Successful laboratory investigations

✓Advance planning

✓Collection of adequate and appropriate specimens

✓Sufficient documentation

✓Biosafety and decontamination

✓Correct packaging

✓Rapid transport

✓Choice of a laboratory that can accurately perform the tests

✓Timely communication of results

Transport of Specimens

1. Rapid, optimally in less than 2 hours.
2. For delays in transport, most specimens should be refrigerated.
3. Exceptions: blood, cerebrospinal fluid (CSF), and specimens to be examined for anaerobes, fastidious organisms such as *Neisseria gonorrhoeae* and *Bordetella pertussis*, and *Trichomonas vaginalis*, all of which should be maintained at room temperature.

Specimen collection: key issues

Consider differential diagnoses

Decide on test(s) to be conducted

Decide on clinical samples to be collected to conduct these tests

- consultation between microbiologist, clinicians and epidemiologist

Specimen rejection criteria

1. Improper transport temperature
2. Improper transport container or medium
3. Prolonged transport time
4. Unlabeled or mislabeled specimen
5. Broken or cracked container
6. Leaking specimen
7. Dried-out specimen
8. Inappropriate specimen for test requested
9. Inadequate volume
10. Specimen in fixative (for culture)

Blood - specimen collection and culture

Blood from properly cleaned and disinfected vein site (70% alcohol and chlorhexidine) sample taken before administration of antimicrobials if at all possible.

For adults, collect 10-20 cc and 1-3 cc for a child for each blood culture set; divide blood into two blood culture bottles, one for aerobes and one for anaerobes; two or three blood cultures (by separate stick) per septic episode is sufficient.

Handling and Transport

Collect into bottles with infusion broth

- change needle to inoculate the broth

Transport upright with cushion

- prevents hemolysis

Wrap tubes with absorbent cotton

Travel at ambient temperature (RT)

- Inoculated blood culture bottles should not be placed in the refrigerator.

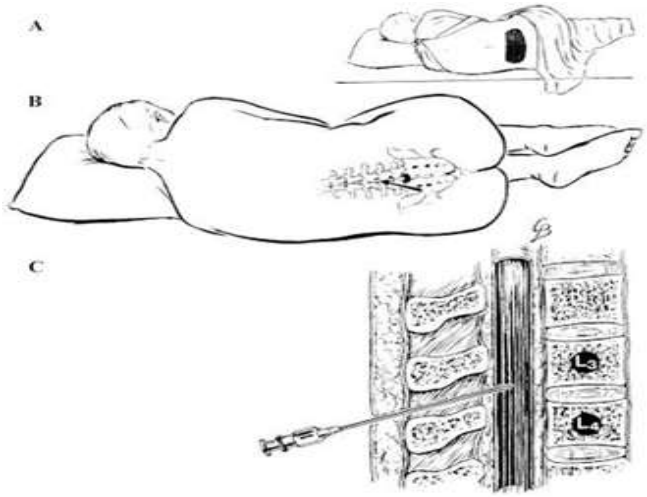
Collected blood should be diluted in blood culture broth in order to obtain blood cultures. Blood cannot be transported before being placed in a blood culture bottle because the syringes do not contain any anticoagulant and the blood will coagulate within a few minutes. Typically, 1-2 ml of blood from a child is added to 20 ml of blood culture broth and 5-10 ml of blood from an adult is added to 50 ml of blood culture broth. It is important to use appropriate ratios of blood to culture broth for optimal bacterial growth. Blood should be cultured in trypticase soy broth (TSB) or brain heart infusion (BHI) broth with a growth supplement (such as IsoVitaleX or Vitox) to support growth of other fastidious organisms such as *H. influenzae.* FOR GENERAL Ideally, inoculated blood culture bottles should be transported to a microbiology laboratory immediately for overnight incubation at 35-37°C with ~5% CO2 (or in a candle-jar) and subsequent culture onto a BAP (Blood agar plate) and CAP (Chocolate **agar** plate).

• Typical pathogens such as S. aureus always considered significant. Typical skin flora such as coagulase negative staphylococcus species, Corynebacterium species, etc., identified in only one set of bottles is highly suggestive of a contaminant.

Collection and transport of CSF

The collection of CSF is an invasive procedure and should only be performed by experienced personnel under aseptic conditions. If bacterial meningitis is suspected, CSF is the best clinical specimen to use for isolation, identification, and characterization of the etiological agents. Suspected agents should include *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* and other pathogens in some cases.

Ensure that the patient is kept motionless during the lumbar puncture procedure, either sitting up or lying on the side, with his or her back arched forward so that the head almost touches the knees in order to separate the lumbar vertebrae during the procedure.



Disinfect the skin along a line drawn between the crests of the two ilia with 70% alcohol and povidone-iodine to clean the surface and remove debris and oils. Allow to dry completely.

Position the spinal needle between the 2 vertebral spines at the L4-L5 level and introduce into the skin with the bevel of the needle facing up. Accurate placement of the needle is rewarded by a flow of fluid, which normally is clear and colorless.

Remove CSF (1 ml minimum, 3-4 ml if possible) and collect into sterile screw-cap tubes. If 3-4 ml CSF is available, use 3 separate tubes and place approximately 1ml into each tube.

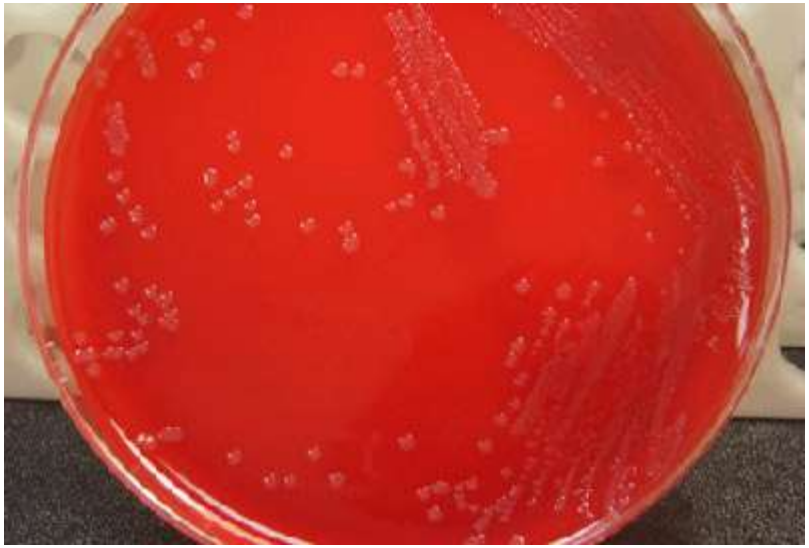
Inoculating and transporting T-I medium

T-I(trans isolate) is a biphasic medium that is useful for the primary culture of meningococci and other etiological agents of bacterial meningitis (*S. pneumoniae* and *H. influenzae*) from CSF. Use a sterile syringe and needle to inoculate 0.5-1.0 ml of CSF into the T-I medium. The remaining CSF should be kept in the collection tube. It should not be refrigerated, but should be maintained at room temperature (20-25°C) before Gram staining and other tests. Incubate inoculated T-I medium at 35-37°C with ~5% CO_2 (or in a candle-jar) and observe daily for turbidity in the liquid phase for up to 7 days.

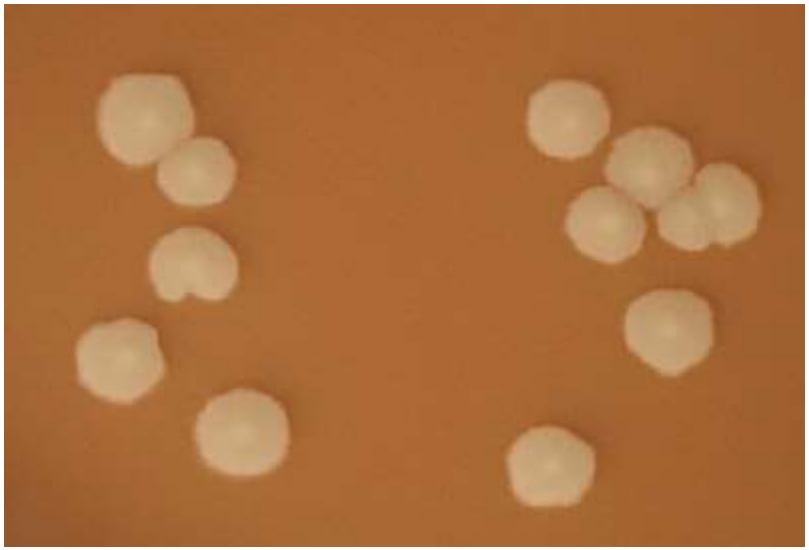
If turbidity is observed, culture onto a blood agar plate (BAP) and a chocolate agar plate (CAP) immediately
If no turbidity is observed, culture onto a BAP and a CAP on day 4 and day 7.
If T-I medium appears to be contaminated, selective media such as Modified Thayer-Martin and chocolate agar with bacitracin may be used.

Slant: Charcoal-starch agar

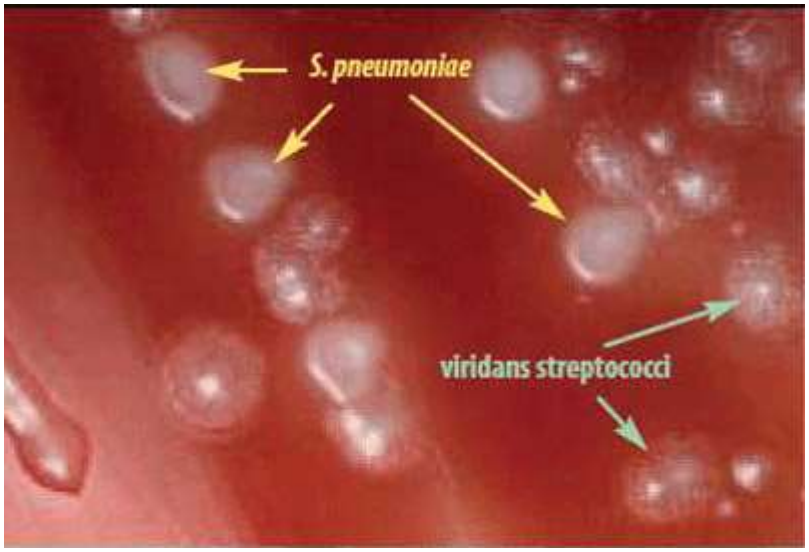
Broth: Soybean-casein digest-gelatin broth buffered at pH 7.2 with 0.1 M 3-(N-morpholino) propane sulfonic acid buffer.



growth of *N. meningitidis* on a BAP



H. influenzae colonies on a CAP



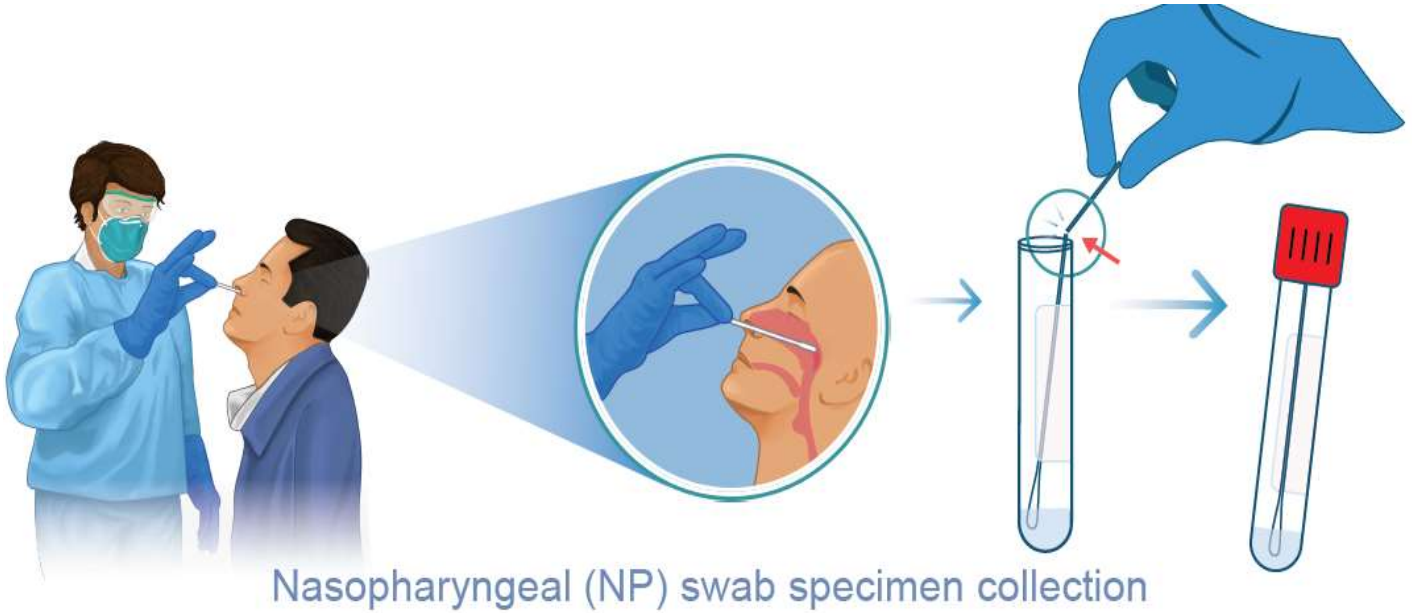
. *pneumoniae* colonies have a flattened and depressed center after 24-48 hours of growth on a BAP, whereas the viridans streptococci retain a raised center

Respiratory Specimen Collection

A. Upper respiratory tract

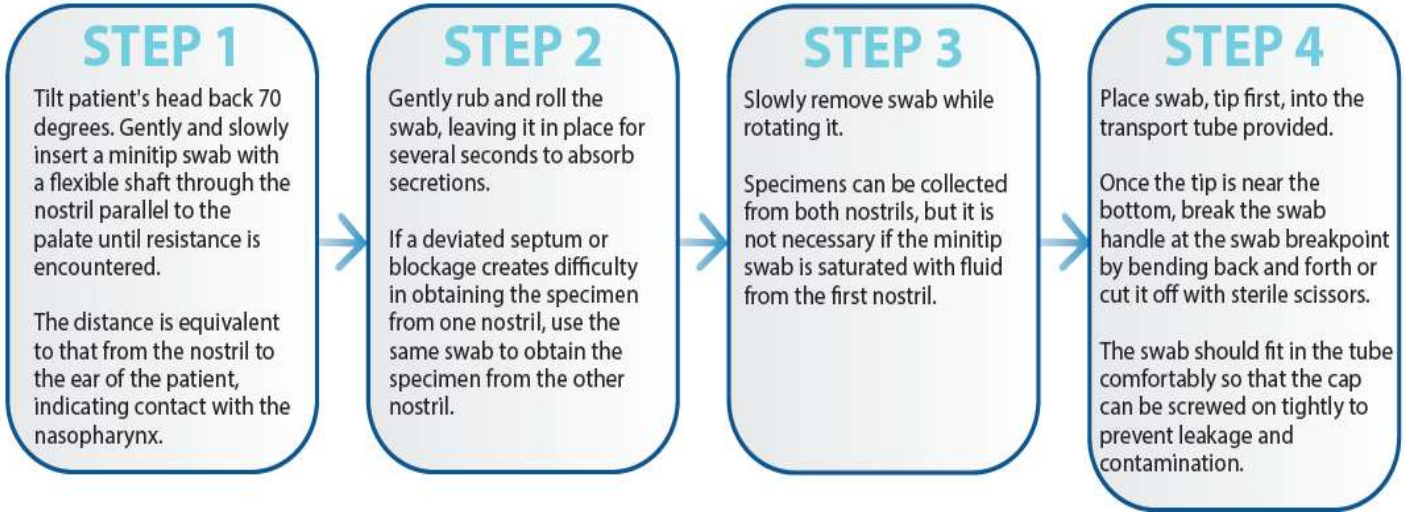
Nasopharyngeal specimen (NP) collection /Oropharyngeal (OP) (throat) specimen collection

Use only **synthetic fiber swabs with thin plastic or wire shafts** that have been designed for sampling the nasopharyngeal mucosa. Do not use calcium alginate swabs or swabs with wooden shafts, as they may contain substances that inactivate some viruses and may inhibit molecular tests.



Nasopharyngeal (NP) swab specimen collection

CDC recommends collecting only the NP specimen, although an OP specimen is an acceptable specimen type. If both NP and OP specimens are collected, combine them in a single tube to maximize test sensitivity and limit use of testing resources



Instructions for collecting an OP specimen

1.Insert swab into the posterior pharynx and tonsillar areas.

2. Rub swab over both tonsillar pillars and posterior oropharynx and avoid touching the tongue, teeth, and gums.

3.Place swab, tip first, into the transport tube provided.

B. Lower respiratory tract

Broncho-alveolar lavage, tracheal aspirate, pleural fluid
Collect 2-3 ml into a sterile, leak-proof, screw-cap sputum collection cup

Due to the increased technical skill and equipment needs, collection of specimens other than **sputum** from the lower respiratory tract may be limited to patients presenting with more severe disease, including persons admitted to the hospital and/or fatal cases.

a. Optimal timing. These specimens may be obtained at any time during the clinical course, but ideally prior to initiation of antimicrobial therapy.
b. Specimen types. Acceptable lower respiratory tract specimens include sputum, tracheal aspirate, BAL fluid, pleural fluid. Specimens with less chance for upper airway contamination (i.e., BAL fluid, pleural fluid) are preferred. c. Specimen collection**. 1. BAL fluid, tracheal aspirate, pleural fluid** Label each specimen container with the patient's name, ID number, the specimen type, and the date the specimen was collected.
c. 2**. Sputum** Educate the patient about the difference between sputum and oral secretions. Have the patient rinse the mouth with water and then expectorate deep cough sputum directly into a sterile screwcap collection cup or sterile dry container

Store respiratory specimens at 2-8°C for up to 72 hours after collection. If a delay in testing or shipping is expected, store specimens at -70°C or below.

Viral Transport Media

Sterile Hanks Balanced Salt Solution (HBSS) 1X with calcium and magnesium ions

Sterile, heat-inactivated fetal bovine serum (FBS)

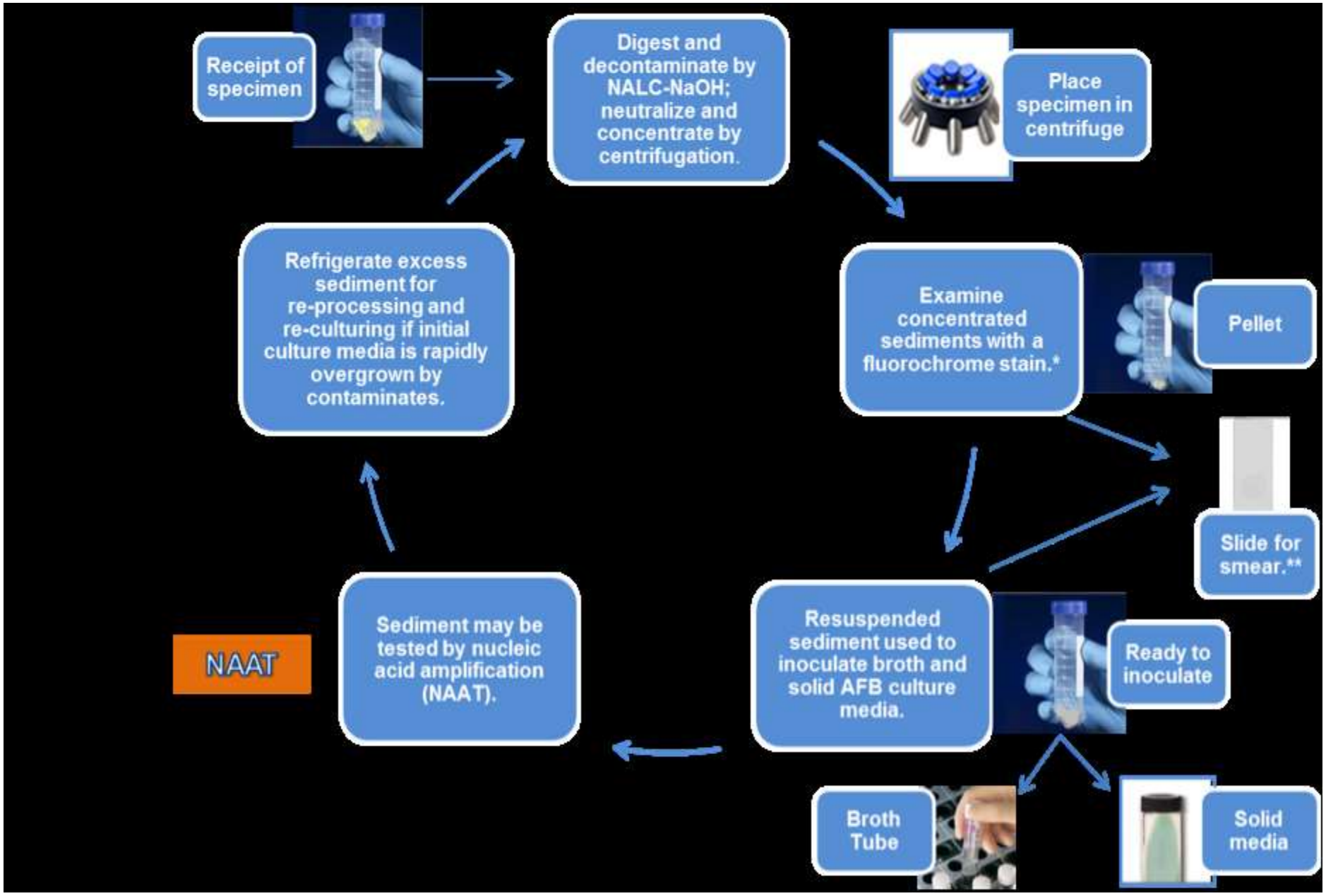
Gentamicin sulfate (50mg/mL) (or similar antibiotic at an appropriate concentration to prevent bacterial contamination and growth) Amphotericin B (250µg/mL).

Specimen Collection Methods for Pulmonary TB Disease

Coughing–Coughing is the most commonly used method of sputum collection. Coughing should be supervised to ensure that sputum is collected correctly. A health-care worker wearing the recommended personal protective equipment should coach and directly supervise the patient when sputum is collected (Figure 4.6). Patients should be informed that sputum is the material brought up from the lungs, and that mucus from the nose or throat and saliva are not good specimens. Unsupervised patients are less likely to provide an adequate specimen, especially the first time.

Sputum Induction–For patients unable to cough up sputum, deep sputum-producing coughing may be induced by inhalation of an aerosol of warm, sterile, hypertonic saline (3%– 5%). Because induced sputum is very watery and resembles saliva, it should be labeled “induced” to ensure that the laboratory staff workers do not discard it

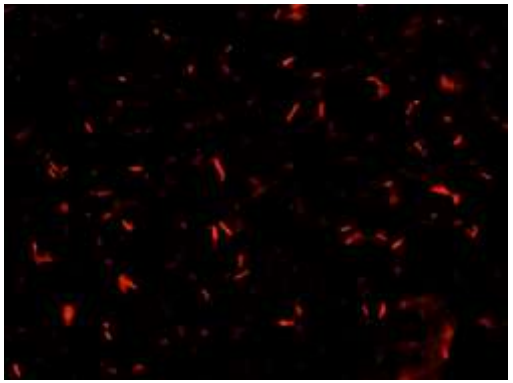
3,000 xg **TB Laboratory Services** N-acetyl-L-cysteine



Procedure for Fluorescent Staining

1.Place slides on staining rack so they are at least 1 cm apart, and flood with auramine or auramine/rhodamine stain and let stand for 20 min.
2. Rinse the stain away with distilled water and tilt slide to drain. Water must be chlorine free.
3. Flood the slide with 0.5% acid alcohol and let stand for 2 min.
4. Wash off the acid alcohol with distilled water.
5. Flood slides with 0.5% potassium permanganate for 1-2 min. Do not allow potassium permanganate to act over 2 min, or it might quench the fluorescence of acid-fast bacilli.
6. Wash off the stain with distilled water.
7. Allow slides to air dry in the slide rack. DO NOT BLOT!
8. Protect smears from light and examine immediately using the fluorescent microscope. If unable to read right away, place slides in covered box.

auramine O stain, organisms fluoresce **bright yellow**, non-specific debris stains **pale yellow**, and the background is almost black. With auramine/ rhodamine stain, organisms fl uoresce yellow-red in an almost black background.



LIQUID CULTURE – MYCOBACTERIA GROWTH INDICATOR TUBE (MGIT)

Sputum specimens are processed (Section 7: Processing Sputum for Smear Microscopy and Qualitative Culture), and inoculated into 7ml MGIT tubes, which are supplemented with OADC (Growth Supplement) and a cocktail of antibiotics (PANTA). The MGIT tubes contain a fluorescent compound embedded in the base of the tube, which is sensitive to the presence of oxygen dissolved in the broth. Initially, the large amount of oxygen quenches the emissions from the compound and little fluorescence is detected. Bacteria present in the concentrated sputum specimens metabolize oxygen in the culture medium, allowing the fluorescence to be detected (365 nm). Blood samples are not suitable for the MGIT system.

The medium components are substances essential for the rapid growth of mycobacteria. **Oleic acid** is utilized by tubercle bacilli and plays an important role in the metabolism of mycobacteria. **Albumin** acts as a protective agent by binding free fatty acids, which may be toxic to Mycobacterium species, thereby enhancing their recovery. **Dextrose** is an energy source. **Catalase** destroys toxic peroxides that may be present in the medium

SOLID CULTURE: LOWENSTEIN JENSEN (LJ) MEDIA

The purpose of this procedure is to isolate and semi-quantify growth of M. tuberculosis on LJ medium. Slants will be inoculated with decontaminated and concentrated sputum specimens Many different solid media are available for cultivating mycobacteria. Most are variations of egg-potato base or albumin-agar base media. There is no general consensus on which medium is best for routine isolation. The advantages of egg-based media such as LJ are: 1) it is easy and economical to prepare, 2) it is associated with lower contamination rates, and 3) isolated colonies with characteristic colony morphology for MTB can be observed. Disadvantages are: 1) when contamination occurs, it often involves total surface of medium, 2) if contamination is slight, it is not evident when mycobacterial growth is confl uent, and 3) drug susceptibility tests are more diffi cult to perform using egg-based media because some drugs must be adjusted to account for their loss by heating or by interaction with certain components of the egg.

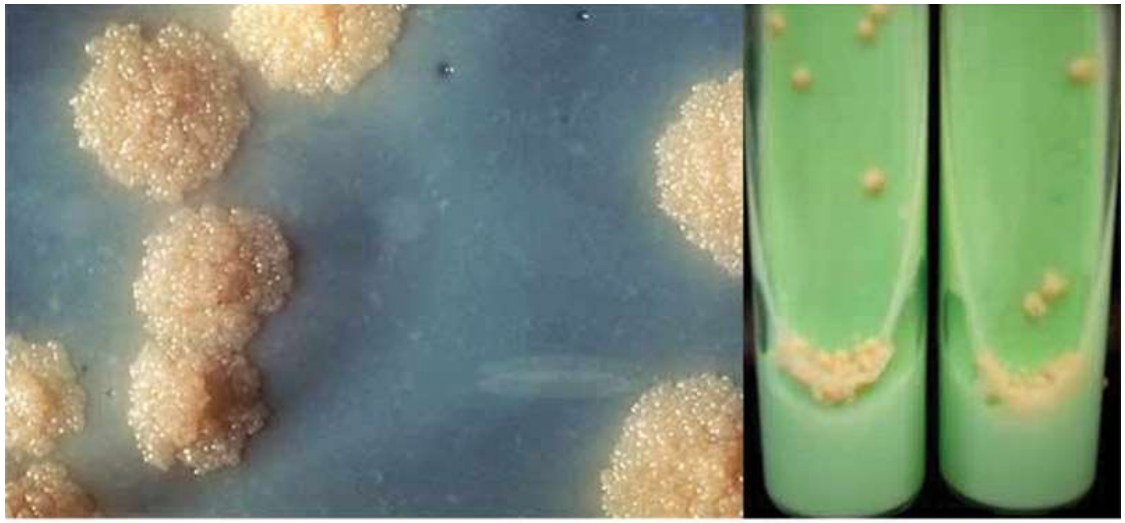


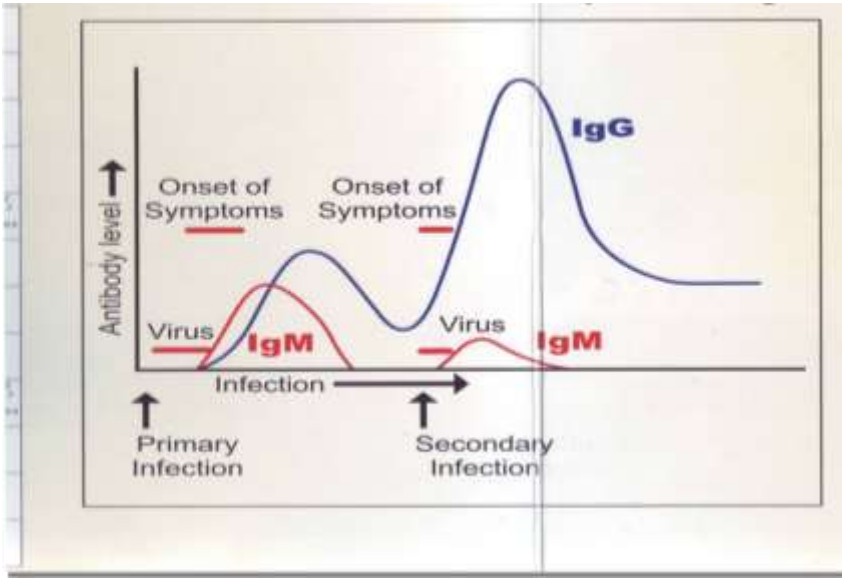
Fig: Cultural Characteristics of *Mycobacterium tuberculosis*

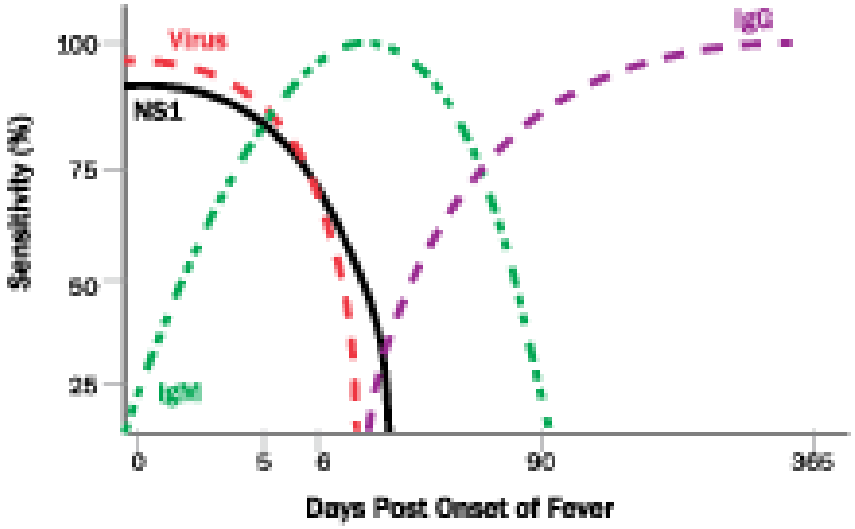
- 4 days to 12 weeks of incubation require

Diagnostic test

Immunological test- ELISA

Immuno response to JE infection



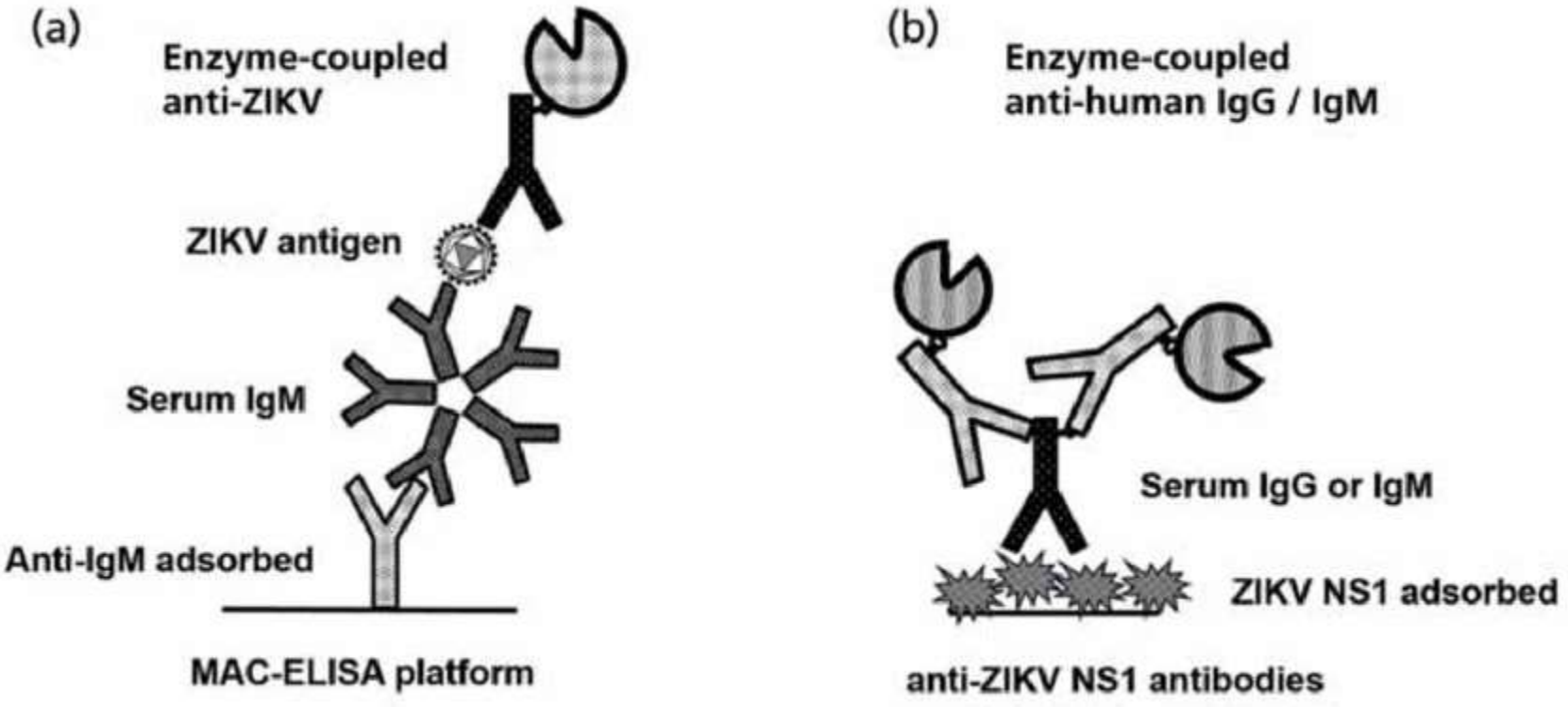


1.NS1 is detectable during the acute phase of dengue virus infections. NS1 tests can be as sensitive as molecular tests during the first 0-7 days of symptoms. After day 7, NS1 tests are not recommended.

2. Dengue virus-specific IgM and neutralizing antibodies typically develop toward the end of the first week of illness. IgM levels are variable, but generally are positive starting 4-5 days after onset of symptoms and continuing for approximately 12 weeks post symptom onset, but may persist longer.

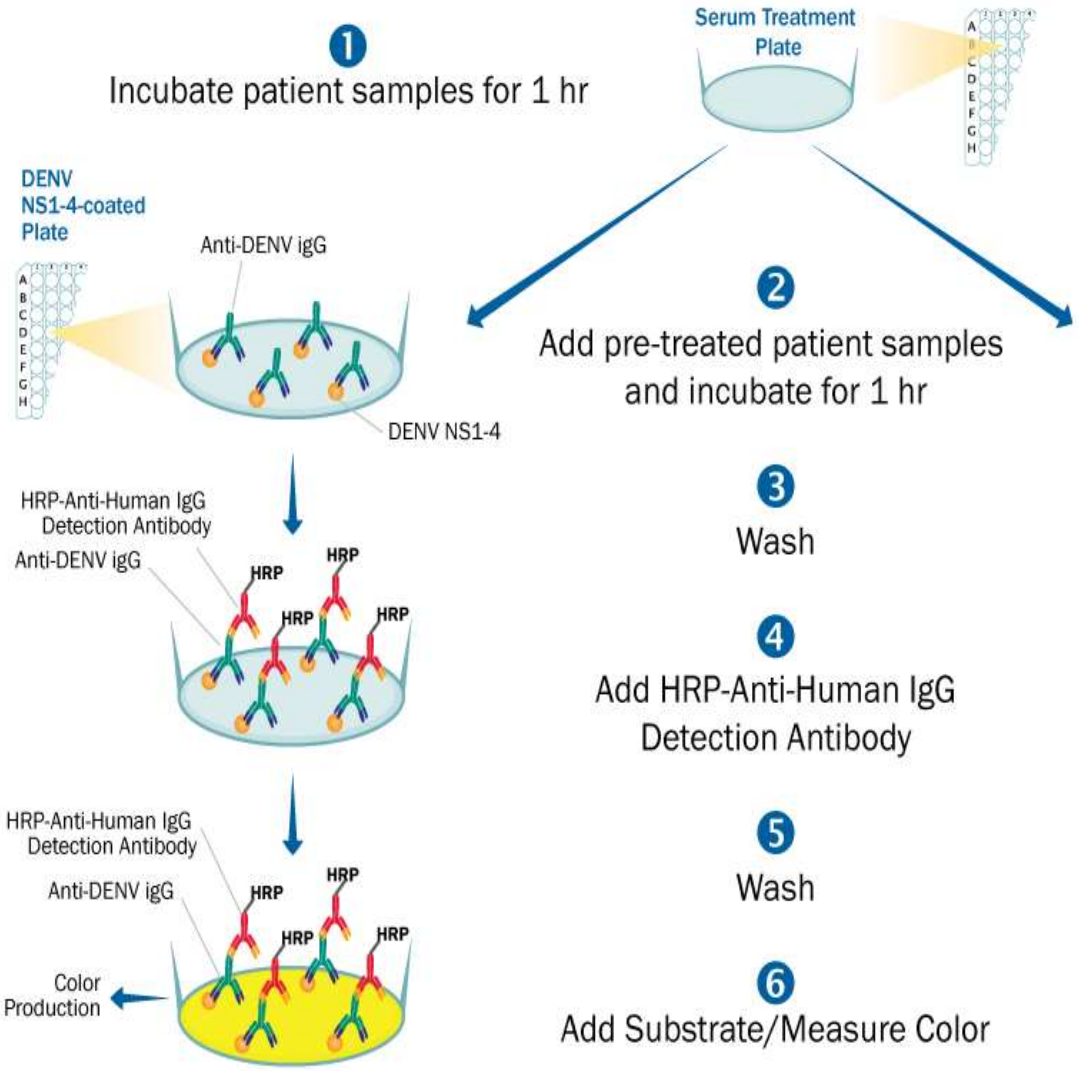
Enzyme-linked immunosorbent assay (ELISA) uses antibodies or antigen and other different immunogens. It is very easy and very sensitive, can detect pico gram level of sample & relies on monoclonal antibody. The MAC-ELISA test for J.E/dengue diagnosis involves the exposure of IgM antibodies in human serum to anti-human IgM that was previously bound to the solid phase. This test is to aid in the diagnosis of human exposure to the Japanese Encephalitis Virus (JEV).

MAC-ELISA has become widely used in the past few years. It is a simple, rapid test that requires very little sophisticated equipment. MAC-ELISA is based on detecting the JE/dengue-specific IgM antibodies in the test serum by capturing them out of solution using anti-human IgM that was previously bound to the solid phase. If the IgM antibody from the patient's serum is JE/dengue specific , it will bind the JE/dengue derived recombinant antigen that is added in subsequent step and can be detected by addition of an enzyme labelled anti-JE/dengue monoclonal antibody. An substrate is added to give a colour reaction .The JE Detect MAC-ELISA consists of one enzymatically amplified "two-step" sandwich-type immunoassay.



TMB (**3,3',5,5'-Tetramethylbenzidine)** substrate is used in ELISA procedures, which utilize horseradish peroxidase conjugates. TMB substrate develops a soluble blue reaction product that can be read at 370 or 655 nm. The reaction is stopped with acid, forming a yellow reaction product which enables accurate intensity measurement at 450 nm.

NS-1 ANTIGEN DETECTION BY ELISA



Reverse transcription polymerase chain reaction (**RT-PCR**), a process of "amplification", is a variant of polymerase chain reaction (PCR), a laboratory technique commonly used in molecular biology to generate many copies of a DNA fragment. In RT-PCR, however, RNA strand is first reverse transcribed into its DNA complement (*complementary DNA*, or *cDNA*) using the enzyme reverse transcriptase, and the resulting cDNA is amplified using traditional or real-time PCR. Reverse transcription PCR is not to be confused with real-time polymerase chain reaction (Q-PCR), which is also sometimes (incorrectly) abbreviated as RT-PCR.

Nucleic Acid Amplification Test (NAAT)

Amplification

Denaturation

High temperature incubation is used to “melt” double- stranded DNA into single strands and loosen secondary structure in single-stranded DNA. The highest temperature that the DNA polymerase can withstand is typically used (usually 95°C). The denaturation time can be increased if template GC content is high.

Annealing

During annealing, complementary sequences have an opportunity to hybridize, so an appropriate temperature is used that is based on the calculated melting temperature (Tm) of the primers(5°C below the Tm of the primer).

Extension

At 70-72°C, the activity of the DNA polymerase is optimal, and primer extension occurs at rates of up to 100 bases per second. When an amplicon in real-time PCR is small, this step is often combined with the annealing step using 60°C as the temperature.

Initial Denaturation	Final Denaturation	Anneling	Extension	Final Extension
95°C	95°C	54°C	72°C	72°C
2min	30 sec	1 min	1min	5 min

4.8 Reverse transcription polymerase chain reaction (RT-PCR)

In RT-PCR, The RNA population is converted to cDNA by reverse transcription (RT), and then the cDNA is amplified by the polymerase chain reaction .The cDNA amplification step provides opportunities to further study the original RNA species, even when they are limited in amount or expressed in low abundance. Common applications of RT-PCR include detection of expressed genes, examination of transcript variants, and generation of cDNA templates for cloning and sequencing.

1 a. **RNA**
RNA consist of Start codon AUG and ends with poly A tail

b. **Oligo dT Primer**
Oligo dT Primer is binding to RNA poly A tail

c. **Reverse Transcriptase and dNTPs**

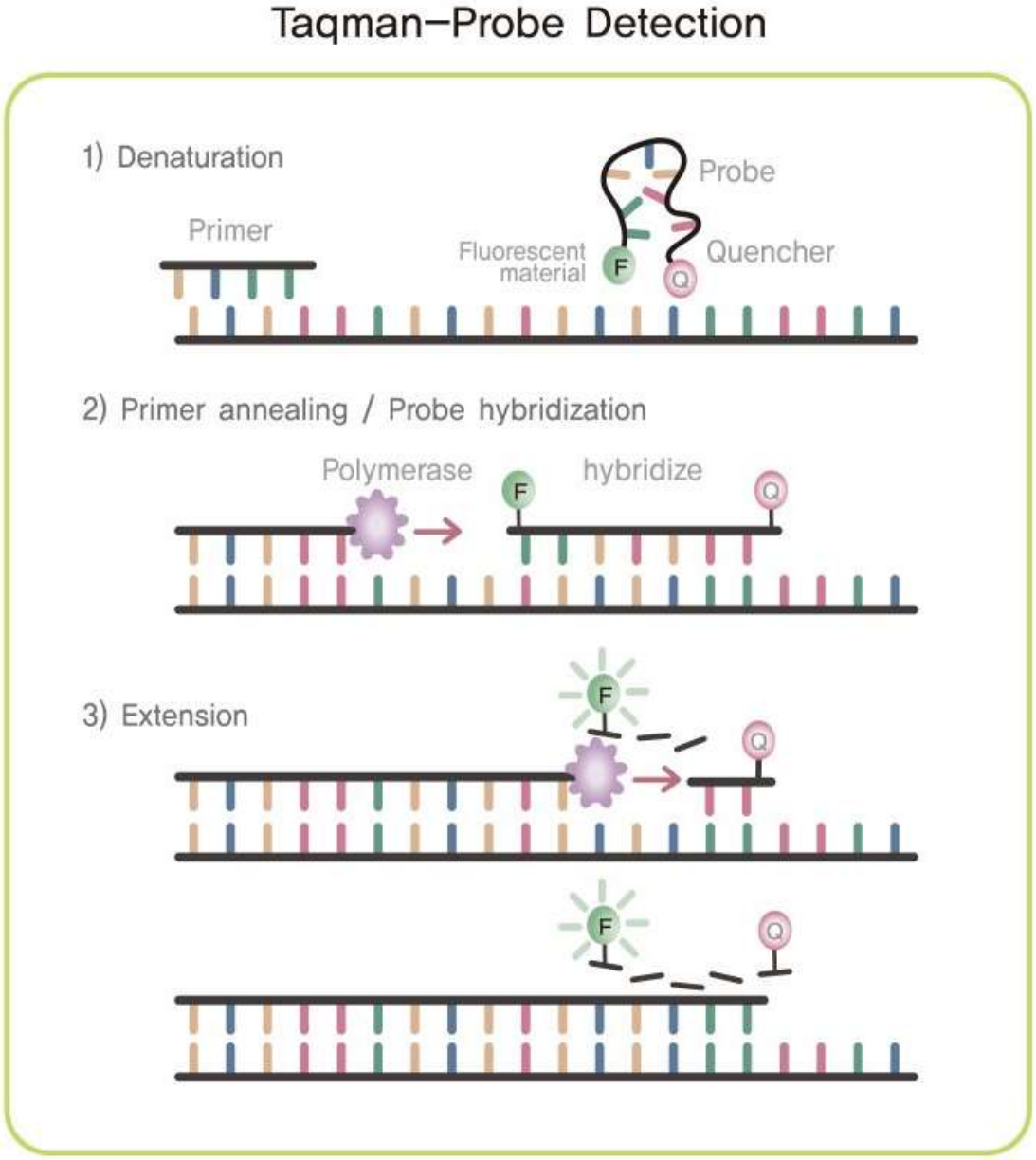
d. **Reverse Transcriptase** is an enzymes binds to oligo dT primer and synthesises the cDNA by adding dNTPs

e. **RNA hybrid formation :**
First - strand cDNA synthesis

f. **complimentary DNA**

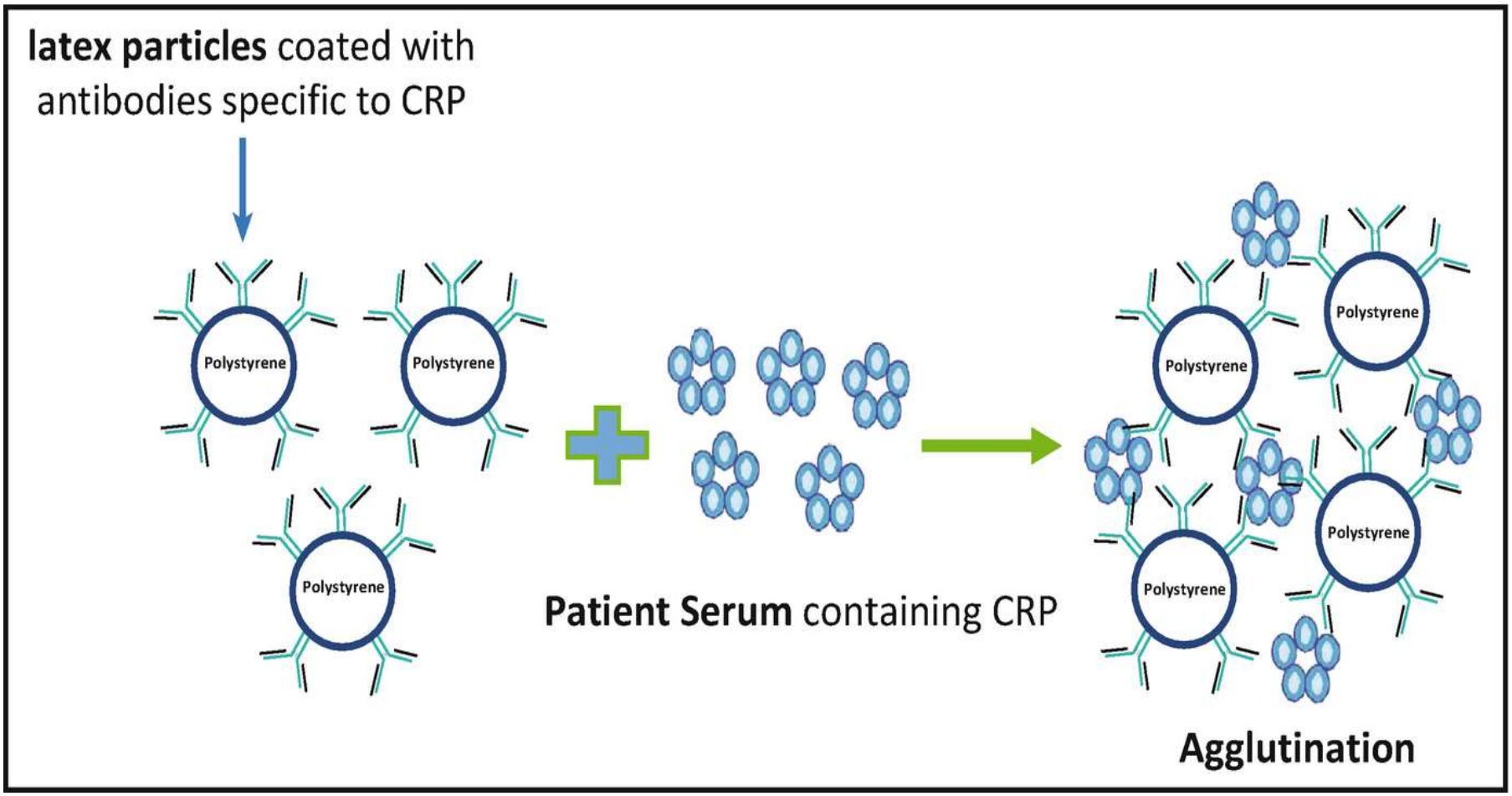
2 a. **Amplification of cDNA** with Specific Primers and Taq Polymerase

Q-PCR


Taqman–Probe Detection
1) Denaturation
Primer
Probe
Fluorescent material
F
Quencher
Q
2) Primer annealing / Probe hybridization
Polymerase
hybridize
F
Q
3) Extension
F
Q
F
Q

LATEX AGGLUTINATION TEST

A group of passive agglutination tests carried out by coating either antigen or antibody on an artificial carrier particle, called latex bead, are known as latex agglutination test (LAT). If antigen and antibodies are homologous, then clumping of beads occur. LAT can be used either to detect antibody or antigen; the latter is sometimes known as Reverse Passive Latex Agglutination Test. Latex beads are polystyrene latex particles; 0.8- 1 µm in diameter. The number of antibody or antigen molecules bound to each latex particle is large, resulting in a high number of exposed potential binding sites. Antigen or antibody present in a specimen binds to the combining sites of the corresponding antibody/antigen exposed on the surfaces of the latex beads, forming cross-linked aggregates of latex beads and antigen/antibody., which are visible as clumps.


latex particles coated with antibodies specific to CRP
Polystyrene
Polystyrene
Polystyrene
Patient Serum containing CRP
Polystyrene
Polystyrene
Polystyrene
Polystyrene
Agglutination

Application

Latex tests are very popular in clinical laboratories for detecting antigen to *Cryptococcus neoformans* in cerebrospinal fluid or serum. It is also used for detection of capsular antigens of Pneumococcus, *Haemophilus influenzae* and Meningococcus.

To confirm the presence of beta-hemolytic *Streptococcus* from culture plates.

Latex tests are continually being developed for a variety of organisms such as for the detection of *Clostridium difficile* toxins A and B, rotavirus, and *Escherichia coli* 0157:H7 from suspect colonies of *E coli*.

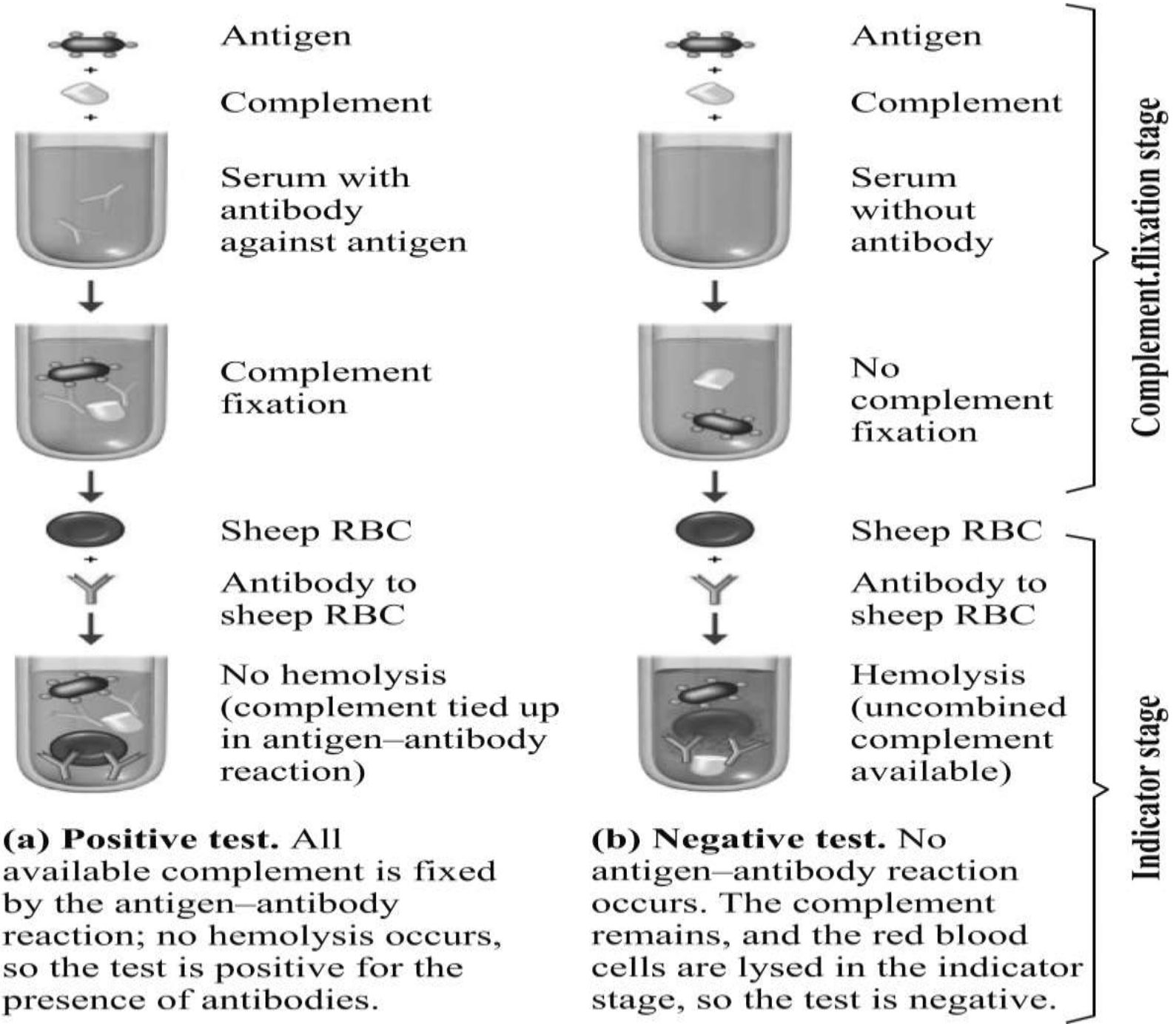
Latex agglutination test using latex particles coated with anti-CRP antibodies is the most widely used method employed worldwide for detection of C-reactive protein. Detection limit of CRP by latex agglutination test is 0.6 mg/dl.

Latex Agglutination Test (LAT) for Antibody Detection is used for detection of ASO (antistreptolysin O antibody).

COMPLEMENT FIXATION TEST consists of two steps:

Step 1: a known antigen and inactivated patient's serum are incubated with a standardized, limited amount of complement. If the serum contains specific, complement activating antibody the complement will be activated or fixed by the antigen-antibody complex. However, if there is no antibody in the patient's serum, there will be no formation of antigen-antibody complex, and therefore complement will not be fixed. But will remain free

Step 2: The second step detects whether complement has been utilized in the first step or not. This is done by adding the indicator system. If the complement is fixed in the first step owing to the presence of antibody there will be no complement left to fix to the indicator system. However, if there is antibody in the patient's serum, there will be no antigen-antibody complex, and therefore, complement will be present free or unfixed in the mixture. This unfixed complement will now react with the antibody- coated sheep red blood cells to bring about their lysis. Thus, no lysis of sheep red blood cells (positive CFT) indicates the presence of antibody in the presence of antibody in the test serum, while lysis of sheep red blood cells (Negative CFT) indicates the absence of antibody in the serum.



(a) Positive test. All available complement is fixed by the antigen–antibody reaction; no hemolysis occurs, so the test is positive for the presence of antibodies.

(b) Negative test. No antigen–antibody reaction occurs. The complement remains, and the red blood cells are lysed in the indicator stage, so the test is negative.

AGGLUTINATION INHIBITION TEST

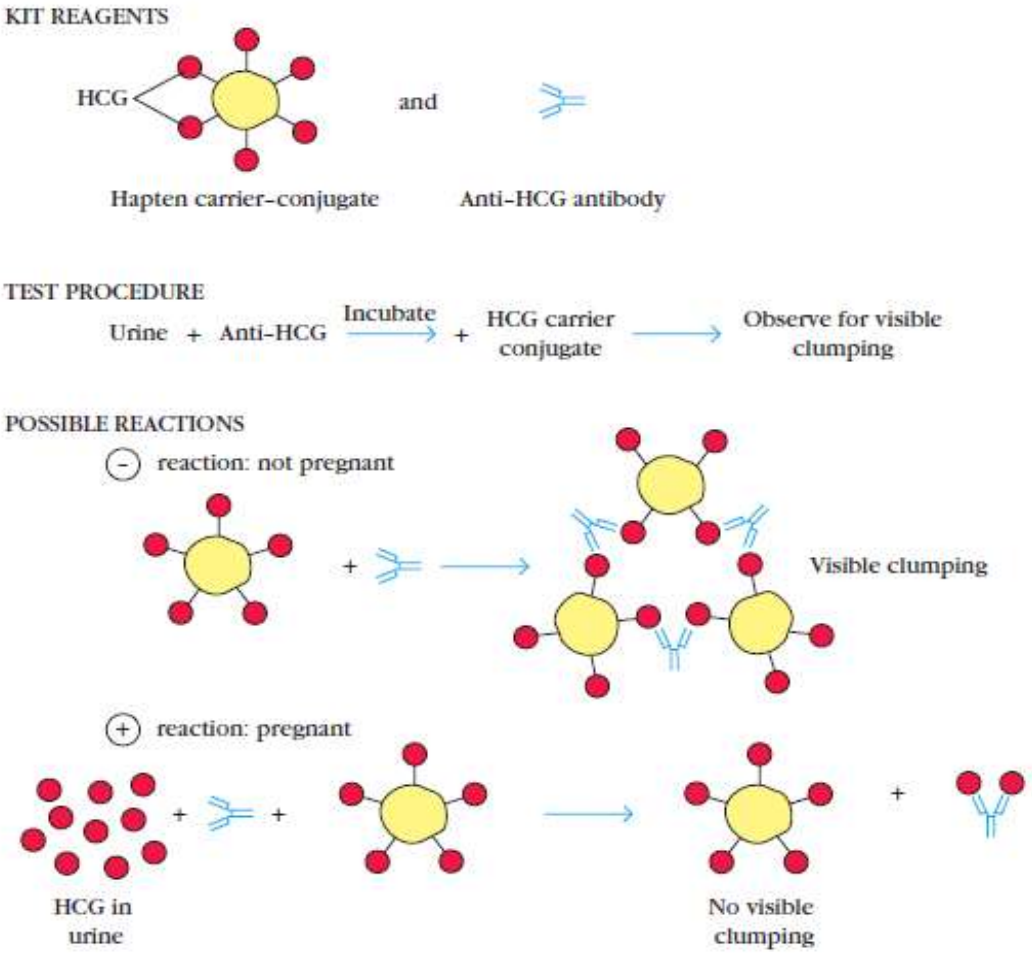
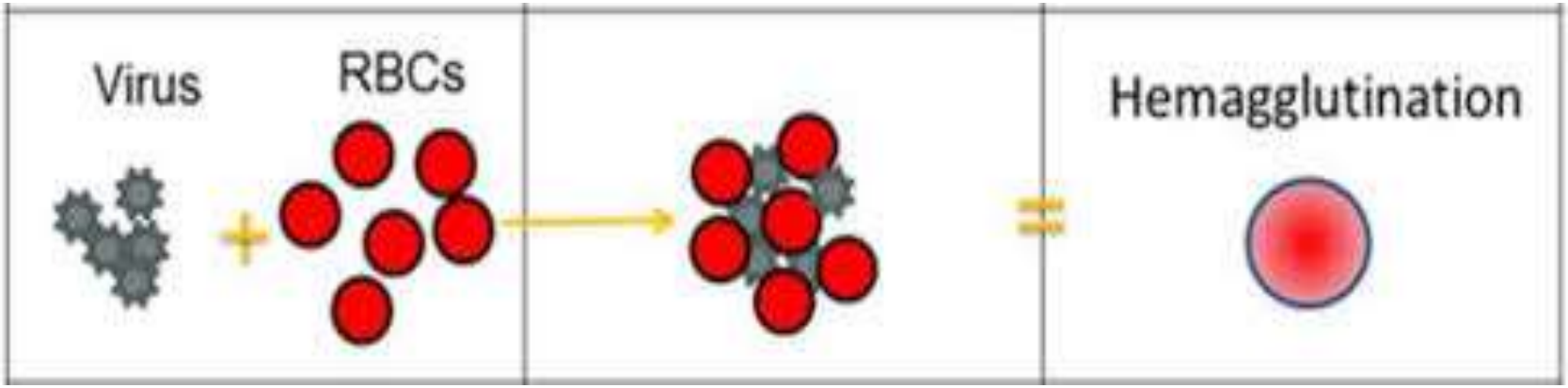
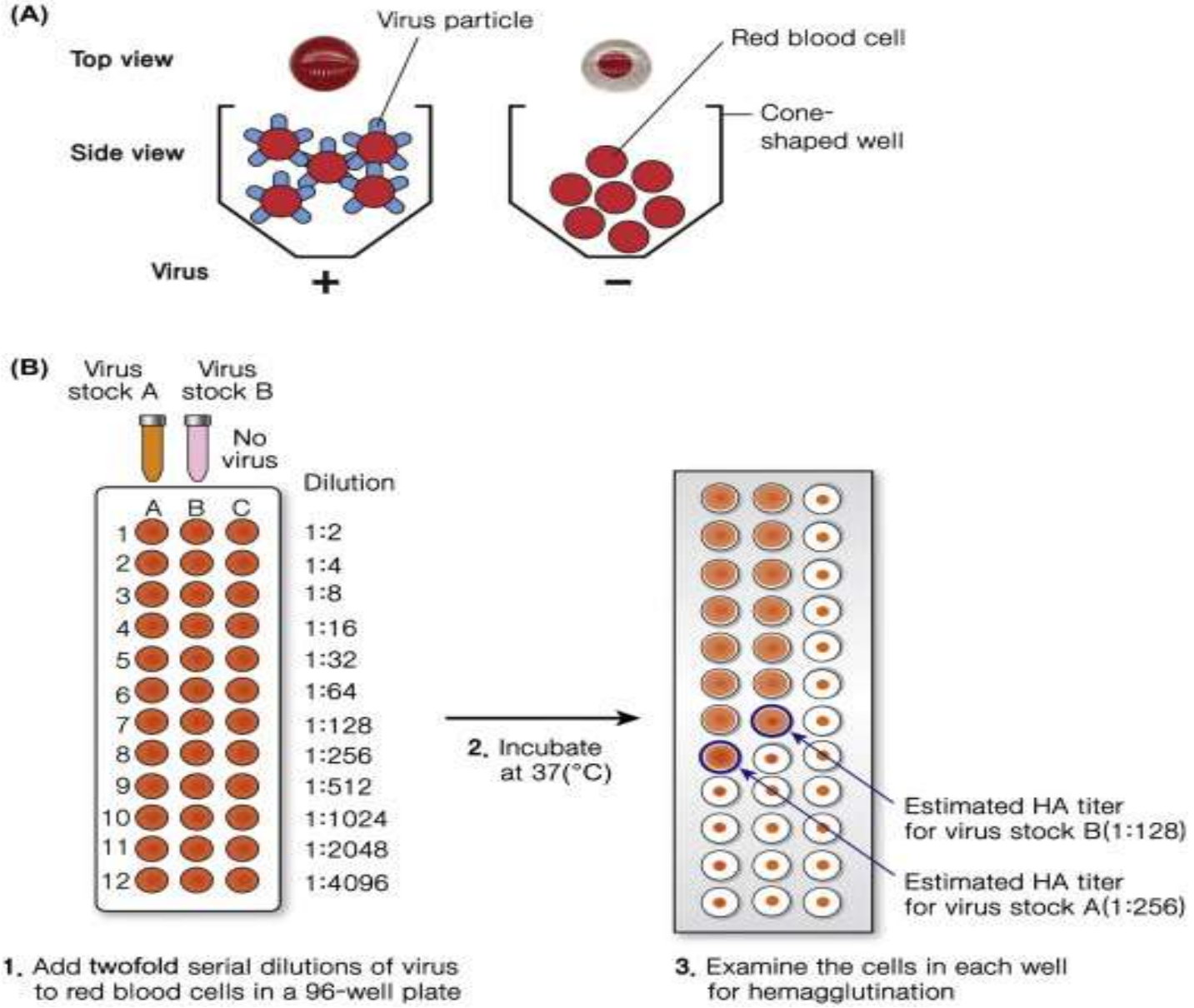


FIGURE 6-8 The original home pregnancy test kit employed hapten inhibition to determine the presence or absence of human chorionic gonadotropin (HCG). The original test kits used the presence or absence of visible clumping to determine whether HCG was present. If a woman was not pregnant, her urine would not contain HCG; in this case, the anti-HCG antibodies and HCG-carrier conjugate in the kit would react, producing visible clumping. If a woman was pregnant, the HCG in her urine would bind to the anti-HCG antibodies, thus inhibiting the subsequent binding of the antibody to the HCG-carrier conjugate. Because of this inhibition, no visible clumping occurred if a woman was pregnant. The kits currently on the market use ELISA-based assays (see Figure 6-10).

Hemagglutinin Inhibition Assay (HI Test)

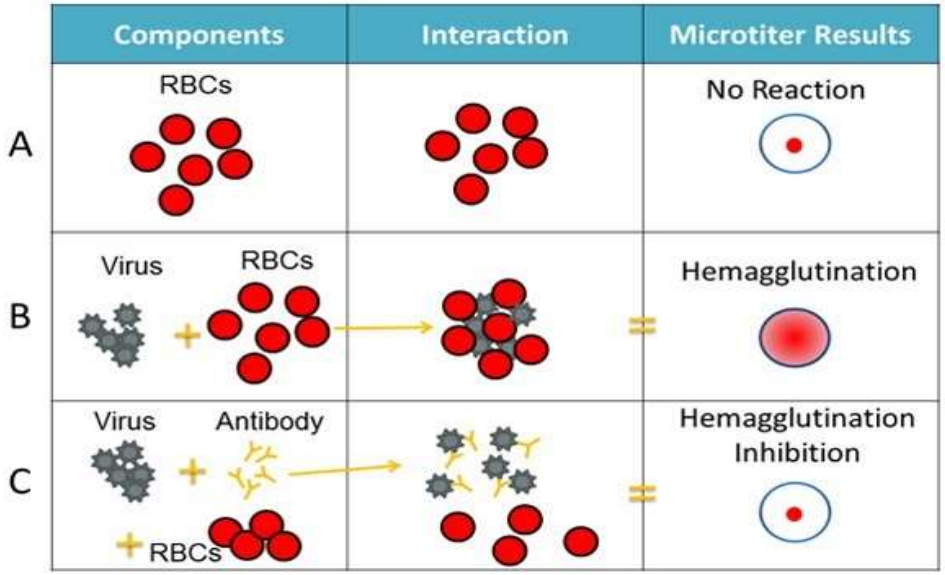
Influenza virus particles have an envelope protein called the hemagglutinin, or HA, which binds to sialic acid receptors on cells.The virus will also bind to erythrocytes (red blood cells), causing the formation of a lattice. This property is called Haemagglutination, and is the basis of a rapid assay to determine levels of influenza virus present in a sample. To conduct the assay, two-fold serial dilutions of a virus or virus containing sample are prepared, mixed with a specific amount of red blood cells, and added to the wells of a plastic tray.




(A)
Top view
Side view
Virus
Virus particle
Red blood cell
Cone-shaped well
+
−
(B)
Virus stock A
Virus stock B
No virus
Dilution
A B C
1 1:2
2 1:4
3 1:8
4 1:16
5 1:32
6 1:64
7 1:128
8 1:256
9 1:512
10 1:1024
11 1:2048
12 1:4096
2. Incubate at 37(°C)
Estimated HA titer for virus stock B(1:128)
Estimated HA titer for virus stock A(1:256)
1. Add twofold serial dilutions of virus to red blood cells in a 96-well plate
3. Examine the cells in each well for hemagglutination

Hemagglutinin Inhibition Assay (HI Test)

Note that some other viruses and some bacteria will also agglutinate red blood cells. To demonstrate that the haemagglutinating agent is influenza disease virus, it is necessary to use a specific virus antiserum to inhibit the haemagglutinating activity. The HA assay can be easily modified to determine the level of antibodies to influenza virus present in serum samples. The basis of the HI assay is that antibodies to influenza virus will prevent attachment of the virus to red blood cells. Therefore haemagglutination is inhibited when antibodies are present. This assay may be modified to include the addition of an antiserum. By using a standard amount of virus, a standard amount of blood cells, and serially diluting the antiserum, one can identify the concentration of the antiserum (the greatest dilution which inhibits haemagglutination).



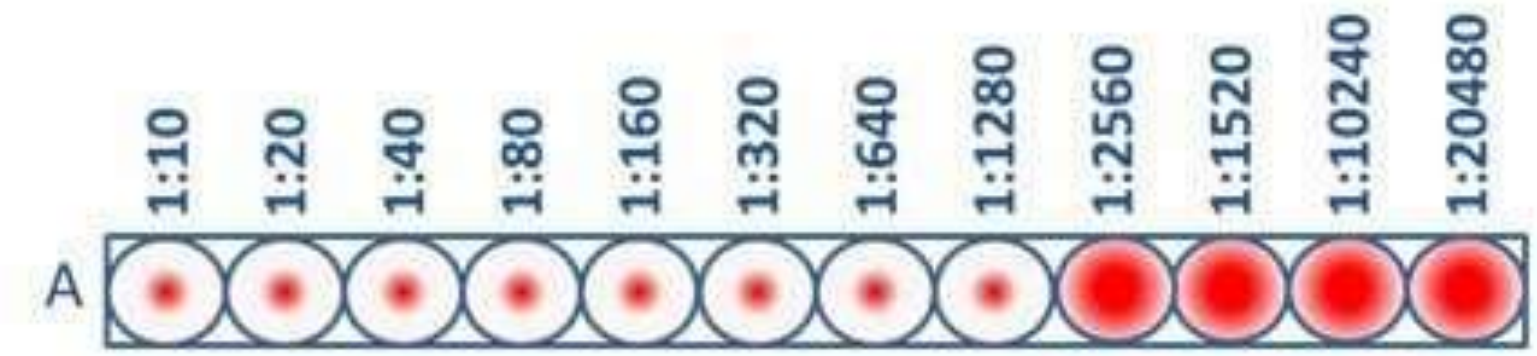
Hemagglutinin Inhibition Assay (HI Test)

Hemagglutination occurs when measles viruses and red blood cells are mixed But, if the serum of a person infected with **measles virus** is mixed with RBC and measles virus, there won't be any agglutination of RBC. This phenomenon is known as **hemagglutination inhibition**. This arises because antibodies present in the serum of that infected person reacts with the measles viruses and neutralize them (positive result).
If the patient's serum do not contain antibodies against surface proteins of test virus, there will be presence of hemagglutination as surface molecules are free to hemaaglutinate RBCs (negative result).

HAI Titer: The highest dilution of serum (Ab) that prevents hemagglutination is called the HAI titer of the serum.

If the serum contains no antibodies that react with measles virus, then hemagglutination will be observed in all wells. Likewise, if antibodies to the measles virus are present, hemagglutination will not be observed until the antibodies are sufficiently diluted.

The HAI test may be complicated by the presence of non-specific inhibitors of viral haemagglutination and naturally occurring agglutinins of the erythrocytes. Therefore, the sera should be treated before use or false positive or negative results may arise.



This virus sample has an HAI titer of 1280, which means that the greatest dilution of antibody that still blocked hemagglutination from occurring was at 1280 dilution. At this dilution, the antibodies were still capable of recognizing and binding to the antigens on the virus.

orthomyxoviruses (influenza), paramyxoviruses (measles, mumps), mononucleosis, abroviruses-togaviruses (including rubella), flaviviruses, and bunyaviruses. Although influenza viruses can be detected by hemadsorption test, typing of the isolate is done most efficiently by hemagglutination inhibition (HAI).