

Practical manual
PLANT VIROLOGY

APP 502 3(2+1)



For

M. Sc. (Ag) Plant Pathology



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**Department of Plant Pathology
College of Agriculture**

**Rani Lakshmi Bai Central Agricultural University
Jhansi, Uttar Pradesh-284003**

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Practical No: 1

Symptoms of virus diseases in plants

Types of Symptoms

Growth Reduction/dwarfing: Growth reduction is the most important symptom of virus infection which reduces yield and quality of produce and often causes partial or total loss of a crop. It results from dwarfing when the plants remain below the ordinary size of its kind and stunting if the growth and development of plant is arrested.

Example: *Barley yellow dwarf virus*, *Peanut dwarf virus* etc.

Mosaic: Dark and light colouration intermingled to each other (no distinct boundary). Mosaic may be green mosaic, pale yellow mosaic, yellow mosaic or golden mosaic. Ex. *Tobacco mosaic virus*.

Vein clearing: In some viral diseases veins become light and translucent. Ex: *Citrus tristeza virus*. **Vein yellowing:** In some viral diseases veins become yellow and a netting appearance of yellow vein symptoms observed. Ex: *Bhendi yellow vein mosaic virus*

Vein banding: Colouration developed around veins and vein becomes thick. Ex: Green vein banding in *Papaya ring spot virus* on watermelon, yellow vein banding in *Grape vine fan leaf virus* in grapes. **Mottling:** In case of mottling light and dark colouration have a distinct boundary.

Yellowing or chlorosis: The term, chlorosis, is used to describe reduced amount of chlorophyll resulting in light color. If the leaf is light green it is referred to as "mild chlorosis" whereas "severe chlorosis" or "yellowing" is used to describe a total depletion of chlorophyll giving a yellow colour.

Rugose: Means "rough leaves". This term covers etch (engraved), crinkling (leaves looking wrinkled) and leaf puckering (blister-like irregularities formed on the leaf). Ex: Etching of tobacco caused *Tobacco etch virus*, Leaf crinkling in urdbean Leaf puckering in cucumber caused by begomovirus

Leaf roll and leaf curl: Leaf rolling is used to describe folding of leaves along their mid axes resulting in a more or less tube-like structure. Ex: *Potato leaf roll virus* (PLRV). When the folding is more irregular or does not result in a tube-like structure it is usually referred to as leaf curling. Ex: *Tomato leaf curl virus* (ToLCV)

Leaf distortion: means that the growth and development of the leaf has been disturbed resulting in changes ranging from small deviations from normal leaf shape to severe deformations.

Epinasty: which is rapid growth of infected organ, as petiole and leaf blade, leading to downward bending or curling of entire part as seen in peas. **Leaf enation:** Abnormal outgrowth of vascular tissue in leaves or in vein or on the stem.

Rosette: An abnormal condition in which the leaves form a radial cluster on the stem. Ex: *Groundnut rosette umbravirus*

Necrosis: Rapid local death is called "Necrosis" and is usually accompanied by blackening or browning, often characteristics of local lesions on inoculated leaves. It also results from degeneration of phloem in vascular bundles, called phloem necrosis. Similarly, vein necrosis, tip necrosis, bud necrosis occur when these parts rapidly react to virus infections. If necrosis proceeds further, whole plant may die rapidly.

Tobacco rattle virus and *Potato mop-top virus* induce internal necrosis of potato tubers which starts from the sites of infection by the viruses.

Stem pitting is localized under development and shallow necrosis of certain tissue resulting from failure of cambium tissue. It appears as numerous sparsely elongated pits in tiny furrows and seen when bark is removed (as in *citrus tristeza virus*).

Stem grooving occurs under similar conditions of phloem necrosis as big deep, ditches or pits in furrows (*Apple stem grooving virus*).

Ring Spotting: On infected leaves, this symptom appears in localized spots. These spots consist of various types of chlorosis and necrosis. The spots may be circular chlorotic areas and are called chlorotic ring spots. In other cases, the necrosis may appear in rings alternating with normal green areas called as necrotic ring spot. Ex: *Tobacco ring spot disease*.



Ring spot



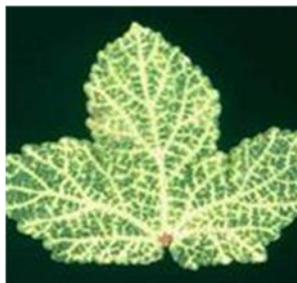
Yellow mosaic virus



Maize streak



Leaf curl



Yellow vein mosaic virus



Mottling



Crinkling



Stunting

Activities: Identification virus symptoms on infected Plants

Material: Polythene bags, Hand lens, Knife, razor blade or scissors, Marker, Labels, Key to symptoms

Procedure:

- Visit a field of different crops and collect infected/suspected plants or uproot the plant keep them in polythene bag and bring to laboratory.
- The samples can be stored in a refrigerator at 4°C until processed.
- Compare and study the symptoms carefully and describe their identity in a tabular form along with their severity (very severe, severe, moderate and mild) and insect vectors associated, if any.

Activities:

1. Collect the different virus infected samples from field and describe their symptoms

Practical No: 2

Bioassay of virus cultures on indicator plants and host differentials

Introduction: Bioassays or biological indexing is one of the earliest active virus tests developed to detect plant viruses. The procedure is based on the ability of certain plants, called indicator plants, to produce symptoms when inoculated with viruses by mechanical inoculation. Indicator plants are chosen for their ability to display relatively distinct disease symptoms when infected.

Objectives: To study the bioassay of virus culture on indicator plants and on the other host for host range studies and for identification of a particular virus.

Materials required: Indicator plants: *Chenopodium quinoa*, *C. amaranticolor*, *Gomphrena globosa*, *Vigna unguiculata*, *Nicotiana benthamiana*, *N. glutinosa* etc. infected planting materials, 0.1 M phosphate buffer, Pestle and mortar, Carborandum, Celite, Mercaptoethanol, Cotton, Water, Blotting Paper

Procedure:

- Infected plants showing symptoms is collected from field, wash the infected leaves in tap water to remove the dust particles adhering to them and dried between the folds of blotting paper.
- The leaves is then macerated in chilled mortar and pestle using potassium phosphate buffer (pH 7.0, 0.05M) at the rate of 1ml/gm of leaf tissue.
- The resultant pulp is squeezed through absorbent cotton and the extract thus obtained is used as standard inoculum.
- To the standard inoculum add celite (600 mesh) at the rate of 0.025 g/ml of the extract and 0.02% mercaptoethanol.
- Apply the inoculum gently on the upper surface of the leaves of any indicator plants with a small piece of absorbent cotton wool.
- Wash the inoculated leaves 1-2 minutes after inoculation to remove the excess of inoculum with a fine jet of distilled water from a squeeze bottle and keep the plants under observation in the glass house.
- Observe the local lesion on indicator plants within 5-6 days and system symptoms on 15 days.
- Repeat the practical on different host to study the host range of particular virus.

Activities:

1. Inoculate the virus on indicator plants and on different host species
2. Study the nature of infection of virus and list out the local lesion and systemic host.

Practical No: 3

Mechanical Transmission of plant viruses through sap and study of disease development

Objectives: To study the mechanical inoculation of virus on Healthy plants.

Materials Required: Infected planting material, 0.1 M phosphate buffer, Pestle and mortar, Carborandum, Celite, Mercaptoethanol, Cotton, Water, Blotting Paper

Procedure

1. 0.1 M phosphate buffer

0.1 M K_2HPO_4 174.18 (MW)

0.1 m KH_2PO_4 136.09 (MW)

- Weigh K_2HPO_4 (8.7 g) and KH_2PO_4 (6.8 g).
- Dissolve each separately in 200 ml distilled water and make up the volume to 500 ml.
- Keep the buffer in refrigerator at 4° C.
- Grind infected leaves with 0.1 M phosphate buffer in chilled pestle and mortar.
- Add small quantity of celite into crude sap and mix properly.
- Wipe the leaves with crude sap using cotton swab.
- Allow it for 5 mins.
- Wash inoculated leaves with water.
- Keep the plants in shade.
- Next day transfer plants onto bench.

Activities: Inoculate the specific virus on specific host and maintain the inoculum for further studies

Practical No: 4

Transmission of plant viruses by vectors and study of disease development

Objectives: Determination of Relative ToLCV transmission efficiencies of *B. tabaci* on healthy seedlings

Materials required: White flies, Cotton plants, Insect proof wooden cages, Muslin cloth, Healthy tomato plants, Plastic bottles, Plastic tubes, aspirator

Procedure:

Maintenance of Whitefly culture

The pure culture of indigenous whiteflies *B. tabaci* is maintain on cotton and keep the plants in insect proof wooden cages. The whiteflies colony is maintained by introducing healthy cotton plants grown in polythene bags in to the cages.

Raising of healthy Tomato Seedlings

Raise the healthy seedlings of Tomato in polyethylene bags filled with soil and compost mixture in 2:1 proportion. Keep the plants in insect proof cages and use throughout the period of investigations.

Preparation of cages for acquisition access feeding by *B. tabaci*

Take the Plastic or Polyvinyl chloride (PVC) bottles tapering towards the narrow mouth and remove the bottom portion of the bottles with the help of a soldering rod and cover with muslin cloth. The narrow mouth of the bottle is cut upto few centimeters above the screw cap and plug with cotton to prevent flies escaping from the bottle during usage.

Preparation of cages for inoculation of plants

Take Plastic tubes remove the bottom of the tube and seal with a black muslin cloth to avoid accumulation of excess moisture inside the cage and also to provide aeration. A small hole (0.5 cm) is made in the middle portion of the tube to facilitate release of whiteflies. The open end of the tube is plugged with cotton after inserting young leaflets into the tube.

Collection of whiteflies

An aspirator made of a glass tube (30 x 0.5 x 40 cm) and a rubber tube of 40 cm length is used for the collection of whiteflies. The leaves colonized with healthy whiteflies are sucked in to the glass tube and gently blown in to the plastic tubes and used for transmission studies.

Transmission of ToLCV using *B. tabaci*

A ToLCV infected Tomato twig is inserted inside the whiteflies containing bottle. Allow the Whiteflies to feed for 24 hr and release at the rate of 10 whiteflies per seedling on the healthy Tomato seedlings covered with cylindrical plastic cages. After 48 hr inoculation access period (IAP), whiteflies are removed from the individual plants. The plants are sprayed with 0.03 per cent Imidachloprid and keep in insect-proof cages for symptom development.

Observations and result:

Practical No: 5

Transmission of plant viruses (cotton leaf curl virus/ mungbean yellow mosaic) by grafting and study of disease development

Introduction: Grafting is the union between cambial tissue of two hosts which are closely related i.e. belonging to the same genus or family. If either the scion or root stock is infected, virus can pass to the healthy one through grafting. The common grafting methods are: approach, wedge or top cleft, tongue and leaf grafting; the former two are easier and convenient Budding is also a common method to transmit viruses. All the virus and virus-like diseases of citrus are graft-transmissible. In approach grafting, slices of uniform size are made on the scion and stock, and united by direct cambial tissues. In wedge or top cleft grafting, wedge is made in the rootstock and scion of the same diameter or size is cut and inserted in it.

Objectives: To study the relative transmission ability of the viruses through grafting

Materials: Infected cotton plants or infected mungbean plants , Healthy plants of cotton, Healthy plants of mungbean, Sterilized knife or blade, distilled water, parafilm or polythene ribbons, plastic bags, beakers.

Procedure:

- Wash the diseased plants (scions) with sterilized/distilled water.
- On the potted test plants (stock), select branches of the same size and approximately same age of scion.
- Make a slanting cut of about 1 cm on the stock, using a sterilized knife,
- Give a wedge shaped cut on scion so that the exposed area on the scion matches the cut area on the stock.
- Insert the wedge shaped end of scion into the cut on stock.
- Immediately tie both by a parafilm or polythene ribbon over the union.
- Cover the plants completely with a plastic bag to maintain humidity.
- Maintain the plant at appropriate temperature (18-25 C) and humid conditions which enhance successful union between stock or scion.
- Include appropriate controls with healthy plants.

Observations and result:

Practical No: 6

Purification of Plant virus

Objectives: To study the Physico-chemical properties and Virus morphology

Materials required: infected leaf samples, Phosphate buffer, 1% beta mercaptoethanol, muslin cloth, n-butanol, PEG, NaCl

Procedure:

- Grind the leaf tissues with liquid nitrogen in a pestle and mortar to fine powder.
- Homogenize the powdered tissues in a blender using 300mL of 0.5M phosphate buffer containing 1% mercaptoethanol.
- Strain the extract through two layers of muslin cloth.
- Add n-butanol (8mL/100mL extract) and stir for 15mins in a cold room.
- Centrifuge the mixture at 10000rpm for 30 min at 4°C and collect the supernatant.
- Add PEG (600MW, 4g /100mL) to the supernatant and stir for 2 h at 4°C.
- Centrifuge at 10000rpm for 15 min, collect the pellet and resuspend in 80 mL of 0.01M phosphate buffer.
- Add NaCl (0.2M) and PEG (4g /100mL) and stir overnight at 4°C.
- Collect the pellet by centrifugation at 10000 rpm for 15 mins and resuspend the pellet in 2mL of 0.01 M phosphate buffer, vortex well and centrifuge at 10000rpm for 2min.
- Collect the milky white supernatant containing virus particles.
- Load the supernatant on a 10-40% sucrose gradient and centrifuge in a swing out bucket rotor at 34000rpm for 1h at 4°C.
- Collect light scattering fraction with the help of a Pasteur pipette from the tubes and examine under an electron microscope.

Observations result:

Practical No: 7

Electron microscopy of plant viruses

Introduction: Electron microscopy provides virologist the basic tool as light microscope does for the bacteriologist. Electron microscope reveals several characteristics and features of virus particles, purity of virus preparations, substructures and inclusion bodies in ultra thin sections of infected tissue.

A series of processes are involved in the electron microscopy such as specimen preparation; preparation of grids, film making, carbon coating, shadow casting, ultra thin sectioning and negative staining, which are beyond the scope of this manual. Handling of the specialized and costly equipment as EM requires highly skilled manpower. However, students must be well acquainted with some basic procedures, which are described briefly.

Objectives: To study the size and shape of different viruses through Electron microscopy

Material:

- Infected tissue (TMV, PVX, PVY) or virus preparations
- Copper grids with formavar/collodion membrane supported by carbon coating.
- Phosphotungstic acid (PTA) or Uranyl acetate (UAc).
- Filter paper.

Procedure:

- Place a drop of 2% PTA, pH 6.8 or UAc, pH 6.4 on an infected tissue and tease the tissue with a fine needle or edge of blade so that the cell exudate oozes out in the stain.
- Alternatively, place a drop of stain on a copper grid and freshly cut edge of virus infected tissue is touched. It is advisable to fix the tissue for 10-20 minutes in 10% formaldehyde to stabilize virus particles.
- Purified or partially purified virus preparation can be mixed with stain and taken on the grid. Drain fluid with a piece of filter paper and allow the grid to dry. Observe the grid in an EM and record detail of virus particles.
- Place the grid on a taped microscopic slide, filmed side up.
- Put one drop of specimen suspension on the grid with a micropipette or syringe. Allow virus particles to settle on the grid for 2-3 minutes.
- Blot dry with a piece of filter paper, and immediately stain the grid with one drop of 2% PTA stain, pH 6.8. Remove the excess fluid after 1-2 minutes with a piece of filter paper, and blot dry and examine under an EM.

Observations and results:

Practical No.8

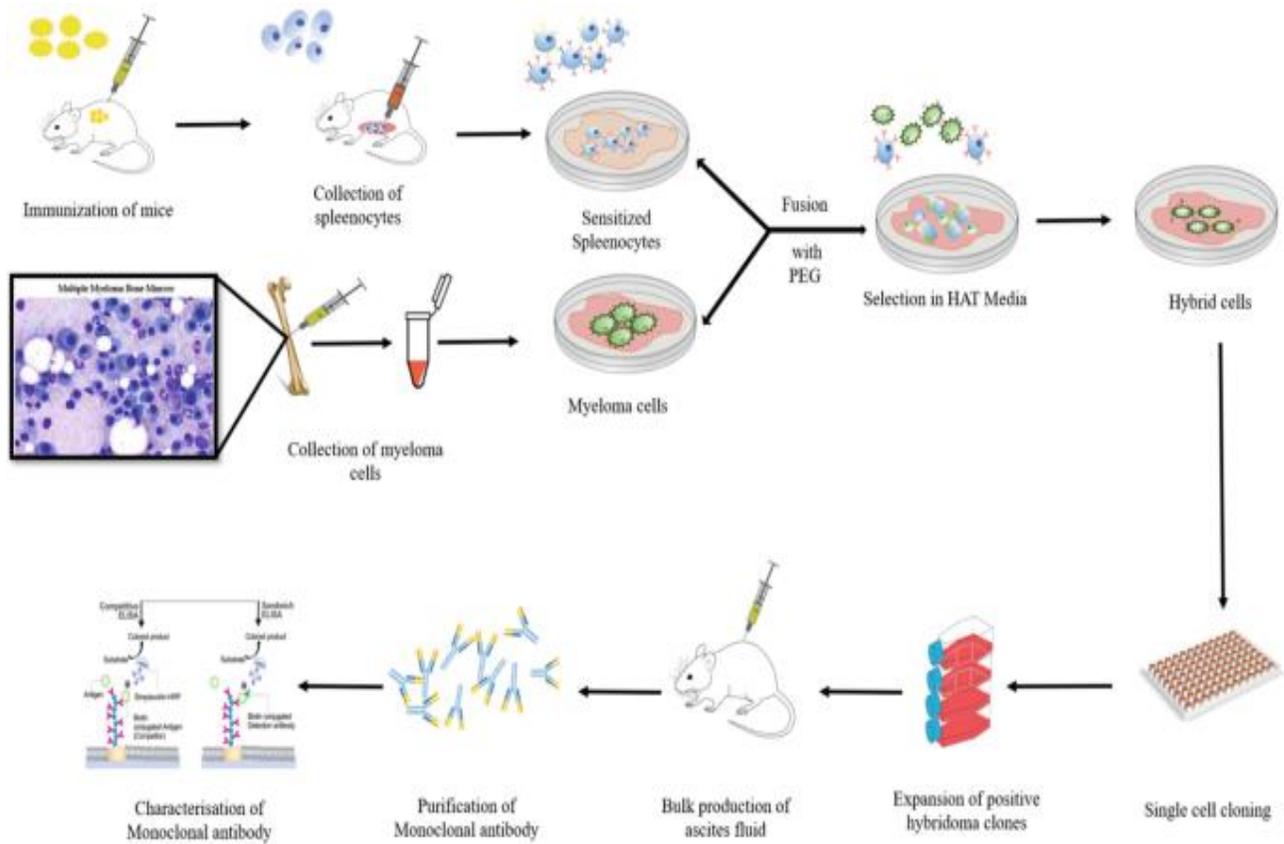
Monoclonal antibody production by Hybridoma technology

Objectives: To learn the monoclonal antibody production

Materials required: Sp2 cells, Immunized mice, Serological Pipettes (5mL, 10mL, 25mL) 200uL Pipette Tips, Micropipette (20uL-200uL), 1.5μL microtubes, 50mL centrifuge Tubes, Dissecting board, 1L Filter Unit Receiver, Inverted Microscope, Washing Solution, 1X PBS, Plating Medium, Dissecting kits, 150mm and 60mm petri dishes, 5mL syringes, Autoclave Bags, Light Duty Tissue Wipers, Isoflurane

Procedure:

1. **Immunization of mice & isolation of splenocytes** - Mice are immunized with an antigen and later their blood is screened for antibody production. This is typically done in two doses, an initial "priming" dose and a second "booster" dose 10 days later. The antibody-producing splenocytes are then isolated for *in vitro* hybridoma production.
2. **Preparation of myeloma cells** - A sample of B cells is extracted from the spleen of the mouse and added to a culture of **myeloma cells** (cancer cells). Myeloma cells are immortalized cells that, once fused with spleen cells, can result in hybridoma capable of unlimited growth. Myeloma cells are prepared for fusion.
3. **Fusion:** Myeloma cells and isolated B cells are fused together to form hybridomas in the presence of polyethylene glycol(PEG), which causes cell membranes to fuse.
4. **Select for the hybridomas:** The myeloma cells are HGPRT- and the B cells are HGPRT+. HGPRT is hypoxanthine-guanine phosphoribosyl transferase, an enzyme involved in the synthesis of nucleotides from hypoxanthine amino acid. The culture is grown in HAT (hypoxanthine-aminopterin-thymine) medium, which can sustain only HGPRT+ cells. The myeloma cells that fuse with another myeloma cell or do not fuse at all die in the HAT medium since they are HGPRT-. The B cells that fuse with another B cell or do not fuse at all die because they do not have the capacity to divide indefinitely. Only hybridomas between B cells and myeloma cells survive, being both HGPRT+ and cancerous.
5. **Clone screening and picking:** Screening is required to decide which hybridoma cells are actually producing the desired antibody. Each hybridoma is cultured and screened after doing SDS-PAGE (sodium dodecyl sulfate - polyacrylamide gel electrophoresis) and Western blots. The probe used is the epitope of the antibody that is desired, which may be labeled by radioactivity or immunofluorescence. Once we are sure that a certain hybridoma is producing the right antibody, we can culture that hybridoma indefinitely and harvest monoclonal antibodies from it.
6. **Functional characterization** - Confirm, validate and characterize (e.g. ELISA) each potentially high-producing colony.
7. **Scale up and wean** - Scale up clones producing desired antibodies and wean off selection agent(s).



Schematic diagram of Monoclonal antibody production by Hybridoma technology

Observations and result:

Practical No: 9

Detection and diagnosis of plant viruses with serological (ELISA) technique

Principle

Enzyme-Linked Immunosorbent Assay (ELISA) covalently links an immunoglobulin molecule to an enzyme, which could retain the immunospecificity of the primary antibody and the catalytic properties of the secondary antibody latter. If an enzyme is linked covalently to the immunoglobulin, the test is classified as direct ELISA. If the enzyme is linked to a molecule, which detects the immunoglobulin the test is called indirect ELISA

Objective: To detect the antigen and antibody reactions.

Buffers and reagents used in ELISA

A) Coating buffer (Carbonate buffer/antigen buffer) (pH 9.6):

Sodium carbonate (Na_2CO_3)	1.59 g
Sodium hydrogen Carbonate (NaHCO_3)	2.93 g
Sodium azide	0.2 g
Distilled water	1.0 L
The diethyl dithiocarbamate (sodium salt) was added at 0.001 M concentration (1.71 g/L).	

B) Phosphate buffer saline (PBS) pH 7.4:

Disodium hydrogen phosphate (Na_2HPO_4 , 2 H_2O)	2.9 g
Potassium di hydrogen phosphate (KH_2PO_4)	0.2 g
Potassium chloride (KCl)	0.2 g
Sodium chloride (NaCl)	8.0 g
Distilled water	1.0 L

C) Washing buffer (PBST):

PBS	1000 ml
Tween-20	0.5 ml

D) Antibody buffer:

PBS-Tween	100 ml
Polyvinyl Pyrrolidone (40000 MW)	2.0 g
Bovine Serum Albumin (BSA)	0.2 g

E) Substrate buffer (pH 9.8):

Diethanolamine	97 ml
Distilled water	800 ml
Sodium azide	0.2 g

Procedure for DAC- ELISA

1. Prepare the antigen and dispense into each well of microtitre plates at the rate of 200 μ l per well using a micropipette and incubate the plates at 37°C for 2-2.5 hr or at 4°C over night.
2. The contents of the plate are poured off and rinsed in PBS-Tween. Washing is carried out by immersing the plate in wash buffer for 3 min. repeat the washing into three times and at the final wash plate is taken to drain off buffer completely.
3. 200 μ l blocking solution is added to each well and incubates at 37°C for one hour to block the unoccupied sites in wells of microtitre plates and wash the plates in PBS-Tween as in step 2.
4. Monospecific Polyclonal antisera is dilute (1:1000 ratio and 1:750 ratio) respectively (v/v) in antibody buffer and add 200 μ l per well. Then incubate the plates at 37°C for 2-2.5 hr or at 4°C over night. After the incubation wash the plates in PBS-Tween as in stp 2.
5. In the next step antirabbit IgG-ALP labeled enzyme conjugate diluted in enzyme buffer (1: 10,000) is added 200 per well. Then the plates are incubated at 37°C for 2 hr and wash the plates with PBS-Tween as in step 2.
6. To develop color, 20 mg tablet of pNPP is dissolved in Substrate buffer and add 200 μ l per well. The plates are incubated at room temperature for 10-30 min for color development. Read the plates at 405 nm by using EL310E ELISA reader (BIO-TEK instruments, USA).

Important critical steps

1. Coating: proper preparation of the samples should be done to improve adhesion to microtiter plates.
2. Washing: Plates should be washed carefully and consistent number of times to reduce background color development in negative control wells.
3. Development: Substrate gets oxidized when it get exposed to light. Hence keep the substrate at dark for better color development.

Observation and results:

Look for the development of yellow colour on wells at the end of the Practical. Read the absorbance at 405nm and record the readings.

Practical No: 10

Total DNA extraction from virus infected plants

Objectives: To extract the DNA from the virus infected plants

Materials required: C-TAB buffer, Chloroform isoamyl alcohol, isopropanol, 70% ethanol, 1X TE buffer

Procedure

1. Grind 100-200 mg of infected leaves using pestle and mortar.
2. Add 1-2 ml CTAB extraction buffer and grind thoroughly.
3. Transfer 0.75 ml of grinded sap into 1.5 ml micro centrifuge tube and incubate at 60° C for 30 min in a water bath.
4. After incubation, add 750 µl of chloroform: isoamyl alcohol (24:1) and mix well to form an emulsion by inverting the tube.
5. Centrifuge the mixture at 13,000 rpm for 10 min.
6. Collect the supernatant and mix thoroughly with 300 µl of isopropanol and incubate at -20° C for at least one hour.
7. Centrifuge the contents centrifuged at 13,000 rpm for 10 min and discard the supernatant without losing pellet.
8. Wash the pellet with 500 µl of 70 per cent ethanol and centrifuge at 14,000 rpm for 5 min.
9. Remove the ethanol and dry the pellet in vacuum drier for 5 min.
10. Finally the dried pellet is resuspended in 100 µl of 1X TE buffer and stored at -20° C.
11. Quantify the DNA by using Spectrophotometer.

DNA extraction buffer composition

2 per cent (w/v) CTAB
1.4 M NaCl
20 mM EDTA
100 mM Tris- HCl (pH 8.0)
0.2 per cent (v/v) β -mercapta ethanol

Activities: Extract the DNA from infected plants and note down the quantity and quality of DNA

Practical No: 11

Polymerase Chain Reaction (PCR) and Gel electrophoresis

Aim: To detect and diagnosis of plant diseases

Introduction: polymerase chain reaction enables researchers to produce millions of copies of a specific DNA sequence in approximately two hours. This automated process bypasses the need to use bacteria for amplifying DNA. PCR is a technique widely used in molecular biology. It derives its name from one of its key components; a DNA polymerase used to amplify a piece of DNA by in vitro enzymatic replication. As PCR progresses, the DNA generated is used as a template for replication. This sets in motion to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating millions or more copies of the DNA piece. PCR can be extensively modified to perform a wide array of genetic manipulations. PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications. These include DNA cloning for sequencing, DNA-based phylogeny, or functional analysis of genes; the diagnosis of hereditary diseases; the identification of genetic fingerprints; and the detection and diagnosis of infectious diseases.

Materials required:

Taq polymerase: Enzyme that extends growing DNA strand complementary to DNA template

MgCl₂: Provides ions needed for enzyme reaction

dNTP's: Nucleotides (Adenine, Cytosine, Guanine, Thymine) building blocks for new DNA strands

Buffer: Maintains optimal pH for enzyme

Primers: Anneal to single-stranded DNA template, provide initiation site for extension of new DNA.
Forward primer: Anneals to DNA anti-sense strand; Reverse primer: Anneals to DNA sense strand

DNA template: the product of our DNA extraction

Procedure:

The PCR usually consists of a series of 30 to 35 cycles. Most commonly, PCR is carried out in three steps, often preceded by one temperature hold at the start and followed by one hold at the end.

Initialization step: This step consists of heating the reaction mixture to a temperature of 94-96°C (or 98°C if extremely thermo stable polymerases are used), which is held for 1-9 minutes. It is only required for DNA polymerases that require heat activation by hot-start PCR.

Denaturation step: This step is the first regular cycling event and consists of heating the reaction mixture to 94-98°C for 20-30 seconds. It causes melting of DNA template and primers by disrupting the hydrogen bonds between complementary bases of the two DNA strands, yielding single strands of DNA.

Annealing step: The reaction temperature is lowered to 50-65°C for 20-40 seconds allowing annealing of the primers to the single-stranded DNA template. Stable DNA-DNA hydrogen bonds are formed only formed when the primer sequence very closely match the template sequence. The polymerase binds to the primer template hybrid and begins DNA synthesis.

Extension / elongation step: The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75-80°C, and commonly a temperature of 72°C is set for elongation. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTP's that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA

fragment to be amplified. As a rule-of-thumb, at its optimum temperature, the DNA polymerase will polymerize a thousand bases in one minute.

Final elongation: This single step is occasionally performed at a temperature of 70- 74°C for 5-15 minutes after the last PCR cycle to ensure that any remaining single stranded DNA is fully extended.

Final hold: This step at 4°C for an indefinite time may be employed for short term storage of the reaction.

Gel electrophoresis:

Principle of electrophoresis

The fundamental driving force of electrophoresis is the voltage applied to the mixture of charged molecules in solution. The separation speed of charged molecules is directly proportional to the surrounding voltage gradient.

Objective: To visualize separated DNA fragments according to their molecular size by applying an electric current to the gel matrix.

Materials required:

- **An electrophoresis chamber and power supply**
- **Gel casting trays**, which are available in a variety of sizes and composed of UV transparent plastic. The open ends of the trays are closed with tape while the gel is being cast, then removed prior to electrophoresis.
- **Sample combs**, around which molten agarose is poured to form sample wells in the gel.
- **Electrophoresis buffer**, usually Tris-acetate- Ethylene Diamine Tetra Acetic Acid (EDTA) (TAE) or Tris-borate-EDTA (TBE).
- **Loading buffer**, which contains something dense (e.g. glycerol) to allow the sample to "fall" into the sample wells, and one or two tracking dyes, which migrate in the gel and allow visual monitoring to track the movement of molecules.
- **Ethidium bromide**, a fluorescent dye used for staining nucleic acids.
- **Transilluminator** (an ultraviolet lightbox), which is used to visualize ethidium bromide-stained DNA in gels.

Procedure:

- agarose powder is mixed with electrophoresis buffer to the desired concentration and then heated in a microwave oven until completely melted. Most commonly, ethidium bromide is added to the gel (final concentration 0.5 ug/ml) to facilitate visualization of DNA after electrophoresis.
- After cooling the solution to about 60°C, it is poured into a casting tray containing a sample comb and allowed to solidify at room temperature.
- After the gel has solidified, the comb is carefully removed without ripping bottom of the wells. The gel, still in its plastic tray, is inserted horizontally into the electrophoresis chamber and just covered with buffer.
- Samples containing DNA mixed with loading dye are then loaded into the sample wells, the lid and power leads are placed on the apparatus, and a current is applied.
- Transmission of electricity is confirmed by observing bubbles coming off the electrodes.
- DNA fragments will migrate towards the positive electrode.

- The distance that the DNA fragments migrate through the gel can be judged by visually monitoring migration of the tracking dyes.
- Bromophenol blue and xylene cyanol dyes migrate through agarose gels at roughly the same rate as double-stranded DNA fragments of 300 and 4000 bp, respectively.
- When adequate migration has occurred, DNA fragments are visualized on a ultraviolet transilluminator.
- It should be noted that DNA will diffuse within the gel over time. Documentation of DNA fragment migration is accomplished shortly after cessation of electrophoresis.

NOTE:

- **Ethidium bromide is a known mutagen and should be handled carefully wearing gloves.**
- **Always wear protective eyewear when observing DNA on a transilluminator to prevent damage to the eyes from UV light.**

Observations and results:

Practical No: 12

RNA extraction from infected plants

Procedure:

TRI reagent method is followed to extract the total RNA from infected plants (Sigma catalogue no. T 9424).

1. Weigh 50-100 mg of the infected leaf tissue.
2. Grind the sample immediately using liquid nitrogen in a pestle and mortar and placed in a sterile micro centrifuge tube.
3. Homogenize the 50-100 mg leaf sample was in 1 ml of TRI reagent.
4. Centrifuge the homogenate at 9,000 rpm for 10 min at 2-8° C.
5. Transfer the supernatant into a fresh micro centrifuge tube and incubate the tube for 5 min at room temperature.
6. Add 0.2 ml of Chloroform to the micro centrifuge tube and shake vigorously for 15 sec.
7. Keep the tube for 2-15 min at room temperature and centrifuge at 9,000 rpm for 15 min at 2-8° C. Three separate phases will form. Colourless upper phase consist of RNA, interphase/ mid phase consisted of DNA and bottom red organic phase consisted of protein.
8. The colourless upper aqueous phase is transferred into a fresh micro centrifuge tube and adds 0.5 ml of Isopropanol and mix properly.
9. Keep the tube for 5-10 min at room temperature and centrifuge at 9,000 rpm for 10 min at 2-8° C.
10. Discard the supernatant and RNA pellet is washed with 75 per cent ethanol with vortexing.
11. The tube is centrifuge at 5,500 rpm for 5 min at 2-8° C.
12. Remove the Ethanol without losing the pellet and dry the pellet for 5-10 min under fan by keeping the lid of the micro centrifuge tube open (The pellet should not be vacuum centrifuged).
13. Finally the dried pellet is resuspended in 20-30 μ l of nuclease free water or DEPC treated water.
14. The pellet is dissolved by repeated pipetting with micro-pipette or by keeping at 55-60° C for 10-15min and store at -80° C.

Reverse transcription

The isolated RNA from the plant samples is used for RT-PCR. First c-DNA is synthesized from viral RNA in a 20 μ l reaction using Bioscript-RT enzyme (BIOLINE catalogue no. BIO- 27036). Reverse transcription is carried out in eppendorf thermo cycler. RT reaction is set up for CMV by adopting manufacturer's instructions which is as follows:

1. 5 μ l of template RNA + 2 μ l of reverse primer + 4 μ l of nuclease free water is added into a 0.2 ml polypropylene tube.
2. The mixture is incubated at 70° C for 5 min.
3. Mixture is immediately chilled on ice for 2 min.
4. All the reaction components including 4 μ l 5 X BIOSCRIPT reaction buffer (BIOLINE), 2 μ l 10mM dNTPs, 2 μ l nuclease free water are then added into the tube and the tube is incubated at 37° C for 1 min.
5. 200 units of Bioscript -RT enzyme (200 U/1 μ l, BIOLINE) was added to the tube and incubated at 42° C for 60 min.
6. The reaction is stopped by heating the mixture at 70° C for 10 min and stored at -20° C. The c-DNA is used for performing PCR.

Reverse transcription polymerase chain reaction: The c-DNA synthesized by reverse transcription is amplified by RT-PCR. PCR reaction mixture of 25 μ l is prepared as follows:

Procedure: 25 μ l of reaction mixture is prepared by adding the following ingredients into the eppendorf tube.

Sterile distilled water	15.4 μ l
10X PCR buffer (Supplied with the enzyme)	2.5 μ l
25 mM MgCl ₂	1.0 μ l
2.5mM dNTP mixture	2.0 μ l
Forward primer	1.0 μ l
Reverse primer	1.0 μ l
<i>Taq</i> Polymerase	0.1 μ l
Viral C-DNA	2.0 μ l
Total	25.0 μ l

After preparing PCR mixture, the DNA template is added and tubes are vortexed briefly and inserted into the wells of a thermal cycler (Eppendorf thermo cycler). The PCR is performed in a thermal cycler using the following PCR conditions:

Initial denaturation	92° C for 3 min	
Denaturation	95° C for 1 min	} 35 cycles
Annealing	60° C for 1 min	
Extension	72° C for 1 min	
Final extension	72° C for 7 min	

After the completion of the reaction, keep the products at 4° C prior to gel analysis. The amplified fragments are electrophoresed in 1 per cent agarose gel.

Result:

Practical No: 13

Exposure to basic bio-informatic tools for viral genome analysis and their utilization in developing detection protocols and population studies

(BLASTn tool, Primer designing software, Bioedit tool, Clustal X/W, MEGA Software)

Objectives: To get the exposure on different bio-informatics tools

NCBI (National centre for Biotechnological information):

NCBI is one of the leading online resources known for providing Biological sequence information. NCBI is maintained by two organizations in US, National Library of Medicine (NLM) and National Institute of science (NIH). As a national resource for molecular biology information, NCBI's mission is to develop new information technologies to aid in the understanding of fundamental molecular and genetic processes that control health and disease. More specifically, the NCBI has been charged with creating automated systems for storing and analyzing knowledge about molecular biology, biochemistry, and genetics.

NCBI is connected to various other sequence databases in order to be more efficient in answering sequence queries. The user queries and sequence information are delivered through NCBI's search tool called the "entrez".

Home Page:

NCBI has a simplified homepage from where the user can navigate to different resources. The left side pane of the Homepage has a site map followed by different categories which narrows down the possibility of finding the right sequence. On the right side, you can see the list of popular resources which is very useful for first time users.

GenBank

The GenBank sequence database is an open access, annotated collection of all publicly available nucleotide sequences and their protein translations. This database is produced and maintained by the National Center for Biotechnology Information (NCBI) as part of the International Nucleotide Sequence Database Collaboration (INSDC). The National Center for Biotechnology Information is a part of the National Institutes of Health in the United States. GenBank and its collaborators receive sequences produced in laboratories throughout the world from more than 100,000 distinct organisms. In more than 20 years since its establishment, GenBank has become the most important and most influential database for research in almost all biological fields, whose data were accessed and cited by millions of researchers around the world. GenBank continues to grow at an exponential rate, doubling every 18 months.

Entrez:

The NCBI database accepts queries and delivers data via a custom made search engine called Entrez. The Home page of NCBI has a search box which directs the user to entrez. Entrez is internally connected to various biological databases which increases the probability of getting the correct information.

BLAST:

BLAST stands for Basic Local Alignment Search Tool. BLAST is a tool that is used to find the sequences homologous to a particular sequence. BLAST compares all the sequences in the database with the one that is searched for and provides many hits which are usually arranged in the increasing order of the scored obtained

BLAST is available at the URL <http://blast.ncbi.nlm.nih.gov/>

BLAST uses PAM and BLOSUM matrices for scoring the alignment.

The home page of NCBI can be seen as follows:

The screenshot displays the NCBI homepage with a blue header containing the NCBI logo and navigation links for 'Resources' and 'How To'. Below the header is a search bar with a dropdown menu set to 'All Databases'. The main content area is divided into three columns. The left column features a vertical navigation menu with categories such as 'NCBI Home', 'Site Map (A-Z)', 'All Resources', 'Chemicals & Bioassays', 'Data & Software', 'DNA & RNA', 'Domains & Structures', 'Genes & Expression', 'Genetics & Medicine', 'Genomes & Maps', 'Homology', 'Literature', 'Proteins', 'Sequence Analysis', 'Taxonomy', 'Training & Tutorials', and 'Variation'. The middle column is titled 'Welcome to NCBI' and includes a mission statement, a list of links for 'About the NCBI', and a 'Get Started' section with links for 'Tools', 'Downloads', 'How-To's', and 'Submissions'. The right column contains 'Popular Resources' (BLAST, Bookshelf, Gene, Genome, Nucleotide, OMIM, Protein, PubChem, PubMed, PubMed Central, SNP) and 'NCBI News' (NCBI will continue to operate SRA, dated 13 Oct 2011). At the bottom, there is a 'New NCBI News Issue' dated 07 Sep 2011. A Facebook widget is also present, encouraging users to find the latest news and participate in community discussions.

Home page of BlastN

 National Library of Medicine
National Center for Biotechnology Information

[Log in](#)

BLAST® » blastn suite [Home](#) [Recent Results](#) [Saved Strategies](#) [Help](#)

blastn | blastp | blastx | tblastn | tblastx

Standard Nucleotide BLAST

BLASTN programs search nucleotide databases using a nucleotide query. more... [Reset page](#) [Bookmark](#)

Enter Query Sequence

Enter accession number(s), gi(s), or FASTA sequence(s) [Clear](#) Query subrange [?](#)

From

To

Or, upload file No file selected. [?](#)

Job Title

Enter a descriptive title for your BLAST search [?](#)

Align two or more sequences [?](#)

Choose Search Set

Database Standard databases (nr etc.) rRNA/ITS databases Genomic + transcript databases Betacoronavirus

Experimental databases [Try experimental taxonomic nt databases](#) [Download](#)
For more info see [What are taxonomic nt databases?](#)

Nucleotide collection (nr/nt) [?](#)

Organism [exclude](#) [Add organism](#)

Optional Enter organism name or id--completions will be suggested

Optional Enter organism common name, binomial, or tax id. Only 20 top taxa will be shown [?](#)

Exclude Models (XM/XP) Uncultured/environmental sample sequences

Optional

Limit to Sequences from type material

Optional

Entrez Query [Create custom database](#)

Optional Enter an Entrez query to limit search [?](#)

Program Selection

Optimize for Highly similar sequences (megablast)

More dissimilar sequences (discontiguous megablast)

Somewhat similar sequences (blastn)

Choose a BLAST algorithm [?](#)

BLAST Search database Nucleotide collection (nr/nt) using Megablast (Optimize for highly similar sequences)

show results in a new window

[+ Algorithm parameters](#)

Output:

The file format of the particular virus after BlastN is shown as follows:


National Library of Medicine
National Center for Biotechnology Information
Log in

BLAST® » [blastn suite](#) » **results for RID-5BTM9X9X016**

[Home](#) [Recent Results](#) [Saved Strategies](#) [Help](#)

[← Edit Search](#)

[Save Search](#) [Search Summary ▾](#)

[? How to read this report?](#) [▶ BLAST Help Videos](#) [↶ Back to Traditional Results Page](#)

Job Title	MK548887.1 Groundnut bud necrosis virus isolate...
RID	5BTM9X9X016 <small>Search expires on 05-07 13:32 pm</small> Download All ▾
Program	BLASTN ? Citation ▾
Database	nt See details ▾
Query ID	Ic Query_63039
Description	MK548887.1 Groundnut bud necrosis virus isolate Guntur...
Molecule type	dna
Query Length	4815
Other reports	Distance tree of results MSA viewer ?

Filter Results

Organism only top 20 will appear exclude

Type common name, binomial, taxid or group name

[+ Add organism](#)

Percent Identity to
 E value to
 Query Coverage to

Descriptions

Graphic Summary

Alignments

Taxonomy

Sequences producing significant alignments [Download ▾](#) [Select columns ▾](#) Show [?](#)

select all 100 sequences selected

[GenBank](#) [Graphics](#) [Distance tree of results](#) [MSA Viewer](#)

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
<input checked="" type="checkbox"/> Groundnut bud necrosis virus isolate Guntur segment M, complete sequence	Groundnut bud...	8892	8892	100%	0.0	100.00%	4815	MK548887.1
<input checked="" type="checkbox"/> Groundnut bud necrosis virus movement protein (NSm) and glycoprotein (G1/G2) genes, complete cds	Groundnut bud...	7430	7430	100%	0.0	94.52%	4815	AY871097.2
<input checked="" type="checkbox"/> Groundnut bud necrosis virus isolate GBNV_2015_001 Groundnut segment M, complete sequence	Groundnut bud...	7273	7273	100%	0.0	93.94%	4813	MF469058.1
<input checked="" type="checkbox"/> Groundnut bud necrosis virus isolate Chilli/Vaddebailu/UHSASB530 segment M, complete sequence	Groundnut bud...	7262	7262	100%	0.0	93.90%	4813	MN735580.1
<input checked="" type="checkbox"/> Groundnut bud necrosis virus isolate Coimbatore Nsm protein and glycoprotein genes, complete cds	Groundnut bud...	7241	7241	100%	0.0	93.83%	4810	MZ505082.1
<input checked="" type="checkbox"/> Groundnut bud necrosis virus isolate Chilli/Kammadanam/UHSASB357 segment M, complete sequence	Groundnut bud...	7238	7238	100%	0.0	93.80%	4817	MN735586.1
<input checked="" type="checkbox"/> Groundnut bud necrosis virus isolate Bangalore segment M, complete sequence	Groundnut bud...	7238	7238	100%	0.0	93.80%	4817	KY006470.1
<input checked="" type="checkbox"/> Groundnut bud necrosis virus isolate brinjal Nagarguda segment M, complete sequence	Groundnut bud...	7230	7230	100%	0.0	93.77%	4816	KX244327.1
<input checked="" type="checkbox"/> Groundnut bud necrosis virus isolate tumkur-ch segment M, complete sequence	Groundnut bud...	7227	7227	100%	0.0	93.75%	4817	MK617532.1
<input checked="" type="checkbox"/> Groundnut bud necrosis virus isolate Tomato/Pamanakalluru/UHSASB127 segment M, complete sequence	Groundnut bud...	7225	7225	100%	0.0	93.75%	4815	MN735601.1
<input checked="" type="checkbox"/> Groundnut bud necrosis virus isolate Solanum nigrum Budamanahalli segment M, complete sequence	Groundnut bud...	7219	7219	100%	0.0	93.73%	4815	KX244329.1
<input checked="" type="checkbox"/> Groundnut bud necrosis virus isolate brinjal Mysore segment M, complete sequence	Groundnut bud...	7214	7214	100%	0.0	93.71%	4816	KX244328.1
<input checked="" type="checkbox"/> Groundnut bud necrosis virus isolate Tomato/Madnal/UHSASB444 segment M, complete sequence	Groundnut bud...	7206	7206	100%	0.0	93.69%	4813	MN735595.1
<input checked="" type="checkbox"/> Groundnut bud necrosis virus isolate Tomato/Tottivaripalli/UHSASB110 segment M, complete sequence	Groundnut bud...	7188	7188	100%	0.0	93.61%	4817	MN735600.1
<input checked="" type="checkbox"/> Groundnut bud necrosis virus isolate Chilli/Uppulurivandlapalli/UHSASB125 segment M, complete sequence	Groundnut bud...	7177	7177	100%	0.0	93.59%	4811	MN735584.1
<input checked="" type="checkbox"/> Groundnut bud necrosis virus isolate Tomato/Madhurai/UHSASB149 segment M, complete sequence	Groundnut bud...	7164	7164	100%	0.0	93.51%	4821	MN735605.1

Practical 14: Making multiple sequence alignment and Phylogenetic tree of sequences by using ClustalW

Multiple sequence alignment

When a protein sequence is newly-determined, an important goal is to assign possible functions to the protein. The first computational step is to search for similarities with sequences that have previously been deposited in the DNA and protein sequence databases. If similar sequences are found, they may match the complete length of the new sequence or only to subregions of the sequence. If more than one similar sequence is found, then the next important step in the analysis is to multiply align all of the sequences. Multiple alignments are a key starting point for the prediction of protein secondary structure, residue accessibility, function, and the identification of residues important for specificity.

A protein sequence is represented by a string of letters coding for the 20 different types of amino acid residues. A protein sequence alignment is created when the residues in one sequence are lined up with those in at least one other sequence. Optimal alignment of the two sequences will usually require the insertion of gaps in one or both sequences in order to find the best alignment. Alignment of two residues implies that those residues are performing similar roles in the two different proteins. This allows for information known about specific residues in one sequence to be potentially transferred to the residues aligned in the other. For example, if the active site residues of an enzyme have been characterized, alignment of these residues with similar residues in another sequence may suggest that the second sequence possesses similar catalytic activity to the first.

CLUSTAL W combines a good hierarchical method for multiple sequence alignment with an easy-to-use interface. The software is free, although a contribution to development costs is required when purchasing the program. CLUSTAL W runs on most computer platforms and incorporates many of the techniques described in the previous section. The program uses a series of different pair-score matrices, biases the location of gaps, and allows you to realign a set of aligned sequences to refine the alignment.

Requirement: Computer system with (legal software) equipped with Internet Connection preferably fast Broadband.

Web resources used: ClustalW: www.ebi.ac.uk/tools/clustalW2/index.html

Principle:

Phenetics is a novel phylogenetic method that takes into account as many variable characters as possible (OTUs = Operational Taxonomic Units), these characters being scored numerically and analyzed by rigorous mathematical methods (numerical taxonomy).

Phylogeny: Phylogeny is the description of biological relationships, especially from evolutionary point of view and usually expressed as a “tree”. There are different ways to represent these trees:

Cladogram: Character-based methods are called cladistic methods. The trees are prepared by considering the various possible pathways of evolution and are based on parsimony or likelihood methods. The resulting tree is called a cladogram.

Dendrogram: A phylogenetic tree generated by taking into account the similarity (coefficient) or dissimilarity seen in the characters of different entities e.g., protein structure or organism species, genera etc., to be useful in their classification.

Construction of Phylogenetic Tree:

In constructing a phylogenetic tree the preliminary step is to compare the nucleotide sequences. This is done by Sequence alignment. Rigorous mathematical approaches have been devised to sequence alignment to be used to convert the data into a phylogenetic tree. The similarity approach aims to maximize the number of matched nucleotides (identical in two sequences compared). Its complementary approach is the Distance method that allows the minimizing the number of mismatches. The final result of the two approaches is the same i.e., the same alignment will be identified as the best i.e., there is a likelihood of identifying the same alignment if there is a relationship. Multiple alignments is often used for the comparison of more than two sequences with the help of clustal W as the popular choice.

Procedure:

1. Go to <http://www.ebi.ac.uk/clustalw/>
2. Put in your e-mail (if you want the results e-mailed to you not necessary) and an alignment title of your choice.
3. Paste sequences in the box below using the FASTA format:
>Name of Sequence #1
>Name of Sequence #2
4. Every sequence MUST have a different name given to it or the alignment will not work. Or Upload a file that includes all your sequences (such as a .doc file) in an acceptable format.
5. Press run button to start alignment reading.
6. When viewing your results, these are the consensus symbols used by ClustalW:
 - a. "*" means that the residues or nucleotides in that column are identical in all sequences in the alignment.
 - b. ":" means that conserved substitutions have been observed.
 - c. "." means that semi-conserved substitutions are observed.
7. If you would like to see your results in color, push the button that displays Show Colors. Click Hide Colors to get rid of color..
8. Click on the button named View Alignment File to see the alignment on a larger scale (ie bigger font).

Practical 15:

Software for primer design

Introduction: The use of software in biological applications has given a new dimension to the field of bioinformatics. Many different programs for the design of primers are now available. Freeware software is available on the internet and many universities have established servers where a user can log on and perform free analyses of proteins and nucleic acid sequences. There are number of simple stand-alone programs as well as complex integrated networked versions of the commercial software available. These software packages may be for complete DNA and protein analysis, secondary structure predictions, primer design, molecular modeling, development of cloning strategies, plasmid drawing or restriction enzyme analyses. Companies engaged in biosoftware development include: Alkemi Biosystems, Molecular Biology Insights, PREMIER Biosoft International, IntelliGenetics Inc., Hitachi Inc., DNA Star, Advanced American Biotechnology and Imaging.

Properties of Primers:

- Length of 18-24 bases
 - 40-60% G/C content
 - Start and end with 1-2 G/C pairs
 - Melting temperature (T_m) of 50-60°C
 - Primer pairs should have a T_m within 5°C of each other
 - Primer pairs should not have complementary regions
-
- **Primer length:** For broad-spectrum studies, primers of typically 18-30 nucleotides in length are the best. Primers should be at least 18 nucleotides in length to minimize the chances of encountering problems with a secondary hybridization site on the vector or insert. Primers with long runs of a single base should generally be avoided. It is especially important to avoid 4 or more G's or C's in a row.
 - **Melting Temperature (T_m):** The optimal melting temperatures for primers in the range 52-58°C, generally produce better results than primers with lower melting temperatures. Primers with melting temperatures above 65°C should also be avoided because of potential for secondary annealing. It is then advisable to do the sequencing reaction with annealing and extension at 60°C. A good working approximation of this value (generally valid for oligos in the 18–30 base range) can be calculated using the formula of Wallace et al. (1979), $T_m = 2(A+T) + 4(G+C)$.
 - **GC Content (T_m and T_a are interrelated):** GC% is an important characteristic of DNA and provides information about the strength of annealing. Primers should have GC content between 45 and 60 percent. For primers with a G/C content of less than 50%, it may be necessary to extend the primer sequence beyond 18 bases to keep the melting temperature above the recommended lower limit of 50°C. GC content, melting temperature and annealing temperature are strictly dependent on one another.
 - **3'-End Sequence:** It is well established that the 3' terminal position in PCR primers is essential for the control of mis-priming. Primers should be "stickier" on their 5' ends than on their 3' ends. A "sticky" 3' end as indicated by a high G/C content could potentially anneal at multiple sites on the

template DNA. A "G" or "C" is desirable at the 3' end but the first part of this rule should apply. This GC clamp reduces spurious secondary bands.

- **Dimers and false priming cause misleading results:** Primers should not contain complementary (palindromes) within themselves; that is, they should not form hairpins. If this state exists, a primer will fold back on itself and result in an unproductive priming event that decreases the overall signal obtained. Hairpins that form below 50°C generally are not such a problem. Primers should not contain sequences of nucleotides that would allow one primer molecule to anneal to itself or to the other primer used in PCR reactions (primer dimer formation).
- **Specificity:** As mentioned above, primer specificity is at least partly dependent on primer length. It is evident that there are many more unique 24 base oligos than there are 15 base pair oligos. However, primers must be chosen so that they have a unique sequence within the template DNA that is to be amplified. A primer designed with a highly repetitive sequence will result in a smear when amplifying genomic DNA. However, the same primer may give a single band if a single clone from a genomic library is amplified.
- **Degenerate Primers:** Degeneracy in primer sequence should also be taken into consideration. Degenerate primers based on the amino acid sequence of conserved regions were also used to search for members of a gene family.
- **Complementary primer sequences:** Primers need to be designed with absolutely no intra-primer homology beyond 3 base pairs. If a primer has such a region of self-homology, "snap back" can occur. Another related danger is inter-primer homology: partial homology in the middle regions of two primers can interfere with hybridization. If the homology should occur at the 3' end of either primer, primer dimer formation will occur.

Activities:

1. Design the primers for predominant viruses
2. Identify the virus from obtained sequence (from your sequences) through BlastN analysis
3. Do multiple sequence analysis and construct Phylogeny by using ClustalW tool

Table 1. Online primer design sites.

Tool name	Description	www
CODEHOP	Consensus Degenerate Hybrid Oligonucleotide Primers; degenerate PCR primer design; will accept unaligned sequences.	http://blocks.fhrc.org/codehop.html
Gene Fisher	Interactive primer design tool for standard or degenerate primers; will accept unaligned sequences.	http://bibiserv.techfak.uni-bielefeld.de/genefisher/
DoPrimer	Easily design primers for PCR and DNA sequencing.	http://doprimer.interactiva.de/
Primer3	Comprehensive PCR primer and hybridization probe design tool; many options but easy to accept defaults at first.	http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi http://www.basic.nwu.edu/biotools/Primer3.html http://www.justbio.com/primer/index.php
Primer Selection	Select PCR primers from nucleotide sequence.	http://alces.med.umn.edu/rawprimer.html
Web Primer	Allow alternative design of primers for either PCR or sequencing purpose.	http://genome-www2.stanford.edu/cgi-bin/SGD/web-primer
PCR Designer	For restriction analysis of sequence mutations.	http://cedar.genetics.soton.ac.uk/public_html/primer.html
Primo Pro 3.4	Reduces PCR noise by lowering the probability of random priming.	http://www.changbioscience.com/primo/primo.html
Primo Degenerate 3.4	Primo Degenerate 3.4 designs PCR primers based on a single peptide sequence or multiple alignments of proteins or nucleotides.	http://www.changbioscience.com/primo/primod.html
PCR Primer Design	An application that designs primers for PCR or sequencing purposes.	http://pga.mgh.harvard.edu/servlet/org.mgh.proteome.Primer
The Primer Generator	The program analyzes the original nucleotide sequence and desired amino acid sequence and designs a primer that either has a new restriction enzyme site or is missing an old one.	http://www.med.jhu.edu/medcenter/primer/primer.cgi
EPRIMER3	Picks PCR primers and hybridization oligos (EMBOSS).	http://bioweb.pasteur.fr/seqanal/interfaces/eprimer3.html
PRIMO	Prediction of forward and reverse oligonucleotide Primers.	http://bioweb.pasteur.fr/seqanal/interfaces/primo.html3 http://atlas.swmed.edu/primo/primo_form.html
PrimerQuest	A primer design tool.	http://www.idtdna.com/biotools/primer_quest/primer_quest.asp
MethPrimer	Design primers for methylation PCRs.	http://itsa.ucsf.edu/~urolab/methprimer/index1.html
Rawprimer	A tool for selection of PCR primers.	http://alces.med.umn.edu/rawprimer.html
MEDUSA	A tool for automatic selection and visual assessment of PCR primer pairs.	http://www.cgr.ki.se/cgr/MEDUSA/
The Primer Prim'er Project	Software suite that completely automates the PCR primer design process.	http://www-nmr.cabm.rutgers.edu/bioinformatics/Primer_Primer_Project/Primer.html
Oligonucleotides for the PCR	Seek oligonucleotides on both sides of an area.	http://www.citi2.fr/bio2/Oligo2lib.html
GAP	Genome- wide Automated Primer finder servers.	http://promoter.ics.uci.edu/Primers/