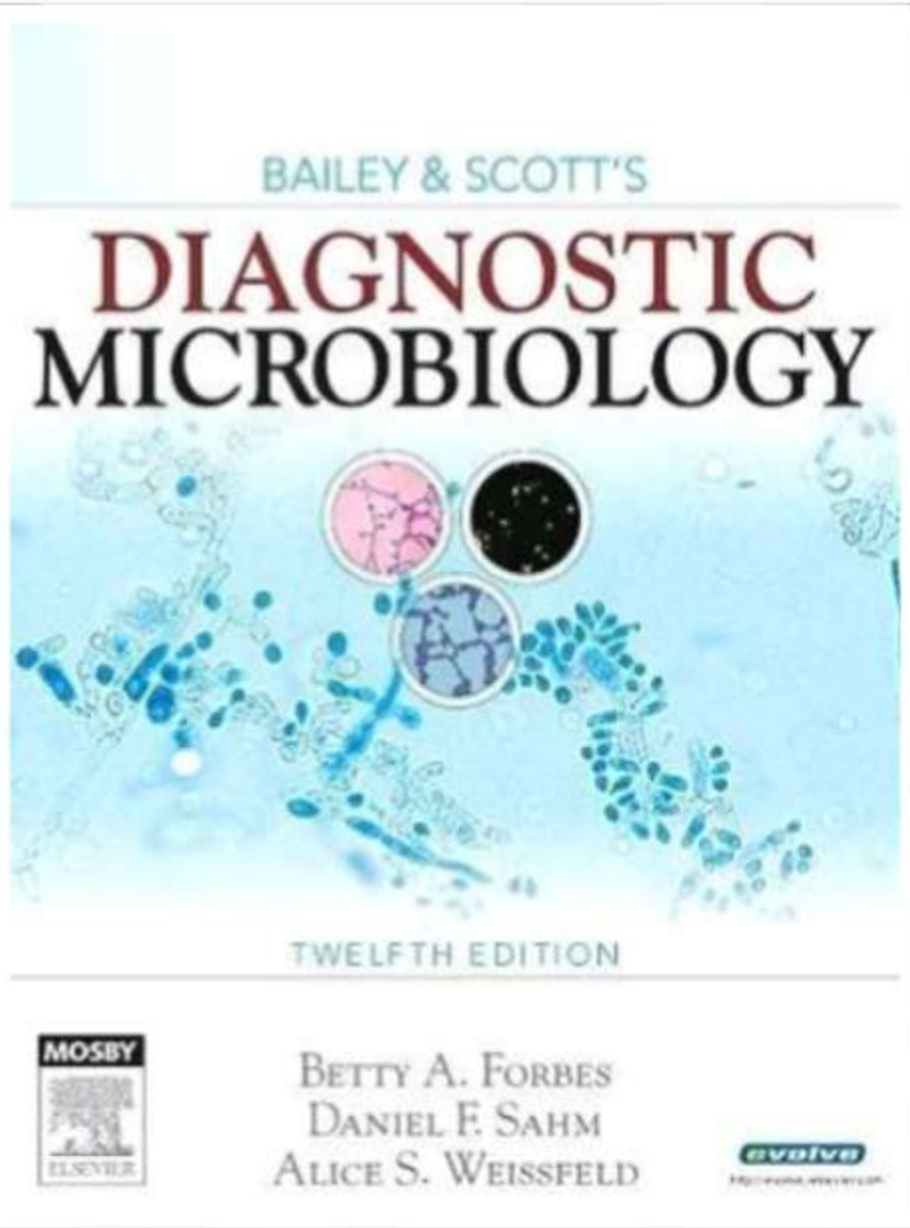




Diagnostic Microbiology 320 MIC

Lecture: 10 Diagnosis of Virus Infections



Specimens for viral diagnosis

Common Pathogenic Viruses	Specimens for Culture	Comments
Respiratory Tract		
Adenovirus; influenza virus; enterovirus (picornavirus); rhinovirus; paramyxovirus; rubella virus; HSV	Nasal washing, throat swab, nasal swab, sputum	Enterovirus is also shed in stool
Gastrointestinal Tract		
Reovirus; rotavirus; adenovirus; Norwalk virus, calicivirus	Stool, rectal swab	Samples are analyzed by electron microscopy and antigen detection (ELISA); viruses are not cultured
Maculopapular Rash		
Adenovirus; enterovirus (picornavirus)	Throat swab, rectal swab	—
Rubella virus; measles virus	Urine	—
Vesicular Rash		
Coxsackievirus; echovirus; HSV; VZV	Vesicle fluid, scraping, or swab, enterovirus in stool	Initial diagnosis of HSV and VZV can be obtained from vesicle scraping (Tzanck smear)
Central Nervous System (Aseptic Meningitis, Encephalitis)		
Enterovirus (picornavirus)	Stool	PCR
Arboviruses (e.g., togaviruses, bunyavirus)	Rarely cultured	Diagnosis is by serologic tests
Rabies virus	Tissue, saliva, brain biopsy	Diagnosis is by immunofluorescence analysis for antigen
HSV; CMV; mumps virus; measles virus	Cerebrospinal fluid	PCR, virus isolation, and antigen are assayed
Urinary Tract		
Adenovirus; CMV	Urine	CMV may be shed without apparent disease
Blood		
HIV; human T-cell leukemia virus; hepatitis B, C, and D viruses	Blood	Serologic antigen or antibody detection (ELISA), PCR, and RT-PCR are performed

Diagnostic Methods in Virology

- B. Direct Examination:** Direct demonstration of the virus, its antigens or nucleic acid.
- widely used
 - fast method of virus diagnosis

Virus or virus antigen is detected in lesions, fluids, tissues or excretions from the patient and a result can be obtained within an 1 or 2 hour of receipt specimen.

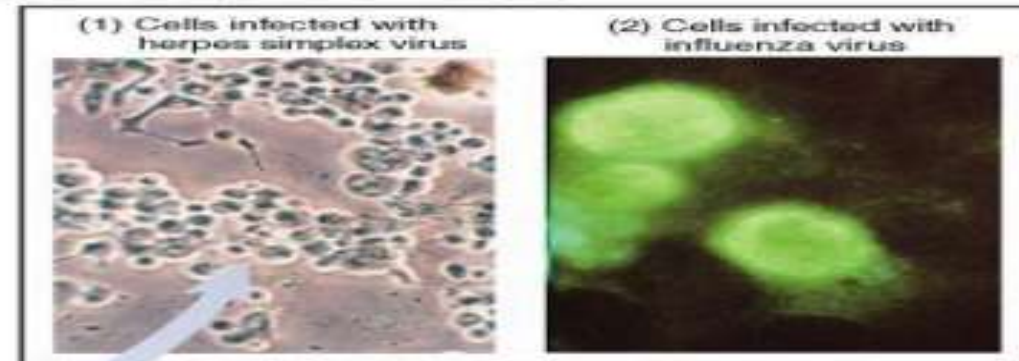
B. Indirect Examination:

Virus Propagation Isolation

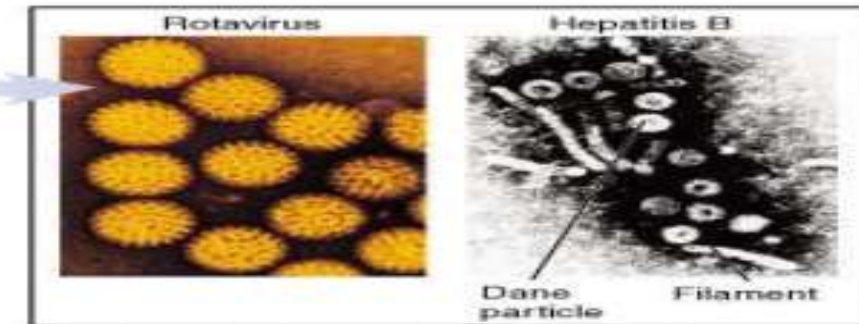
- Chick embryo
- Cell culture (cell line)

C. Animal inoculation

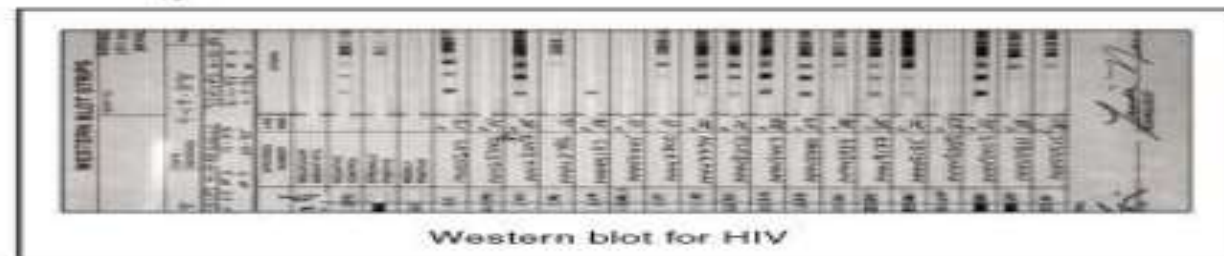
D. Serology



(b) Cells taken from patient are examined for evidence of viral infection, such as cytopathic effects (1) or virus antigen detected by fluorescent staining (2).



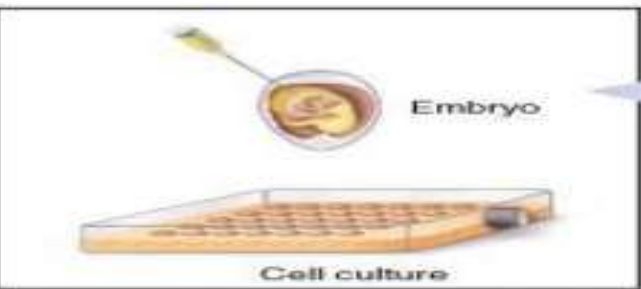
(c) Electron microscope is used to view virus directly. Viruses are sufficiently unique in structure that they can be differentiated to family or genus.



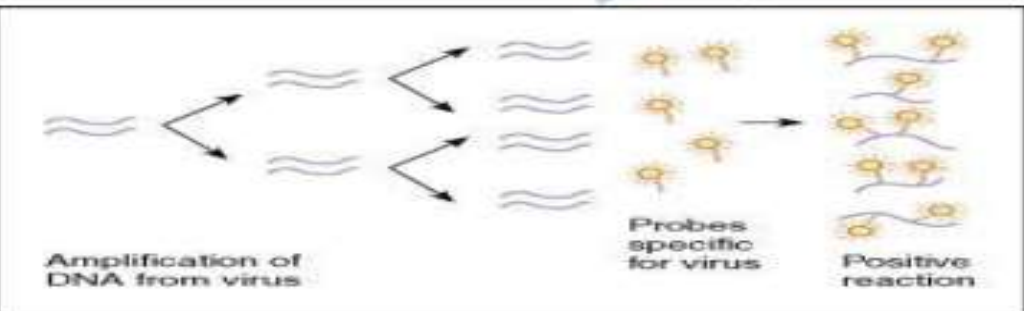
(d) Serological testing for antibodies.



(g) ELISA methods



(f) Culture techniques: Viruses require a living host to multiply.



(e) Genetic analysis (PCR): Detection of viral nucleic acid using specific probes.

A- Direct Examination

- 1- Serology (Antigen Detection) → immunofluorescence, ELISA etc.
- 2- Electron Microscopy → morphology of virus particles
number of virus particles
immune electron microscopy
- 3- Light Microscopy → histological appearance
changes in cell morphology
inclusion bodies
- 4- Viral Genome Detection → hybridization with specific nucleic acid probes
polymerase chain reaction (PCR)



B- Indirect Examination

1- Cell Culture



cytopathic effect (CPE)
haemabsorption
immunofluorescence

2- Eggs



pocks on chorioallantoic membrane (CAM)
haemagglutination
inclusion bodies

3- Animals



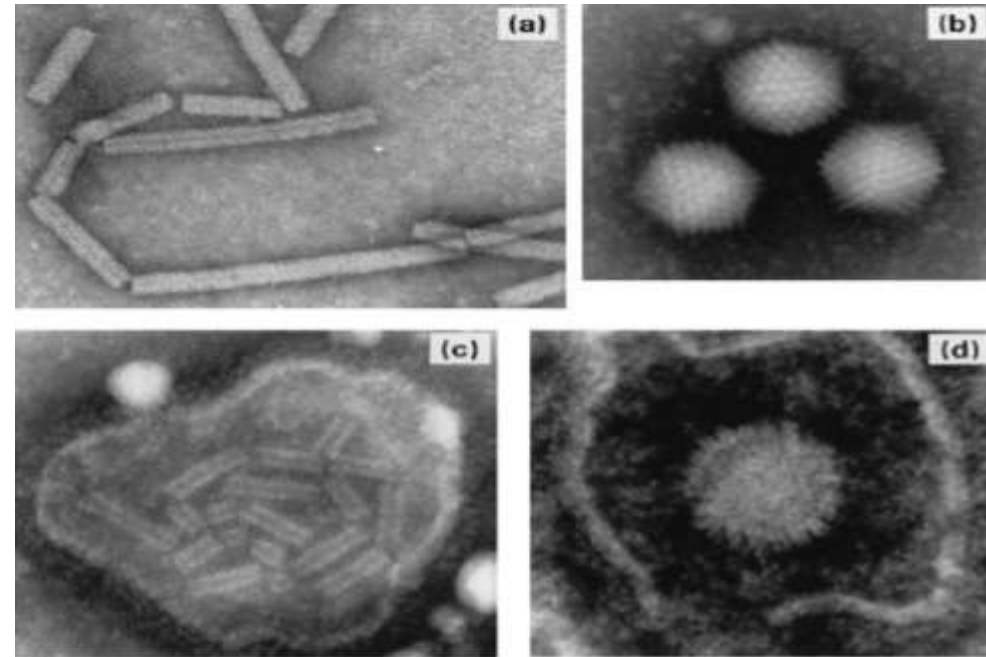
disease or death

Electron Microscopy (EM)

- 10^6 virus particles per ml required for visualization.
50,000 - 60,000 magnification normally used.

Viruses may be detected in the following specimens:

- Faeces → Rotavirus, Adenovirus
- Vesicle Fluid → HSV
- Skin scrapings → papillomavirus



Immune Electron Microscopy

The sensitivity and specificity of EM may be enhanced by immune electron microscopy.

There are two variants:-

- **Classical Immune electron microscopy (IEM)** - the sample is treated with specific anti-sera before being put up for EM. Viral particles present will be agglutinated and thus congregate together by the antibody.
- **Solid phase immune electron microscopy (SPIEM)** - the grid is coated with specific anti-sera. Virus particles present in the sample will be absorbed onto the grid by the antibody

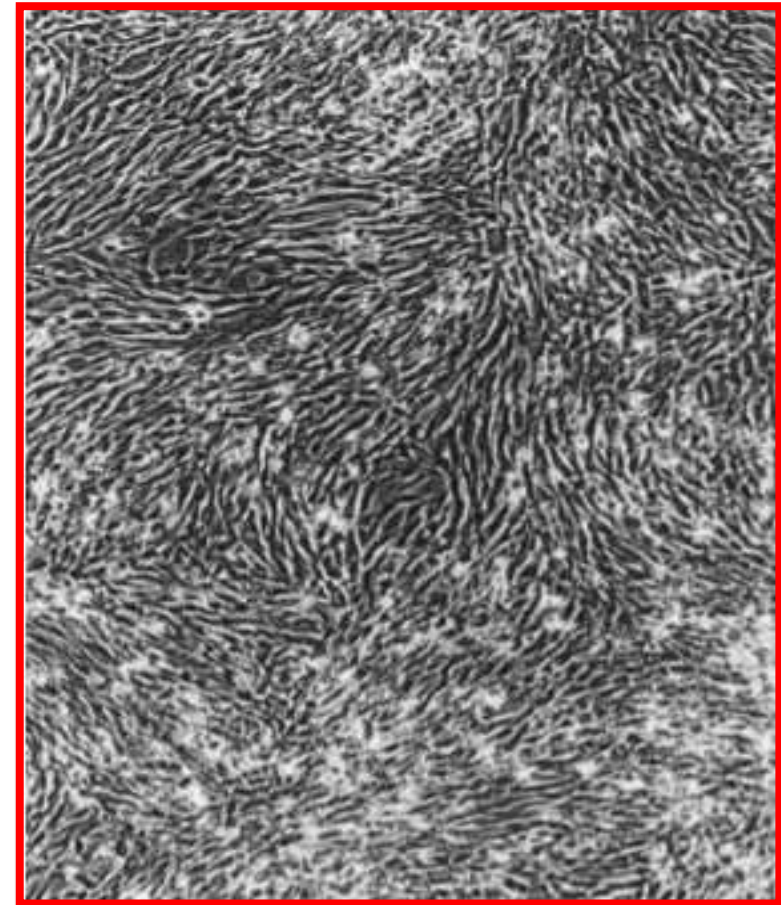
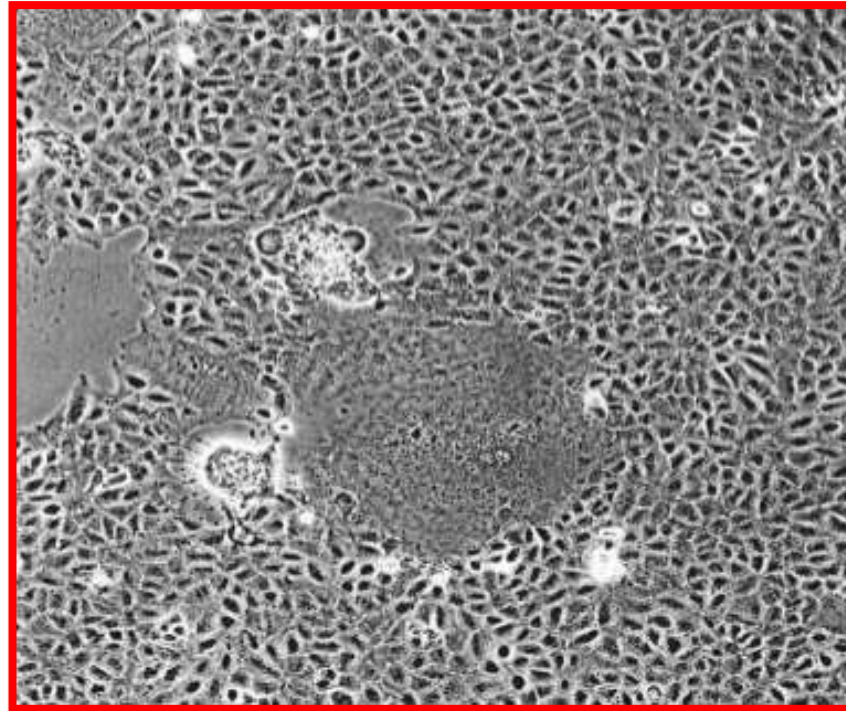
Problems with Electron Microscopy

- Expensive equipment
- Expensive maintenance
- Require experienced observer
- Sensitivity often low



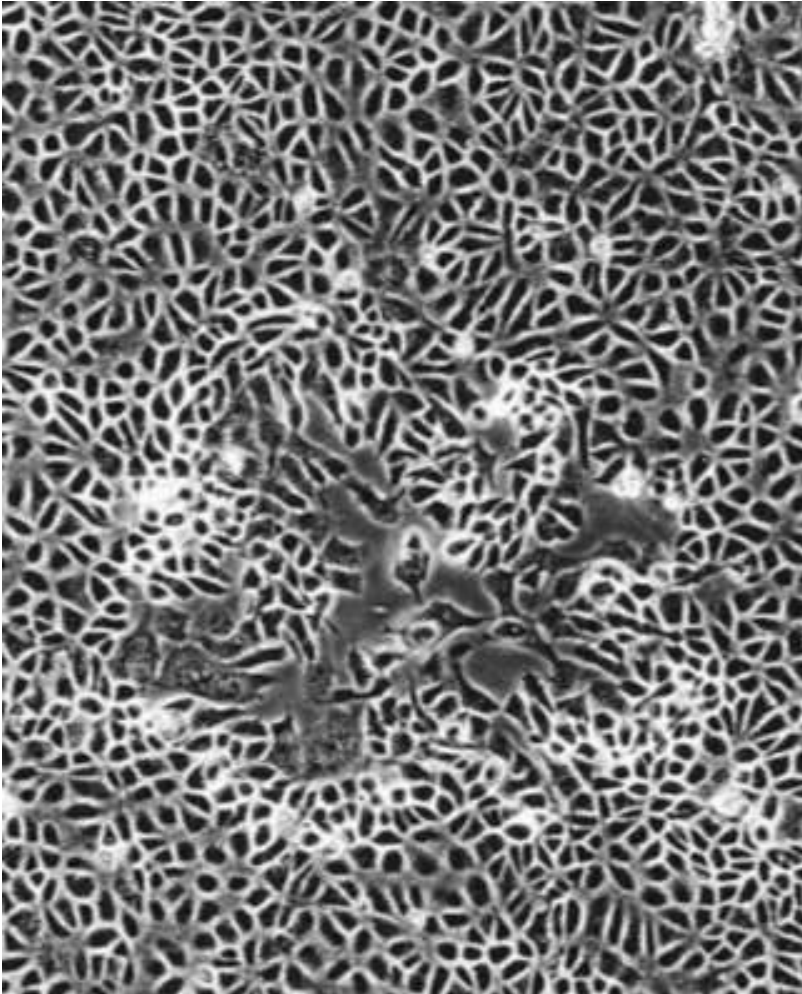
Epithelial-like
(human lung carcinoma, A549)

Measles
on human lung carcinoma

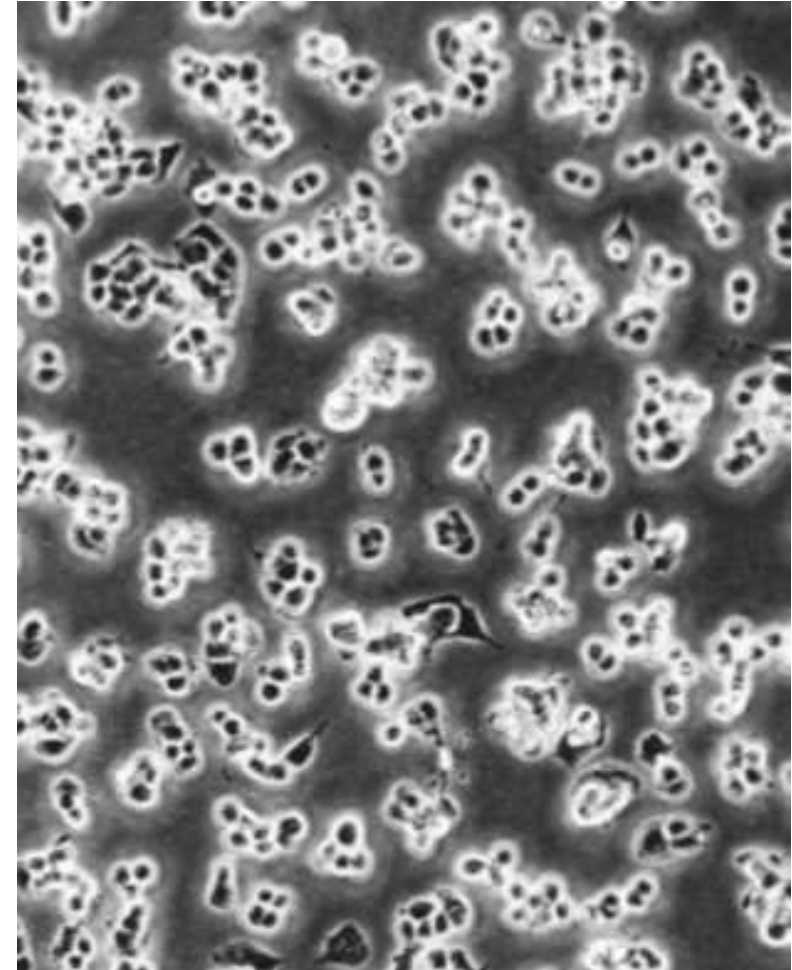


Fibroblast like
(baby hamster kidney, BHK)
www.freelivedoctor.com

Vaccinia on monkey kidney



Low multiplicity of infection



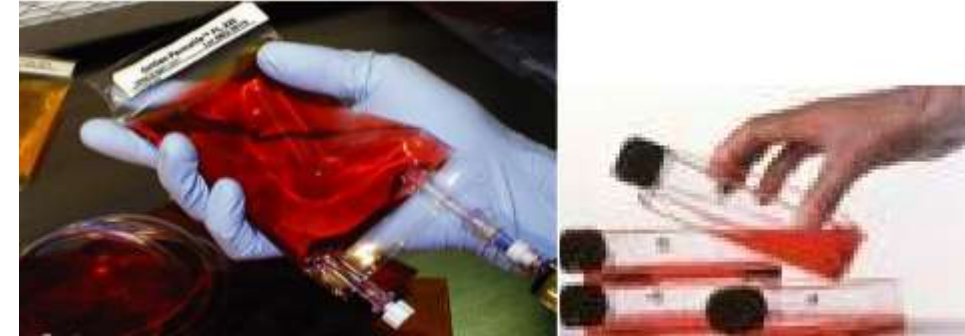
High

Virus Isolation

Cell Cultures are most widely used for virus isolation.

There are 3 types of cell cultures:

1. **Primary cells** - Monkey Kidney
2. **Semi-continuous cells** - Human embryonic kidney and skin fibroblasts
3. **Continuous cells (Hela)**



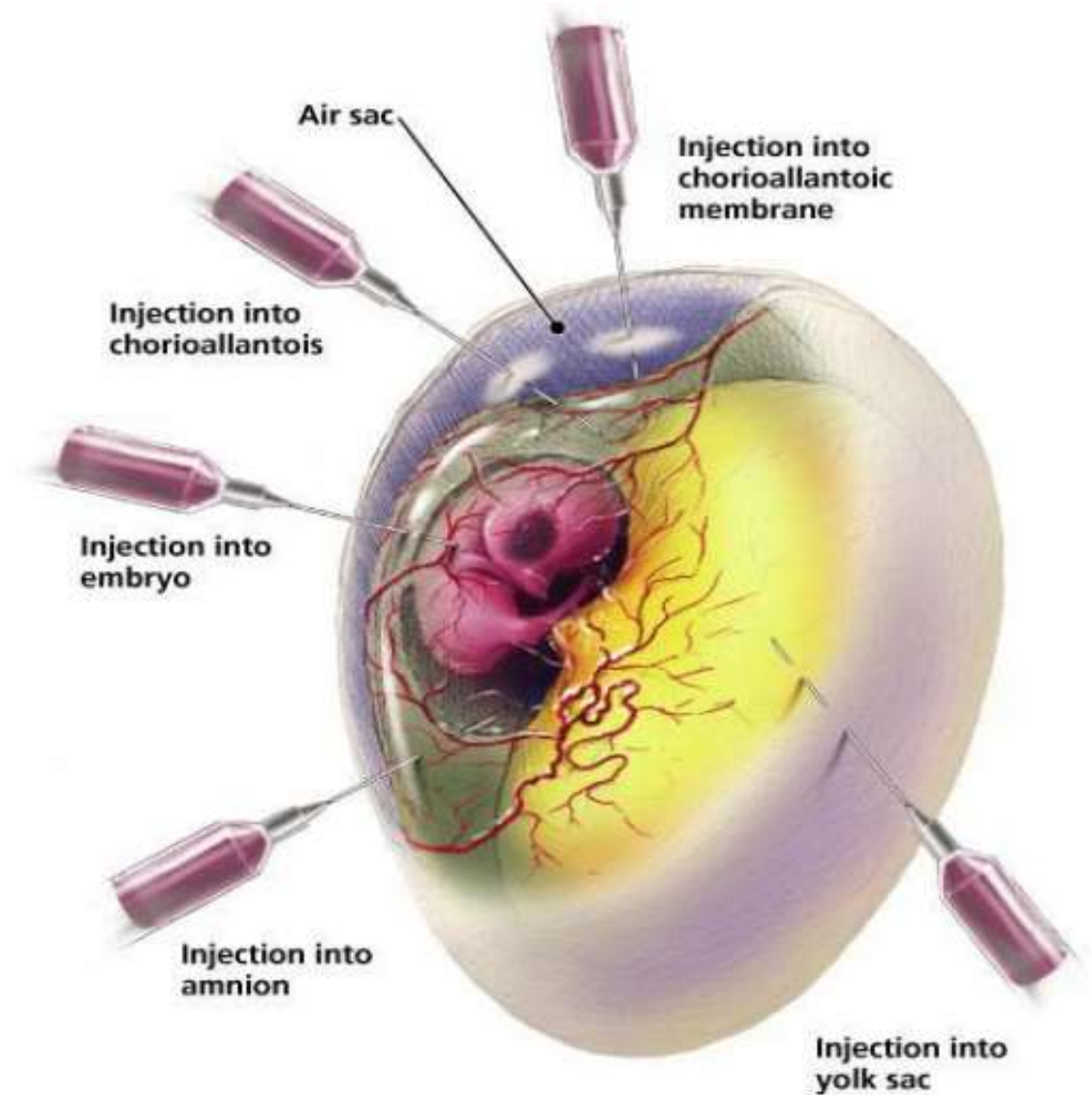
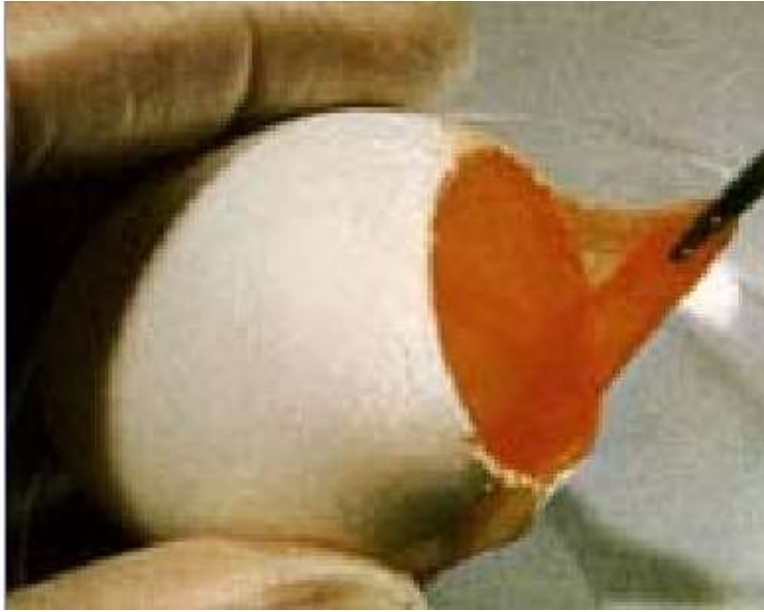
Primary cell culture are widely acknowledged as the best cell culture systems available since they support the widest range of viruses. However, they are **very expensive** and it is **often difficult to obtain a reliable supply**.

Continuous cells are the most easy to handle but the range of viruses supported is often limited.

Virus Isolation

- In the laboratory, solid samples are minced, homogenized, centrifuged at low speed to remove cellular debris that may be toxic to the cultured cells, and sterilized by 0.2 μm filter.
- A backup aliquot is stored at 4 or -70 C.
- Next step is the inoculation of a system supporting virus replication:
cell cultures, embryonating eggs, organ cultures, laboratory animals or host animals.

Virus Isolation (chick embryo)



Cell Cultures

Growing virus may produce:

1. Cytopathic Effect (CPE):

such as the ballooning of cells or syncytia formation, may be specific or non-specific.

2. Haemadsorption:

cells acquire the ability to stick to mammalian red blood cells.

Types of Cytopathic Effects (CPE):

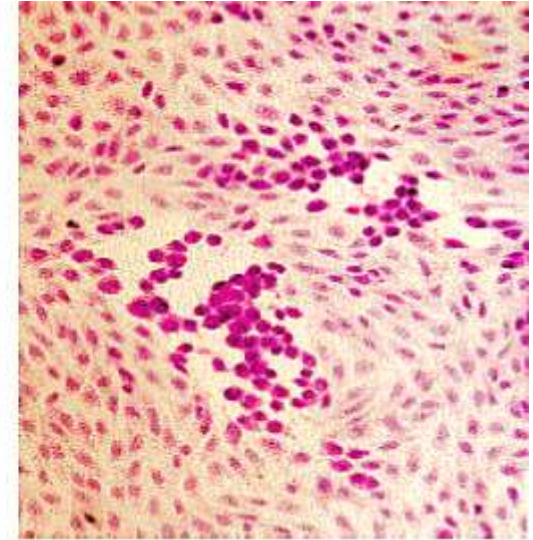
Cytopathic Effect (1)

Cytopathic effect of enterovirus and HSV in cell culture: note the ballooning of cells.

(Virology Laboratory, Yale-New Haven Hospital, Linda Stannard, University of Cape Town).

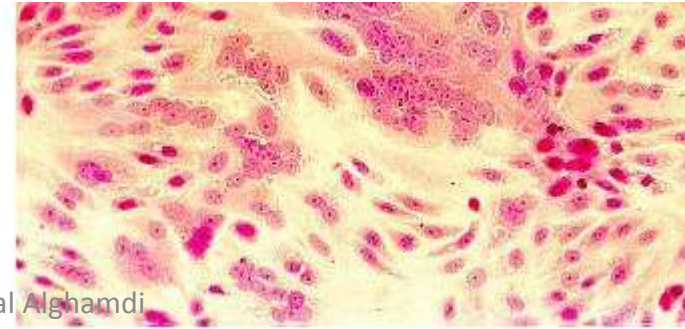
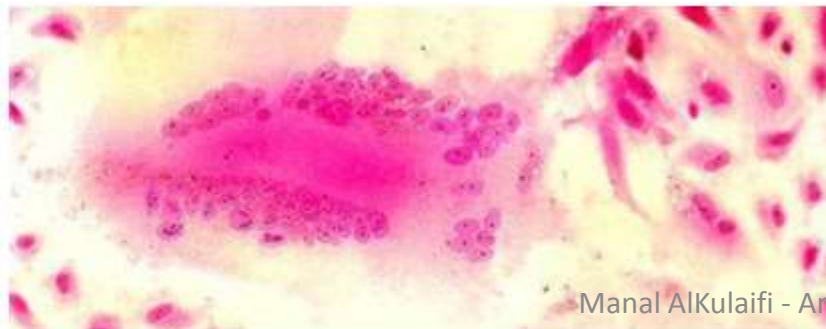


Fig. 1. Cytopathic effects of enterovirus 71 in rhesus monkey kidney cells

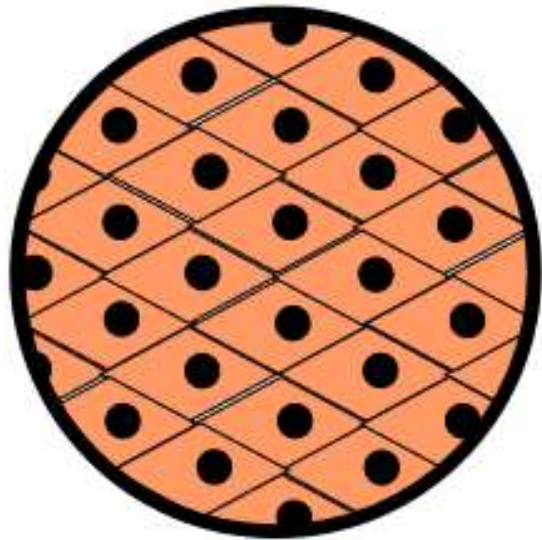
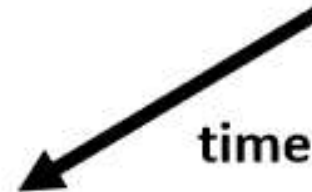
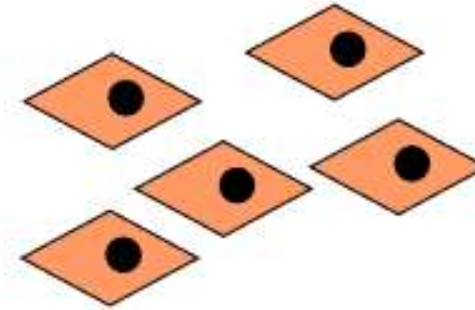
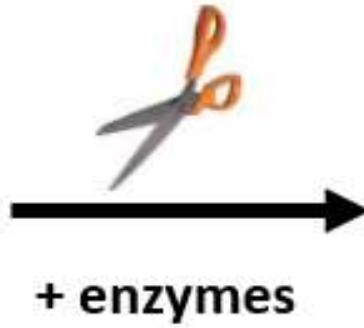
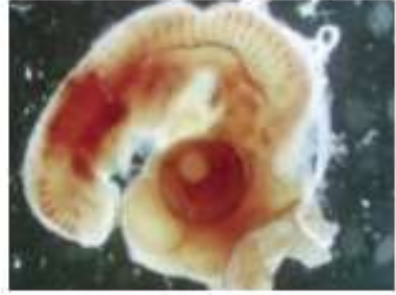


Cytopathic Effect (2)

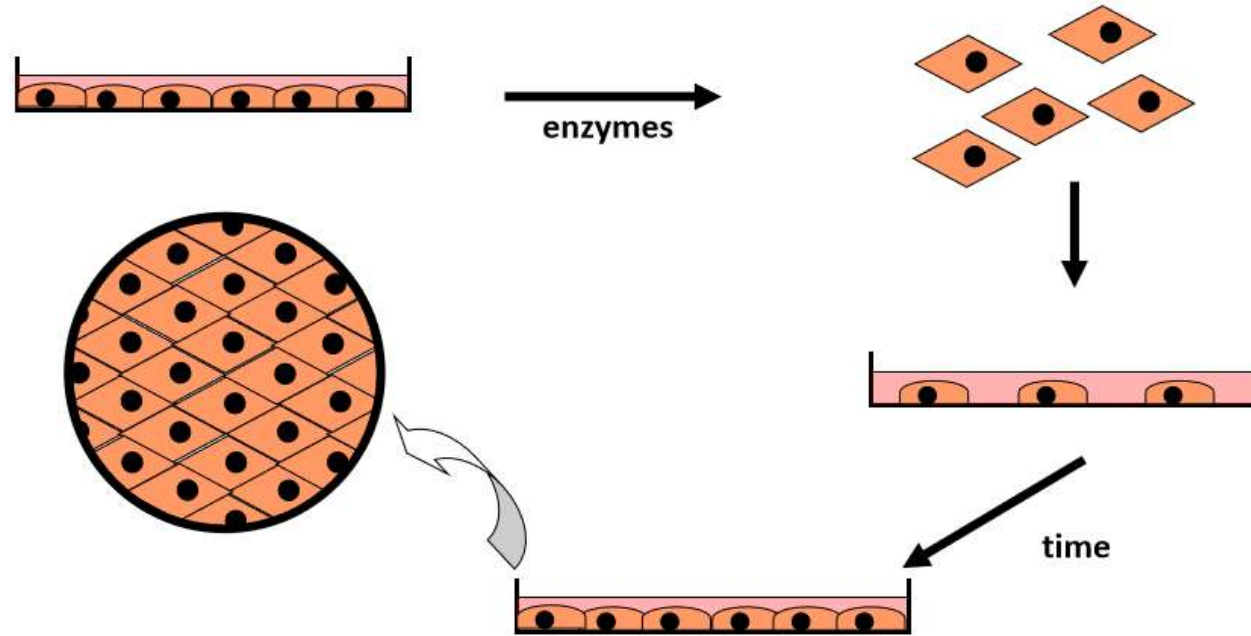
Syncytium formation in cell culture caused by RSV and measles virus.(courtesy of Linda Stannard, University of Cape Town, S.A.)



Primary cell culture



Subculture



Growth of cells in culture. A primary culture is defined as the original plating of cells from a tissue, grown to a confluent monolayer, without subculturing. A cell strain (solid line) is defined as a euploid population of cells subcultivated more than once in vitro, lacking the property of indefinite serial passage. Cell strains ultimately undergo degeneration and death, also called crisis or senescence. A cell line (dashed line) is an aneuploid population of cells that can be grown in culture indefinitely. Spontaneous transformation or alteration of a cell strain to an immortal cell line can occur at any time during cultivation of the cell strain. The time in culture and corresponding number of subcultivations or passages are shown on the abscissas. The ordinate shows the total number of cells that would

Cultured cells

a) Primary

- Heterogeneous – many cell types
- Closest to animal
- Technical hassle

b) Diploid cell strain

- Relatively homogeneous – fewer cell types
- Further from animal
- Technically less hassle

c) Continuous cell line

- Immortal • Most homogeneous • Genetically weird – furthest from animal
- Hassle free • Suspension or monolayer

Problems with cell culture

- Long period (up to 4 weeks) required for result.
- Often very poor sensitivity, sensitivity depends on 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.

Haemadsorption:

Syncytial formation caused by mumps virus and haemadsorption of erythrocytes onto the surface of the cell sheet.

(courtesy of Linda Stannard, University of Cape Town, S.A.)

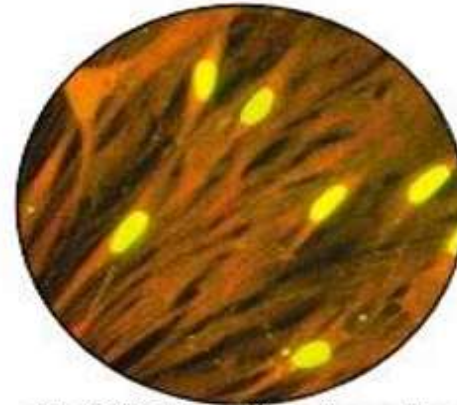
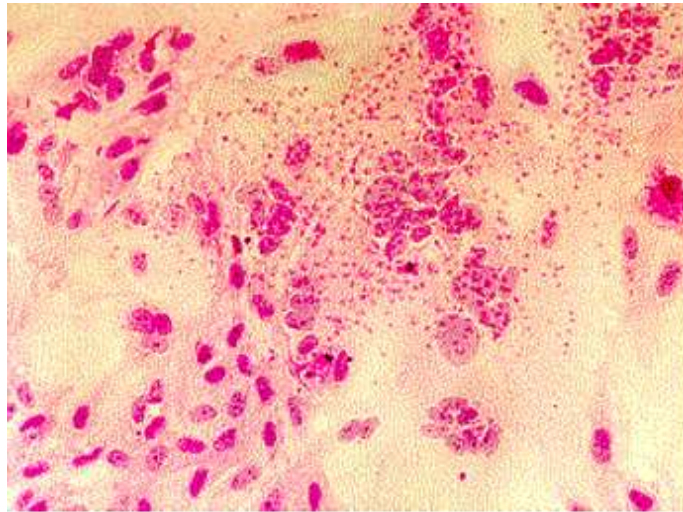
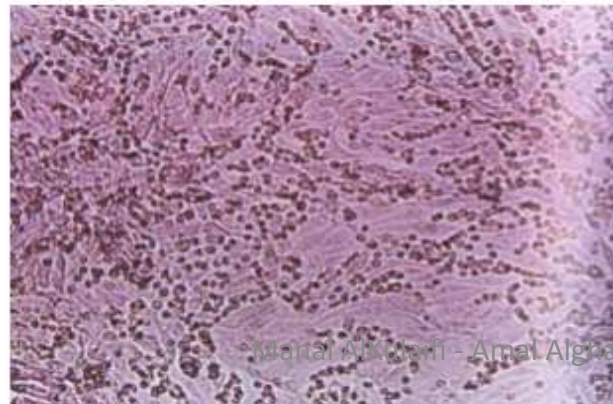
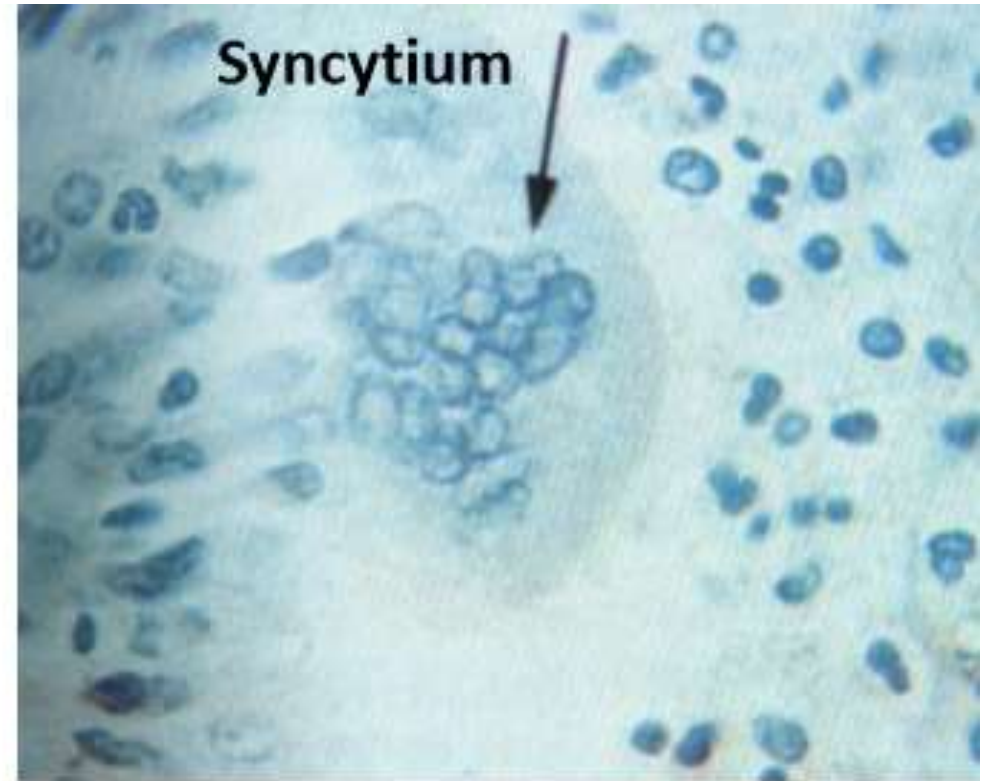
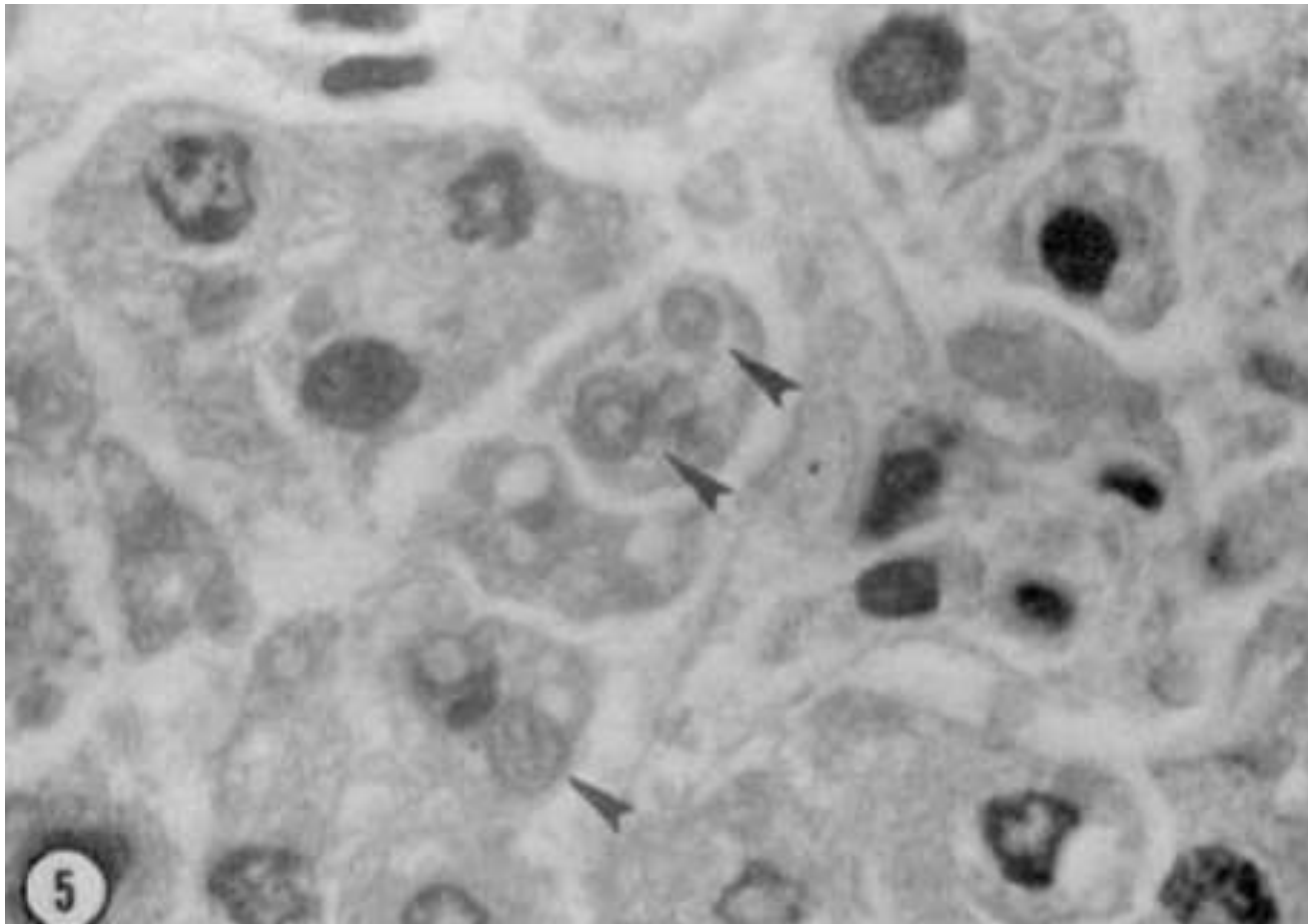


Fig. 2. CMV centrifugation culture fixed and stained 16 hrs after inoculation showing viral proteins in nuclei of infected human fibroblast cells

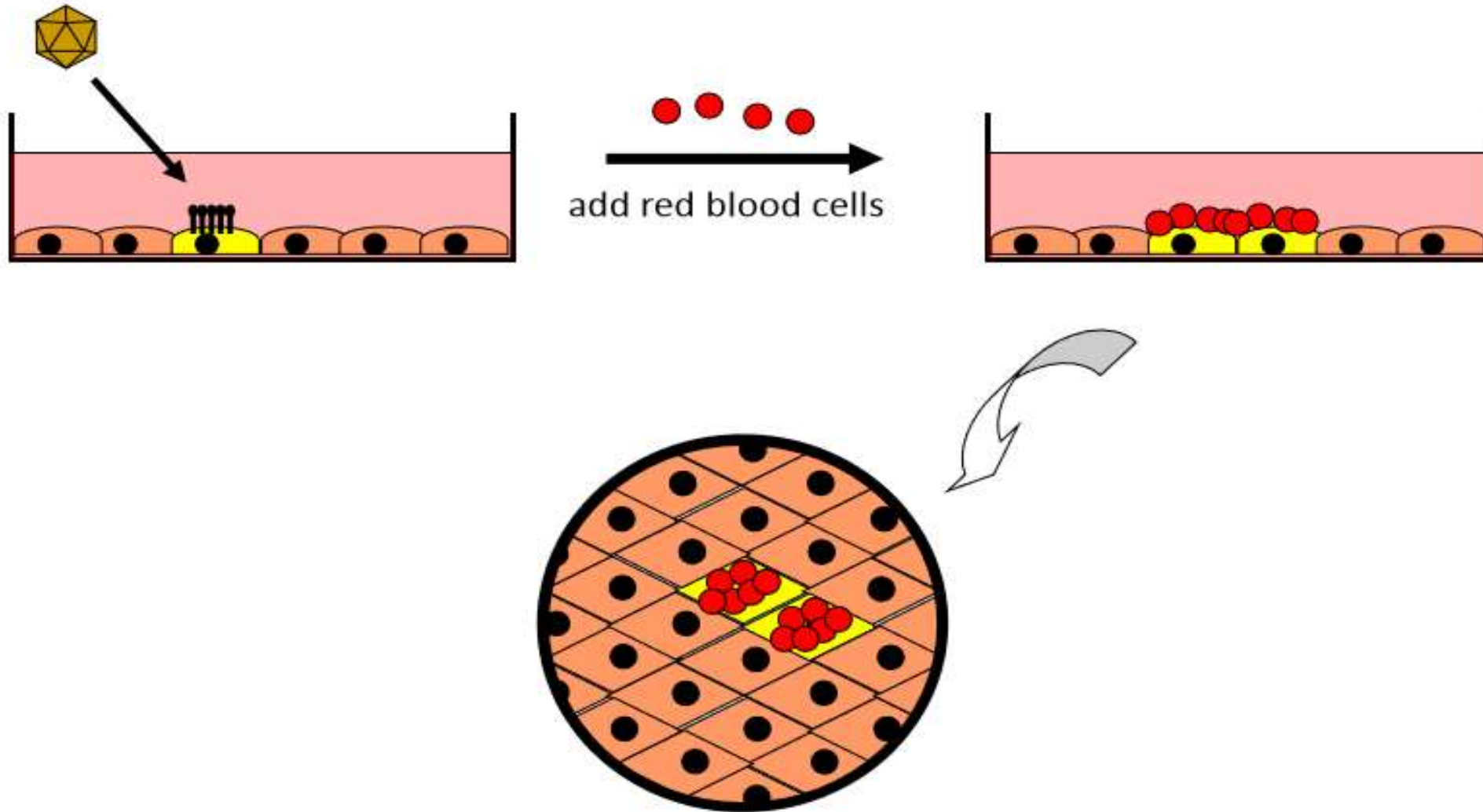


Haemadsorption:



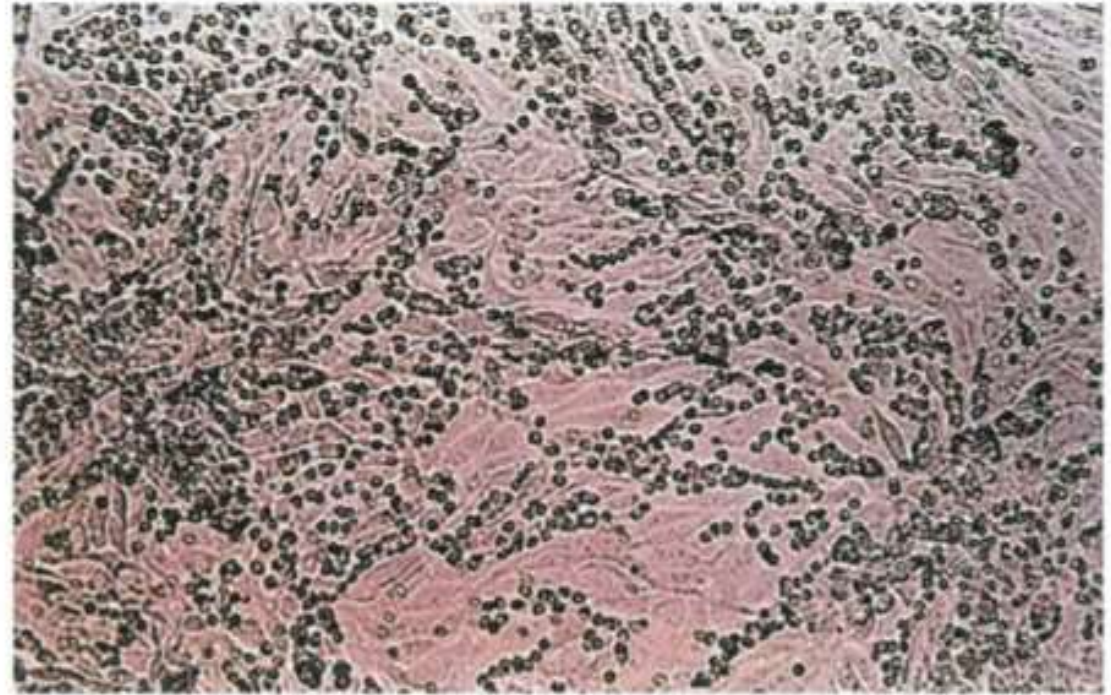
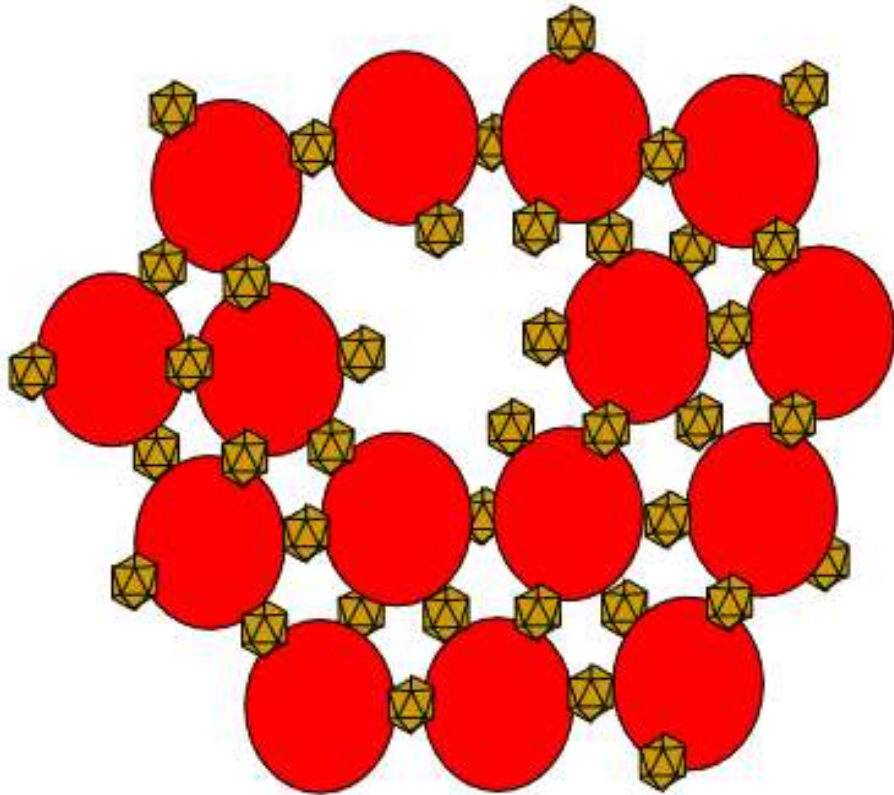
Ebola virus infection,
inclusion bodies (arrows)

Haemadsorption

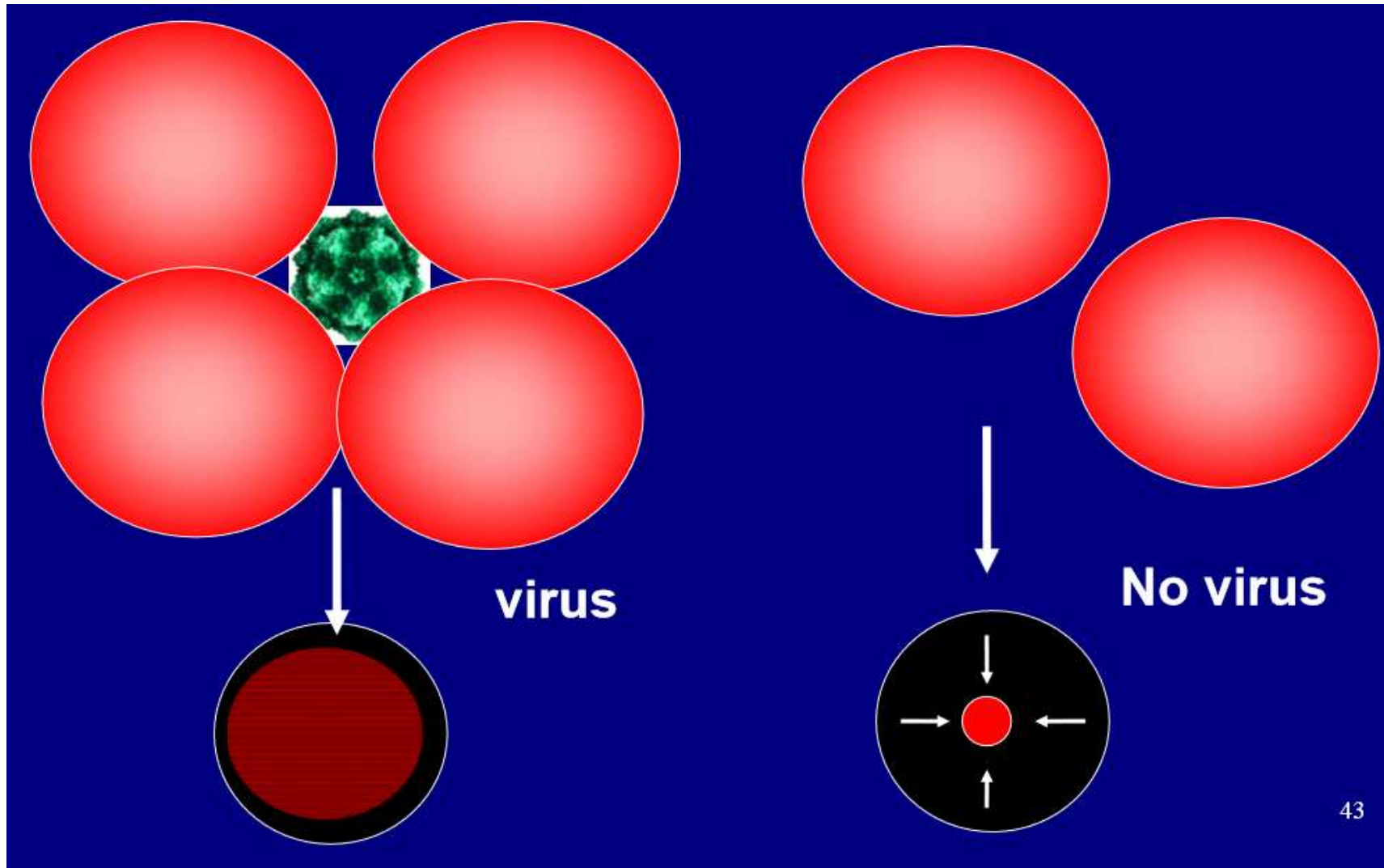


Haemadsorbtion

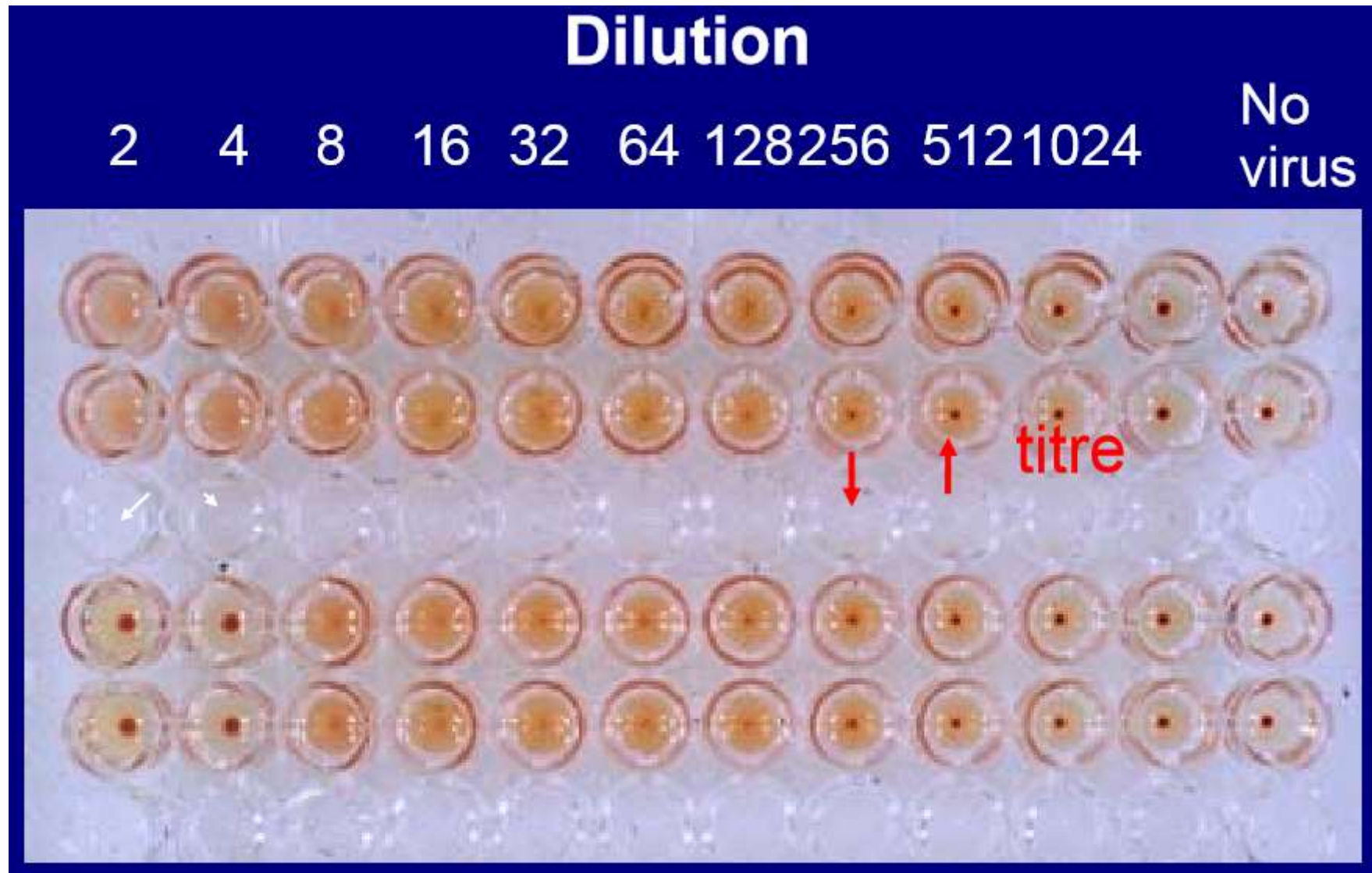
- Hemadsorption of erythrocytes to cells infected with influenza viruses. This virus express a hemagglutinin, which bind erythrocytes of selected animal species.



Haemagglutination: to Detect Viral Antigen



Haemagglutination: to Detect Viral Antigen



Serology

- Detection of rising titers of antibody between acute and convalescent stages of infection, or the detection of IgM in primary infection.

Classical Techniques

1. Complement fixation tests (CFT)
2. Haemagglutination inhibition tests
3. Immunofluorescence techniques (IF)
4. Neutralization tests
5. Counter-immunoelectrophoresis

Newer Techniques

1. Radioimmunoassay (RIA)
2. (ELISA)
3. Particle agglutination
4. Western Blot (WB)
5. RIBA, Line immunoassay

Serology

Criteria for diagnosing Primary Infection

- 4 fold or more increase in titre of IgG or total antibody between acute and convalescent sera
- Presence of IgM


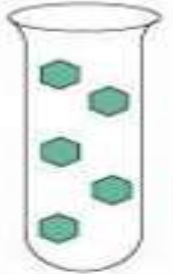
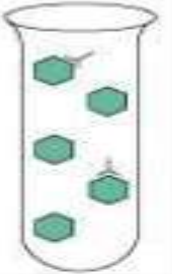
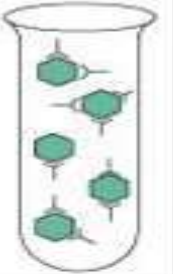
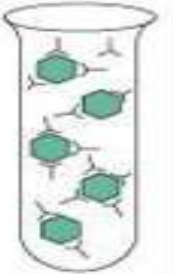
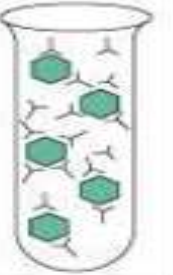







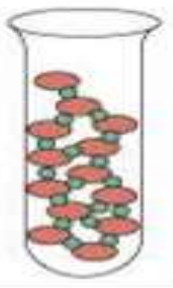
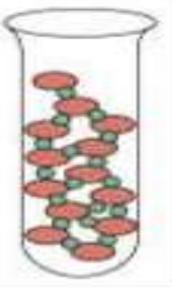
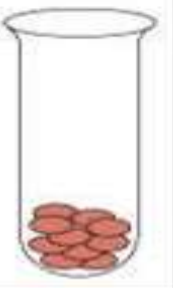
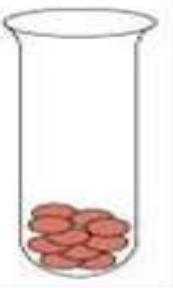
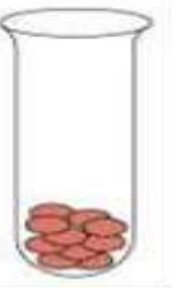




Criteria for diagnosing Reinfection

- 4 fold or more increase in titre of IgG or total antibody between acute and convalescent sera
- Absence or slight increase in IgM

Serology

- Neutralization
- Hemagglutination inhibition
- Western blot
- ELISA, radioimmune assay (RIA)

Antibody Detection: Neutralization & Haemagglutination

Patient serum (dilution)	0	0	1/1000	1/100	1/10	1
Virus concentration	0	5000 pfu	5000 pfu	5000 pfu	5000 pfu	5000 pfu
Virus concentration						
CELL CULTURE serum/virus mixture	 No virus	 CPE	 CPE	 No CPE	 No CPE	 No CPE
		Infection Hemagglutination		Neutralization Hemagglutination inhibition		
HEMAGGLUTINATION REACTION serum/virus mixture						
	  Virus	 Antibody	 Erythrocyte			

In the assay shown, 10 fold dilutions of serum were incubated with virus.

Aliquots of the mixture were then added to cell cultures or erythrocytes.

In the absence of antibody, the virus infected the monolayer (indicated by CPE) and caused hemagglutination.

In the presence of the antibody, infection was blocked (neutralization), and hemagglutination was inhibited, allowing the erythrocytes to pellet.

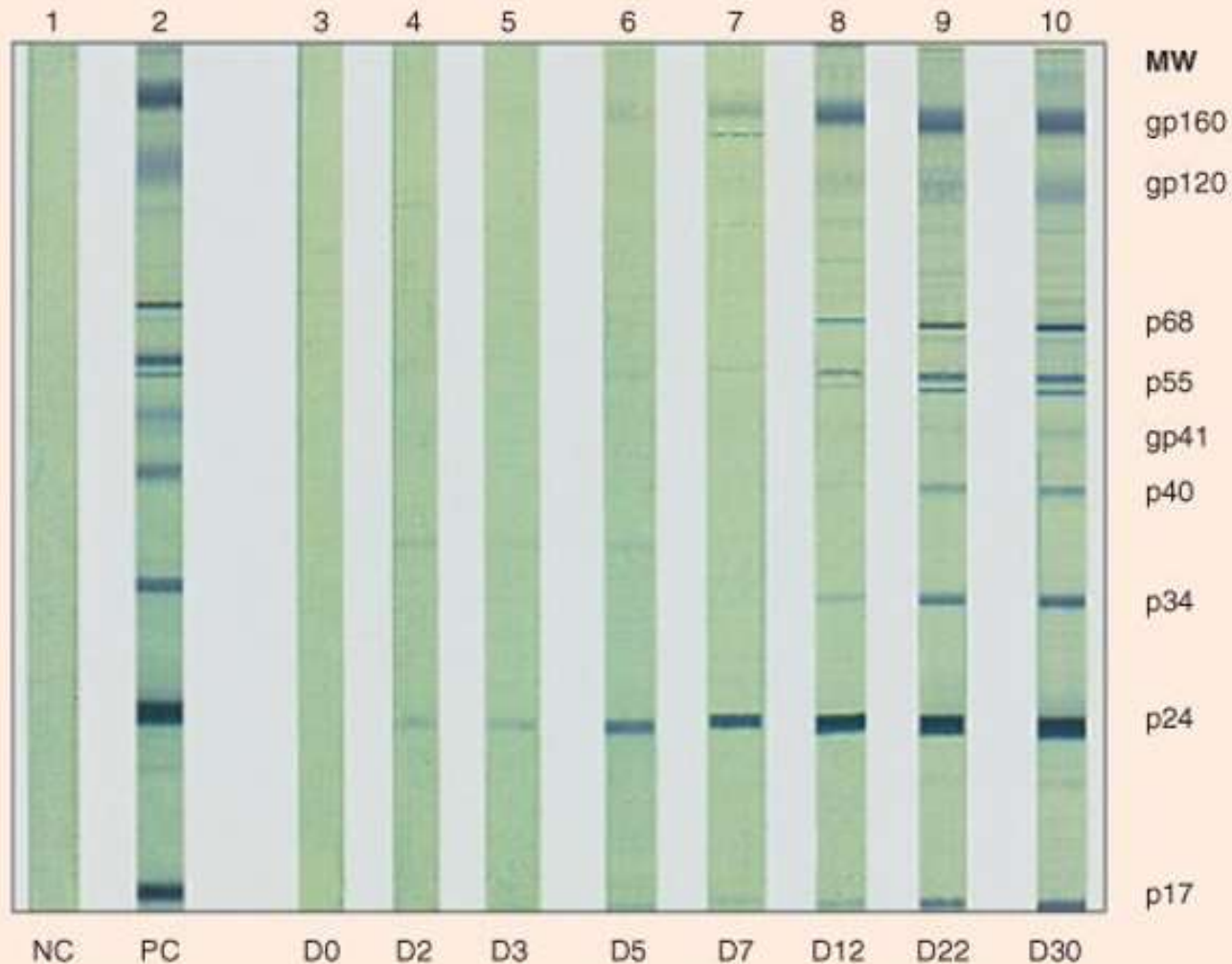
Antibody Detection: Western Blot

Western blot analysis of HIV antigens and antibody.

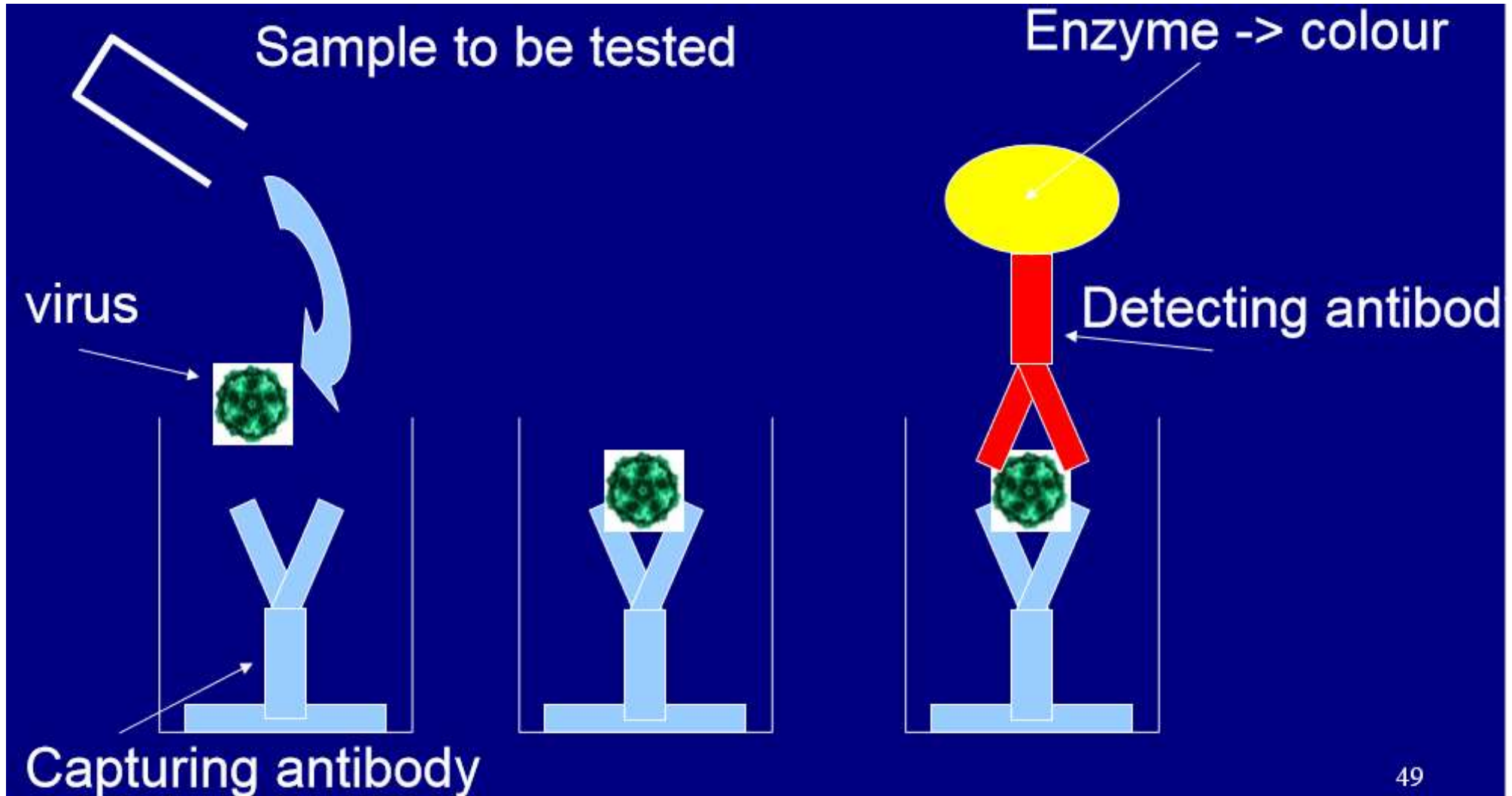
HIV protein **antigens** are separated by electrophoresis and blotted onto nitrocellulose paper strips.

The strip is incubated with patient antibody, washed to remove the unbound antibody, and then reacted with enzyme-conjugated antihuman antibody.

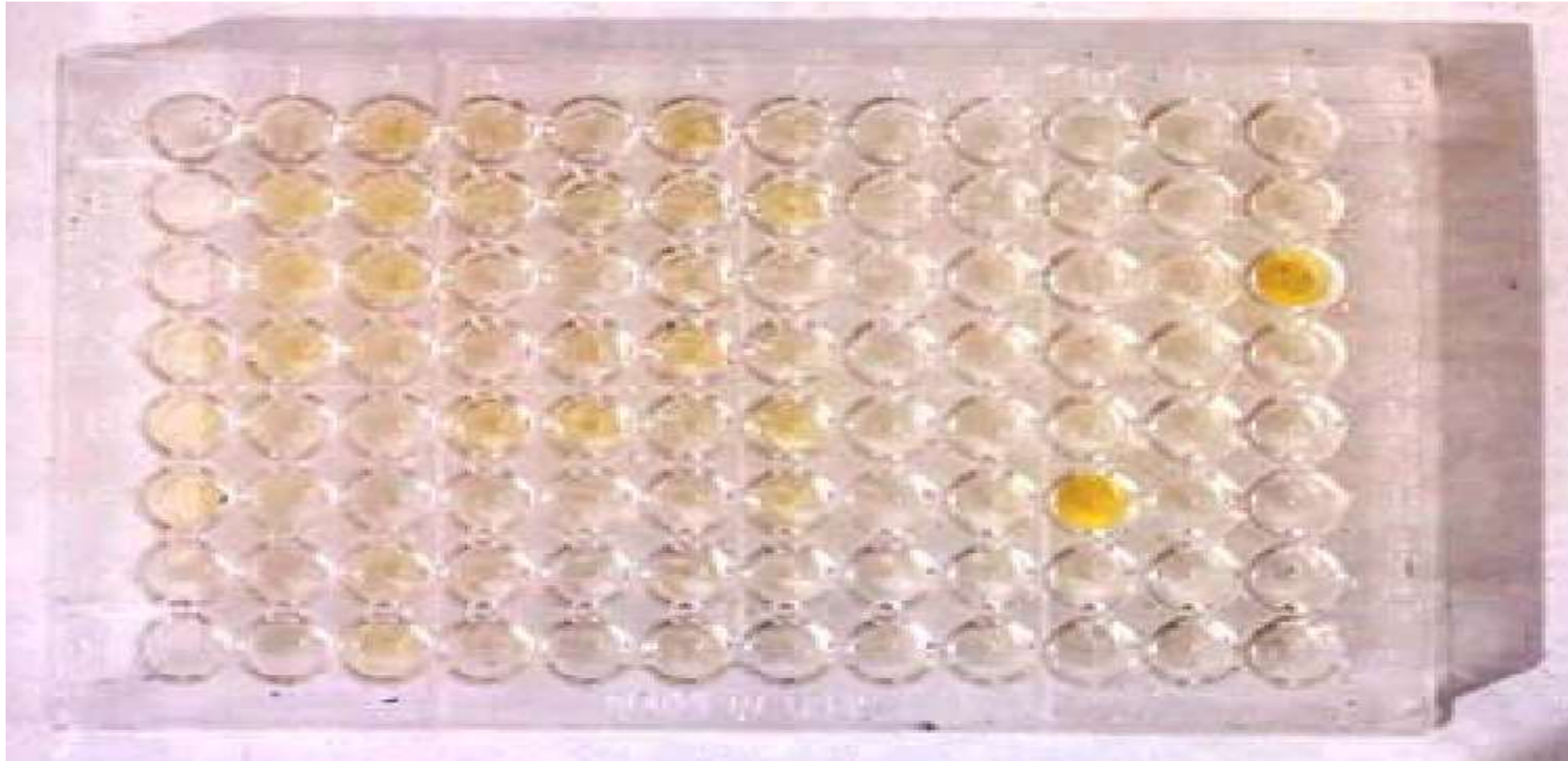
Serum from an HIV- infected person binds and identifies the major antigenic proteins of HIV.



HIV Antigen Detection: ELIZA



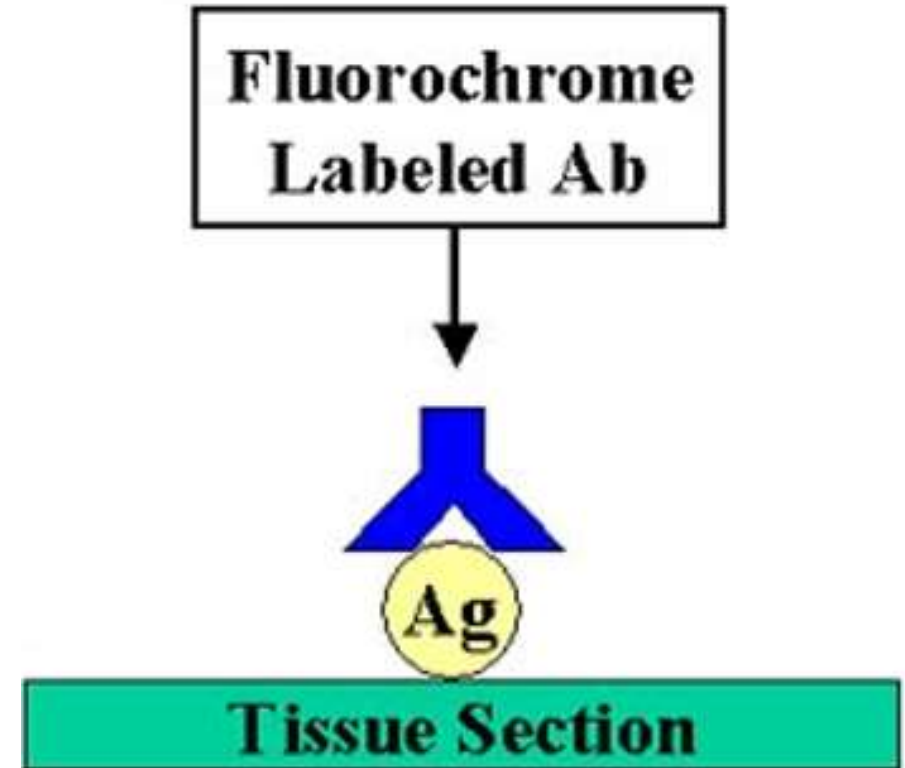
Microplate ELISA for HIV: coloured wells indicate reactivity



Virus Antigen Detection: Immunofluorescence

Antibody binds to antigen in fixed cells;

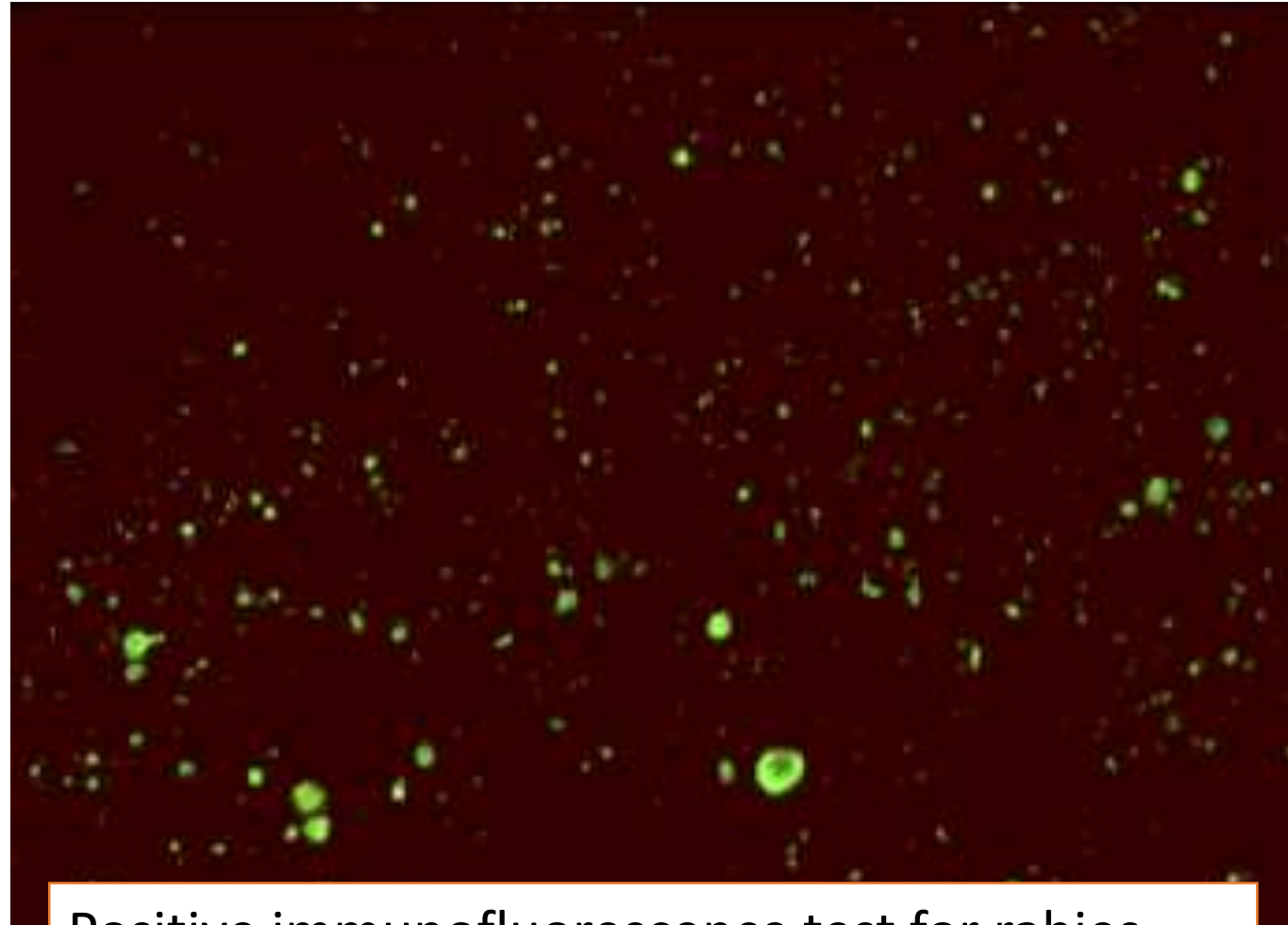
- fluorescein-labeled anti-IgG binds;
- fluoresces by UV microscopy



Virus Antigen Detection: Immunofluorescence



BHV-1 antigens
in neuron in trigeminal ganglion



Positive immunofluorescence test for rabies
virus antigen. (Source: CDC)

Usefulness of Serological Results

- How useful a serological result is depends on **the individual virus**.
- *For example*, for viruses such as **rubella** and **hepatitis A**, the onset of clinical symptoms coincide with the development of antibodies. **The detection of IgM or rising titres of IgG in the serum** of the patient would indicate **active disease**.
- However, **many viruses often produce clinical disease before the appearance of antibodies** such as **respiratory and diarrheal viruses**. So in this case, any serological diagnosis would be retrospective and therefore will not be that useful.
- There are also **viruses which produce clinical disease months or years**. e.g. **HIV**. In this case, **the presence of antibody** is sufficient to make a definitive diagnosis.

Problems with Serology

- Long period of time required for diagnosis for paired acute and convalescent sera.
- Mild local infections may not produce a detectable humoral immune response.
- Extensive antigenic cross-reactivity between related viruses e.g. HSV may lead to false positive results.
- immunocompromised patients often give a reduced or absent humoral immune response.
- Patients given blood or blood products may give a false positive result due to the transfer of antibody.

Assay of viruses

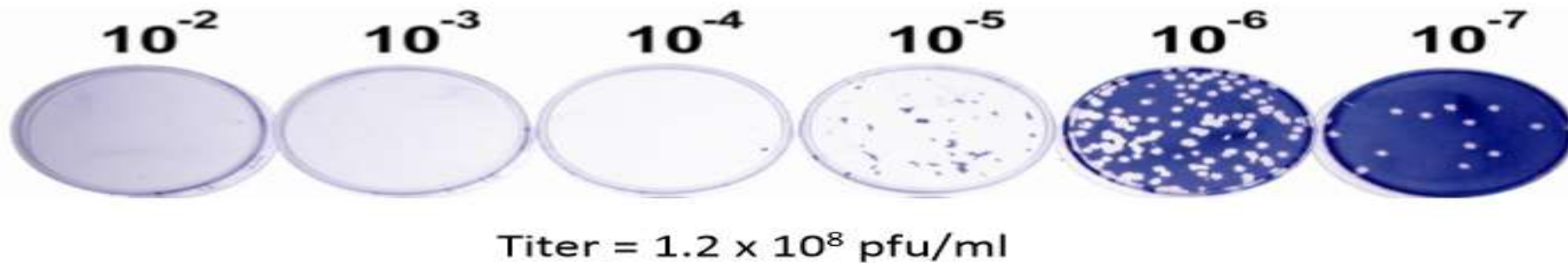
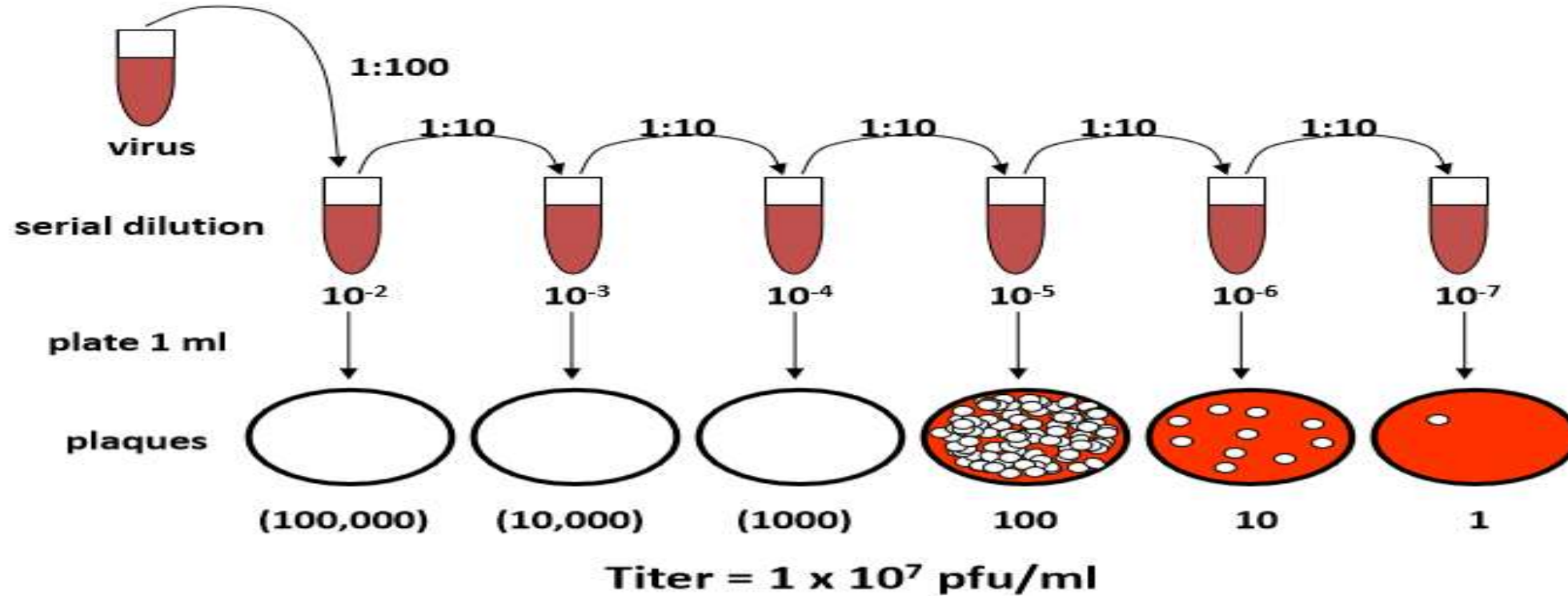
– Biological

- Plaque assay
- Transformation
- Endpoint Method

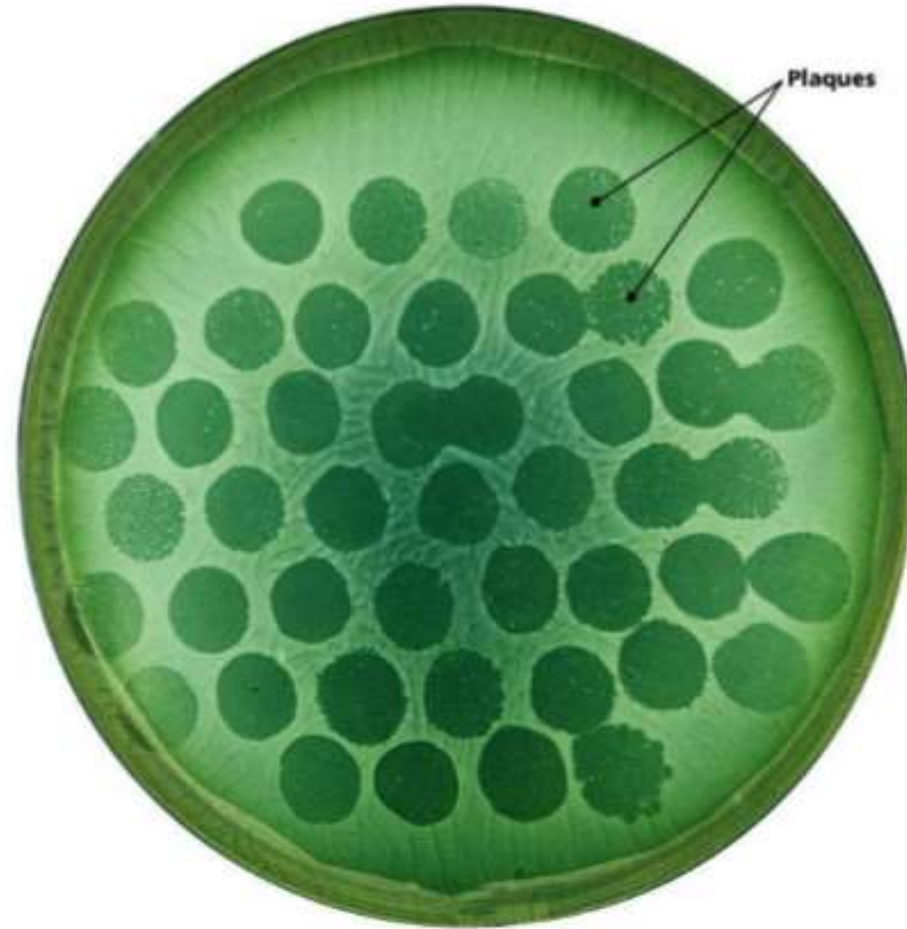
– Physical and biochemical.

- Hemagglutination.
- Direct particle count.
- Immunological tests for proteins.
- Assay for nucleic acid (PCR).
- Enzymatic (reverse transcriptase for retroviruses).

Plaque Assay: Method



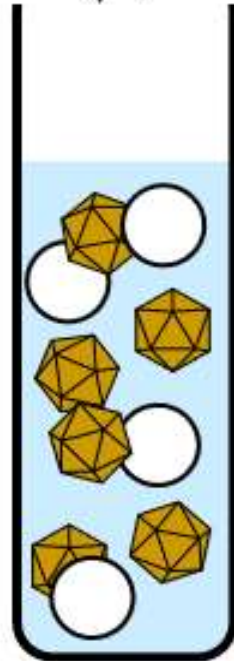
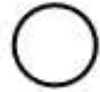
Plaque Assay : Result



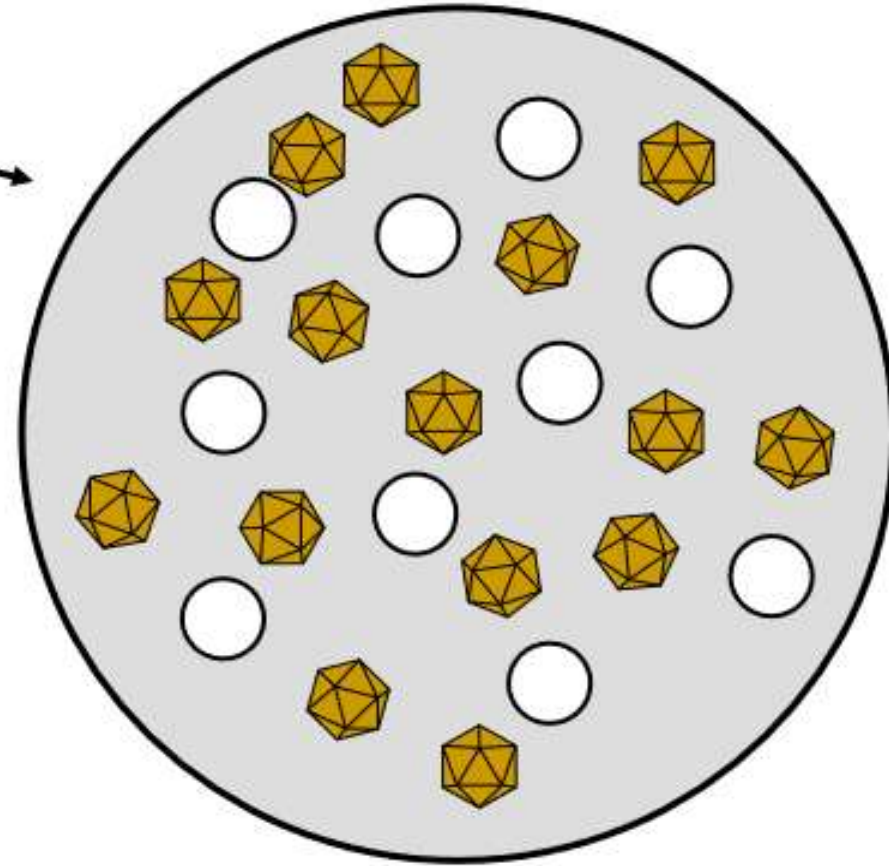
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Direct Particle Count

Beads ($10^4/\text{ml}$)



1.5×10^4 virus/ml



10 beads \Rightarrow 1 μl

15 virus $\Rightarrow 1.5 \times 10^4$ virus/ml

Assay for Viral Protein & Nucleic Acid

Proteins

Protein patterns (electrophoresis)

Enzyme activities (e.g., reverse transcriptase)

Hemagglutination and hemadsorption

Antigen detection (e.g., direct and indirect

immunofluorescence, enzyme-linked immunosorbent assay, Western blot)

Nucleic Acids

Restriction endonuclease cleavage patterns

Size of RNA for segmented RNA viruses (electrophoresis)

DNA genome hybridization in situ (cytochemistry)

Southern, Northern, and dot blots

PCR (DNA)

Reverse transcriptase polymerase chain reaction (RNA)

Real-time PCR

Branched-chain DNA and related tests (DNA, RNA)

PCR, Polymerase chain reaction.

Molecular Methods

- Methods based on **the detection of viral genome** are also commonly known as molecular methods. It is often said that molecular methods is the future direction of viral diagnosis.
- However in practice, although the use of these methods is indeed increasing, the role played by molecular methods in a routine diagnostic virus laboratory is still small compared to conventional methods.

Classical Molecular Techniques

- **hybridization** are examples of classical techniques. They depend on the use of **specific DNA/RNA probes** for hybridization.
- The specificity of the reaction depends on the conditions used for hybridization. However, the sensitivity of these techniques is not better than conventional viral diagnostic methods.
- However, since they are usually expensive than conventional techniques, they never found widespread acceptance.

Polymerase Chain Reaction

PCR allows the **in vitro amplification of specific target DNA sequences** in an extremely sensitive technique.

- it is based on an **enzymatic reaction** involving the use of synthetic oligonucleotides flanking the target nucleic sequence of interest.
- These oligonucleotides act as primers for the **thermostable Taq polymerase**. Repeated cycles (usually 25 to 40) of denaturation of the template DNA (at 94°C), annealing of primers to their complementary sequences (50°C), and primer extension (72°C) result in the exponential production of the specific target fragment.
- **Detection and identification of the PCR product** is usually carried out by agarose gel electrophoresis, restriction enzyme analysis, or DNA sequencing.

Polymerase Chain Reaction

Advantages of PCR:

Extremely high sensitivity, may detect down to one viral genome per sample volume - Easy to set up - Fast turnaround time

Disadvantages of PCR :

Extremely liable to contamination - High degree of operator skill required - Not easy to set up a quantitative assay- A positive result may be difficult to interpret, especially with latent viruses, where any seropositive person will have virus present in their blood irrespective whether they have disease or not.- These problems are being addressed by the arrival of commercial closed systems which requires minimum handling. The use of synthetic internal competitive targets in these commercial assays has facilitated the accurate quantification of results. However, these assays are very expensive.

Diagnostic Methods for most Common Human Viruses

VIRUS	METHOD
Adenovirus (gastrointestinal)	ELISA
Adenovirus (respiratory)	Antigen, PCR, culture
Cytomegalovirus	Antigen, PCR, culture
Enteroviruses (echo, coxsackie, polio)	PCR, culture
Epstein-Barr virus	PCR, serology
Hepatitis A virus	Serology
Hepatitis B virus	Antigen, PCR, serology
Hepatitis C virus	PCR, serology
Herpes simplex virus	Antigen, PCR, culture
HIV	Antigen, PCR, culture, serology
Influenza virus	ELISA, antigen, culture
Parainfluenza virus	Antigen, PCR, culture
Respiratory syncytial virus	ELISA, antigen, PCR, culture
Rotavirus	ELISA
Varicella-zoster virus	Antigen, PCR

ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction.

Summary

- **Four main clinical diagnostic techniques:**

- Culture, serology, antigen detection, nucleic acid detection.

- **Virus culture**

- Not all viruses can be cultured – Cultured cell types – Cytopathic effect

- **Virus quantitation**

- Biological

- Physical

- **Basic serological techniques**