Komar University of Science and Technology
Medical Laboratory Science Department

MEDICAL VIROLOGY

A LABORATORY MANUAL

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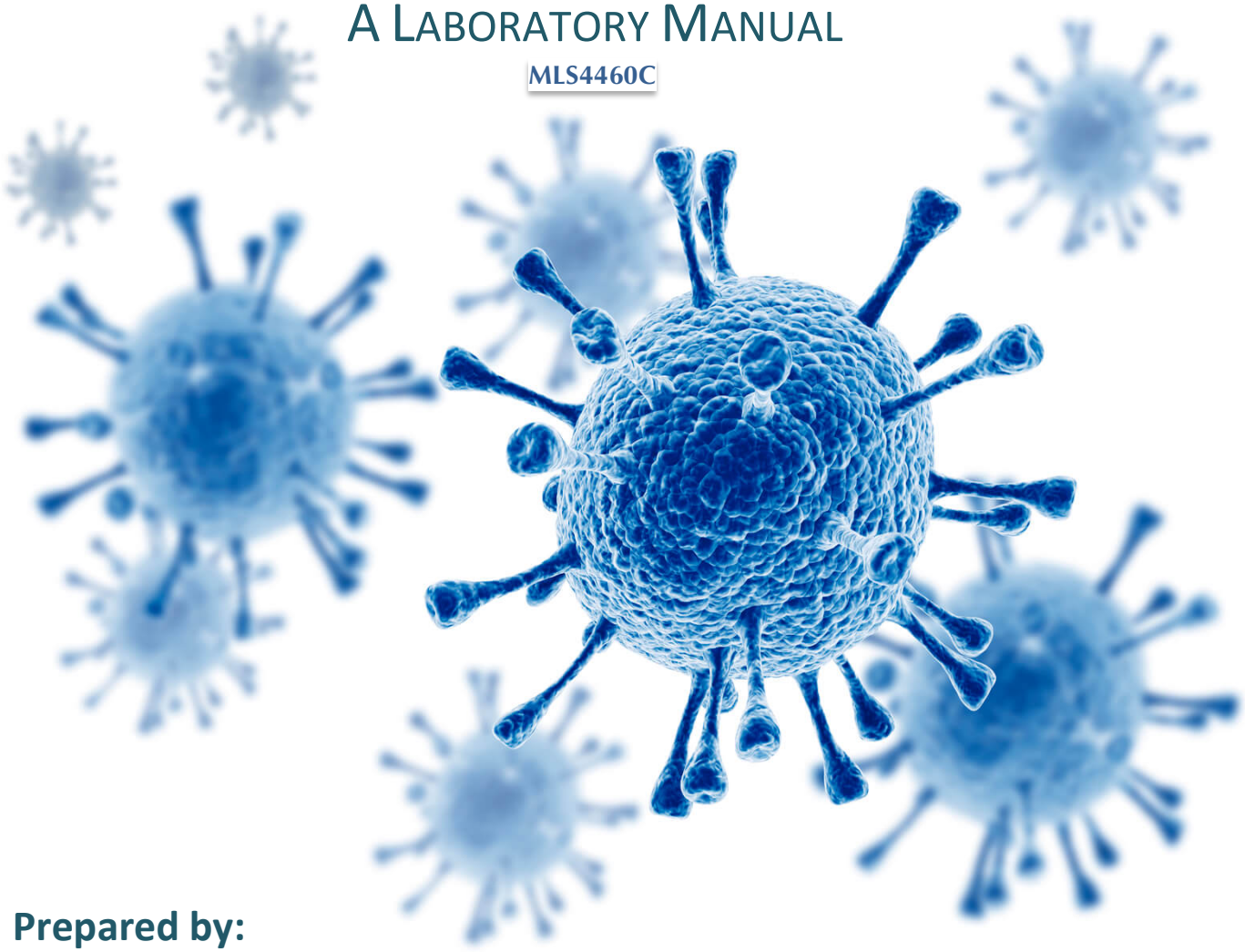

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Introductory

Virology is a fascinating and rapidly developing subject, and is worthy of study purely because viruses are interesting! Furthermore, virology is a branch of science that is of immense relevance to mankind for a host of reasons, not least of which the threats to human health are caused by viruses. There is a continuing need for trained virologists, and it is hoped that this course will play a small role in helping to fulfil this need. Methods used in virology are introduced in order to provide an appreciation of the nature of the techniques that have been used to achieve our current level of knowledge and understanding of viruses.

Virology is a huge subject and uses a wide range of methods. Many of the techniques of molecular biology and cell biology are used, and constraints on space permit us to mention only some of them. Much of the focus of this course is on methods that are unique to virology. Many of these methods are used, not only in virus research, but also in the diagnosis of virus diseases of humans, animals and plants.

Virology Labs

Week 1:

1.1 Laboratory safety guidelines and aseptic techniques

1.1.1 General Laboratory Safety Guidelines:

- You should be aware that laboratories handling microbes, whether for research or diagnostic purposes, are classified in terms of the level of containment they provide, usually on a scale of 1 to 4.
- Thus routine clinical or teaching laboratories are classified as containment level 1, and those dealing with the most hazardous pathogens as containment level 4.
- You should handle all patient samples as potentially biohazard material. This means universal precautions should be followed at all times.

1.1.2 Safety guidelines when working in the laboratory:

- DO NOT smoke, eat, drink and applying cosmetics in the laboratories.
- Wear protective closing (lab coat, gloves. If you have a cut/abrasion, also wear a band-aid).
- Avoid spillage and aerosol formation.
- Hands should be washed immediately and thoroughly if contaminated with blood or other body fluids.
- Gloves should be removed before handling a telephone, computer keyboard, etc., and must NOT be worn outside the immediate work area.
- Hands should always be washed immediately after gloves are removed.
- You should wash your hands after completing laboratory activities and before leaving the area.
- All protective clothing should be removed prior to leaving the lab.
- All biohazardous material should be discarded in a biohazard bag to be autoclaved.

- All counter and table tops should be disinfected with a proper disinfecting solution:
    - At the beginning of the day.
    - If you should spill a patient sample.
    - At the end of the day.
- Wipe off visible contamination by using a towel or gauze pad moistened with acceptable decontaminant. Ensure label and bar code are not obscured before advancing specimen for analysis.

1.1.3 Common Methods of Inactivating Viruses for Various Purposes

Viruses may be inactivated for various reasons, such as to:

1. Sterilize laboratory supplies and equipment
2. Disinfect surfaces or skin
3. Make drinking water safe
4. Produce inactivated virus vaccines

Different methods and chemicals are used for these purposes:

1. Sterilization: Is the process where all forms of microorganisms are killed. This can also be accomplished by physical or chemical means.

A. The physical methods of sterilization are incineration, moist heat or autoclaving, dry heat, filtration, and ionizing radiation.

- Incineration is a method commonly used for medical waste.
- Moist heat is used for sterilization of media and supplies by autoclaving at 121°C for 15 minutes and for sterilization of medical waste by autoclaving at 132°C for 30 to 60 minutes.
- Hot air oven at 160 ° C for 60 min
- Filtration using a 0.45µm filter can be used to remove bacteria from solutions; however, this will not remove viral agents.

B. Chemical Methods of Sterilization: The most common chemical method is ethylene oxide; however, glutaraldehyde and peracetic acid are also used.

2. Disinfection: Disinfection is the process where most, but not necessarily all, microorganisms are destroyed. This can be accomplished by physical or chemical means.

A. Physical methods of disinfection are boiling at 100°C for 15 minutes, pasteurizing, or UV light irradiation.

B. Chemical methods of disinfection are more commonly used: The choice of disinfectant used should be based upon its effectiveness against expected pathogens.

- **At concentrations of 60% to 80%, ethanol** is active against enveloped viruses (e.g., herpesviruses, vaccinia virus, and influenza virus) and many nonenveloped viruses including (adenovirus, enterovirus, rhinovirus, and rotaviruses, but not hepatitis A virus or poliovirus.
- **Quaternary ammonium compounds:** are also effective surface disinfectants. They are fungicidal, bactericidal, and virucidal against enveloped viruses
- **Chlorine bleach** is an effective disinfectant and is recommended for surface disinfection. It has a broad spectrum of activity being bactericidal, fungicidal, sporicidal, tuberculocidal, and virucidal. A 1:10 dilution of 5.15% to 6.25% sodium hypochlorite (household bleach) can be used for decontamination of blood spills. The length of contact time required depends on the kind of the infectious agent, the organism load, the location of the spill, and the volume of the spill disinfectant employed.

1.2 Specimen Collection, Transport and Storage

Proper specimen collection is essential for interpreting test results because of the wide range of viruses, the complexity of virus–host interactions, and changes in testing methodology. The selection of an appropriate specimen is vital to a correct test result; this includes not only the source of the specimen but also the timing and volume of collections.

1.2.1 Specimen collection:

1.2.1.1 Specimen collection rules:

- Microbiology specimens are to be received in uncontaminated containers that are intact and are consistent with laboratory specimen collection policy.
- Consider all blood samples as coming from patients potentially infected (e.g., with human immunodeficiency virus (HIV) or hepatitis), and handle appropriately.
- If any concern exists about external contamination, carefully disinfect the outside of the tubes or bottles before inserting them into the blood culture instruments.
- Change and discard gloves after cleanup and decontamination of the immediate area. Document the external contamination for reporting purposes.
- Viral specimens with damaged or leaking containers may need to be discarded before opening, and request another specimen.
- Label the sample container with name, age, sex, date and the type of test to be performed.

Suitable specimens are dependent on the methods used for testing. Molecular, antigen, culture, serology, or histopathology assays each have different performance characteristics and volume requirements. Importantly, each method detects viral infections in different ways and therefore might require different specimen sources (Table 1).

1.2.1.2 Repeat specimen collection:

- In some cases, submission of a single specimen is sufficient for clinical and laboratory assessment, For example, a dermal specimen positive for HSV should be considered a meaningful result that requires immediate clinical attention.
- In other cases repeat or additional specimen collection may be warranted for confirmatory testing, such as HIV diagnosis, where a positive or negative result can have significant implications for the patient
- For therapeutic, rather than diagnostic, purposes, laboratories should be prepared to receive multiple specimens from a single patient over time.

Table 1: Specimen Information for Some Viral Infections

Kind of Infection	Viral Pathogen	Nucleic Acid Testing	Serology	Culture	Other Comments
Respiratory disease	Influenza virus	Nasal swab		Nasal swab	
	Herpes simplex virus	Respiratory washes BAL Throat swab Tissue		Respiratory washes BAL Throat swab Tissue	
Hepatic infections	Hepatitis A virus	Serum Stool Saliva	Serum		Serology is the current gold standard method of diagnosis.
	Hepatitis B virus	Serum	Serum		Quantitative molecular assays are available for monitoring disease progression and efficacy of therapy. Both antigen and antibody markers may be detected from serological studies.
	Hepatitis C virus	Serum	Serum		Reactive serology is most commonly confirmed with a quantitative viral load assay and can be used for monitoring disease progression and efficacy of therapy.
AIDS	Human immunodeficiency virus types 1 and 2	Blood, plasma, serum	Blood, plasma, serum. Serology with an antigen/antibody immunoassay is recommended as the initial screen.		Serology results should be confirmed with an HIV-1/2 differentiation immunoassay. Nucleic acid assays may be used for diagnosis of acute HIV infection as well as for monitoring.

1.2.2 Transport:

The type of transport medium, and transportation time to the laboratory are important considerations for viability of the specimen matrix, the pathogen, viral antigens, and nucleic acid.

1) Send specimen to lab immediately at (2°C - 8°C) (testing sensitivity decreases over time).
2) Some specimen require viral transport medium (VTM).

Most transport media consist of a buffered isotonic solution with:

a) Some type of protein, such as albumin, gelatin, or serum, to protect less stable viruses and prevent drying.
b) Antibacterial and antifungal agents are added in some transport systems to inhibit contaminating bacterial and fungal biota.

Specimens that require viral transport medium:

- Respiratory specimens such as nasal wash and bronchoalveolar lavage specimens
- Conjunctival, genital, rectal, and vesicle swabs and tissue specimens

Specimens that do not require VTM:

- Blood, bone marrow, cerebrospinal fluid (CSF), amniotic fluid, urine, pericardial fluid, and pleural fluid.

Notes:

- Tubes containing 2-3 mL VTM are used for swab specimens, while those with 5-7 mL VTM are suitable for tissue samples.
- VTM do not interfere with either immunodiagnostic or molecular diagnostic procedures.
- The transport container should be unbreakable and able to withstand freezing and thawing.

1.2.3 Specimen processing:

- Body fluid specimens like CSF, urine, synovial fluid, secretions, and other aspirates can be applied directly to antigen assays and culture cell lines.
- For PCR-based assays, nucleic acid is generally extracted prior to testing. Although nucleic acid extraction from specimens requires an additional processing step, time, and expense, it can enhance detection of viral pathogens by removing inhibitors from the patient sample.

1.2.4 Storage:

In general, all specimens should be kept at:

- Handling and storage for short period (2 - 8°C) (Most viruses persist longer at cooler temperatures, and even highly labile pathogens such as CMV, RSV, and VZV can survive transit for 1 to 3 days if maintained at 4°C)
- Freezing (-70°C) for long period storage.

Notes:

- Unlike viral culture, where each hour of delay before inoculation translates to progressively lower probability of successful isolation, molecular and antigen testing abrogates the need for viral viability.
- Viruses that are enveloped (herpesviruses and HIV) are relatively labile compared to those without envelopes.
- A single freeze-thaw cycle may decrease the titer of HSV by 100-fold in cell culture.
- Freezing patient specimens (−20°C) or (-70°C) and refrigerating (4°C) nucleic acid extracts of specimens are both reasonable for at least 16 months.

1.2.5 Review questions:

- What is the effect of bacterial growth on virus activity?
- Why cerebrospinal fluid sample does not require VTM?

Review questions for previous courses:

- **Problem 1**: You have a 0.01% solution. You want a 0.001% solution and you need 25 ml.
- **Problem 2**: Prepare 50 ml of a 3% solution from a 4% solution.
- **Problem 3**: Prepare 100 ml of 70% alcohol from 95% alcohol.
- **Problem 4**: Prepare a 45 ml of a 2% suspension of RBCs from a 5% suspension.
- **Problem 5**: You want a 1:128 dilution of serum and you need 4 ml. How would you perform this dilution in several steps?

Week 2: Isolation of viruses

3.1 Background

Viruses are obligate intracellular parasite. In order to replicate they require living cells in the form of cultured cells, embryonated hen's eggs, or laboratory animals. Cell Culture has long been considered the "gold standard" for viral diagnosis. This technique is most often used to confirm optimal performance of other methods, to validate newer methods, or to aid the discovery of new viruses, rather than as the primary diagnostic method.

The term cell culture is technically used to indicate culture of cells in vitro; the cells are not organized into a tissue. While the term tissue culture or organ culture is used to denote growth of tissues or an organ in a way that preserves the architecture or function of the tissue or organ.

The first step of viral infection is attachment to specific cell type, and this is happened because of ligand and receptor interaction, which is very specific. This process is known as cell tropism. Based on this specificity cells culture techniques are used in studying viruses. Because of viral activities in cell lines, different changes will happen called cytopathic effects (CPE), which are characteristic for each type of virus.

Cell culture techniques are used for different purposes including studying viruses. Cells should be grown to a desire number by using suitable conditions and growth medium. After such growth the cells should be maintained in a live but not further growth. This situation is achieved by replacing the growth medium with maintenance medium. In order to maintain cell viability, they must periodically be removed, diluted, and placed into a new container. This process is referred to as passaging.

Purpose of cell culture technique in virology:

Cell culture techniques are used in different aspects of studying viruses such as:

1. Determining the host cell(s) in which virus replicates (cell tropism)
2. Isolation of virus particles.
3. Preserving viruses for further use.

4. Studying the changes in cell morphology because of viral infection and how quickly viruses cause cell damage.
5. Testing the ability of specific antibody that block viral attachment.
6. Studying the effect of different chemicals on virus infectivity.
7. Viral vaccine production.

Table 2. Types of cell cultures commonly used in a clinical virology laboratory:

Cell culture	Example	Number of passaging
Primary	Kidney tissues from monkeys (RhMK), rabbits (RK), etc. Embryos from chickens (CE), guinea pigs (GPE), etc.	**1-2**
Diploid (semicontinuous)	Human embryonic lung (MCR-5) or human newborn foreskin (HFF)	**20-50**
Continuous	Human epidermoid carcinoma of lung (A549), mink lung (ML or Mv1Lu)	Indefinite

Requirements for isolation of viruses in cell culture:

For inoculation of cell cultures:

1. Laminar flow hood
2. Centrifuge
3. Pipettes, automatic pipetting device, pipette jar and discard can

4. Disposable gloves
5. Disinfectant
6. Sterile glass and plastic ware
7. Maintenance of cell cultures

Maintenance of cell cultures:

1. Culture media
2. Serum
3. Antibiotics
4. 4°C refrigerator
5. Test tube racks, and/or rotating drum
6. Shell vial racks
7. Room air incubators
8. CO_2 incubator
9. Water bath
10. Upright and inverted microscopes

Preservation and storage of viruses:

1. Freezer vials
2. Ultra-low temperature freezer (- 70°C)
3. Dimethyl sulfoxide as stabilizer

3.4 Procedure: Inoculation of cell culture with virus sample

1. When the cell sheets have developed sufficiently, the cell culture fluid is aseptically removed from each tube or bottle.
2. The monolayers are to be washed 2-3 times with warm PBS to remove the proteins and the dead cells.
3. The virus materials are inoculated (The cell monolayers are infected with virus material).
4. Incubation at 37 °C for 1 hour.

5. Replace the inoculum with maintenance medium, then incubation at 37 °C.
6. After infection at 24 hours intervals, the monolayers are examined for CPE.

3.5 Detection of Virus-Induced Effects:

1. CPE: Cytopathic effects due to virus activities are manifested in different ways and they are characteristic for each type of virus.

The degree of CPE is usually graded from + to ++++ based on the percentage of the cell monolayer infected: 25% of the cell monolayer (+), 50% (++), 75% (+++), and 100% (++++).

There are two important points that should be emphasized regarding CPE induced by virus:

1. The rate at which CPE progresses may help to distinguish similar viruses; for example, HSV progresses rapidly to involve the entire monolayer of several cell systems. In contrast, two other herpesviruses, CMV and VZV, grow primarily in HDF cells and progress slowly over a number of days or weeks.
2. The type of cell culture(s) in which the virus replicates is important factor in identification.

2. Hemadsorption (HAd): Parainfluenza and sometimes influenza virus replication may not induce distinctive cellular changes; however, these viruses express hemagglutinins that are expressed on the infected cell membrane and have an affinity for red blood cells (RBCs). The addition of a guinea pig RBC suspension to the infected cultures allows the RBCs to adsorb onto the infected cells

3. Production of plaques by viruses

4. Direct Immunofluorescence

2.3 Determining cell number and viability with a hemocytometer and staining

2.3.1 Purpose:

In order to study viral activity in cell culture, cell lines should be maintained viable and checked for contamination, and these testes are named viability test and sterility test respectively. Determining the number and percentage of viable cells in culture is important in standardization of culture conditions and in performing accurate quantitation experiments. A hemocytometer is a thick glass slide with a central area designed as a counting chamber.

2.3.2 Materials:

1. 70% (v/v) ethanol
2. Cell suspension
3. 0.4% (w/v) trypan blue or 0.4% (w/v) nigrosin.
4. Hemocytometer with coverslip.
5. Hand-held counter

2.3.3 Procedure:

- **Prepare hemocytometer:**
- **Prepare cell suspension**
- **Load hemocytometer**
- **Vital Staining & cell counting:**

1) Mix 0.1 ml of dilute cell suspension + 0.5 ml of (0.4% (w/v) Trypan Blue or Nigrosin) in a tube for 5 minutes
2) Observe the cell suspension under the microscope by using standard counting chamber.
3) Calculate % of viable cells. $$\% \text{ viable cells} = \frac{\text{number of unstained cells}}{\text{total number of cells}} \times 100$$

*Adjust the cells to a certain concentration with growth medium to a standard number of cells/ml.

2.3.4 Result:

Record the following results:

1. % Viable cells
2. Number of viable cells/ml

3.6 Review Questions:

1. After adding the virus material the cell monolayer should be incubated for 1 hour, why?
2. After adding viral sample to monolayer cells which type of medium is added, and why?
3. How can you differentiate dead cells from viable cells?
4. What is the purpose of using trypan blue stain in this lab?
5. Why cell cultures require passaging?

Week 3: Rapid Culture Methods

Centrifugation Culture (Shell Vial Technique)

3.1 Background:

The rapid diagnosis of viral infections is important in patient management. However, conventional virus isolation requires observation of monolayer cultures for CPE, which can take days to weeks to appear. The application of centrifugation cultures to viral diagnosis can shorten time to diagnosis to 1 to 2 days. It has long been recognized that low-speed centrifugation of cell cultures enhances infectivity of viruses.

In 1984 the use of centrifugation cultures followed by staining with a monoclonal antibody at 24 hours post- inoculation was first reported for CMV. The overall sensitivity of the shell vial technique varies with the type of specimen, the length and temperature of centrifugation, the virus, the cell cultures, the antibody employed, and the time of fixation and staining. The use of young cell monolayers and inoculation of multiple shell vials enhance the recovery rate. Toxicity, particularly problematic with blood and urine specimens, can lead to cell death and the loss of the monolayer, necessitating blind passage of the specimen or specimen reinoculation.

3.2 Purpose:

The shell vial technique usually combines cell culture to amplify virus in the specimen, centrifugation to enhance viral infectivity, and early detection of virus- induced antigen (before CPE) by the use of high-specificity antibodies.

3.3 Inoculation of Shell Vials (Traditional Single Cell Culture Type, Stained for One Virus):

Staining of shell vials and identification of virus isolates:

3.3.1 Materials:

1. Antibodies to specific viral types, usually fluorescein labeled
2. Cold acetone
3. Cell cultures grown on coverslips in shell vials, sensitive to the suspected viruses
4. Low-speed centrifuge with adapters for shell vials
5. Humidified chamber
6. Rotator or rocker
7. Suction flask and vacuum source
8. PBS
9. Teflon-coated microscope slides
10. Forceps
11. Incubator
12. Mounting medium
13. Fluorescence microscope

3.3.2 Procedure:

14. Prepare two shell vials.
15. Remove cap and aspirate medium from shell vial.
16. Inoculate prepared specimen onto monolayer, 0.2 to 0.3 ml per vial.
17. Replace cap and centrifuge (30 to 60 minutes at 700 · g).
18. Aspirate inoculum for blood, urine, and stool samples, then rinse with 1 ml of medium to reduce toxicity.
19. Add 1.0 ml of maintenance medium to each shell vial and incubate at 35°C for 1–2 days.

Fixation of coverslips in shell vials:

1. Before fixation, inspect the coverslips for toxicity, contamination, and so forth. If necessary, passage the cell suspension to a new vial and repeat incubation before staining.
2. If monolayer is intact, aspirate medium from shell vials and rinse once with 1.0 ml of phosphate-buffered sa- line (PBS) (pH 7). If monolayer appears fragile, do not rinse with PBS.

3. Aspirate medium completely, add 1.0ml of 100% cold acetone or 50/50 acetone/methanol to each shell vial and allow cells to fix for 10 minutes.
4. Aspirate the acetone and allow the coverslips in the shell vial to dry completely.

Staining of coverslips.

1. Add 1.0 ml of PBS to each coverslip, then aspirate the PBS.
2. Pipet 150 ml (five drops) of antibody reagent (ap- propriately titrated and diluted) into the shell vial. Replace the cap.
3. Rock the tray holding shell vials to distribute the re- agent; then check to see that coverslips are not floating above the reagent.
4. Place rack holding the shell vials in a humidified chamber in the 35°C incubator.
5. Incubate for 30 minutes.
6. Add 1.0 ml of PBS to the shell vial, then aspirate. Repeat wash step two additional times.

For direct assays (primary antibody is labeled), go directly to step 9. For indirect assays (primary antibody is not labeled):

7. Pipet 150 ml (five drops) of labeled conjugate onto the monolayer.
8. Repeat steps 3 to 6, except do not aspirate the last 1.0 ml of PBS.
9. Using forceps and a wire probe, remove coverslip, and blot on tissue or absorbent paper (e.g., Kimwipe).
10. Add one drop of mounting fluid to a properly labeled slide and place coverslip on mounting fluid with cell side down, being careful not to trap air bubbles.

3.3.3 Reading procedure:

- Coverslips are examined using a 20X objective with a fluorescence microscope equipped with the appropriate filters to maximize detection of the fluorescein

isothiocyanate (FITC) label (or a light microscope if a peroxidase label is used).

- A known positive control is run for each viral antigen with each assay.
- Noninfected monolayers are fixed and stained as negative antigen controls. For indirect IF, normal goat serum, or PBS plus FITC conjugate, is used as a negative serum control.

3.3. 4 Mixed (Co-Cultivated) Cell Cultures and Monoclonal Antibody Pools:

In order to detect more viruses with fewer cell cultures:

1. Antibodies to more than one virus, often with two or three different fluorescent labels, were pooled.
2. Two to three different cell cultures were combined in one vial, thus described as co-cultivated cells

There are at present a variety of combinations of cultures to choose from, depending upon the suspected viruses:

R-mix (Mv1Lu and A549) and R-mix Too (Madin Darby Canine Kidney [MDCK] and A549) are commonly used with monoclonal antibody pools to rapidly detect selected respiratory viruses—that is, adenovirus; influenza A and B; parainfluenza 1, 2, and 3; and respiratory syncytial virus (RSV).

Week 4: Viral Antigen Detection by Immunofluorescence Assay

4.1 Purpose:

Like most serological tests, the fluorescent antibody (FA) technique can be used to identify the presence of either antigen or antibody in a sample. Direct tests (DFA) identify the presence of antigens, while indirect tests (IFA) detect the presence of antibody in a sample. FAs are useful in diagnosing many viral infections.

4.2 Principle:

Fluorescent antibodies are labeled with fluorescein isothiocyanate (FITC) dye, which fluoresces when illuminated with UV light. In a DFA, a sample containing the suspected antigen is fixed to a microscope slide. The fluorescent antibody is added and allowed to react with the antigen (Figure 1). After rinsing to remove unbound antibody, the slide is viewed with a fluorescent microscope with a UV light source. If the suspected antigen is present, the labeled antibodies will have bound to it and will emit an apple green color.

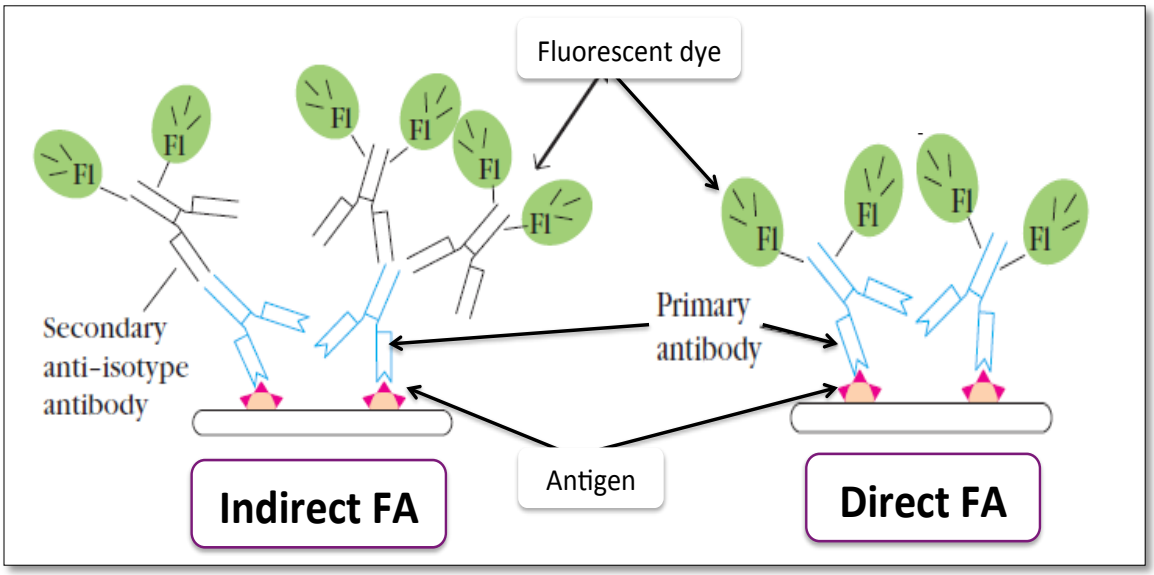


Figure 1: Schematic diagram of direct and indirect fluorescent antibody.

IFAs are used to detect antibodies in a sample. In this form of the test, the specific antigens (viral infected cells) are fixed to a microscope slide. Dilutions of the patient's

sample are added to several slides and given time to react with the antigen. The FITC-labeled antibody is an anti-gamma globulin antibody, so if there is patient antibody bound to antigen on the slide, the fluorescent antibody will bind to it. After rinsing to remove any unbound fluorescent antibodies, the slide is viewed under a fluorescent microscope with a UV light source. If the suspected antibody is present, the labeled antibodies will fluoresce and appear apple green (Figure 2).

4.3 Procedure:

4.3.1 Specimen Collection and Processing

Specimens that are considered most appropriate for analysis by immunofluorescence include nasopharyngeal swabs, aspirates, or washes, bronchoalveolar lavage samples, swabs or scrapings from vesicular lesions, tissue biopsy specimens (e.g., lung, liver, and brain), blood leukocytes, conjunctival cells, corneal scrapings, and urine sediment.

4.3.2 Preparation of slides:

4.3.2.1 Preparation of slides from nasopharyngeal aspirates and washes, tissue aspirates, and swabs submitted in viral transport medium:

1. Centrifuge the specimen at 600 × *g* for 5 min to pellet the cells.
2. The cell pellet is suspended with 5 ml of sterile PBS (pH of 7.0 - 7.6) and centrifuged as described above.
3. All but 100 to 200 µl of the PBS is removed; a uniform suspension of cells in the remaining fluid is made by gently pipetting up and down.
4. A drop of the cell suspension is placed into one or more wells of a Teflon-coated multiwell glass slide and air-dried or using slide warmer.

4.3.2.2 Preparation of slides directly from swabs:

This is usually the case with swabs obtained from vesicular lesions caused by HSV, VZV, or enterovirus infections.

1. A fresh vesicular lesion should be chosen and opened with a sterile scalpel.
2. The base of the vesicle is swabbed firmly with a cotton or Dacron (not calcium alginate) swab.
3. The swab is then rolled (not rubbed) onto the appropriate number of wells of a

Teflon-coated glass slide. (Rubbing the swab onto the slide may result in damage to the cells, making the slide difficult to read.)

4. Slides should be air dried completely before fixation.

4.3.3 Fixation

Prior to staining, the specimen must be properly fixed, fixative agents are:

- Cold acetone (for 10 min) (Mostly used)
- A mixture of methanol-acetone or ethanol-acetone (If plastic multiple-well cell culture plate (polystyrene) is used)

Notes:

- It is important to ensure that fresh acetone is used for fixing the specimens. Acetone that appears to be cloudy should be discarded.
- Methanol alone as a fixative is not compatible with many FA reagents.
- Water is not recommended as a diluent in place of methanol or ethanol.
- Plastic multiple-well cell culture plate (polystyrene) is not compatible with 100% acetone.

4.3.4 Staining

The following procedures should be used as a guide for performing both DFAs and IFAs:

1. During the staining process, slides should be placed in a humidified chamber to prevent evaporation of reagents.
2. The specimen is completely overlaid with the primary antibody, after which the slide is placed in the humidified chamber and incubated at 37°C. The length of incubation (15 to 30 min)
3. The primary antibody must be removed by washing. Immersion in a Coplin jar containing fresh PBS is the most gentle and efficient method. A series of one or two washes of 1-min duration is sufficient if the primary antibody is a MAb. Poly- clonal antibodies may require longer washing. **If the primary antibody is directly coupled to the fluorochrome (DFA), skip to step 7**.
4. Remove excess PBS from the slide, being careful not to touch the areas of the

slide containing the specimen.

5. Overlay the specimen with the labeled secondary antibody and incubate as described above.

   A mixture of these monoclonal antibodies can be added at the same time to detect a panel of viruses (e.g. respiratory viruses, all at one go).
6. Wash the slide as described in step 3
7. If PBS is used for washing, the slides should be mounted immediately. Excess PBS should be blotted or shaken from the slide, again taking care not to disturb the specimen. If the slides are to be air dried before mounting, rinse in distilled water and then dry completely. A drop of mounting medium is placed on the specimen area followed by a glass coverslip. Mounting medium, usually supplied with commercial kits, contains buffered glycerol, pH 9.0, with photobleach inhibitors to prolong fluorescence.

4.4 Results:

Reading and interpretation of results for DFA and IFA require critical evaluation to ensure reliable results. Staining patterns are highly dependent on the type of virus being detected as well as on the specificity of the primary antibody being used. The control slides supplied with commercial kits may be used as an indicator of expected results.

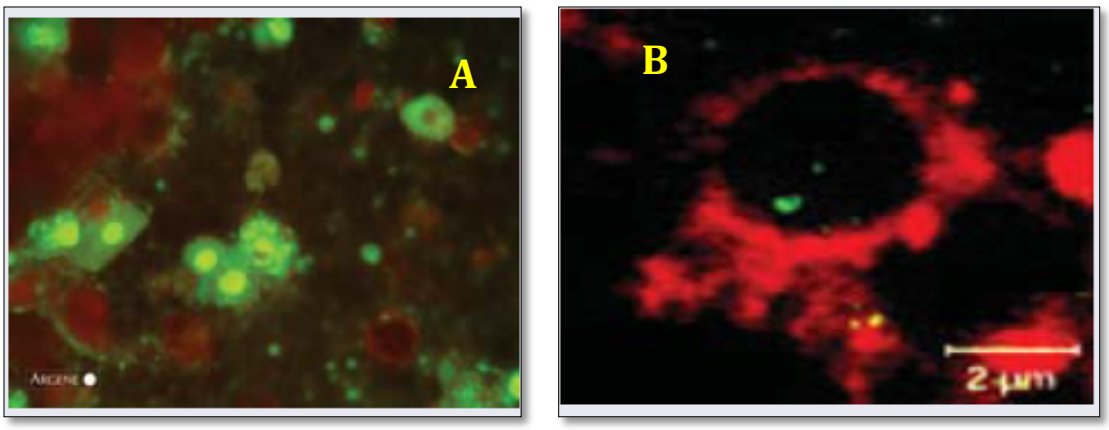


Figure 2: (A) Virus infected cells detected using a virus specific antibody labeled with a fluorescent dye. (B) An endosome labeled red containing virus protein (labeled green) in an infected cell.

Week 5: Viral Antigen Detection by Immunochromatographic Assays

One step hepatitis B surface antigen (HBsAg) test

5.1 Background:

Hepatitis B virus (HBV) is an enveloped, double-stranded DNA virus belonging to the Hepadnaviridae family and is recognized as the major cause of blood transmitted hepatitis together with hepatitis C virus (HCV). Infection with HBV induces a spectrum of clinical manifestations ranging from mild, inapparent disease to fulminant hepatitis, severe chronic liver diseases, which in some cases can lead to cirrhosis and carcinoma of the liver. Classification of a hepatitis B infection requires the identification of several serological markers expressed during three phases (incubation, acute and convalescent) of the infection. Now several diagnostic test are used for screening, clinical diagnosis and management of the disease.

Hepatitis B surface antigen or HBsAg, previously described as Australia antigen, is the most important protein of the envelope of Hepatitis B Virus. The surface antigen contains the determinant "a", common to all known viral subtypes and immunologically distinguished in two distinct subgroups (ay and ad). HBV has 10 major serotypes and four HBsAg subtypes have been recognized (adw, ady, ayw, and ayr). HBsAg can be detected 2 to 4 weeks before the ALT levels become abnormal and 3 to 5 weeks before symptoms develop. The serological detection of HBsAg is a powerful method for the diagnosis and prevention of HBV infection and ELISA has become an extensively used analytical system for screening of blood donors and clinical diagnosis of HBV in infected individuals.

5.2 Principle

Immunochromatographic assays are faster and easier to read immunoassays. In this systems, HBsAg in the sample (Whole blood, serum or plasma) flowing through a porous

material by capillary action encounters anti-HBsAg antibody labeled with "visualizing particles," such as colloidal gold nanoparticles, carbon black or blue polystyrene—sometimes called the "signal" or "detection" antibodies. Where HBsAg and labeled anti-HBsAg antibody bind, colored immune complexes flow through a region where the complexes encounter goat anti-HBsAg antibody is immobilized in the test region (T) on nitrocellulose membrane, and anti-HBsAg antibody (anti-antibody) in control line (C) resulting in a clearly visible pink or blue line, depending on the label used (Figure 3 & 4).

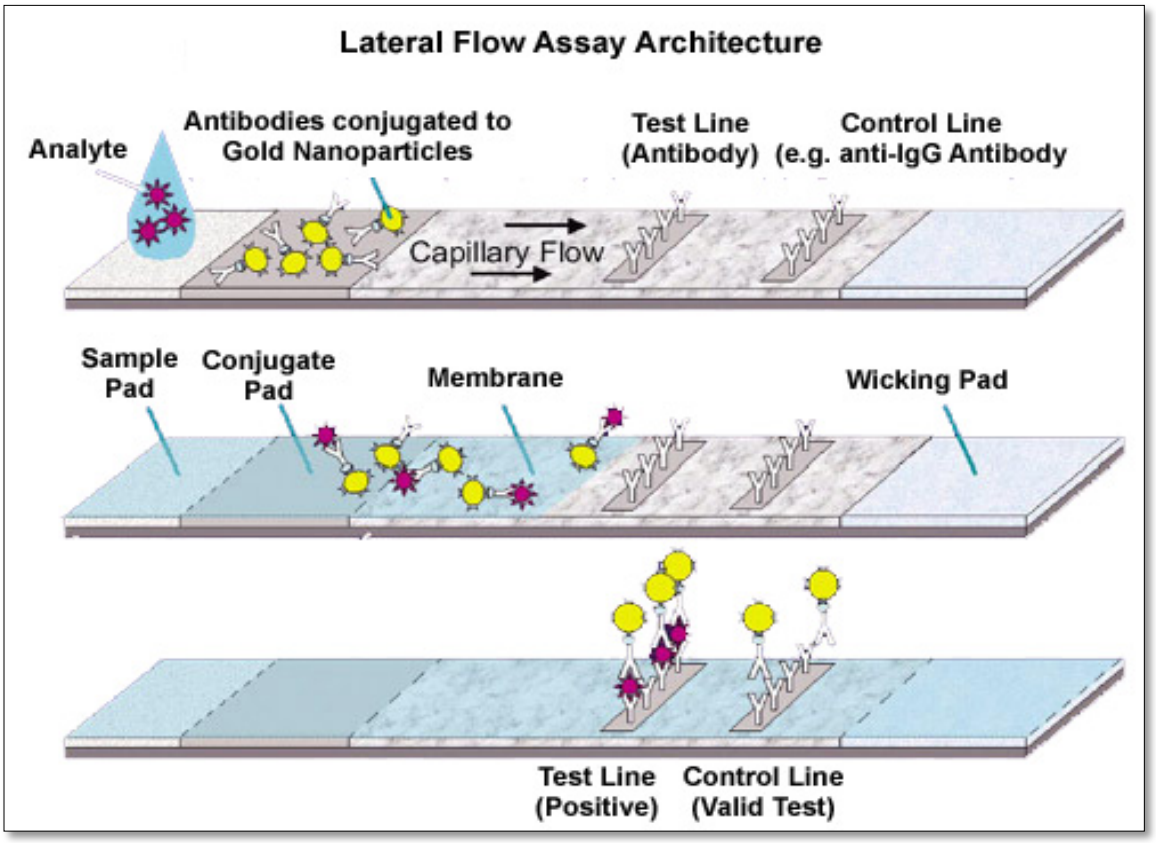


Figure 3: Principle of Immunochromatographic test (looking for antigen in the sample).

5.3 Materials:

1. Sample (whole blood, serum or plasma)
2. Test cards or strips individually foil pouched with a desiccant. (Kit)
3. Sample dispense plastic dropper

4. Timer

5.4 Procedure:

5.4.1 Specimen collection

Whole blood

- Collect whole blood in tubes with anticoagulant, do not use hemolyzed blood samples.
- Whole blood should be used immediately.

Serum or Plasma

- Collect serum or plasma specimens following regular clinical laboratory procedures.
- Hemolyzed specimens or specimens with very high fat level are not suitable
- Storage:
    - A specimen should be refrigerated if not used the same day of collection. Specimens should be frozen if not used within 3 days of collecting.
    - Avoid freezing and thawing the specimens more than 2-3 times before using.
    - 0.1% of sodium azide can be added to specimen as preservative without affecting the results of the assay.

5.4.2 Test procedure

1. Read the entire procedure carefully prior to performing any tests.
2. Allow test devices and serum samples to equilibrate to room temperature prior to testing. Remove test from the sealed pouch.
3. Identify the test cards or strips for each specimen or control.
4. For test cards: Dispense 100 µl (3 drops) of the specimen or control into the sample well.
5. For test strips: Apply at least 80 µl of the specimen to the sample pad behind the  mark at the bottom of the test strip.
6. Interpret test results at 15 minutes.

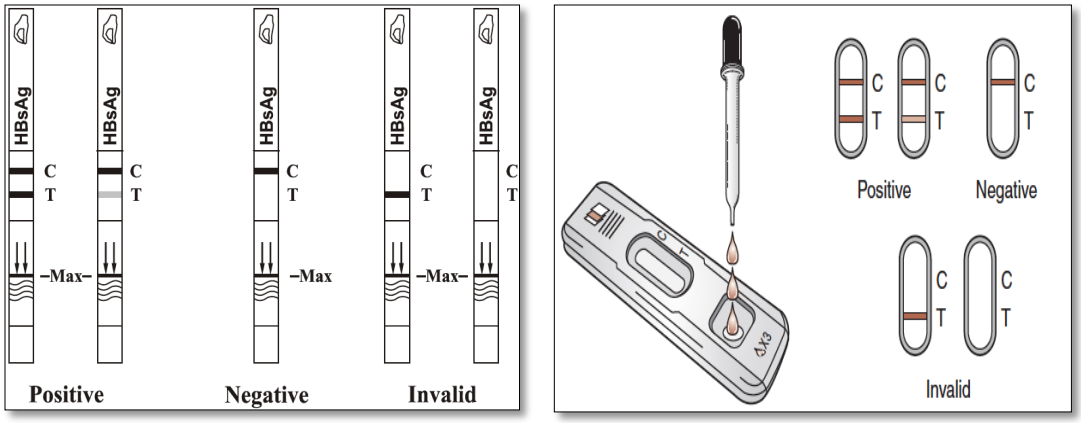


Figure 4: Interpretation of results of immunochromatographic test.

Note:

- HBsAg concentration ≥ 5 ng/ml develop the band in about 5-10 min, while HBsAg concentration 1 ng/ml determined in 15 min.
- Do not interpret result after 20 minutes.

5.5 Interpretation of results

- **Negative:** Only a single line appears in the control region (C). There is no line in the test region (T).
- **Positive:** Two distinct lines will appear within 15 minutes, one in the test region (T) and another in the control region (C).
- **Invalid:** A total absence of colored lines in both regions is an indication of procedure error and/or that test reagent deterioration has occurred (Figure 4).

Notes: It is normal to have a slightly lightened control band with very strong positive samples as long as it is distinctly visible.

Week 6: Viral Antigen Detection by Enzyme Immunoassay (EIA)

Hepatitis B surface antigen detection (HBsAg) by ELISA

6.1 Background

6.1.1 ELISA:

- ELISA is a commonly used technique for the determination of antibodies or antigens.
- Two research groups developed the format of the ELISA in the 1960s independently at the same time.
- ELISA is nearly highly **sensitive (>99%)** and **specific (>99%)**.
- ELISAs are routinely **used in** scientific research, veterinary medicine, environmental and agricultural applications, and in healthcare.
- There are three main types of ELISA; direct, indirect and competitive.
- Often the antigens of bloodborne pathogens such as HIV or hepatitis B (HBV) are detected with such systems. Neither HIV nor HBV proliferates in standard cell cultures.
- Microwell-based EIAs for detection of antigens of several nonculturable gastrointestinal viral pathogens (rotavirus and adenovirus) are available.

6.1.2 Hepatits B surface antigen HBsAg: look at week 5 (5.1)

6.2 Purpose:

This method mainly used in clinical diagnosis of hepatitis B infection in humans. This method is known as **(Direct, Sandwich or Capture)** ELISA, because the antigen of the virus directly is detected.

6.3 Principle:

The principle of direct ELISA is that antibodies (Ab) are bound to the solid phase, after adding the tested samples into the wells if corresponding Ag found can attach to the bounded Ab on a solid phase, after washing (Automated), all unbounded materials are washed away. A set of conjugated (Enzyme-Ab), then substrate is used to indicate any changes in the color that indicates positive results, then adding stop solution to the mixture. The intensity of the color changes is determined by an instrument (ELISA Reader), which determines the exact amount of Ag in the tested sample (Figure 5).

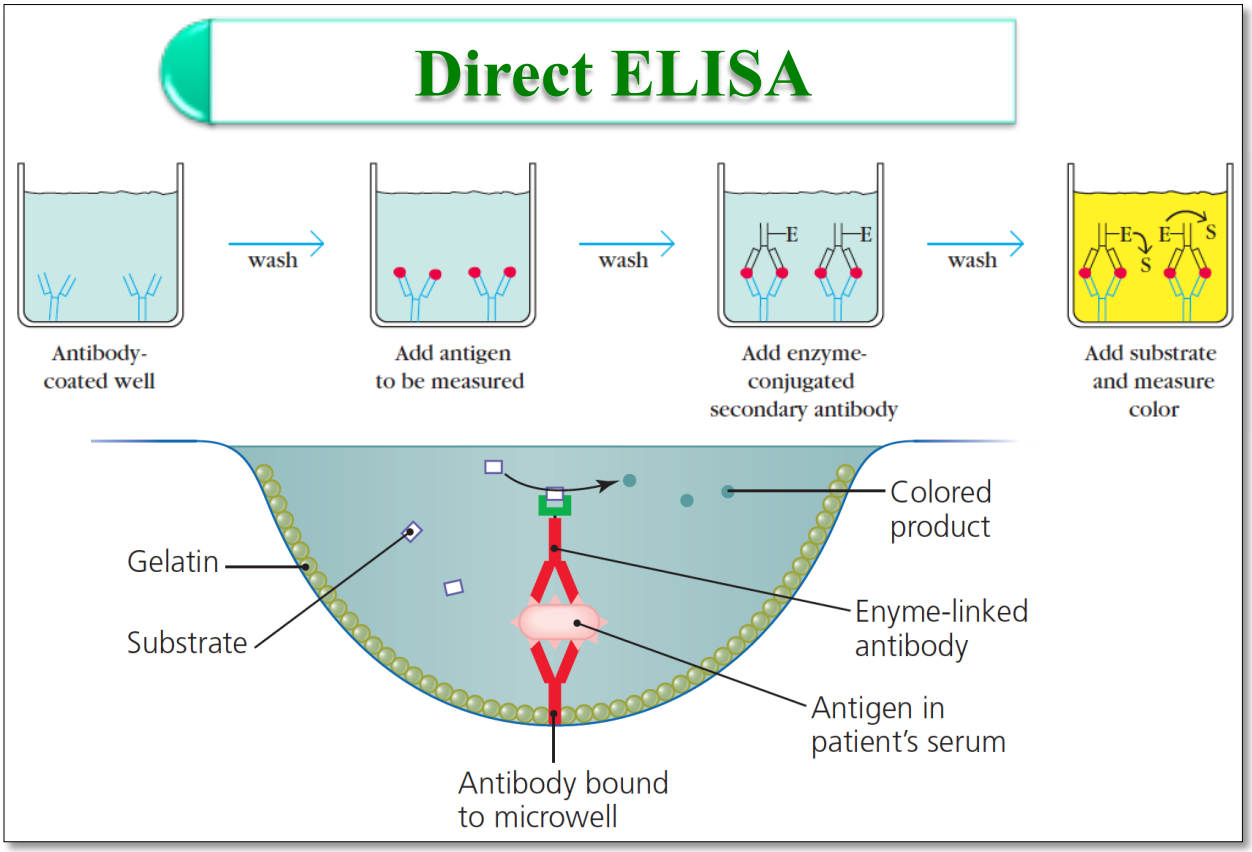


Figure 5: Main steps of direct ELISA.

6.4 Materials:

- ELISA kit

- Washing buffer
- Negative control
- Positive control (HBsAg)
- Conjugate (anti-HBsAg antibody labeled with HRP-enzyme)
- Chromogenic solution A (Urea peroxide solution)
- Chromogenic solution B (TMB solution)
- Stop solution (Sulfuric acid)
- Microtiter plate coated with anti-HBsAg antibody
- Plastic sealable bag
- Cardboard plate cover

- Sample from the patient (Serum or Plasma)
- Distilled Water
- Absorbent paper or clean towel
- Micropipettes (single or multichannel) and their tips
- Waste container
- Incubator or water bath (37°C)
- Microwell plate reader

6.5 Procedure:

1. Bring all kit components and the sample to room temperature.
2. Shake all reagents before use.
3. Prepare 1X wash buffer from (preheated (37°C) 20X wash buffer)
4. Prepare a record (plate map) identifying the placement of the controls and specimens in the microwells.
5. Add 50 µl of patient sample (at least two replicates), positive control and negative control to the titre wells.
6. Add 50 µl of conjugate (antibody labelled with enzyme), mix the mixture by gentle shaking
7. Apply cover seal and incubate at 37°C for 1 hour
8. Discard the liquid in the well, dry the outside of the wells with filter paper, and

wash the wells with the diluted washing buffer. Caution: To prevent overflow don't add too much washing buffer to any of the wells. Wait for 5-10 seconds before discarding the washing buffer from the wells. Repeat washing the wells 5 times.

9. Add 50µl of chromogenic solution A into loaded wells, and then add chromogenic solution B. Mix, tape, label and incubate at 37°C for 15 minutes in dark place.
10. Terminate the reaction with 50µl of stop solution in each well.
11. Read the plate at 450nm for OD values

6.6 Result analysis

- **Cut-off value: COV= mean value of negative control OD values x 2.1**
- Sample OD>=COV is positive, < COV is negative
- Lowest negative mean OD value is 0.05
- Borderline: (COV = 0.9-1.1): Specimens with absorbance to Cut-off ratio between 0.9 and 1.1 are considered borderline and retesting of these specimens in duplicates is required to confirm the initial results.

Record the results as:

Sample Code	ELISA Result (OD)	Positive or Negative	Comments

6.7 Attention:

1) Samples and test kit need to be used under room temperature.
2) Shake reagents before using.
3) Slowly draw and discharge samples with micropippettor.
4) Change tips between solutions.
5) Filter paper and tape can't be reused.
6) Wash wells carefully to prevent overflow.
7) Plate needs to be read within 10 minutes after adding the termination solution.
8) Retesting in duplicates of any initially reactive sample is recommended.
9) Repeatedly reactive samples could be considered positive.
10) Any positive sample should be immediately discarded (autoclaved).

Week 7: Antibody Detection Methods:

Background:

For communicable diseases, clinical management, and public health response, it is often important to know the body's immune response following exposure and infection with pathogens. Although humoral and cell-mediated immunity both play roles in the body's specific immunity against viral pathogens, testing antibody response for humoral immunity is much more common and is also easier in clinical virology laboratories than testing for cell-mediated immunity because of the convenience of antibody serological testing methods.

There is a long history of using various serologic methods in clinical virology laboratories for antibody detection. Some methods, such as complement fixation test and immunodiffusion test, have been gradually phased out and replaced by faster and less laborious methods. In this manual, we will focus on antibody detection methods used in clinical virology laboratories: neutralization, hemagglutination inhibition, indirect immunofluorescence, Immunochromatographic test, enzyme immunoassay, and Western blot.

Antibody detection tests can be used to:

- Diagnose current or past infection, acute or chronic disease
- Evaluate immune status
- Predict prognosis, such as with hepatitis B virus (HBV) infections in combination with antigen detection, and for human immunodeficiency virus (HIV) infections in combination with CD4 cell counts and virus load testing.

7.1 Hemagglutination Inhibition (HI) Test for Influenza Virus

4.2.1 Purpose:

HI is commonly used to detect serum antibodies to viruses that express hemagglutinin (HA) such as influenza virus, arboviruses, adenovirus, measles virus, and hantavirus. Anti-HA Antibodies have the ability to bind HA will prevent hemagglutination.

4.2.2 Principle

Serological diagnosis by the HI test is accomplished by making serial dilutions of a patient's serum, mixing each dilution with a fixed amount of viral HA, and then adding an RBC suspension. The animal species from which the indicator RBCs are collected is dependent on the viral HA used in the assay. If the serum contains antibody, the virus will be bound and unable to agglutinate the RBCs. If there are no specific antibodies in the patient's serum, the virus will agglutinate the RBCs (Figure 6).

The reciprocal of the highest dilution of the patient's serum that will completely inhibit agglutination is termed the HI titer. The most common use of the HI test in laboratories today is for subtyping of influenza virus.

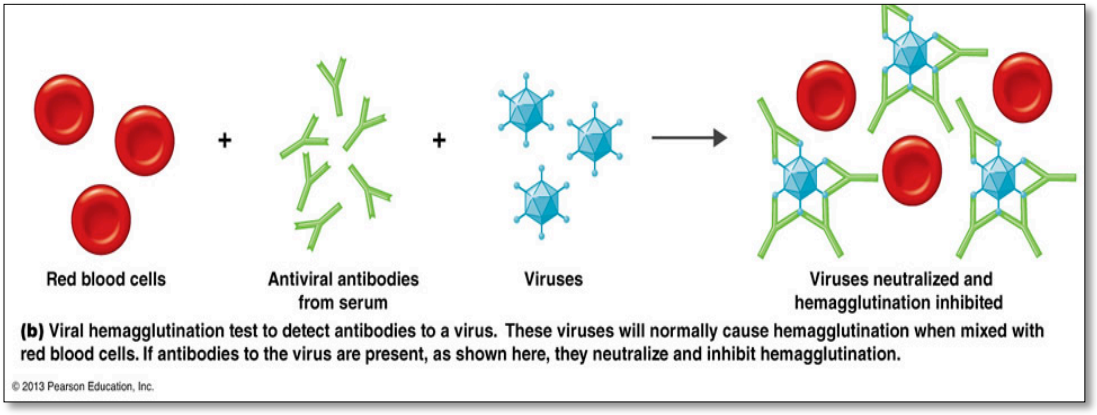


Figure 6: Principle of viral hemagglutination inhibition test

4.2.3 Materials

1. Influenza virus (The virus is prepared from cell culture, embryonated chicken eggs, or animal tissue depending on the target viruses)
2. The patient serum
3. RBC suspension (The RBCs are prepared from appropriate animal or human blood, depending on the target virus)
4. PBS
5. Disposable microtiter plates, "U" type
6. Micropipettes with their tips.
7. Water bath (37°C)

4.2.4 Procedure:

Before Running HI test, the viral HA titer is first determined by the hemagglutination assay, where serial dilutions of virus or HA are mixed with RBCs to yield the HA titer of the virus corresponding to the highest dilution showing hemagglutination of RBCs

a) The patient serum is pre- treated to remove non-specific viral inhibitors and RBC agglutinins.
b) A predetermined amount of virus or standardized HA is added to serial dilutions of pretreated test serum in wells and incubated for 30 minutes.
c) Then red blood cells are added to the virus-serum mixture and incubated for 30 minutes.

4.2.5 Result:

The presence of specific anti- HA antibodies will inhibit hemagglutination, which would otherwise occur between the virus and the RBCs.

- If the serum contains no antibodies to the virus, then hemagglutination will be observed in all wells.
- If antibodies to the virus are present, then hemagglutination will be inhibited. The

highest dilution of serum that prevents hemagglutination is called the HI titer of the serum (Figure 7).

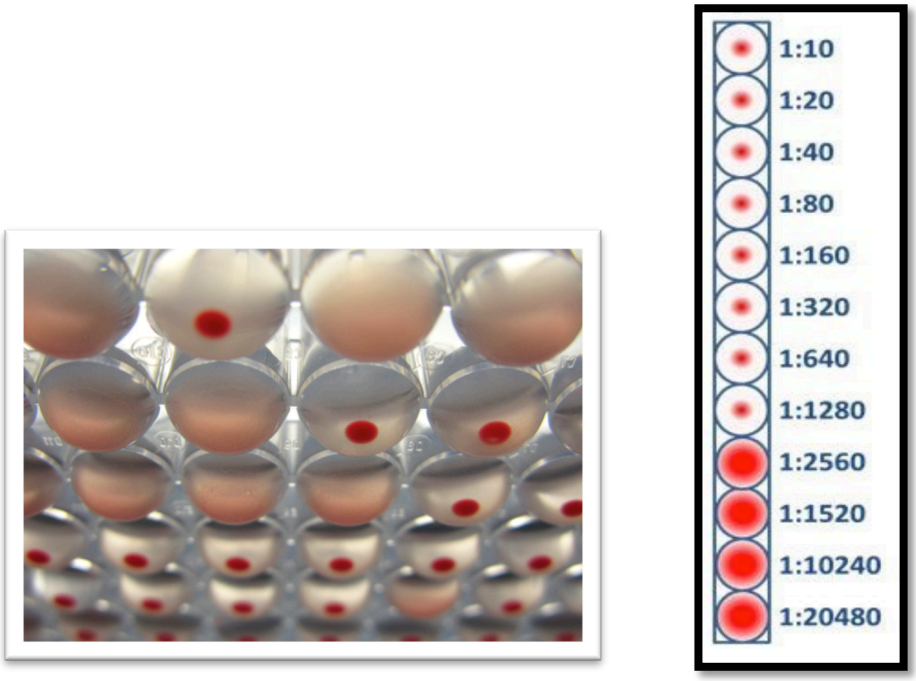


Figure 7: Hemagglutination inhibition assay

Applications:

- **Vaccine efficacy studies:** where sera from individuals who have received influenza vaccine are tested for the presence of strain-specific antibodies to the particular influenza virus in question.
- Indicates presence of antibody in human serum to the virus being tested. Paired acute-convalescent sera yielding a 4- fold rise by the HI assay is indicative of recent or current infection.
- In addition to influenza, the HI assay has been commonly used for paramyxoviruses and arboviruses, as well as for serodiagnosis and serosurveillance of hemorrhagic fever with renal syndrome caused by Hantaan virus.

Quality Assessment:

It is imperative to run in parallel with each test the proper controls for the virus used, the RBCs used, and the serum samples being tested.

- **The virus control** ensures the hemagglutination capacity of the virus with the test RBCs.
- **The RBC control** determines whether the RBCs in use agglutinate in PBS on their own without virus.
- **The serum control** consists of the test serum diluted at 1:10 and added to RBCs. If the serum alone agglutinates the RBCs, this may indicate interfering agglutinins in the test serum that may yield incorrect results.

Limitations :

1. Standardizing the virus concentration each time a test is performed.
2. Obtaining a reliable source of fresh RBCs, appropriate preparation and storage of RBC suspension.
3. Need for experienced analysts for interpretation of test results.
4. Species choice of RBCs is critical for accurate HI results.

7.2 Virus Neutralization Test

4.3.1 Background:

Neutralization is the loss of infectivity, which ensues when antibody binds to a cognate epitope on the virus particle. The antibody–antigen reaction is so specific that it is unaffected by the presence of other proteins. Hence antibodies need not be extracted from crude serum, and impure virus preparations can be used to observe neutralization.

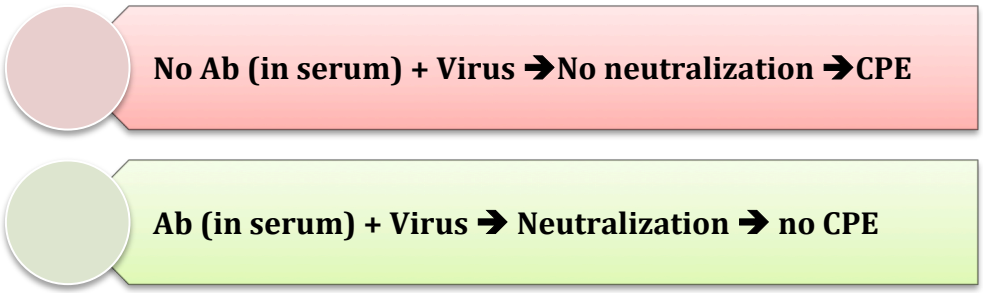
However not all antibodies which bind to a virus particle are capable of neutralizing its infectivity. Neutralization is an epitope-specific phenomenon.

4.3.2 Purpose:

Neutralization assays can be used to identify antibody response or the specific virus.

4.3.3 Principle:

- Neutralization of virus is defined as the loss of infectivity though reaction of the virus with specific antibody.
- Virus and serum are mixed under appropriate conditions and then inoculated into cell culture, eggs or animals.
- The presence of unneutralized virus may be detected by reactions such as CPE, haemadsorption, haemagglutination, plaque formation and disease in animals.



4.3.4 Materials:

1. For viral identification: Well-characterized, pretitered antiserum or well-standardized immune serum pools are used.
2. To measure antibody response to a virus: a Well-characterized, pretitered virus is required (Virus is frequently measured in units between 1-100 of 50% tissue culture infectious dose ($TCID_{50}$)).
3. To monitor for viral inactivation (neutralization): A living host system is required (Target cells grown in 96 well tissue culture plates or other suitable assay format)

4.3.5 Procedure:

- Serial 1:1 or 1:2 dilutions of antibody, human serum, antiserum or tissue culture supernatant are made in a microtiter plate or other suitable format.
- Target dose of specific virus is added to antibody and co-incubated for 1 hour at

room temperature or 37°C.

- Include virus, test serum and cell controls in defined wells.
- Transfer co-incubated antibody - virus to wells containing monolayers of target cells and allow incubation for 24 hours at 37°C.

Quality Assessment:

Each time the test is performed, internal controls that give expected results must be included for the test to be valid. These controls include:

- **The virus control** demonstrating that the virus replicates in the host cells of the test as expected.
- **A test serum control** demonstrating that it is not toxic to the host cells by itself.
- **A cell control** demonstrating that uninoculated host cells do not exhibit any effects that could interfere with reading or interpreting the test results.

Additionally, these standards should be periodically checked for run to run reliability.

4.3.6 Result:

- Infectivity is identified by the presence of a cytopathic effect on target cells, or alternatively reduction of proliferative effects. Cytopathic effects can be assayed in varied ways as described in cell culture lab. Plaque reduction neutralization testing has long been considered the standard for viral assays.
- Neutralizing titer can be defined as the reciprocal of the highest dilution of serum or antibody at which there is no cytopathic effect.

Applications:

- Determine infection: single or paired specimens to determine evidence of current or recent infection, infection or exposure at some time, or no evidence of exposure for the given specimen(s).
- For viruses with many serotypes, neutralization may be the only method for dis- criminating infection with a particular serotype
- Determining and evaluating immunity to a specific virus (successful vaccination

to viruses such as rubella and mumps have been based on plaque reduction neutralization tests, and to measure immunity or protection, as for rabies virus)

4.3.7 Other variation of NT to determine virus-neutralizing antibody (VNA):

- ELISA-based microneutralization test (microNT-ELISA) (Figure 8)
- Fluorescent Antibody Virus Neutralization Tests (FAVN).

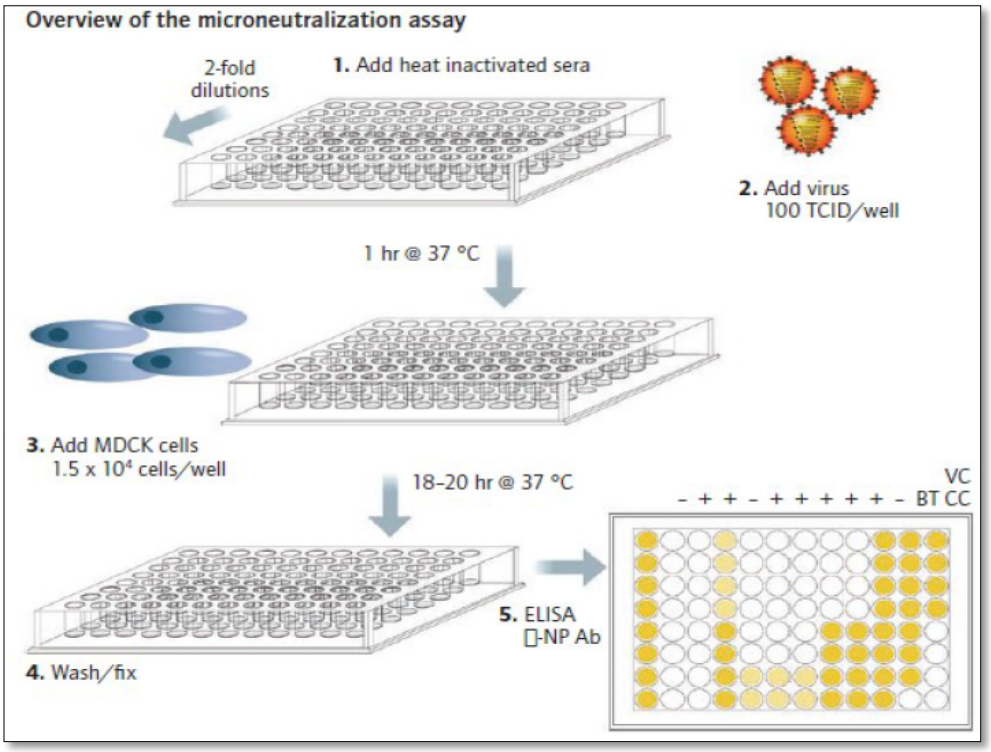


Figure 8: Overview of the microneutralization assay

4.3.8 Review Questions:

- Write two applications of virus neutralization assay.
- No CPE in neutralization assay, if you are looking for antibody in the sample means ---------- (positive result or Negative result).

Week 8: Antibody Detection Methods:

8.1 Antibody Detection Methods: IC Rapid Anti-HCV Test:

8.1.1 Purpose:

The general method of detecting infection with hepatitis C virus (HCV) is to observe the presence of antibody to the virus by ELISA followed by western blot for confirmation. This immunochromatographic test is rapid and simple, which can detect anti-HCV antibody in whole blood, serum and plasma. The result can be obtained within 15 minutes.

8.1.2 Principle:

The assay starts with a sample applied to the sample well and the addition of the provided sample diluent immediately. HCV antigen-Colloidal Gold conjugate embedded in the sample pad reacts with the HCV antibody present in serum or plasma, forming conjugate/HCV antibody complex. As the mixture is allowed to migrate along the test strip, the conjugate/HCV antibody complex is captured by an antibody-binding protein A immobilized on a membrane forming a colored band in the test region. A negative sample does not produce a test line due to the absence of Colloidal Gold conjugate/HCV antibody complex. The antigens used in the test are recombinant proteins corresponding to highly immunoreactive regions of HCV. A colored control band in the control region appears at the end of the test procedure regardless of the test result. This control band is the result of CoIloidal Gold conjugate binding to an anti-HCV antibody immobilized on the membrane. The control line indicates that the Colloidal Gold conjugate is functional. The absence of the control band indicates that the test is invalid

8.1.3 Materials:

1. Sample (whole blood, serum or plasma)
2. Test cards or strips individually foil pouched with a desiccant. (Kit)
3. Sample dispense plastic dropper
4. Sample Diluent

5. Timer

8.1.4 Sample collection and storage:

Same as previously described in one step HBsAg detection test.

8.1.5 Assay Procedure:

Same as previously described in one step HBsAg detection test, except

- Dispense 1 drop (10 µl) of the sample
- Add 2 drops of the sample diluent (Figure 9)

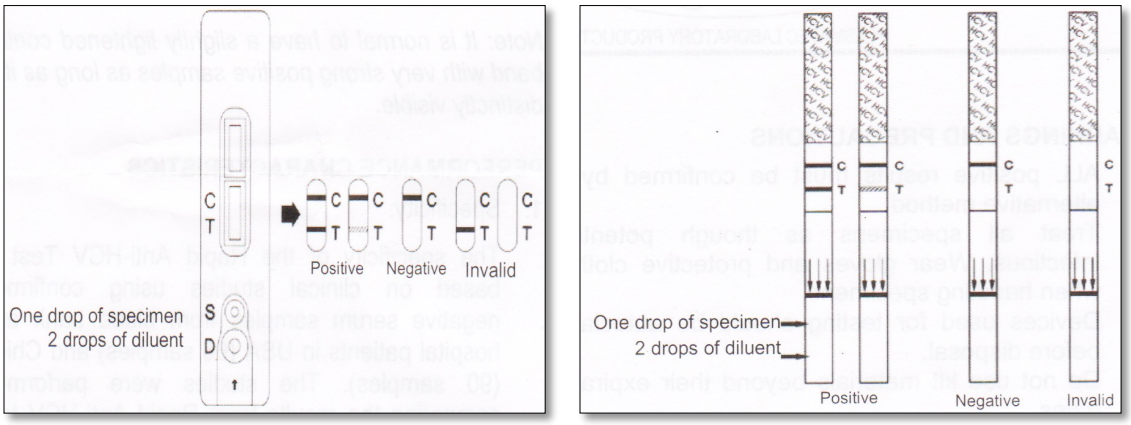


Figure 9: Interpretation of results of immunochromatographic test.

8.1.6 Limitation of Immunochromatographic tests:

1. This test is qualitative NOT quantitative.
2. Only samples that are clear with good fluidity can be used in this test.
3. Fresh samples are best but frozen samples can be used. If a sample has been frozen, it should be allowed to thaw in a vertical position and checked for fluidity.
4. Do not agitate the sample. Insert a pipette just below the surface of the sample to collect the Specimen.

8.1.7 Warnings and precautions:

1. Treat all specimens as though potentially infectious.
2. All positive results must be confirmed by an alternative method.
3. Devises used for testing should be autoclaved.

8.2 Anti-HCV antibody detection by ELISA

8.2.1 Purpose:

This method mainly used in clinical **diagnosis of specific antibody** in human body. This method is known as **indirect ELISA**, because indirectly the entry of antigens (HCV) is confirmed by looking for specific anti-HCV antibody, which is produced in response to the entry of their corresponded antigen.

8.2.2 Principle:

The principle of indirect ELISA is that antigens (Ag) is bound to the solid phase, after adding the tested samples into the wells if corresponding Ab present can attach to the bounded Ag on the solid phase, after washing (Automated), all unbounded materials are washed away. A set of conjugated (Enzyme-Ab), then substrate is used to indicate any changes in the color that indicates positive results, then adding stop solution to the mixture. The intensity of the color changes is determined by an instrument (ELISA Reader), which determines the exact amount of Ab in the tested sample (Figure 10).

8.2.3 Materials:

- ELISA kit
  - Washing buffer
  - Negative control
  - Positive control (anti-HCV antibody)
  - Specimen diluent
  - Conjugate (anti-human IgG antibody labeled with HRP enzyme)
  - Chromogenic solution A (Urea peroxide solution)
  - Chromogenic solution B (TMB solution)
  - Stop solution (Sulfuric acid)
  - Microtiter plate, wells coated with recombinant HCVantigen
  - Plastic sealable bag

- Cardboard plate cover

- Sample from the patient (Serum)
- Distilled Water
- Absorbent paper or clean towel
- Micropipettes (single or multichannel) and their tips
- Waste container
- Incubator or water bath (37°C)
- Microwell plate reader

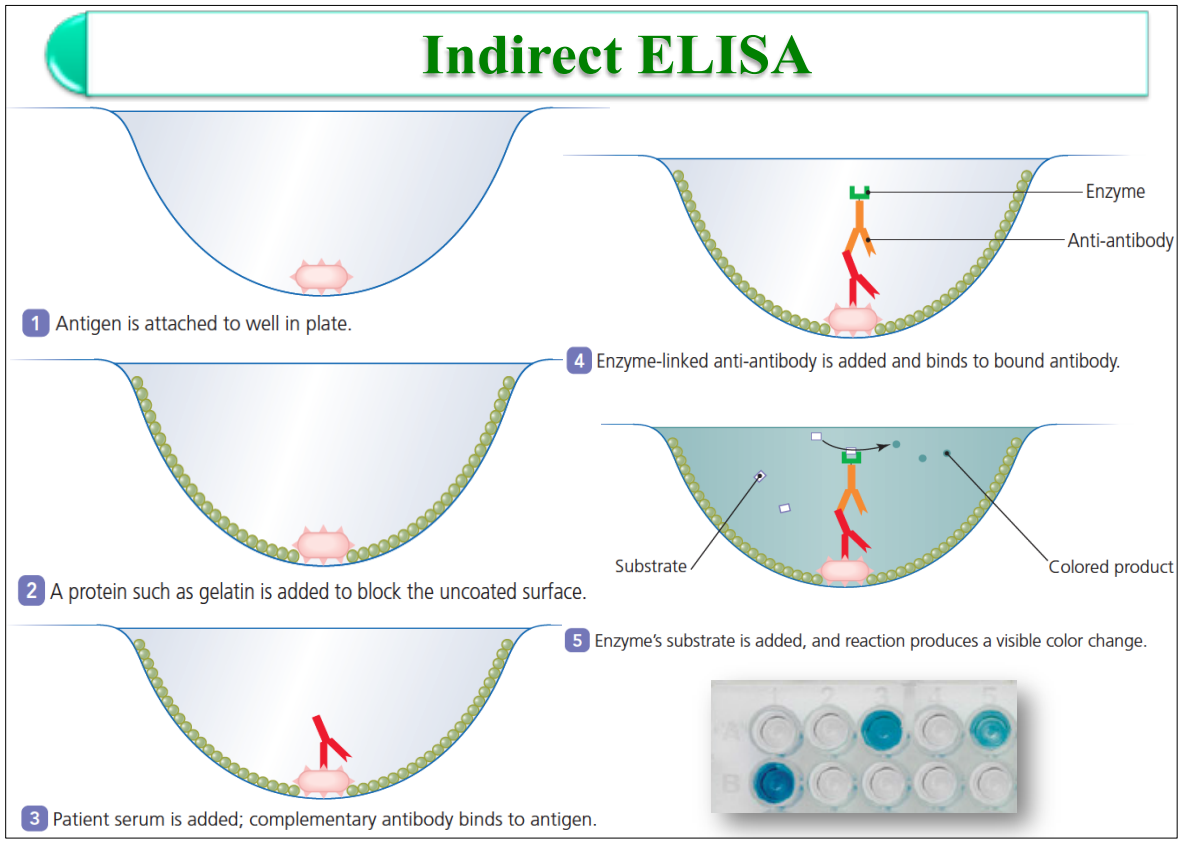


Figure 10: Main steps of indirect ELISA.

8.2.4 Procedure:

1. **Reagent Preparation:** Bring all kit components and the sample to room temperature. Shake all reagents before use. Prepare 1X wash buffer from (preheated (37°C) 20X wash buffer)
2. **Numbering the wells:** Prepare a record (plate map) identifying the placement of the controls and specimens in the microwells. Three wells for NC, two wells for PC and one blank (neither samples nor HRP-conjugate should be added into the blank well).
3. **Adding Diluent:** Add 100 µl of specimen diluent into wells except the blank.
4. **Adding Sample:** Add 10 µl of patient sample (at least two replicates), positive control and negative control to the titre wells. Mix by tapping the plate gently.
5. **Incubation (1):** Apply cover seal and incubate at 37°C for 30 minutes.
6. **Washing (1):** Remove and discard the plate cover. Wash each well 5 times with diluted Wash buffer. Each time, allow the microwells to soak for 30- 60seconds. After the final washing cycle, turn the strips plate onto blotting paper or clean towel, and tap it to remove any remainders.
7. **Adding HRP-Conjugate:** Add 100 µl of conjugate (antibody labelled with enzyme) to each well except the blank.
8. **Incubation (2):** Apply cover seal and incubate at 37°C for 30 minutes.
9. **Washing (2):** Remove and discard the plate cover. Wash each well 5 times with diluted Wash buffer.
10. **Coloring:** Add 50µl of chromogenic solution A into loaded wells, and then add 50µl of chromogenic solution B. Mix, tape, label and incubate at 37°C for 15 minutes in dark place. (Positive wells are blue)
11. **Stopping Reaction:** Terminate the reaction with 50µl of stop solution in each well and mix gently. (Positive wells are yellow).
12. **Measuring the Absorbance:** Calibrate the plate reader with the Blank well and read the absorbance at 450nm. Calculate Cut-off value and evaluate the results.

8.2.5 Result analysis

Calculation of Cut-off value: Cut-off value (COV) = *Nc + 0.12

***Nc** = the mean absorbance value for three negative controls.

Important: If the mean OD value of the negative control is lower than 0.02, take it as 0.02.

(S = the individual optical density (OD) of each specimen)

- **Negative Results (S/COV <1):** samples giving an optical density lower than the Cut-off value are considered negative.
- **Positive Results (S/COV ≥1):**
- **Borderline (S/C.O. =0.9-1.1):** Retesting of these samples in duplicates is recommended to confirm the results. Repeatedly positive samples could be considered positive.

*Follow-up and supplementary testing of any anti-HCV positive samples with other analytical system (e.g. Western Blot) is required to confirm the diagnosis

Record the results as:

Sample Code	ELISA Result (OD)	Positive or Negative	Comments

Week 9: Antibody Detection: Anti-HIV1/2 detection by ELISA

9.1 Purpose:

Testing for the presence of HIV antigens or antibodies from serum is the best way to obtain serological evidence of infection from individuals that may be harboring the HIV virus. Detection of antigen can be evidenced in both acute phase and symptomatic phases of AIDS. However, **the HIV-1 and HIV-2 antibodies can be identified all through the entire infection period** - beginning at the acute phase all the way through the last stages of AIDS.

9.2 Principle:

Polystyrene microwell plate pre-coated with recombinant HIV antigens indicated in *E. coli* (recombinant HIV-1gp41, gp120, and recombinant HIV-2 gp-36) make up this two-step incubation antigen sandwich enzyme immunoassay kit (HIV-1 & 2 ELISA).

At the time of the first incubation step, patient's plasma or serum sample is added. If specific HIV1/2 antibodies are present, they will be captured inside the wells. After this, microwells are washed to remove unbound serum proteins. Added after this stage is the second set of recombinant antigens conjugated to the enzyme (HRP-Conjugate) and indicating the same epitopes as the pre-coated antigens. While the second incubation occurs, these antigens will bind to the captured antibody. The chromogen solutions can be added to the wells, but not before the microwells are washed again to remove unbound conjugate. In the wells where the antigen-antibody-antigen (HRP) sandwich immunocomplex is taking place, the colorless chromogens are hydrolyzed by the bound HRP-conjugate to a blue-colored product. After the reaction is stopped with sulfuric acid, the blue color turns yellow.

9.3 Materials:

- ELISA kit
    - Wash buffer

  - Negative control
  - Positive control (HIV 1) (anti-HIV 1 antibody)
  - Positive control (HIV 2) (anti-HIV 2 antibody)
  - Conjugate (HRP conjugated to HIV1/2 antigens)
  - Chromogenic solution A (Urea peroxide solution)
  - Chromogenic solution B (TMB solution)
  - Stop solution (Sulfuric acid)
  - Microtiter plate, wells contain recombinant HIV 1/2 antigens
  - Plastic sealable bag
  - Cardboard plate cover
- Sample from the patient (Serum)
- Distilled Water
- Absorbent paper or clean towel
- Micropipettes (single or multichannel) and their tips
- Waste container
- Incubator or water bath (37°C)
- Microwell plate reader

9.4 Procedure:

1. **Reagent Preparation:** Bring all kit components and the sample to room temperature. Shake all reagents before use. Prepare 1X wash buffer from (preheated (37°C) 20X wash buffer)
2. **Numbering the wells:** Prepare a record (plate map) identifying the placement of the controls and specimens in the microwells. Three wells for NC, two wells for PC (One for HIV1 and one HIV2 controls) and one blank (neither samples nor HRP-conjugate should be added into the blank well).
3. **Adding Sample:** Add 100 µl of patient sample, positive control and negative control to the titre wells. Mix by tapping the plate gently.
4. **Incubation (1):** Apply cover seal and incubate at 37°C for 30 minutes.
5. **Washing (1):** Remove and discard the plate cover. Wash each well 5 times with

diluted Wash buffer. Each time, allow the microwells to soak for 30- 60 seconds. After the final washing cycle, turn the strips plate onto blotting paper or clean towel, and tap it to remove any remainders.

6. **Adding HRP-Conjugate:** Add 100 µl of conjugate to each well except the blank.
7. **Incubation (2):** Apply cover seal and incubate at 37°C for 30 minutes.
8. **Washing (2):** Remove and discard the plate cover. Wash each well 5 times with diluted Wash buffer.
9. **Coloring:** Add 50µl of chromogenic solution A into loaded wells, and then add 50µl of chromogenic solution B. Cover the plate with plate cover, Mix by tapping the plate gently, and incubate at 37°C for 15 minutes in dark place. (Positive wells are blue)
10. **Stopping Reaction:** Terminate the reaction with 50µl of stop solution in each well and mix gently. (Positive wells are yellow).
11. **Measuring the Absorbance:** Calibrate the plate reader with the Blank well and read the absorbance at 450nm. Calculate Cut-off value and evaluate the results.

9.5 Result analysis:

Calculation of Cut-off value: Cut-off value (COV) = *Nc + 0.12

***Nc** = the mean absorbance value for three negative controls.

Important: If the mean OD value of the negative control is lower than 0.02, take it as 0.02.

(S = the individual absorbance (OD) of each specimen)

- **Negative (S*/C.O. <1):** Samples giving absorbance less than the Cut-off value are negative for this assay, which indicates that no HIV 1/2 antibodies have been detected with this HIV 1/2 ELISA kit,
- **Positive (S/C.O.≥1):** Indicates that HIV 1/2 antibodies have probably been detected using this HIV 1/2 ELISA kit.
- **Borderline (S/C.O. =0.9-1.1):** Retesting of these samples in duplicates is recommended to confirm the results. Repeatedly positive samples could be

considered positive for antibodies to HV 1/2.

*Follow up and supplementary testing of any positive samples with other analytical system (e.g. WB, PCR) is required before establishing of the final diagnosis.

Record the results as:

Sample Code	ELISA Result (OD)	Positive or Negative	Comments

Week 10: Anti-HIV Antibody Detection by Western Blot

10.1 Purpose:

Western blots are used to identify proteins in a sample. (Similar techniques called the Southern blot and Northern blot are used for identifying DNA and RNA in samples, respectively.) This technique has applications in research in detecting specific protein(s) in a cell, tissue, organ, or body fluid. This technique is also used for detection of different antibodies against multiple antigens in a complex mixture. Compared to other tests, western blot can detect more types of antibodies or antigens at the same time. One important use of this technique in virology is to identify HIV antibodies in a patient's serum or plasma. Because it is more expensive and requires greater skill than other serological tests, it is only used to confirm the results from samples that are repeatedly positive for HIV antibodies in preliminary ELISA screening.

10.2 Principle:

This test is used to detect antibodies and antigens. When looking for specific antibodies in a sample, known antigens (proteins) are used. If detection of antigens in a sample is requested, their corresponded antibodies should be prepared and used as reagent. The basic steps of western blot technique if looking for antibody are:

First: Polyacrylamide gel electrophoresis: Purified viral proteins are placed into wells in polyacrylamide gel and separated by gel electrophoresis according to their size, charge and conformation. Before electrophoresis proteins are treated with Sodium dodycyle sulfate (SDS). Each of the proteins in the solution is resolved into a single band, producing invisible protein bands.

Second: Blotting: The most common method is electroblotting. Simply, the gel is sandwiched together with the membrane between layers of absorbent material. The proteins are moved into the membrane and remain fixed in the same position as the original bands (but are not visible). The nitrocellulose is then cut into strips, which are ready for use (Figure 11). (The manufacturer does the electrophoresis and blotting steps if

a commercial kit is used.)

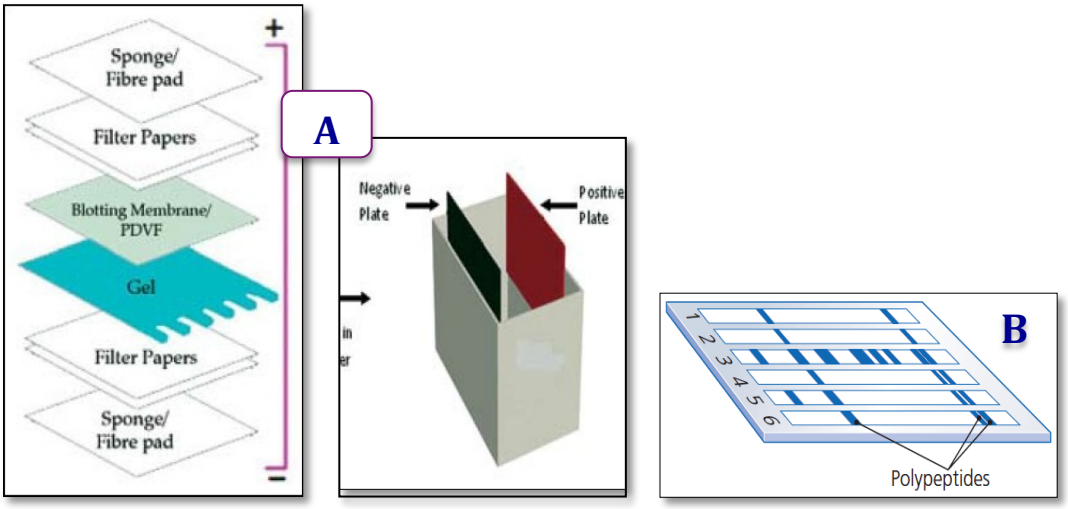


Figure 11: A. Western blot electrobloting. B. The nitrocellulose is then cut into strips

Third: Immunoblotting (Labeled immunoassays): A tray with troughs is used to hold the strips so that several tests may be run simultaneously. After careful preparation, each sample (suspected of containing antibody) is applied to a strip and allowed to react for the prescribed time (usually 12 or 24 hours). This incubation allows any antibody in the sample to bind to the known antigens in the strip. After rinsing to remove unbound antibody, the strips are exposed to an anti-immunoglobin antibody, which is labeled with an enzyme or a fluorescent chemical. The strips are rinsed again to remove any unbound labeled antibody.

Visualization of the bands depends on the type of labeled antibody. If an enzyme is used, then substrate is added. If a fluorescent chemical is used, then the strip is observed under UV light. Comparing band patterns on each strip to positive and negative controls allows determination of whether the sample contains the relevant antibodies.

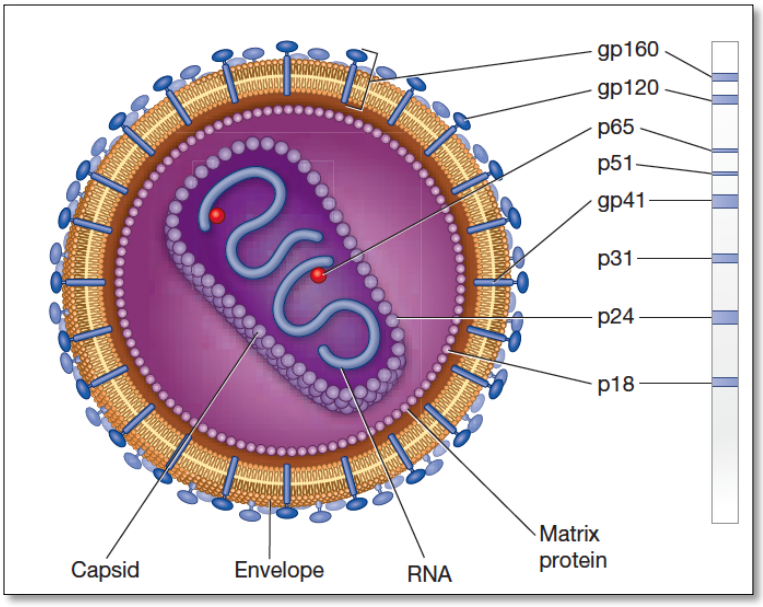


Figure 12: Schematic diagram of HIV

10.3 Applications of (WB) in virology:

- Generally it is used as a confirmatory or supplemental test to help verify the specificity of positive results obtained from other assays used to initially screen for virus-specific antibodies.
- Commercial kits are available for **HIV-1** and **HIV-2**, **hepatitis C** virus (HCV), and **human T-cell leukemia** virus types I (HTLV-I) and II (HTLV-II).
- Immunoblots utilizing recombinantly derived proteins immobilized to nitrocellulose strips have been described for HCV, HSV-2, hantavirus, and dengue virus.
- Western blot assays are able to detect anti-HEV IgM antibody.

10.4 Interpretation of Results:

- **Positive result:** reactivity to two or more of the following: p24, p41, gp120/160 (Figure 12 & 13).
    - Patient is infected
- **Indeterminate result:** Reactivity to other bands.

- Either a false positive or an early infection; repeat test in 3 and 6 months: if positive, negative or still indeterminate, likely not infected

- **Negative result:** No reactivity to any of the antigens.
  - Not infected

A

MOLECULAR WEIGHT	GENE	ANTIGEN	DESCRIPTION
gp 160	ENV	Polymeric form of gp41	Broad diffuse glycoprotein
gp 120	ENV	Outermembrane	Diffuse glycoprotein
p66	POL	Reverse Transcriptase	Discreet band
p55	GAG	Precursor protein	Discreet band
p51	POL	Reverse Transcriptase	Discreet band just below p55
p39	GAG	Fragment of p55	Discreet band
gp41	ENV	Transmembrane	Diffuse glycoprotein
p31	POL	Endonuclease	Doublet
p24	GAG	Core protein	Broad band
p17	GAG	Core protein	Broad band

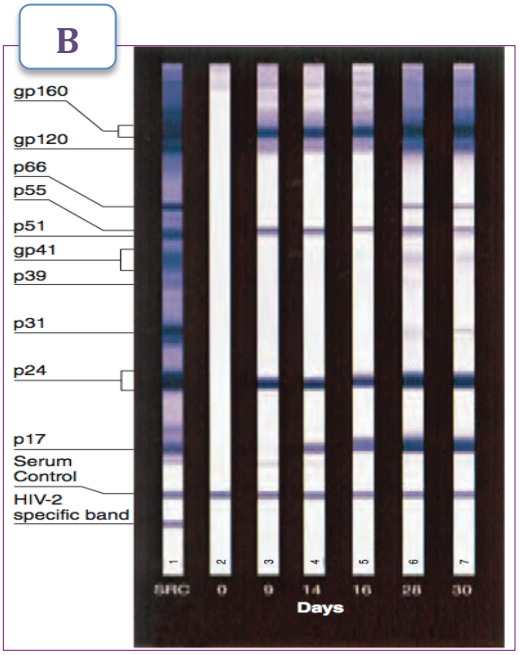


Figure 13: A. Main immunogenic components of HIV ordered based on their sizes. B. Successive tests on an HIV+ patient over 30 days reveal an increase in band intensity over time. This is due to continued formation of anti-HIV antibodies.

Note:

- If proteins are not blotted on separated nitrocellulose membrane strips, the test requires optimizing the experimental conditions (i.e. protein isolation, buffers, type of separation, gel concentration, etc.).

Week 11: Nucleic Acid Extraction for Polymerase Chain Reaction (PCR)

DNA (HBV) and RNA (HCV) extraction:

11.1 Purpose:

All nucleic acid amplification techniques require genome. In order to prepare nucleic acids for amplification or other purposes they should extracted from cells. Different commercial kits are available according the type of nucleic acid (DNA or RNA) to be worked on, and the type of specimen, which extracted from.

11.2 Preparation of Clinical Specimens for PCR:

Viral gene detection methods do not rely on persistence of viral infectivity within the clinical specimen and in one respect this is a major advantage over traditional methods of virus detection.

- Specimens should be transported and stored in the refrigerator or freezer prior to analysis, but less meticulousness is required than to achieve virus isolation.
- However, viral RNAs are susceptible to nucleases, present in all biological material, and certain specimen types (e.g. intraocular fluids and urine) contain inhibitors of PCR.
- PCR is therefore susceptible to false-negative results, and specimens for qualitative and especially qPCR require careful preparation.
- Each assay needs to be evaluated for individual specimen type and patient group. For blood samples, the anticoagulant heparin is contraindicated because it inhibits the PCR reaction. It is generally recommended that for viral quantification ethylenediamine tetraacetic acid (EDTA) anticoagulated blood is separated as soon as possible, after which the plasma can be stored frozen until analysis.
- If multiple tests are to be undertaken on one sample, it should be aliquoted on receipt to avoid multiple freeze-thawing. A number of different nucleic acid extraction methods are available.
- The choice depends on the nature of the clinical specimen and whether the target

is RNA or DNA.

11.3 Materials

DNA/RNA Extraction From Serum

1. Proteinase K stock solution at 2 mg/ml in sterile distilled water.
2. Phenol:Chloroform:Isoamyl alcohol (25:24:1, v/v).
3. Yeast t-RNA at 0.8 mg/ml.
4. Glycogen 20 mg/ml.
5. Ethanol at 100 and 70%.
6. Chloroform.
7. Plasticware.

Note: Instead of the above, one could use one of the following commercially available extraction kits:

- QIAamp DNA Blood Mini or Midi kit.
- QIAamp Viral RNA mini kit or PurescriptR Cell and Tissue RNA extraction kit (Gentra/Flowgen) or other similar kits.
- TRIzolR and TRIzolR LS reagent (InVitrogen).

11.4 Method:

Extraction of Nucleic Acids

DNA from Serum or Plasma

1. Mix in a 1.5-ml Eppendorf tube 50 ml of serum/plasma with 20 ml of proteinase K (2 mg/ml), 2.5 ml of t-RNA (0.8 mg/ml), 1.7 ml of SDS (25%), 100 ml of 2× buffer (50 mM sodium acetate, 5 mM EDTA) and make up to 200 ml with H_2O.
2. Incubate at 65°C for 2 h.
3. Add an equal volume of phenol:chloroform:isoamyl alcohol and invert several times to mix.
4. Centrifuge at 12,000×g for 10 min.
5. Transfer the top aqueous phase into a new tube and mix with an equal volume of chloroform.

6. Centrifuge as above and remove the top aqueous layer into a new tube.
7. Add one-tenth volume of 3 M sodium acetate (pH 5.2) and two volumes of ethanol before placing at −80°C for 1 h.
8. Centrifuge as above, but this time at 4°C. Add 500 ml of 70% ethanol and re-spin for 5 min.
9. Air-dry the pellet and re-suspend the DNA in 20 ml of H_2O.
10. Store at −20°C until use.

Extraction of RNA from Serum/Plasma

The protocol is the same as that described for DNA extraction, except that the t-RNA step is omitted. Instead, after chloroform extraction, 1 ml of glycogen is added to the aqueous layer that is removed to act as carrier. This is followed by ethanol precipitation. The final RNA pellet is re-suspended in 20 ml of RNAse-free water and stored at −80°C until use.

Week 12: Nucleic Acid Amplification by PCR (DNA and RNA Viruses)

12.1 Conventional PCR

12.1.1 Purpose:

The polymerase chain reaction (PCR) is a relatively simple and convenient method of amplifying (copying) even as little as one molecule of DNA. Once multiple copies of the DNA are made, they can be used in various ways ranging from research, diagnostics, and forensics. The PCR may also be used to amplify RNA, which must first be converted into cDNA by the enzyme reverse transcriptase (RT-PCR). PCR and other molecular amplification techniques have now been applied to the diagnosis of virtually all human viruses and, in general, the sensitivity of these assays far exceeds that of other virus detection systems. A number of commercial kits and automated systems are now available.

12.1.2 Principle:

In 1983, the Cetus scientist Kary Mullis developed an ingenious "in vitro" nucleic acid amplification technique termed the *polymerase chain reaction* (PCR). This process is designed to make multiple copies of (amplify) a desired gene or other short DNA fragment. In the process, the double-stranded DNA sequence to be amplified (the **template**) is separated with heat and then replicated using free nucleotides, two commercially prepared **primers**, and a polymerase. The primers (short nucleic acid molecules) are selected to be complementary to positions on opposite strands of the DNA molecule. The points at which they attach flank the area to be replicated. DNA Polymerase is then able to attach the free nucleotides to complementary bases on the template and make the desired copy.

The PCR process involves 20 to 40 cycles of three different activities: **denaturation** (DNA strand separation), **annealing** (of primers), and **extension** (DNA replication). In denaturation, the temperature of the reaction tube is raised to 92–96°C for one to a few minutes to separate the DNA strands. Following this, the temperature is lowered to 45–

65°C, which allows the primers to anneal to their complementary sequences on opposite template strands. For the extension phase, the temperature is raised to 72°C, for a few seconds to a few minutes, to allow the polymerase to synthesize DNA complementary to the template (elongate primer) (Figure 14).

This process is performed automatically by a thermocycler programmed by the lab worker. By continuing the process and using the products of one cycle as templates for the next cycle, the number of DNA target strands doubles each cycle and can be amplified a million-fold, all in a few hours. (For 20 cycles, each strand will be replicated 2^{20} = 1,048,576 times!).

Important notes:

- Primers must be added to the PCR reaction mixture in great excess to flood the mixture and promote primer-DNA annealing and to discourage DNA-DNA re-annealing.
- Sufficient quantities of free deoxyribonucleotides (dATP, dCTP, dTTP, and dGTP) needed for extension.
- Buffers to maintain the appropriate pH, magnesium required by the polymerase, and other salts to create the proper osmotic balance.
- Because of the high temperatures required for the denaturation phase, the thermotolerant ***Taq*DNA polymerase**, isolated from the thermophile *Thermus aquaticus* is used to catalyze the extension.

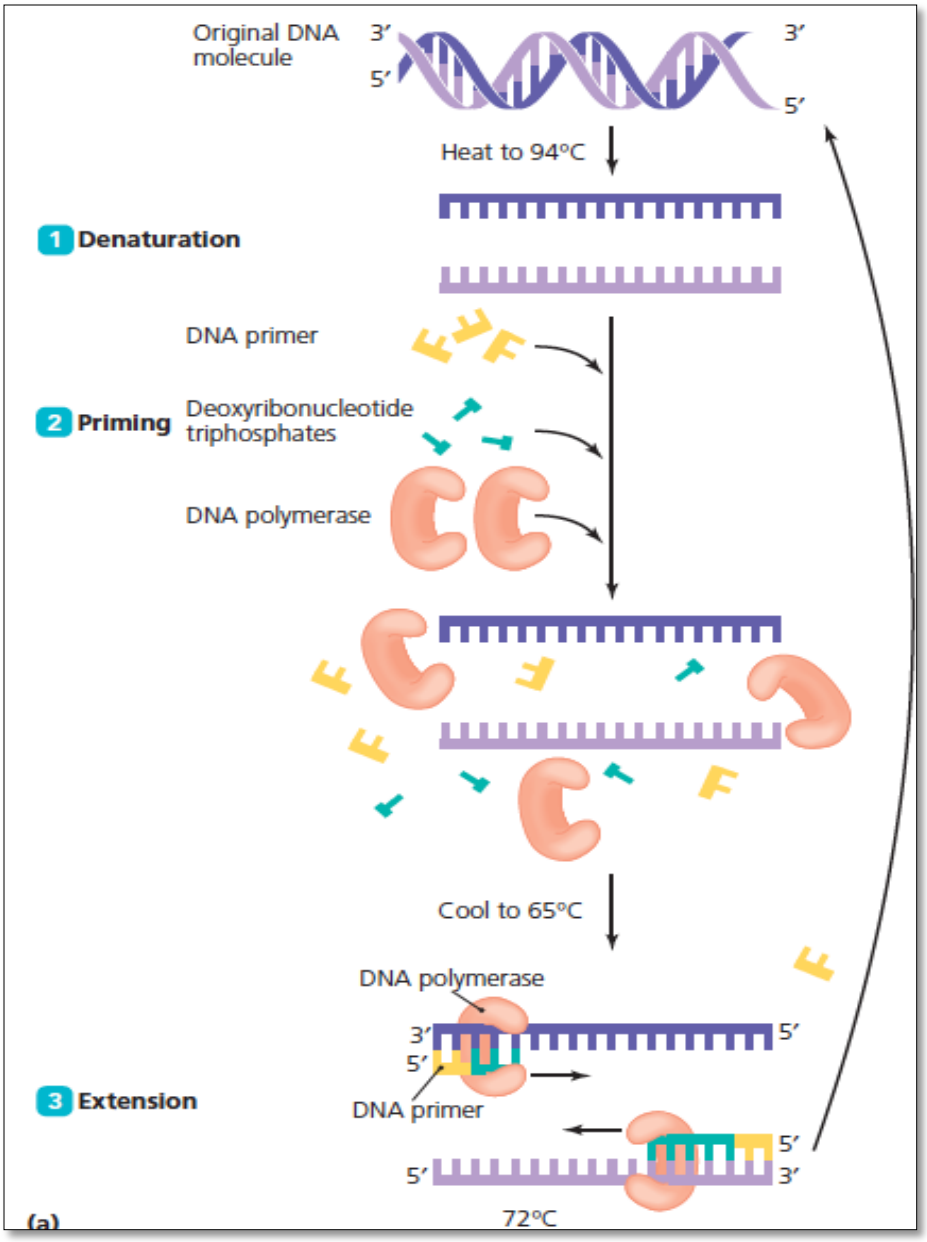


Figure 14: Three main steps of polymerase chain reaction

12.1.3 Materials:

DNA PCR

1) Programmable thermal cycler.
2) Sterile distilled water (use as fresh).
3) Solution A: containing $MgCl_2$ and a source of buffer/salt (usually KCl and Tris-HCl)

4) 10X Reaction buffer.
5) 5 m*M* dNTPs: dilute 50 m*M* stock solution of dNTPs (i.e., dA TP , dCTP , dGTP , and dTTP) in distilled water. Store at -20°C for up to 1 mo.
6) 15 pmol primers 1 (forward) and 2 (reverse).
7) 5 U/µL *T aq* polymerase.

12.1.4 Procedure:

DNA PCR:

1. Into a sterile tube, prepare the following master mix according to the number of samples to be analyzed:
    a. 10 µL of 10X reaction buffer
    b. 10 µL of 5 m*M* dNTPs
    c. 15 pmol primer 1
    d. 15 pmol primer 2
    e. 0.5 µL (2.5 U) *Taq* polymerase
    f. Distilled water to a volume of 98 µL per sample
2. Aliquot 98 µL of master mix into a PCR reaction tube. Add 2 µL genomic DNA (50 µg/mL) to give a final volume of 100 µL. PCR may be done in much smaller volumes down to 10 µL, especially for analytical PCR, by reducing the reaction components proportionately.
3. Always perform a blank control alongside the samples by replacing the genomic DNA with dH_2O. This will check for contamination.
4. Denature the template for 5 min at 94°C.
5. PCR at the following settings for 30 cycles:
    a. Denaturation: 94°C for 1 min.
    b. Annealing: 55 – 65°C for 1 min (adjust temperature according to the calculated T_m of the primers.
    c. Extension: 72°C for3min (optimum temperature for *Taq* polymerase activity)
6. Analyze 10 µl of the sample on a 1–2% agarose gel containing ethidium bromide.

Attention:

- Always wear, and frequently change, gloves to prevent contamination
- Solutions and buffers must be stored in sterile containers.
- Always prepared with fresh distilled water.

12.2 Reverse transcription PCR

12.1 Background:

Reverse-transcription PCR (RT-PCR) is an extension of polymerase chain reaction (PCR) in which template RNA is first reverse-transcribed into complementary DNA (cDNA). This cDNA then undergoes amplification by PCR.

Routine methods for diagnosing influenza infection (including virus culture and antigen detection) are both sensitive and specific. However, molecular techniques to directly detect influenza A or B genetic material in respiratory samples or viral cultures can greatly facilitate the investigation of outbreaks of respiratory illness. In addition, such techniques can also be highly useful in the rapid identification of human influenza A subtypes, including those with the potential to cause a pandemic.

Molecular assays should be used, in conjunction with other diagnostic assays and clinical and epidemiological information, to:

- Detect influenza virus type A or B in symptomatic patients from viral RNA in respiratory specimens and virus culture.
- Determine the subtype of human influenza A viruses.
- Presumptively identify virus in patient respiratory specimens or viral cultures, which may be infected with influenza A of subtype H5 (Asian lineage).
- Detect potentially novel or newly evolving influenza A viruses.

RT -PCR is necessary for the amplification of specific influenza gene targets, because the genome of influenza viruses consists of eight single-stranded RNA segments. RT -PCR can be used for the detection of influenza viruses in respiratory samples (such as nasopharyngeal swabs and aspirates, oropharyngeal aspirates or washes, throat swabs, sputum, tracheal aspirates or broncheoalveolar lavage) taken from patients with influenza-like illness or for the characterization of viruses grown in cell culture or embryonated eggs.

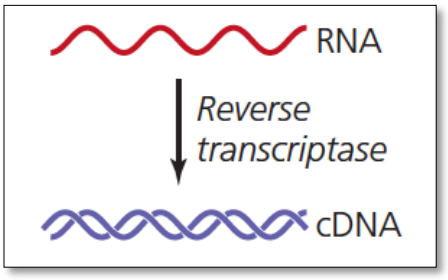
12.2.1 Purpose: Reverse transcription PCR was introduced to amplify RNA targets.

- To detect RNA viruses (influenza virus, HCV and HIV).

- In order to detect transcribed microbial DNA marker of active infection.
- Detecting viable microbial species.
- Monitoring the effectiveness of antimicrobial therapy
- Commercially kits are available for detection of HCV RNA and for quantitation of HIV-1 and HCV RNA in clinical specimens.
-

12.2.3 Principle: In this method:

- Template RNA is first reverse-transcribed into complementary DNA (cDNA)
- The cDNA is amplified by PCR.
- Four basic reagents needed to produce cDNA: mRNA as template, dNTPs, reverse transcriptase and primers.



12.2.4 Viral RNA extraction

The performance of RT-PCR amplification based assays depends upon the amount and quality of sample template RNA. A number of commercially available extraction procedures have been shown to generate highly purified RNA when following the manufacturer's recommended procedures.

1. Extract viral RNA from the clinical specimen(s) using an appropriate extraction kit in accordance with the manufacturer's instructions.
2. Use viral RNA immediately in real-time or conventional RT-PCR – or aliquot intosmall volumes and freeze at -70 °C until use.

12.2.5 Conventional RT -PCR reactions typically require:

- A pair of oligonucleotides (known as primers)

- Four deoxyribonucleoside triphosphates (dNTPs)
- Template RNA
- Reverse transcriptase
- Taq DNA polymerase.

Conventional RT-PCR amplification conditions

Reaction step	Temperature	Time
Reverse transcription	42–50 °C	30 minutes
RT inactivation/Taq activation	95 °C	2–15 minutes
PCR amplification (35–45 cycles) Denaturation Primer annealing Template extension	 95 °C 45–60 °C[a] 72 °C	 15 seconds 30 seconds 30–120 seconds[b]
Hold	4 °C	hold

[a] Primer annealing temperature will depend upon the specific assay used.
[b] Template extension at 72 °C may not be necessary for all assay designs.

Note: The recent availability of improved "one-step" RT -PCR strategies have decreased the number of pipetting steps required, making the process technically easier and less susceptible to contamination.

12.3 Nested PCR:

12.3.1 Purpose:

This technique is designed to increase the sensitivity of the PCR reaction and is particularly useful for the amplification of small quantities of target. The logic behind nested PCR is; if the wrong amplicon was amplified by mistake, the probability is very low that it would also be amplified by the specific internal primers.

12.3.2 Principle:

This involves the use of two sets of primers. The first set (external primers), which flanks the region of interest, allows for a first round of amplification. A small aliquot from the first round of PCR is then used as the target for a second round of amplification, primed by a second pair of primers that lie internal to the first (Figure 15).

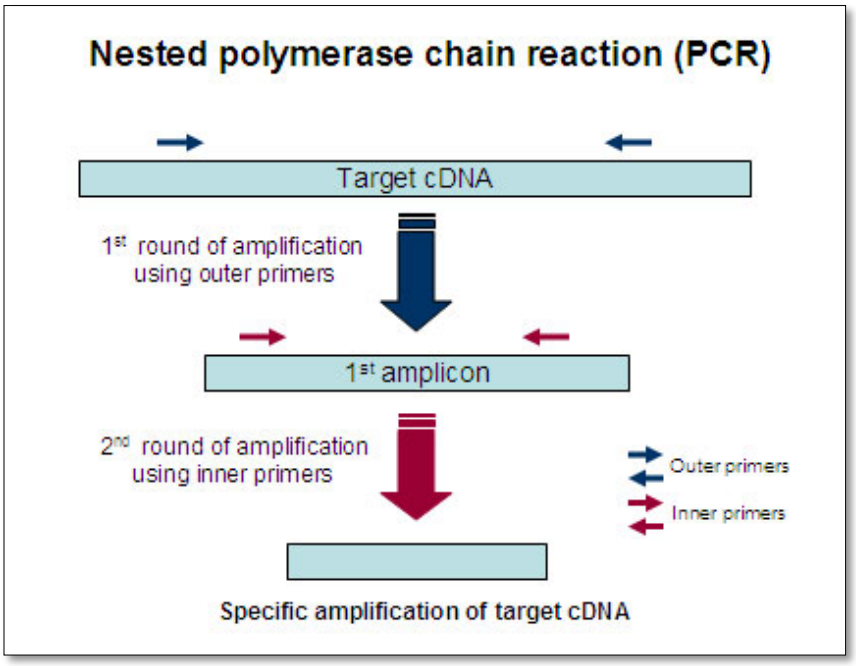


Figure 15: Nested polymerase chain reaction main steps

Limitation: Because of opening of PCR reaction tube between PCR reactions, the chance of contamination will increase.

12.4 Multiplex PCR:

12.4.1 Purpose:

- Screen for different pathogenic strains or isolates in one assay, which reduce time and cost.
- Differentiate pathogenic and non-pathogenic strains or isolates from each other using target specific primer pairs in combination.

12.4.2 Principle:

Multiplex PCR is an amplification reaction in which two or more sets of primers specific for different targets are introduced in the same tube, allowing multiple target sequences to be amplified simultaneously. Primers used in multiplex reactions must be designed carefully to have similar annealing temperatures and to lack complementarity, in order to avoid dimerization (Figure 16).

12.4.3 Attention:

- Primers must have similar or nearly similar annealing temperatures
- The size of amplicons to be amplified must be different, in order to achieve clear separate bands.

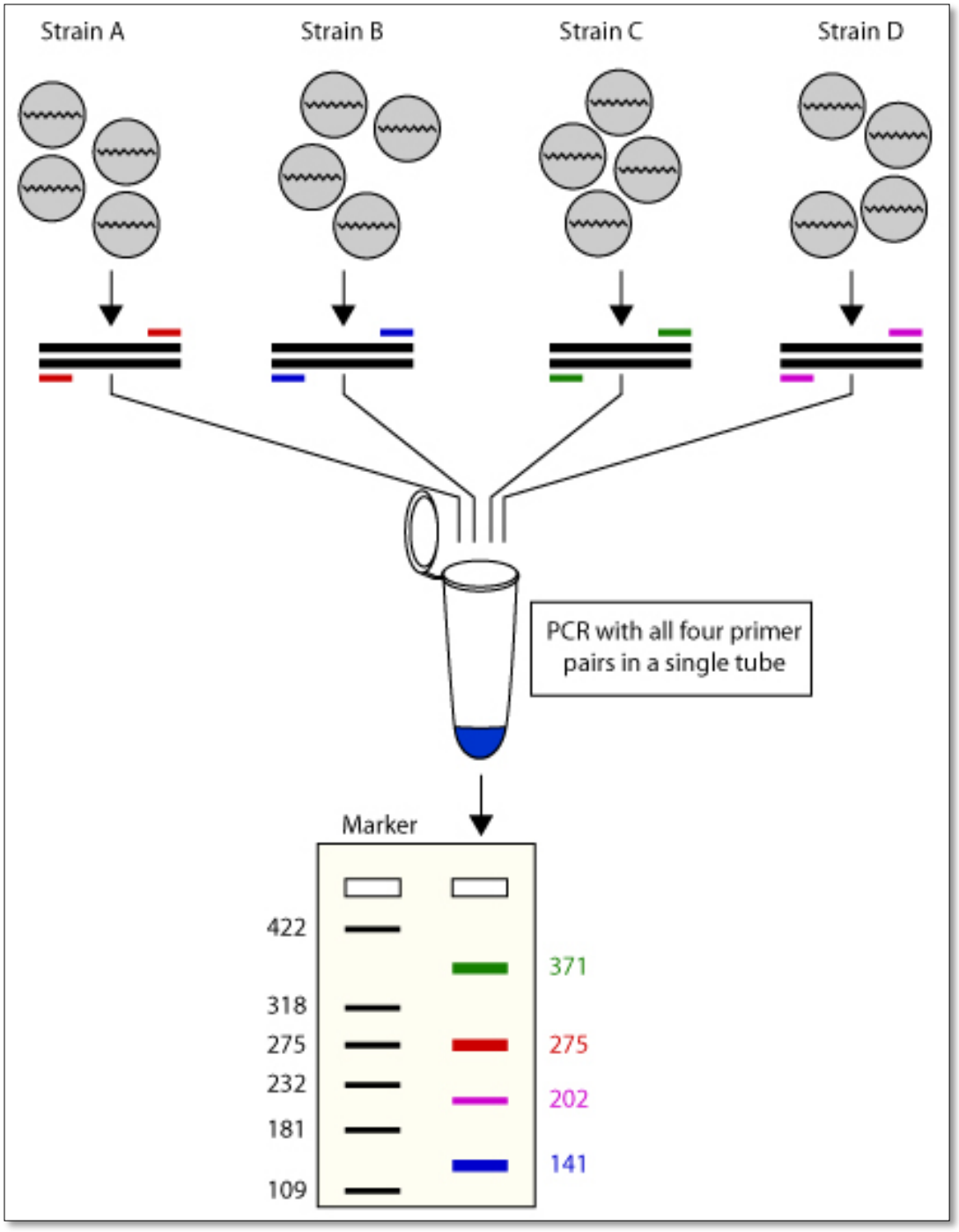


Figure 16: Multiplex polymerase chain reaction main steps

Week 13: Visualization of PCR products by Agarose Gel Electrophoresis:

13.1 Purpose:

- Detection of specific ampilcon after amplification.
- Separation and sometimes purification of different DNA fragments or proteins according to their size, charge and conformation. The DNA fragments could be used in recombinant DNA technology.

13.2 Principle:

Electrophoresis is a technique used to separate and sometimes purify macromolecules, especially proteins and nucleic acids that differ in size, charge, or conformation. When charged molecules are placed in an electric field, they migrate toward either the positive or negative pole according to their charge. Nucleic acids have a consistent negative charge imparted by their phosphate backbone and migrate toward the anode. Nucleic acids are electrophoresed within a matrix or gel (Figure 17).

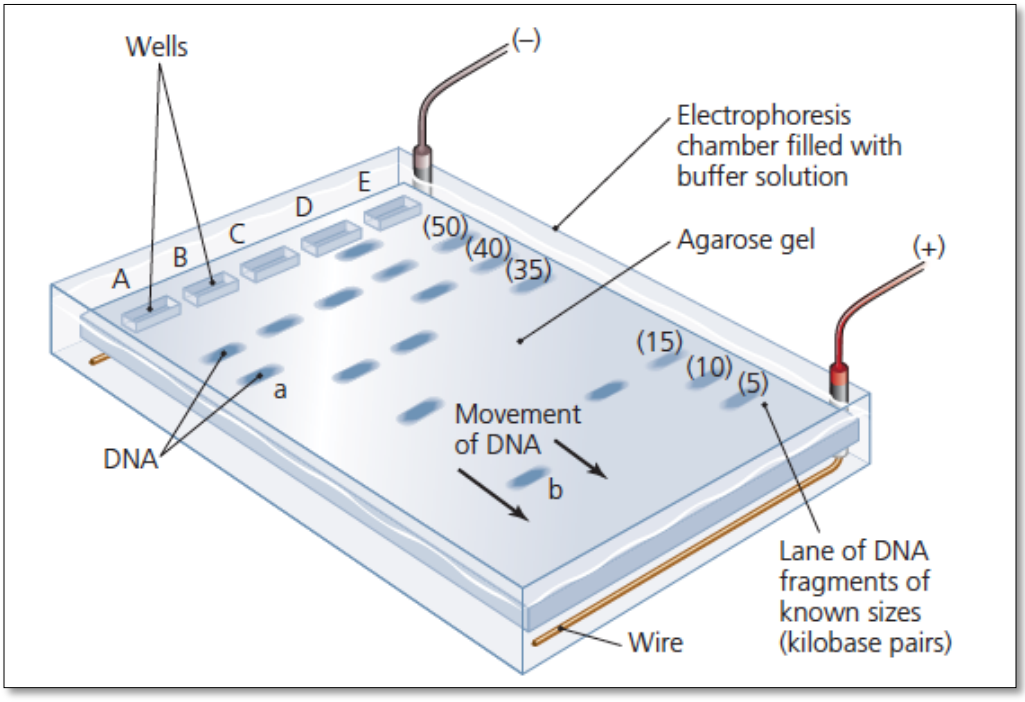


Figure 17: Agarose gel electrophoresis

The gel is immersed within an electrophoresis buffer that provides ions to carry a current and some type of buffer to maintain the pH at a relatively constant value. Agarose is typically used at concentrations of 0.5–2 %. Agarose gels have a large range of separation, but relatively low resolving power. By varying the concentration of agarose, fragments of DNA from about 200 to 50,000 bp can be separated using standard electrophoretic techniques (Table 3).

Table 3: Resolution of agarose gel

Agarose %	Mol wt range (kb)	Comments
0.2	5–40	Gel very weak; separation m 20–40 kb range improved by increase in ionic strength of running buffer (i.e., Loenings E); only use high-melting point agarose
0.4	5–30	With care can use low-melting point agarose
0.6	3–10	Essentially as above, but with greater mechanical strength
0.8	l–7	General-purpose gel separation not greatly affected by choice of running buffer, bromophenol blue runs at about 1 kb
1	0.5–5	As for 0.8 %
1.5	0.3–3	As for 0.8 %, bromophenol blue runs at about 500 bp
2 0	0.2–l.5	Do not allow to cool to 50 °C before pouring
3 0	0.l–l	Can separate small fragments differ mg from each other by a small amount; must be poured rapidly onto a prewarmed glass plate

13.3 Materials:

1. Agarose (powder)
2. Running buffer 1X
3. Distilled water
4. Screw capped bottle
5. Sensitive balance
6. Microwave
7. Electrophoresis chamber.
8. Gel trey and comb
9. Ethidium bromide
10. Micropipette and their tips (2 µl-20 µl and 20 µl-200 µl)

11. Tape

13.4 Method:

13.4.1 Melting of Agar

- An appropriate amount of powdered agarose is weighed carefully into a conical flask.
- One-tenth of the final volume of 10 concentrated running buffer is added, followed by distilled water to the final volume (i.e., 1 ml of 10 buffer in 9 ml of distilled water to make 1 of 10 ml).
- Cover the container with plastic wrap. Pierce a small hole in the plastic for ventilation.
- Heat the solution in the microwave oven on high power until it comes to a boil. Watch the solution closely; agarose foams up and boils over easily.
- Remove the container (protect your hand with a pot holder or folded paper towel) and gently swirl it to resuspend any settled agar.
- Continue this process until the agar dissolves completely.
- Cool the agar until you can comfortably touch the flask and add ethidium bromide solution to give a final concentration of 5 pg/ml.
- The gel mixture is ready to be poured into the gel apparatus.

13.4.2 Pouring the Gel

1. Place tape across the ends of the gel form and place the comb in the form.
2. Pour cooled agar into the form. The agar should come at least half way up the comb teeth.
3. Immediately rinse and fill the agar flask with hot water to dissolve any remaining agar.
4. When the agar has solidified, carefully remove the comb. Remove the tape from the ends of the gel form.

13.4.3 Setting up the gel & loading the samples

1. Place the gel in the electrophoresis chamber.

2. Make sure that the wells are closest to the negative (black) electrode.
3. Fill with 1X running buffer to just cover the wells.
4. Make a written record of which sample you will load in each well of the gel.
5. Load 10 µl of each sample in each well.
6. Be careful not to puncture the bottoms of the wells as you load each sample.
7. Load the DNA ladder (DNA fragments of known sizes) in one well (Mostly first well is used).
8. Adding more 1X running buffer gently until the gel completely overlaid.
9. Place the lid on the chamber and connect the electrode leads to the power supply.
10. Connect the black lead to the negative terminal and the red lead to the positive terminal.

13.4.4 Running and Analyzing the Gel

0 Turn on the power supply and adjust the voltage to 50–100 V.

1 The gel is usually run between 1 and 3 h, depending on the percentage of the gel and length.

2 Once the dyes have moved through the gel, turn off the power supply, disconnect the electrode leads, and remove the chamber lid.

3 Remove the gel from the electrophoresis chamber and analyze your results.

13.5 Result:

- The products can be viewed on a UV transilluminator.
- The bands (Amplified amplicons) are compared to the DNA ladder to determine their sizes.
- Unexposed bands to UV can be purified and used for further purposes.

Week 14: Quantitative Molecular Methods in Diagnostic Virology (Real time PCR)

14.1 Background:

Real-time PCR or Q-PCR is a variation of the standard PCR technique used to quantify DNA or messenger RNA (mRNA) in a sample. It is called "real-time PCR," because it allows us to monitor the increase in the amount of DNA as it is amplified. Furthermore, real time PCR enables both detection and quantification of a specific sequence in a DNA sample. Quantification of amplified product is obtained using fluorescent probes and specialized machines that measure fluorescence while performing temperature changes needed for the PCR cycles. This technique is based on the detection of the fluorescence produced by a reporter molecule, which increases, as the reaction proceeds. This occurs due to the accumulation of the PCR product with each cycle of amplification. These fluorescent reporter molecules include dyes that bind to the double-stranded DNA (SYBR Green) or sequence-specific probes (TaqMan and molecular beacon) (Figure 18).

A variety of fluorogenic molecules are used. These include nonspecific dyes such as SYBR green, which binds to the minor groove of double-stranded DNA, and amplicon-specific detection methods using fluorescently labeled oligonucleotide probes, which fall into three categories: TaqMan or hydrolysis probes; fluorescence energy transfer (FRET) probes; and molecular beacons. The signal from these probes is proportional to the amount of product DNA present in the reaction and is plotted against the PCR cycle. Use of a threshold value allows determination of positive and negative reactions. The signal is measured through the closed reaction tube using fluorescent detectors; hence, the assay is performed in "real time". When used with a standard curve, real-time PCR assays can be quantitative, allowing for determination of organism concentration. These assays are commonly used for viral load quantification of HIV, hepatitis C virus, hepatitis B virus, and CMV.

14.2 Purpose:

Real-time PCR or Q-PCR is used to detect and quantify DNA or RNA (messenger RNA or RNA virus) in a sample.

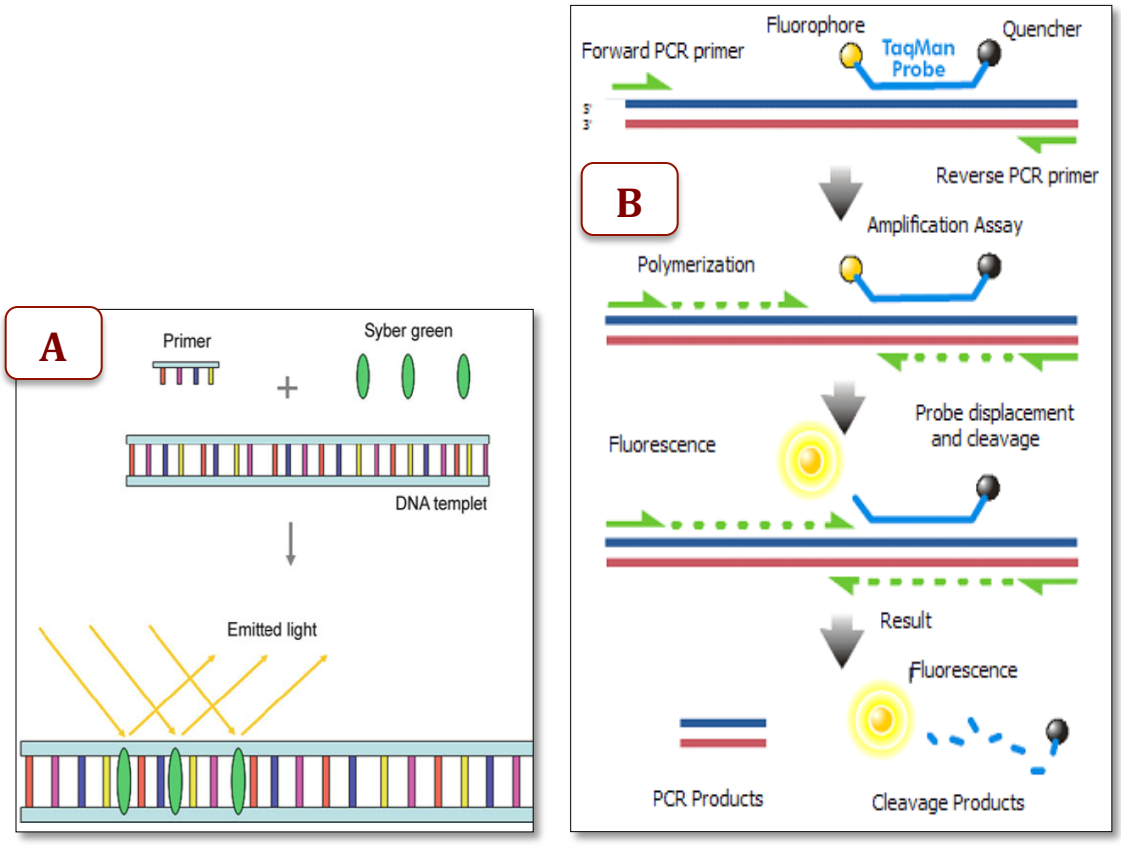


Figure 18: These fluorescent reporter molecules. A. SYBR Green a fluorescent dye that bind to the double-stranded DNA. B. TaqMan is a sequence-specific probes.

14.3 Detection and quantification of RNA viruses

14.3.1 Reaction materials

	Materials	Amount (µ)		Materials	Amount (µ)
1	RNA (10–100 ng) (Sample)	30.0	6	Primer R (10 mM)	2.5
2	10 TaqMan buffer	5.0	7	TaqMan probe (10 mM)	1.0
3	MgCl2 (25 mM)	5.0	8	Taq Polymerase (5 U)	0.5
4	dNTPs (10 mM)	2.0	9	Reverse Transcriptase (20 U)	0.5
5	Primer F (10 mM)	2.5	10	RNase inhibitor (20 U)	1.0

General Recommendations for Real-Time RT-PCR

The optimal concentrations of the real-time PCR reagents are as follows:

1. Magnesium chloride concentration should be between 4 and 7 mM.
2. Concentrations of dNTPs in TaqMan reaction should be 200 µM of each dNTPs.
3. Typically 1.25 U of Taq DNA polymerase is used in a 50-ml reaction mixture. This is the minimum requirement; if necessary, optimization can be done by increasing this amount by 0.25 U increments.

14.3.2 Cycling Parameters

1. Reverse transcription at 48°C for 30 min.
2. Taq activation 95°C for 10 min. PCR profile:
3. Denaturation at 95°C for 15 s.
4. Annealing/extension at 60°C for 1 min (repeated 40 times).

14.3.3 Method:

Reaction Set and Thermal Cycling

1. Prepare the reaction mix (according to the components given in Sect. 14.2.1) and load it on the multi-well plate.
2. Set up the cycling parameter (shown in Sect. 14.2.2)
3. Run the program and analyze the results.

Table 4. FDA-approved quantitative assays by type, a list of FDA-approved viral load assays:

Target	Test or Reagent (Manufacturer)	Method
HIV-1	**Cobas AmpliPrep/ Amplicor HIV-1 Monitor v. 1.5 (Roche)**	**Real-time RT-PCR**
HCV	**Realtime HCV Assay (Abbott)**	**Real-time RT-PCR**
HBV	**RealTime HBV PCR (Abbott)**	**Real-Time PCR**
CMV	**artus CMV RGQ (Qiagen)**	**Real-time PCR**