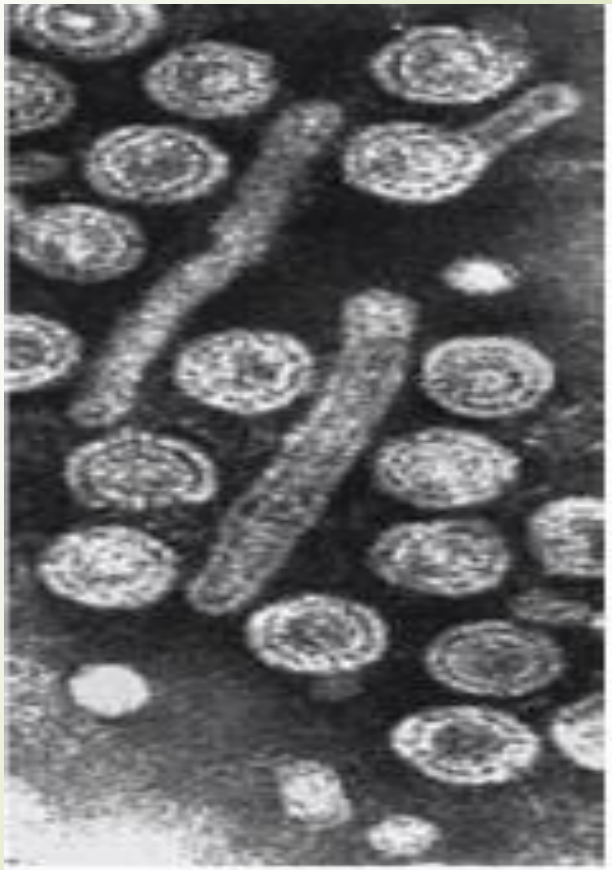
Methods of Laboratory Diagnosis of Viral Infectious



Viral Diagnostics in the Clinical Laboratory

- Over 60% of all infectious disease cases seen by a physician are due to viral infections.
- Quality of patient specimens and their transport to the laboratory is important.

Storage and Collection of Biological Specimens for Viral Testing

Respiratory tract infections: Nasal and bronchial washings, throat and nasal swabs, sputum
Eye infections: throat and Conjunctival swab/scraping
Gastrointestinal tract infections: stool and rectal swabs
Vesicular rash: vesicle fluid, skin scrapings
Maculopapular rash: throat, stool, and rectal swabs
CNS (encephalitis and meningitis cases): stool, tissue, saliva, brain biopsy, cerebrospinal fluid
Genital infections: vesicle fluid or swab
Urinary tract infections: urine
Blood borne infections: blood

Three General Approaches for Laboratory Diagnosis of Viral Infections

- Direct detection
  - –Microscopy or staining
- Virus Isolation
  - –PCR
- Serology
  - –Antibodies

Direct Examination

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

Indirect Examination

1. Cell Culture cytopathic effect (CPE)

2. Eggs haemagglutination

inclusion bodies

3. Animals disease or death

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, Vero, Hep2, LLC-MK2, MDCK

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.

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.

Confirmation of the identity of the virus may be carried out using neutralization, haemadsorption-inhibition or immunofluorescence tests.

Cytopathic Effect (1)

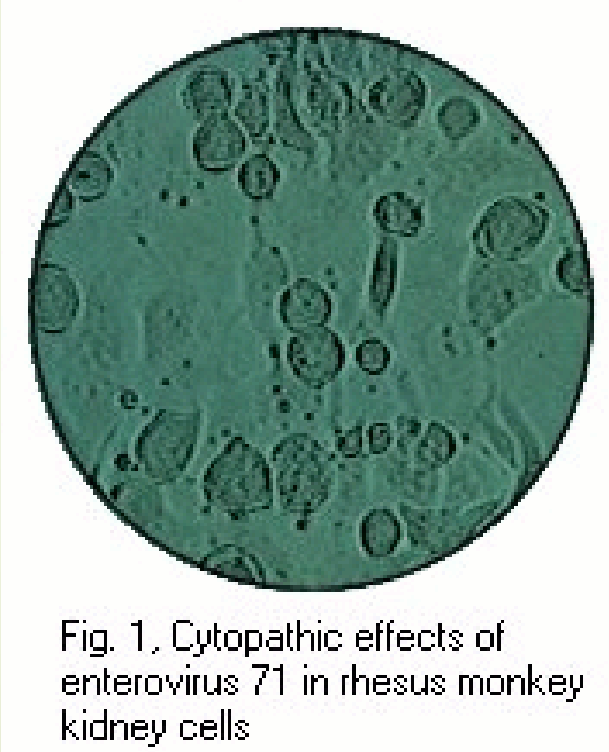
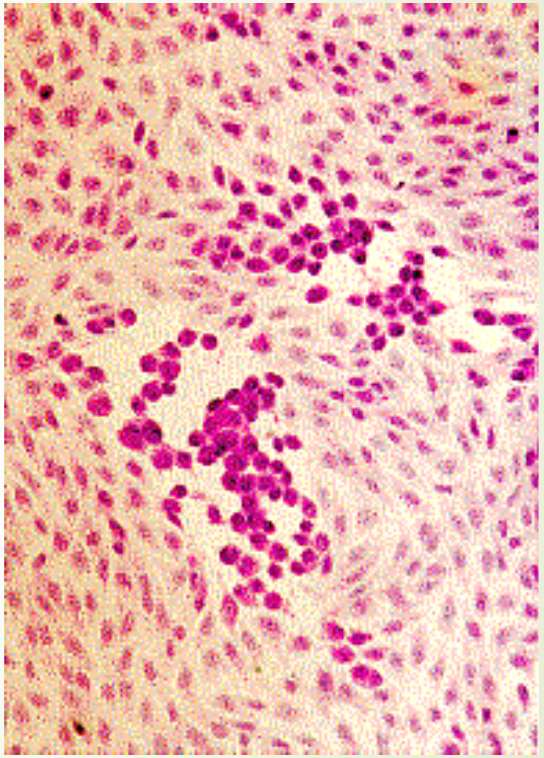
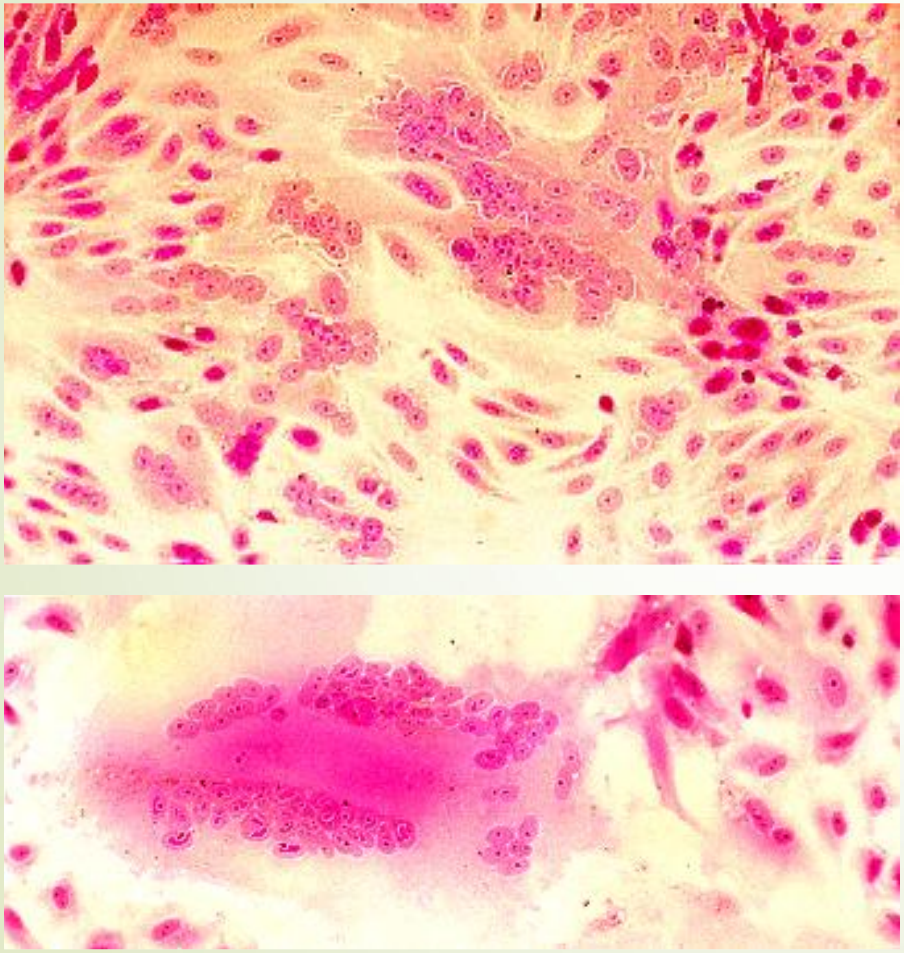


Fig. 1, Cytopathic effects of enterovirus 71 in rhesus monkey kidney cells



Cytopathic effect of enterovirus 71 and HSV in cell culture: note the ballooning of cells.
(Virology Laboratory, Yale-New Haven Hospital, Linda Stannard, University of Cape Town)

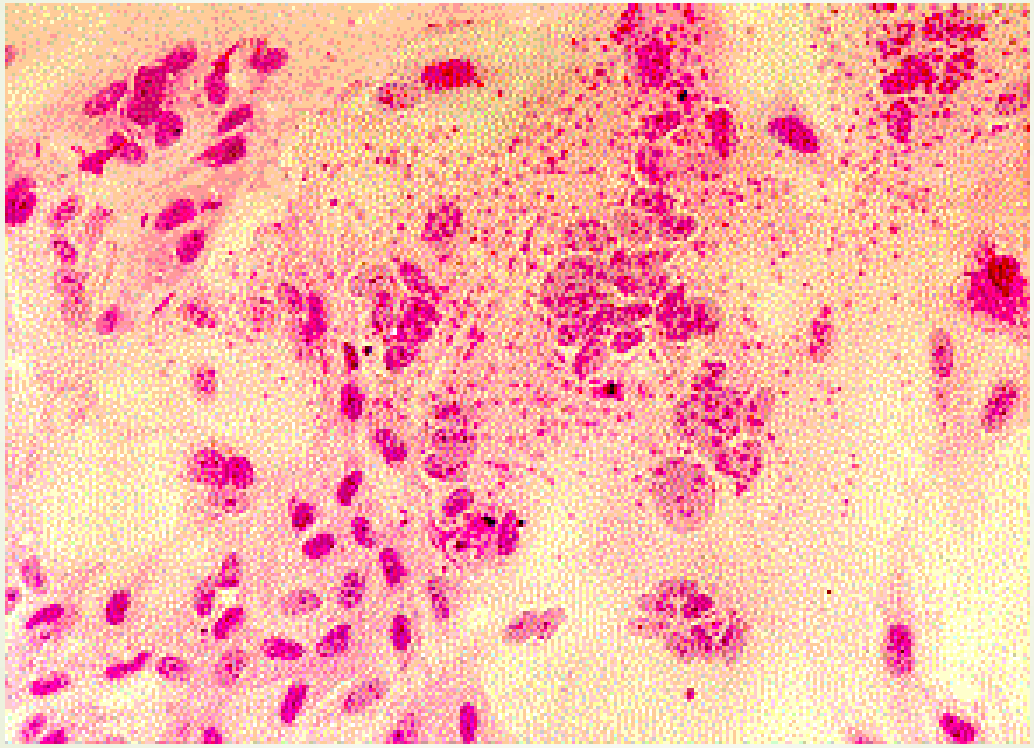
Cytopathic Effect (2)



Syncytium formation in cell culture caused by RSV (top), and measles virus (bottom).

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

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.)

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, Diarrhoeal viruses, parvovirus, papillomavirus.

Rapid Culture Techniques

Rapid culture techniques are available whereby viral antigens are detected 2 to 4 days after inoculation. The CMV DEAFF (detection of early antigen fluorescent foci) test is the best example, where by

- The cell sheet is grown on individual cover slips in a plastic bottle.
- Following inoculation, the bottle then is spun at a low speed for one hour (to speed up the adsorption of the virus) and then incubated for 2 to 4 days.
- The cover slip is then taken out and examined for the presence of CMV early antigens by immunofluorescence.

DEAFF test for CMV

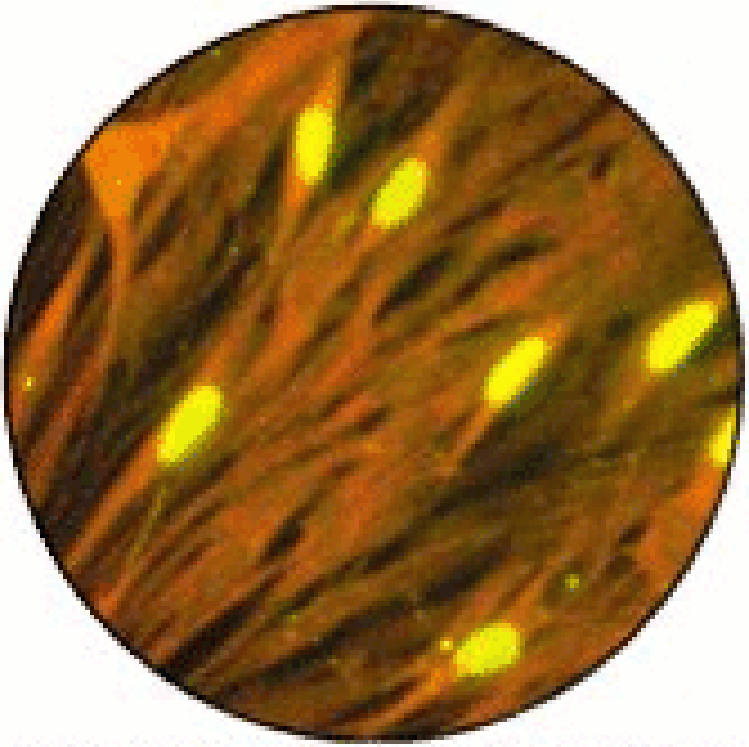


Fig. 2, CMV centrifugation culture fixed and stained 16 hrs after inoculation showing viral proteins in nuclei of infected human fibroblast cells

(Virology Laboratory, Yale-New Haven Hospital)

Microscopy

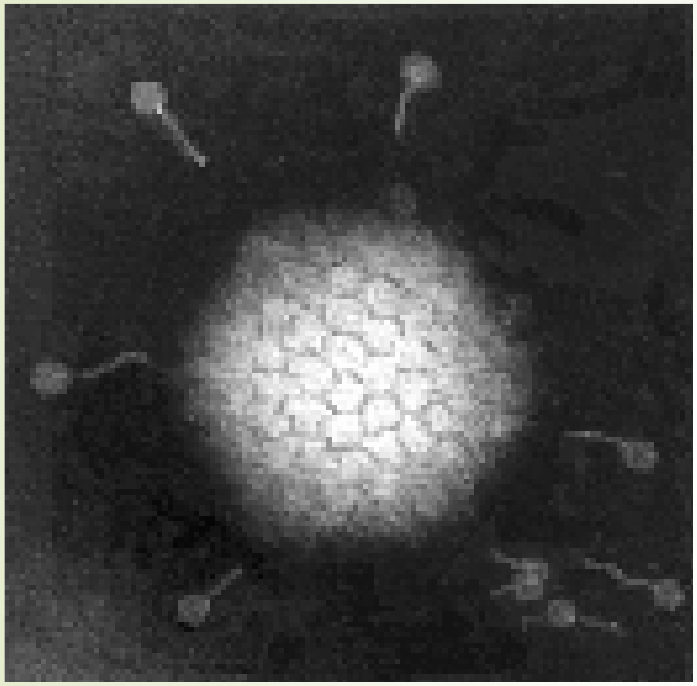
- Light Microscopy – elementary bodies
- Electron Microscopy Rota viral detection
- Florescent Microscopy Direct / Indirect

Electron Microscopy

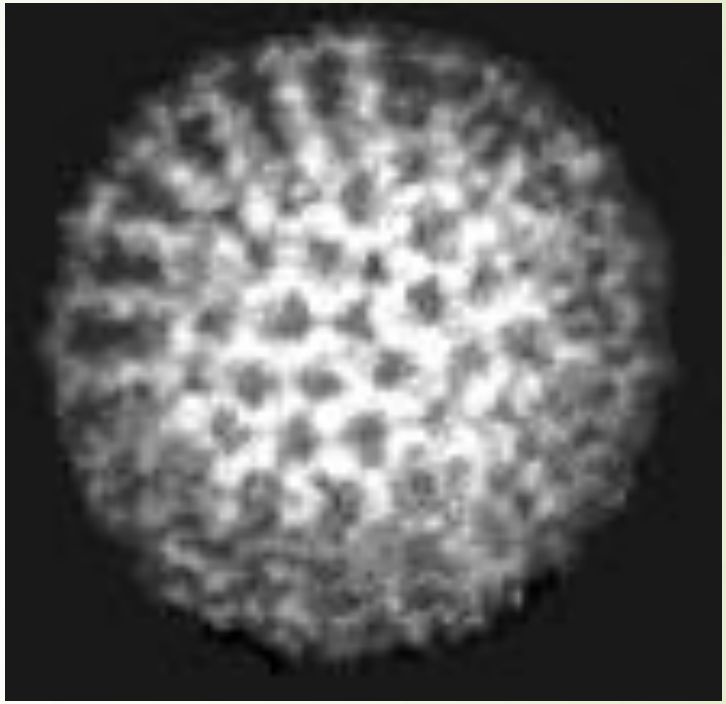
10^6 virus particles per ml required for visualization, 50,000 - 60,000 magnification normally used. Viruses may be detected in the following specimens.

Faeces	Rotavirus, Adenovirus
	Norwalk like viruses
	Astrovirus, Calicivirus
Vesicle Fluid	HSV
	VZV
Skin scrapings	papillomavirus, orf
	molluscum contagiosum

Electronmicrographs



Adenovirus



Rotavirus

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

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

Serology

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

Classical Techniques	**Newer Techniques**
1. Complement fixation tests (CFT)	1. Radioimmunoassay (RIA)
2. Haemagglutination inhibition tests	2. Enzyme linked immunosorbent assay (EIA)
3. Immunofluorescence techniques (IF)	3. Particle agglutination
4. Neutralization tests	4. Western Blot (WB)
5. Counter-immunoelectrophoresis	5. RIBA, Line immunoassay

Serology

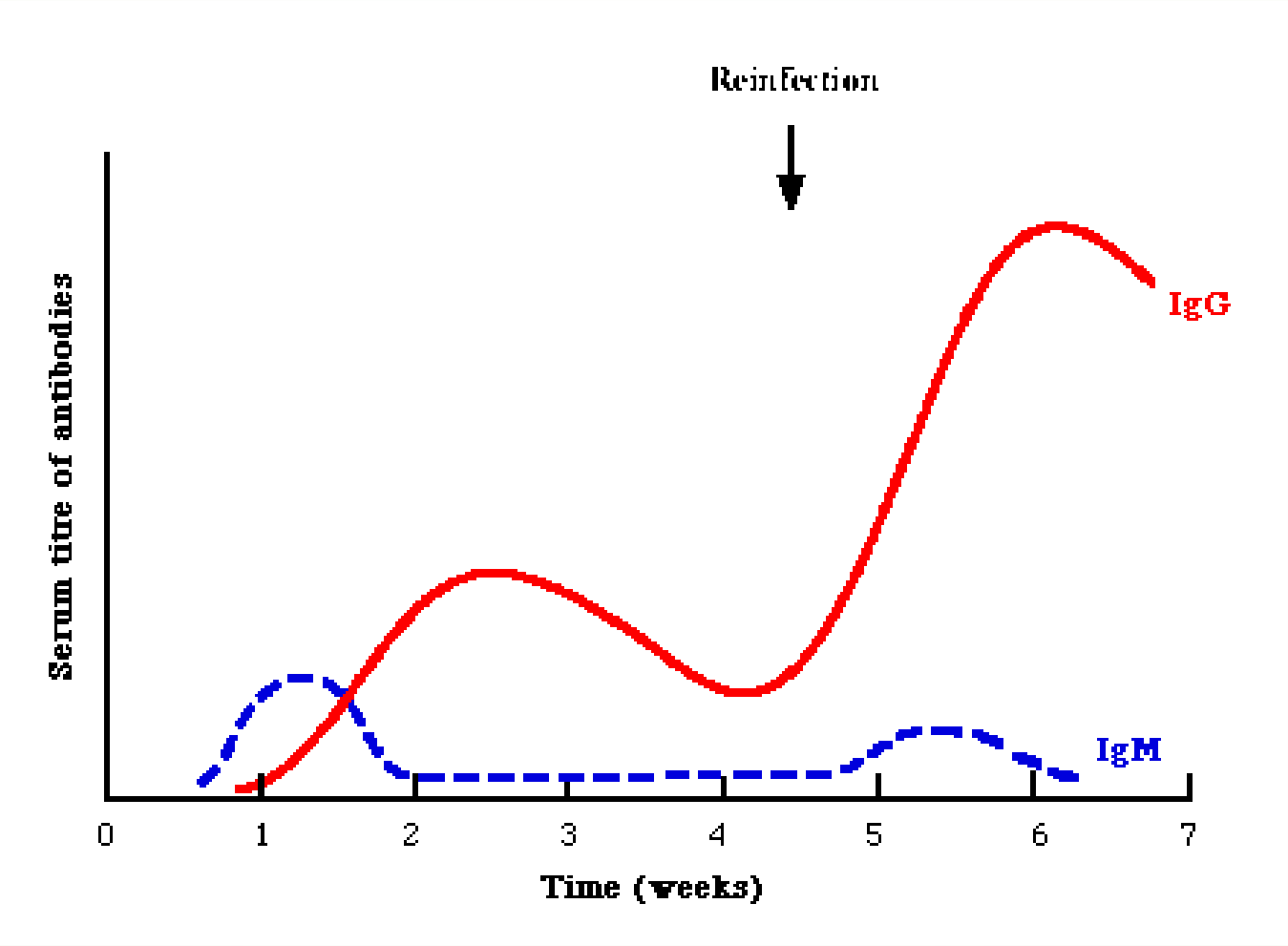
Criteria for diagnosing Primary Infection

- 4 fold or more increase in titre of IgG or total antibody between acute and convalescent sera
- Presence of IgM
- Seroconversion
- A single high titre of IgG (or total antibody) - very unreliable

Criteria for diagnosing Reinfection

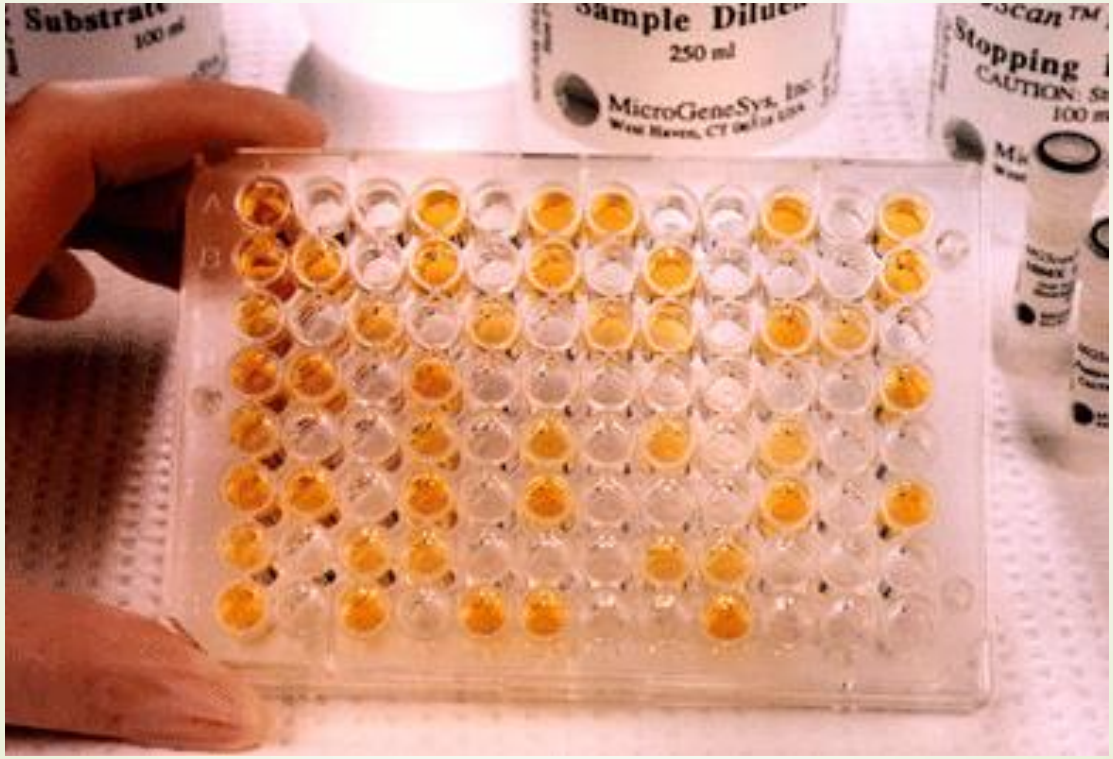
- fold or more increase in titre of IgG or total antibody between acute and convalescent sera
- Absence or slight increase in IgM

Typical Serological Profile After Acute Infection



Note that during reinfection, IgM may be absent or present at a low level transiently

ELISA for HIV antibody



Microplate ELISA for HIV antibody: coloured wells indicate reactivity

ELISA Procedures

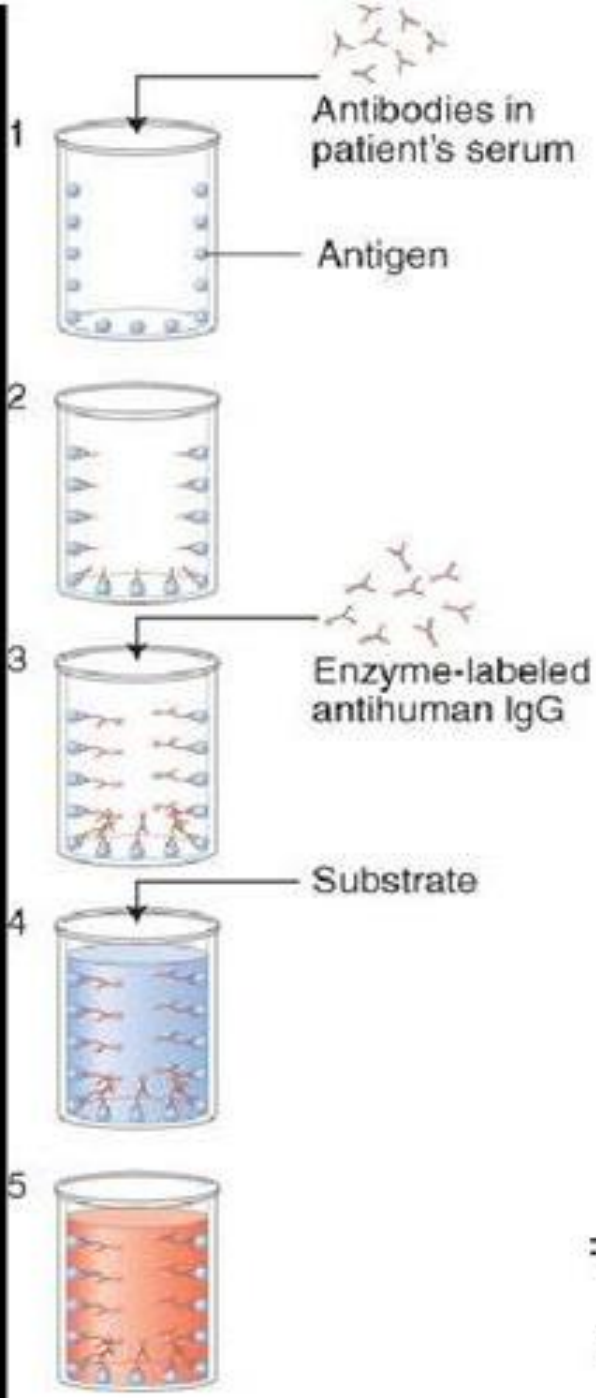


Figure 5-19a

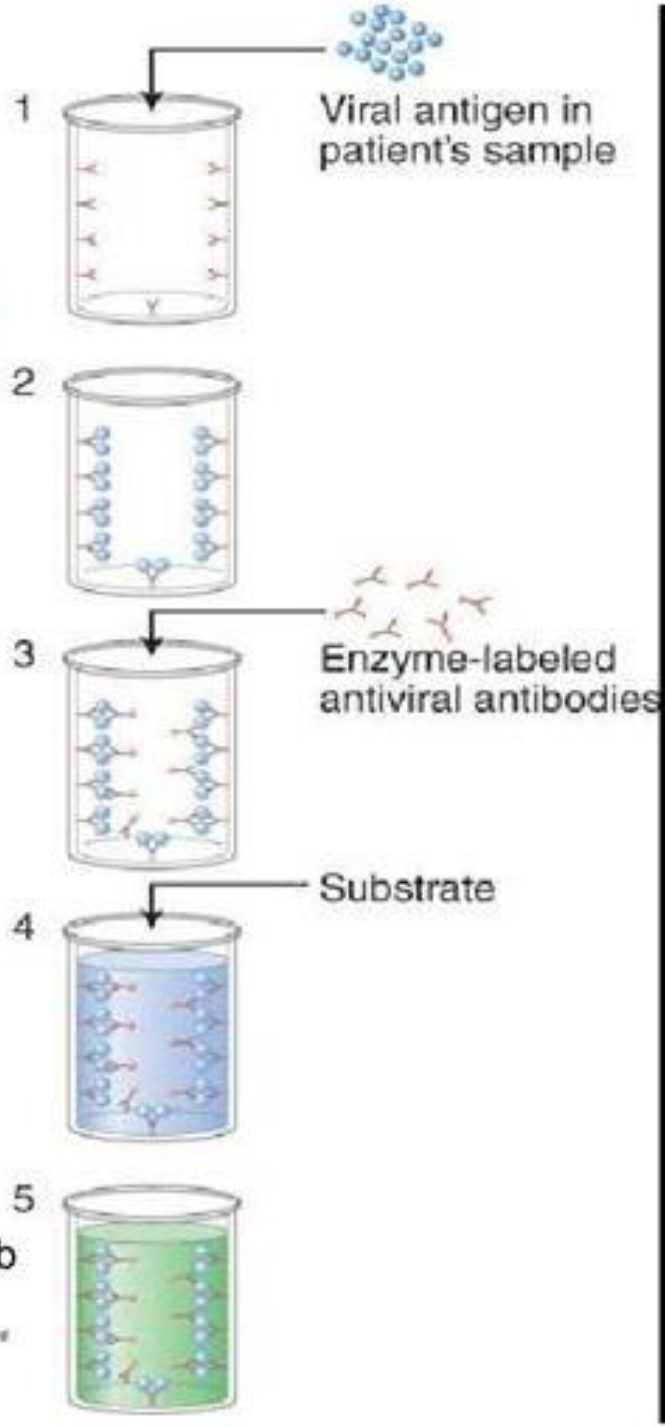


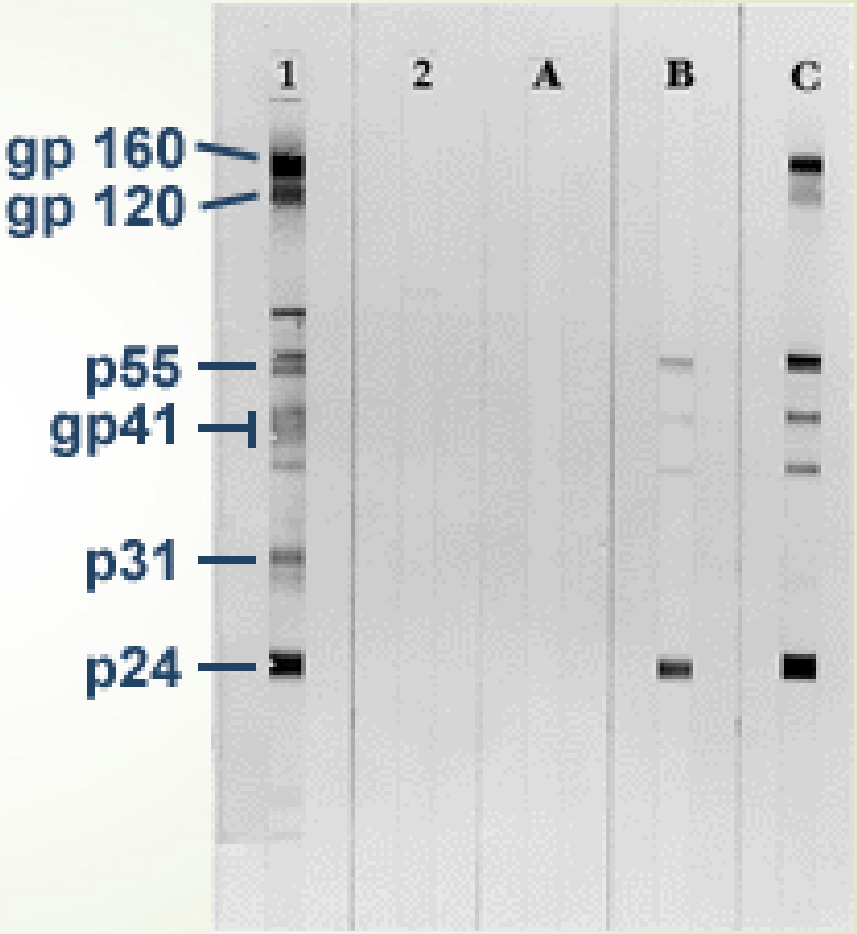
Figure 5-19b

Modified from Specter, S. C., R. L. Hodinka and S. A. Young. Clinical Virology Manual, Third Edition. ASM Press, 2000.

Western Blot

HIV-1 Western Blot

- Lane1: Positive Control
- Lane 2: Negative Control
- Sample A: Negative
- Sample B: Indeterminate
- Sample C: Positive



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 diarrhoeal 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 after seroconversion e.g. HIV and rabies. In the case of these viruses, the mere 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 such as HSV genitalis may not produce a detectable humoral immune response.
- Extensive antigenic cross-reactivity between related viruses e.g. HSV and VZV, Japanese B encephalitis and Dengue, may lead to false positive results.
- immunocompromised patients often give a reduced or absent humoral immune response.
- Patients with infectious mononucleosis and those with connective tissue diseases such as SLE may react non-specifically giving a false positive result.
- Patients given blood or blood products may give a false positive result due to the transfer of antibody.

CSF (cerebrospinal fluid) antibodies

- Used mainly for the diagnosis of herpes simplex and VZV encephalitis
- CSF normally contain little or no antibodies
- presence of antibodies suggest meningitis or meningoencephalitis

$$\frac{\text{CSF antibody titre}}{\text{Serum antibody titre}} > \frac{1}{100}$$ is indicative of meningitis

- Diagnosis depends on the presence of an intact blood-brain barrier

Rapid Diagnosis Based on the Detection of Viral Antigens

Nasopharyngeal Aspirate	RSV
	Influenza A and B
	Parainfluenza
	Adenovirus
Faeces	Rotaviruses
	Adenoviruses
	Astrovirus
Skin	HSV
	VZV
Blood	CMV (pp65 antigenaemia test)

Immunofluorescense

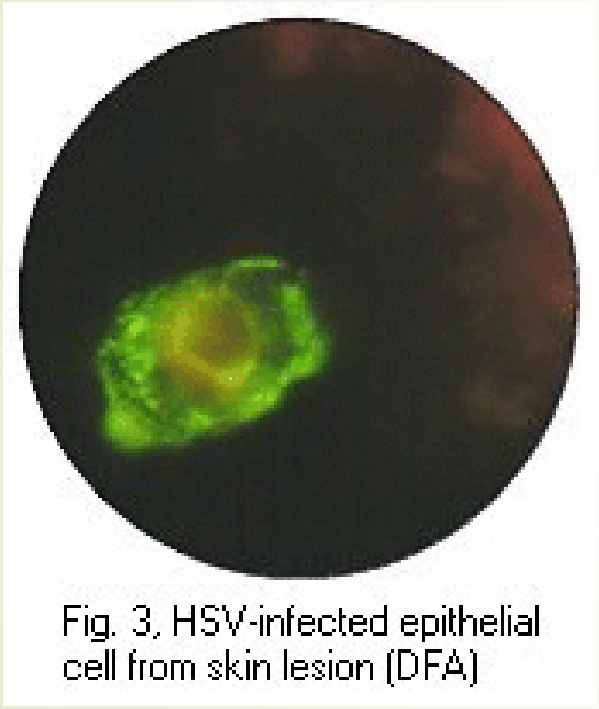
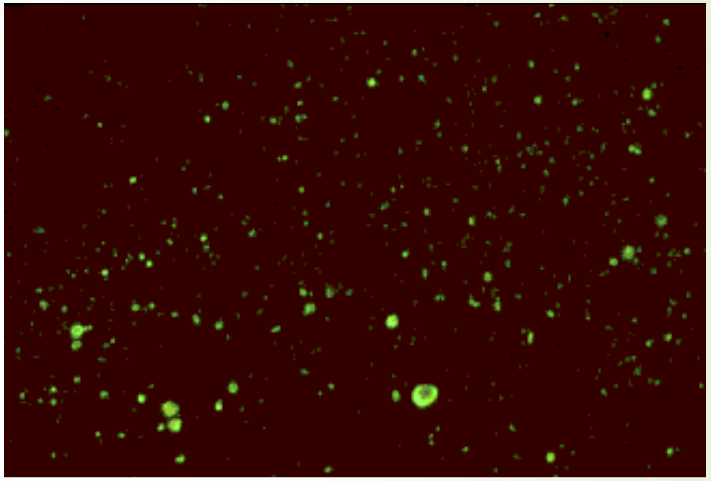


Fig. 3, HSV-infected epithelial cell from skin lesion (DFA)

(Virology Laboratory, Yale-New Haven Hospital)



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

CMV pp65 antigenaemia test

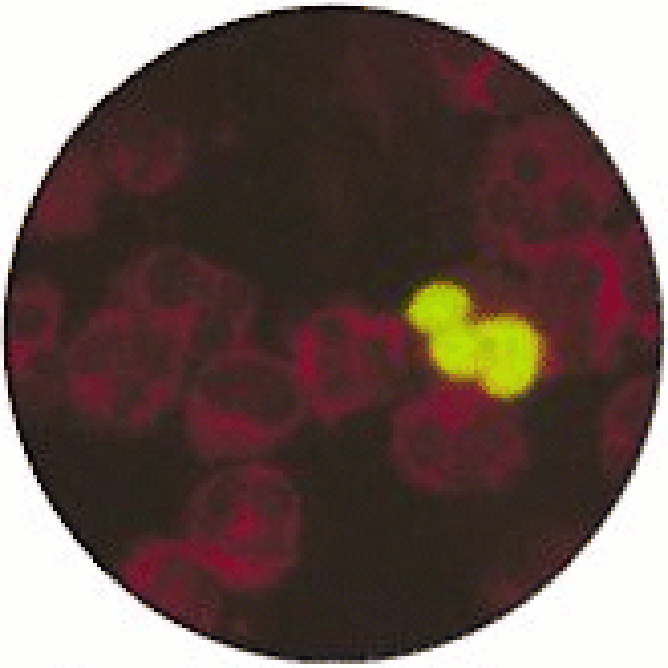


Figure 4 CMV pp65 antigens detected in nuclei of peripheral blood neutrophils

(Virology Laboratory, Yale-New Haven Hospital)

Advantages and Disadvantages

Advantages

- Result available quickly, usually within a few hours.

Potential Problems

- Often very much reduced sensitivity compared to cell culture, can be as low as 20%. Specificity often poor as well.
- Requires good specimens.
- The procedures involved are often tedious and time-consuming and thus expensive in terms of laboratory time.

Specimens for Routine Tests

Clinical Category	Blood	Throat swab	Faeces	CSF	Other
1. Meningitis	+	+	+	+	
2. Encephalitis	+	+	+	+	Brain biopsy
3. Paralytic disease	+	+	+	+	
4. Respiratory illness	+	+			Nasopharyngeal aspirate
5. Hepatitis	+				
6. Gastroenteritis			+		
7. Congenital diseases	+				Urine, saliva
8. Skin lesions	+		+		Lesion sample e.g. vesicle fluid, skin scrapping
9. Eye lesions					Eye swab
10. Myocarditis	+				Pericardial fluid
11. Myositis	+		+		
12. Glandular fever	+				
13. Post Mortem	+				Autopsy

After use, swabs should be broken into a small bottle containing 2 ml of virus transport medium. Swabs should be sent to the laboratory as soon as possible without freezing. Faeces, CSF, biopsy or autopsy specimens should be put into a dry sterile container.

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.
- It is certain though that the role of molecular methods will increase rapidly in the near future.

Classical Molecular Techniques

- Dot-blot, Southern blot, in-situ hydridization 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 more tedious and expensive than conventional techniques, they never found widespread acceptance.

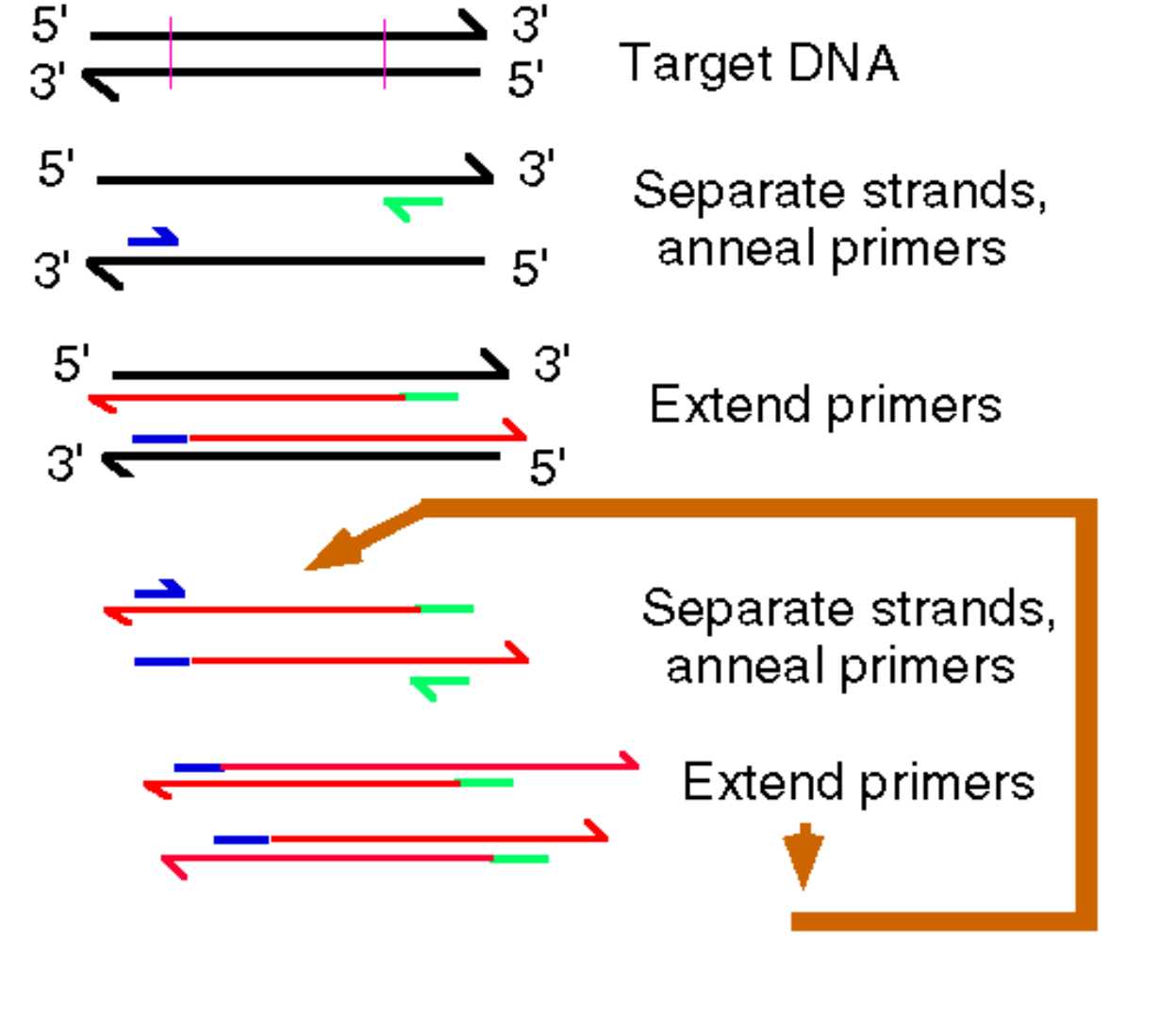
Polymerase Chain Reaction (1)

- PCR allows the in vitro amplification of specific target DNA sequences by a factor of 10^6 and is thus 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^{o}C), annealing of primers to their complementary sequences (50^{o}C), and primer extension (72^{o}C) result in the exponential production of the specific target fragment.
- Further sensitivity and specificity may be obtained by the nested PCR.
- Detection and identification of the PCR product is usually carried out by agarose gel electrophoresis, hybridization with a specific oligonucleotide probe, restriction enzyme analysis, or DNA sequencing.

Polymerase Chain Reaction (2)

- 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 such as CMV, 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 such as the Roche Cobas Amplicor 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.

Schematic of PCR



Each cycle doubles the copy number of the target

Other Newer Molecular Techniques

- Branched DNA is essentially a sensitive hydridization technique which involves linear amplification. Whereas exponential amplification occurs in PCR.
- Therefore, the sensitivity of bDNA lies between classical amplification techniques and PCR. Other Newer molecular techniques depend on some form of amplification.
- Commercial proprietary techniques such as LCR, NASBA, TMA depend on exponential amplification of the signal or the target.
- Therefore, these techniques are as susceptible to contamination as PCR and share the same advantages and disadvantages.
- PCR and related techniques are bound to play an increasingly important role in the diagnosis of viral infections.
- DNA chip is another promising technology where it would be possible to detect a large number of viruses, their pathogenic potential, and their drug sensitivity at the same time.

Comparison between PCR and other nucleic acid Amplification Techniques

Method	Target Amplification	Signal Amplification	Thermocycling	Sensitivity	Commercial Examples
PCR	Exponential	No	Yes	High	Roche Amplicor
LCR	No	Exponential	Yes	High	Abbot LCX
NASBA	Exponential	No	No	High	Organon Teknika
TMA	Exponential	No	No	High	Genprobe
Qß-Replicase	No	Exponential	No	High	None
Branched DNA	No	Linear	No	Medium	Chiron Quantiplex