum containing these antibodies to a suspension of virus and then inoculating the mixture into the susceptible cell culture. The presence of neutralizing antibodies is demonstrated if the cell culture fails to develop a cytopathic effect while control cell culture which have a received virus plus serum free antibodies develop CPEs. To establish diagnosis, one looks for a significant virus in antibody titer (4-fold) or greater is desirable during the course of the infection. In recurrent infections e.g., herpes simplex high antibody titer are commonly detected in serial serum samples, the diagnoses rise between acute and convalescent sera is not registered. A positive test in a single sample of the serum is not of diagnostic value in acute infections unless the antibodies belong to the IgM class. Neutralizing antibodies can persist for years (IgG) and their presence may indicate past infection. Although NT is simple in their principle, it's expensive in time, materials and standardized for each viral agent. But, NT application is very important to determine and evaluate the vaccine efficiency that must be given protection against for viral infection.

Antigen, Antibody reaction can be explained the HA, HI and NT assays by: