LABORATORY CLASSES

SCHOOL OF BIOLOGICAL SCIENCES

NISER
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Laboratory Safety Guidelines
School of Biological Sciences
NISER, BBSR, India

The School of Biological Sciences, NISER is committed to providing a safe environment for all. However, laboratory safety is a mutual responsibility and requires full participation and cooperation of all involved persons - students, researchers, and faculty and staff members. The following Lab Safety Guidelines have been established for your protection as a student in the school of biological sciences. These rules will be rigidly and impartially enforced. Noncompliance may result in a grading penalty and/or dismissal from lab.
Personal Protection

1. Safety glasses must be worn at all times in the Lab. This is the policy of NISER and a national safety requirement. The glasses must be of the impact protection type with splash guards and must meet required specifications. Other eye/face protection may be required with specific procedures. The laboratory in-charge has approved safety glasses. The glasses must be worn at all times within the lab space - even while working on the computer or writing in your lab book.

2. Contact lenses are discouraged. The safety of wearing contact lenses in laboratories has been hotly debated over the last several years. NISER is preparing guidelines indicating that contact lenses can be worn if and only if proper protective eyewear is also worn. The school of biological sciences recognizes that some eye conditions require contacts for certain vision correction therapies. However, students who choose to wear contacts must recognize the inherent increased risks - they are difficult to remove if chemicals get in the eye, they have a tendency to prevent natural eye fluids from removing contaminants, and sudden displacement can cause visual problems that create additional hazards. Soft contact lenses are especially problematic because they can discolor and also absorb chemical vapors causing damage before the wearer is alerted to the problem. If you choose to wear contacts, please tell your lab instructor and individual will be liable for any personal damages caused as a result of wearing the contact lens.

3. Appropriate gloves will be provided when needed. Use of gloves is required for handling certain chemicals and biological. Gloves are very expensive. Do not change gloves needlessly. For some uses double gloving may be necessary, your instructor will guide you for such need.

4. Appropriate clothing is required. Your clothing is a barrier between your skin and chemicals. You must be covered to the knee - also no bare midriffs or shoulders. Knee length shorts are acceptable, anything above the knee - shorts, skirts, or dresses are not. Lab coats are.

5. Shoes must be worn. No sandals, thongs, open toed or open heeled shoes.

6. Roll up sleeves and tie up loose clothing and long hair when working with equipment, open flame, any chemicals or biological substances.

7. Do not eat, drink (including sport bottles and water bottles), or store food in the labs.

8. Smoking or use of other tobacco products is prohibited.

9. Wash hands after working with chemicals.

10. It is the recommendation of this department that all students of reproductive age, especially women who have recently conceived or are anticipating conception
during the semester, discuss the course content and reagents with their physician if they are concerned about reproductive toxins.

**General Lab Rules**

1. Conduct yourself in a responsible manner at all times in the laboratory.

2. When first entering a lab room, do not touch any equipment, chemicals, or other materials in the laboratory area until you are instructed to do so.

3. Read all instructions carefully and plan your work. Understand the experiment and if in doubt, ask.

4. Follow the written lab procedure - laboratory activity at this level is not meant to be creative. Improper combinations or amounts of chemicals can be very dangerous. No unauthorized experiments are to be performed. If you are curious about trying a procedure not covered in the experimental procedure, consult with your laboratory instructor.

5. Lab tables should be as uncluttered as possible to allow work space and avoid accidents. Also, keep the aisles clear to prevent tripping over your gear, and so that other people can pass unhampered. Place book bags, pocketbooks, etc. under the lab tables. In some labs, seats or stools are not to be used during labs - students need to be mobile to avoid possible spills and are not to place themselves under the edge of the lab bench where chemicals may spill.

6. Lab activities require your undivided attention. No music allowed in student labs. Radios (including Walkman type) and other entertainment devices are not permitted. No mobile phone use.

7. Lab computers are for laboratory business only - no unrelated Internet surfing or checking e-mails.

8. Treat chemicals with respect and understand the chemicals you are using. Material Safety Data Sheets (MSDSs) are available in the binders in each room or consult your instructor or the laboratory manager to find them. Do not remove the MSDSs from the binders. Bring the binder to the Biology Lab Manager to request a copy.

9. Learn where the safety and first-aid equipment is located. This includes fire extinguishers, fire blankets, and eyewash stations.

10. Notify the instructor immediately in case of an accident, no matter how small it seems.

11. Students are never permitted in the Biology storage rooms or preparation areas unless given specific permission by their instructor or head of the department/institution.

12. Handle all living organisms used in a laboratory activity in a humane manner. Preserved biological materials are to be treated with respect and disposed of properly.
13. Leave the lab area clean. Put equipment and chemicals away and wipe off the bench top.

Laboratory Usage Education: At a glance

- Know your laboratory well before you actually start working.
- Know functioning/handling of all instruments before actually using them.
- Know what you should not do with the instrument.
- Enter into logbooks of instruments after each use.
- Know the chemicals that you will be handling in each of your study.
- Keep the glassware and plastic wares you need for your study at your disposal well ahead of your actual study time.
- Know the nature and handling of chemicals/reagents that you will be handling.
- Know the dos and do-not in the lab before functioning.
- Know the trouble shooting of instruments well.
- Know the handling of the waste you generate from your study.
- Keep record of your day to day activities in a record book where you enter all information: positive/negative.
- Inform your PI about your progress of study time to time.
## LAB DOS AND DON’TS

<table>
<thead>
<tr>
<th>LAB DOS</th>
<th>LAB DON’TS</th>
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<tbody>
<tr>
<td>1. Know the location of all safety and emergency equipment used in the lab.</td>
<td>1. <strong>NEVER</strong> experiment on your own.</td>
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<tr>
<td>2. Know use of fire extinguishers and their locations.</td>
<td>2. <strong>Do not eat or drink in the lab room</strong> at any time.</td>
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<tr>
<td>3. Know the location of the closest telephone.</td>
<td>3. <strong>Do not chew gum or eat candy during</strong> lab exercises.</td>
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<tr>
<td>4. Familiarize yourself with all lab procedures before doing the lab exercise.</td>
<td>4. <strong>NEVER</strong> add water to an acid or vice-versa.</td>
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<tr>
<td>5. Report <strong>ALL</strong> accidents, hazards or chemical spills to the instructor (<strong>no matter how small</strong>).</td>
<td>5. <strong>Do not</strong> wear contacts in the lab without proper eye protection.</td>
</tr>
<tr>
<td>6. Keep your work area clean and clutter free.</td>
<td>6. <strong>NEVER</strong> smell, taste or touch chemicals.</td>
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<tr>
<td>7. Tie back all long hair and remove dangling jewelry during lab.</td>
<td>7. <strong>NEVER</strong> work in the lab alone.</td>
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<tr>
<td>8. Always be sure that electrical equipment is turned in the &quot;off&quot; position before plugging it into a socket.</td>
<td>8. <strong>NEVER</strong> use electrical equipment around water.</td>
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<tr>
<td>9. Handle all animals with care</td>
<td>9. <strong>NEVER</strong> mix chemicals before asking the instructor.</td>
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<tr>
<td>10. Use extreme care when handling sharp objects.</td>
<td>10. <strong>NEVER</strong> return unused chemicals to the original container.</td>
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<tr>
<td>11. Dispose of all chemicals, broken glass and other lab materials into the proper containers as directed by the instructor.</td>
<td>11. Absolutely <strong>NO PLAY</strong> is allowed in the lab area!</td>
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<tr>
<td>12. When heating liquids in a test tube, always point the test tube away from anyone.</td>
<td>12 <strong>NEVER</strong> leave the lab area without washing your hands.</td>
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<tr>
<td>13. Keep all materials away from open flames.</td>
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Disposal of Wastes:

1. Do not dispose off chemicals in the sink. (Rule of Thumb: If you don’t want to drink it, don’t dump it in the sink). Follow your instructor’s directions for disposal. Be sure to dispose of chemicals in the proper waste collector. Do not mix chemical waste without being instructed to do so. **Any container that is used to collect chemical waste must be properly labeled and closed at all times unless actively pouring into it.**

2. Properly dispose of animal tissue in the **MARKED** Biohazard bags. Never throw animal tissue in lab garbage cans. Your instructor will provide necessary detail.

3. Dispose of broken glass in the cardboard "broken glass box" in your lab. Place “Sharps” (scalpels, needles, razorblades, etc) in the sharps boxes.

4. Color codes for waste disposal

   RED: PLASTIC WARES, SYRRINGES, GLOVES, GEL

   YELLOW: GAUGE, CLOTH, COTTON, CULTURE, PLATE CULTURE DISH

   BLUE: BLADE, SHARP OBJECTS, SCISSOR, GLASS, NEEDLES

*Do not place general trash in the any of the specialized collection containers.*

*Do not let the potential hazards listed above make you afraid to participate in the lab. If instructions are followed and care is taken, the likelihood of an accident is greatly reduced. Labs are usually the most fun part of any science course.*
<table>
<thead>
<tr>
<th>COLOUR</th>
<th>ITEMS</th>
</tr>
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</table>
| ![Red Icon] | PLASTICWARE  
SYRINGES  
GLOVES  
GELS |
| ![Yellow Icon] | GAUGE  
CLOTH  
COTTONCULTURE  
PLATE CULTURE DISH |
| ![Blue Icon] | BLADE  
SHARP OBJECTS  
SCISSOR  
GLASS |
Who to Contact

If you have any questions, the following people are your safety resources:

- Your instructor
- Members of Biology Faculty
- Biology Scientific Officer
- Respective Lab Operators, SBS
First Semester
BIOLOGY
LAB
Laboratory Safety and Introduction to the Biology Lab

Remarks:

Scheduling: First week
Number of students: 60 @15 students/batch/day
Required chemicals: Safety googles, gloves, Spill kit
Required consumables-glass ware: NR
Required consumables-plastic ware: NR
Required equipment: NR

NR: Not required
Experiment 1

Aim of the Exp.: Classification of the Animal Kingdom and identification of animals of different phyla.

Principle: Classification is a method of orderly arrangement of organisms into related groups according to their similarities and dissimilarities, which helps in easy identification of animals and plants. Animal forms and classifications are of great importance to the biologist because it make easy to identify different organisms or different group of animals by studying representatives from each group. Biological classification is based on the binomial system of nomenclature introduced by C. Linnaeus. The first name is the generic name and the second is the specific name.

The general structure of an animal, the arrangement of its organ systems, and the integrated functioning of its parts are referred to as its body plan. Although animal body plans are extremely varied, they can be seen as variations on four key features.

1) The symmetry of the body
2) The structure of the body cavity
3) The segmentation of the body
4) External appendages that move the body.

Depending upon these morphological and anatomical features, the animal kingdom is divided into groups or taxas as follows:

| Kingdom | Phylum | Class | Order | Genus | Species |

The overall shape of an animal can be described by its symmetry. An animal is said to be symmetrical if it can be divided along at least one plane into similar halves. Animals that have no plane of symmetry are asymmetrical. Two types of symmetries are there, radial and bilateral. Radially symmetrical animals can be divided into similar halves by any plane that contains the main axis. A bilaterally symmetrical animal can be divided into left and right halves (mirror image) by a single plane that passes through the midline of its body i.e. anterior to the posterior part.

The animals can be divided into three types according to their body cavity:
- Acoelomate: they lack an enclosed fluid filled body cavity, e.g. flat worms
- Pseudocoelomate: they have a body cavity which is actually fluid filled space in which the internal organs are suspended.
Coelomate: They have a true body cavity that develops within the mesoderm, the coelome is enclosed by both the sides, outside and inside by the mesoderm. Segmentation includes control of movement and also facilitates specialization of different body regions. The appendages that project externally from the body greatly enhance an animal's ability to move around.

Remarks:

Scheduling: week 2, 3, 4
Number of students: 60 @15 students/batch/day
Required chemicals: NR
Required consumables-glass ware: NR
Required consumables-plastic ware: NR
Required equipment: Museum Specimens of different phyla of Animal kingdom
NR: Not required
VISIT TO THE MUSEUM

Scheduling: 5th week
Number of students: 60 @ 15 students/batch/day
Required chemicals: NR
Required consumables-glass ware: NR
Required consumables-plastic ware: NR
Required equipment: NR

OTHER REQUIREMENTS: Institute Bus to make the to and fro trip to National Museum of Natural History, Bhubaneswar, near Acharya Bihar.
Experiment 2: Spectrophotometry

Aim of the experiment: To validate the Beer-Lambert’s Law.

**Principle:** The basic principle of spectrophotometry follows the Beer-Lambert’s law which states that when a ray of monochromatic light of intensity (Io) passes through a solution, the intensity of transmitted light (I) is always less than initial intensity of light mainly due to absorption of light by the solution. The relationship between Io and I depends upon the concentration (c) and the length (l) of the absorbing solution.

Absorbing medium with length l and concentration c will have $I = Io e^{-kl}$

Or, $I / Io = e^{-kl}$

$I / Io = T$ (Transmission)

Taking logarithm

$\log I / Io = -kl$

Or, $\log Io / I = kl$

$\log_{10} Io / I = kcl$

Or, $\log_{10} Io / I = kcl$

$\log_{10} Io / I$ is known as Extinction (E) or Absorbance (A).

Now in any system which obeys Beer-Lambert’s law and which has a constant length, then extinction (E) against concentration will give a straight line passing through the origin while transmission (T) against concentration will give a negative exponential curve.

**Procedure:**

1. Prepare a stock solution of Bromophenol Blue (0.01%).
2. Dilute the stock solution 10 times (Now it is 0.001% stock solution 2).
3. From stock solution 2, prepare different concentration of BPB in 10 test tubes in increasing order.
4. Take absorbance and transmittance of solutions against distilled water blank in ascending order at 600 nm.
5. Plot a graph of absorbance and transmittance against concentration.

**Remarks:**

**Scheduling:** 6th wk  
**Number of students:** 60 @15 students/batch/day  
**Required chemicals:** Bromo phenol Blue  
**Required consumables-glass ware:** Test tubes (150/day), Beakers (15+2), Measuring cylinder (5),  
**Required consumables-plastic ware:** Tips 10ml (15+2), Tips 5ml (15+2)  
**Required equipment:** Accu-Pipettes 10ml (15+2), Accu-Pipettes 5ml (15+2), Spectrophotometer (3+2), Cuvettes (8), balance (1+1). Millipore system
Experiment 3: Study of Buffers

Aim of the Experiment: Preparation of buffers and titration curves.

Principle: Titration is a technique used to determine the concentration of an unknown acid or base. In acid-base chemistry, titration is often used to determine the pH of a certain solution. When a strong base is mixed with a solution of an acid and the pH measured, a plot of the base added against pH recorded can be obtained and this is known as a titration curve.

A buffer is defined as a chemical solution that resists changes in pH upon the addition of acid or alkali. Commonly buffers are mixtures of a conjugate acid and a conjugate base. It plays a crucial role in the maintenance of constant pH, essential for various biochemical reactions. A buffer can be prepared by using Henderson-Hasselbalch equation:

\[
pH = pK_a + \log \frac{[Base]}{[Acid]}
\]

Procedure:

1. Make different titration curve using strong acid / strong base, weak acid/ strong base.

2. Prepare 50 ml of 50 mM phosphate buffer of pH 7.0.

3. Prepare 50 ml of 1.5 M Tris Buffer of pH 8.8.

Remarks:

Scheduling: -------7th wk
Number of students: 60 @15 students/batch/day
Required chemicals: HCl, H₂SO₄, NaOH, KOH, Phosphate Buffer, Tris Buffer, PH Tablets (PH 4, 7 and 9)
Required consumables-glass ware:
Required consumables-plastic ware: Tips 10ml (50tips), Tips5ml(50tips), Tips200microlit(50tips),Tips1ml(50tips)
Required equipment: Magnetic stirrer, Magnetic beads, Accu-pipette-10ml(15+2), Accu-pipette-5ml(15+2), Accu-pipette-1ml(15+2), Accu-pipette-200microlit.( 15+2), balance (1+1).PH meter (2), Millipore system
Others: gloves, graf papers, weighing boats,
Experiment 4: Benedict’s test for reducing sugar

**Principle**
If a suspension of copper hydroxide in alkaline solution is heated, then black cupric oxide is formed:
\[ \text{Cu(OH)}_2 \rightarrow \text{CuO} + \text{H}_2\text{O} \]
However, if a reducing substance is present, then rust brown cuprous oxide is precipitated:
\[ 2\text{Cu (OH)}_2 \rightarrow \text{Cu_2O} + 2\text{H}_2\text{O} + \frac{1}{2} \text{O}_2 \]
In practice, an alkaline solution of a copper salt and an organic compound containing alcoholic –OH is used rather than above suspension. Under these conditions, the copper forms a soluble complex and the reagent is stable. Carbohydrates with a potentially free aldehyde or ketone group have reducing properties in alkaline solution. In addition, monosaccharides act as reducing agent in weekly acid solution.
Benedict modified the original Fehling’s test to produce a single solution which is more convenient for tests, as well as being more stable, than Fehling’s reagent.

**MATERIALS**
1. Benedict’s Reagent-
   Dissolve 173g of Sodium citrate and 100 g sodium carbonate in about 800ml of warm water. Filter through a fluted filter paper into a 1000 ml measuring cylinder and make up to 850ml with water. Meanwhile, dissolve 17.3g of copper sulphate in about 100 ml of water and make upto 150 ml. Pour the first solution into a 2 liter beaker and slowly add the copper sulphate solution with stirring.
2. Glucose solution (or any test solution)
   10 g/liter and 1 g/liter

**METHOD**
Add five drops of the test solution to 2ml of Benedict’s reagent and place in a boiling water bath for 5 min. Examine the sensitivity of Benedict’s test using increasing dilutions of glucose.

**Remarks:**

Scheduling: ------8th wk
Number of students: 60 @ 15 students/batch/day
Required chemicals: Sodium Citrate, Sodium Carbonate, Copper Sulphate, Glucose
Required consumables-glass ware: Test tube, Beakers, Measuring cylinder
Required consumables-plastic ware: NR
Required equipment: Water bath, Thermometer, Accu-Pipette10ml (15+2), Accu-pipette5ml(15+2), balance (1+1), Accu-pipette 1000ml(15+2), Millipore system
Others: gloves, graf papers, weighing boats
Experiment 5: Iodine test for Polysaccharides

Carbohydrates are compounds containing aldehyde or ketone derivatives of higher polyhydric alcohols.

Starch or amylum is a carbohydrate consisting of a large number of glucose units joined together by glycosidic bonds. This polysaccharide is produced by all green plants as an energy store. It is the most important carbohydrate in the human diet and is contained in such staple foods as potatoes, wheat, maize (corn), rice, and cassava.

Pure starch is a white, tasteless and odorless powder that is insoluble in cold water or alcohol. It consists of two types of molecules: the linear and helical amylose and the branched amylopectin. Depending on the plant, starch generally contains 20 to 25% amylose and 75 to 80% amylopectin. Glycogen, the glucose store of animals, is a more branched version of amylopectin.

Starch is processed to produce many of the sugars in processed foods. When dissolved in warm water, it can be used as a thickening, stiffening or gluing agent, giving wheatpaste.
Chemical Test for Starch or Iodine:

Iodine test: This test distinguishes polysaccharides and other carbohydrates. Only iodine element in the presence of iodide ion will give the characteristic blue black color in presence of soluble starch solution. Neither iodine element alone nor iodide ions alone will give the color result.

Polysaccharides will react with iodine to form a blue, red, violet, or purple product which is a positive indicator of the presence of a polysaccharide.

Amylose in starch is responsible for the formation of a deep blue color in the presence of iodine. The iodine molecule slips inside of the amylose coil.

PRINCIPLE

Iodine forms colored adsorption complexes with polysaccharides, starch gives a blue color with iodine while glycogen and partially hydrolyzed starch react to form red-brown colors.
Reagents:

Iodine - KI Reagent: Iodine is not very soluble in water; therefore the iodine reagent is made by dissolving iodine in water in the presence of potassium iodide. This makes a linear tri-iodide ion complex with is soluble. The tri-iodide ion slips into the coil of the starch causing an intense blue-black color.

Solution:

1. Iodine solution: 5mM/litre
2. Potassium Iodide Solution: KI (30g/litre)
3. Starch, Glucose, Fructose, Lactose, Sucrose, Inulin (10g/l)

Procedure:
1. Label 7 test tubes (1-7) and place them on a test tube rack.
2. To each test tube add 1 ml (20 drops) of each of the solutions of fructose, glucose, lactose, starch, sucrose, your unknown, and water.
3. Add 2 drops of iodine in each test tube. Mix. Record your observation on the table below.
Remarks:

<table>
<thead>
<tr>
<th>Tube</th>
<th>SOLUTION</th>
<th>IODINE SOLUTION</th>
<th>OBSERVATION</th>
<th>INERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
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</tbody>
</table>

**Scheduling:** ------9th wk  
**Number of students:** 60 @15 students/batch/day  
**Required chemicals:** Pottassium iodide, Starch, Glucose, Sucrose, HCl (1N)  
**Required consumables-glass ware:** Test tubes, Beakers  
**Required consumables-plastic ware:** Tips (5ml, 10 ml, 100 pcs)  
**Required equipment:** Accu-pipette 10ml, Accu-pipette 5ml, balance (1+1=2), Millipore system  
**Others:** gloves, graf papers, weighing boats,
Experiment 6: Quantitative determination of carbohydrates

(Estimation of Carbohydrate by the anthrone method)

PRINCIPLE

The anthrone reaction is the basis of a rapid and convenient method for the determination of hexoses, aldopentoses and hexuronic acids, either free or present in polysaccharides. The blue-green solution shows an absorption maximum at 620 nm, although some carbohydrates may give other color. The reaction is not suitable when proteins containing a large amount of tryptophan are present, since a red color is obtained under these conditions. The extinction depends on the compound investigated, but is constant for a particular molecule.

MATERIAL
1. Anthrone reagent (2 g/liter in conc. H₂SO₄) care!!!!
2. Glucose (0.1 g/liter)
3. Glycogen (0.1g /liter)
4. Any other carbohydrate

METHOD
Add 4 ml of the anthrone reagent to 1ml of a protein-free carbohydrate solution and rapidly mix (care!!!). Place the tube in a boiling water bath for 10 min with a marble on top to prevent loss of water by evaporation, cool and read the extinction at 620 nm against a reagent blank. Prepare standard curves for the glucose and glycogen solutions and compare them. Remember that glucose exists as the glycoside form (C₆H₁₀O₅) in glycogen of mol wt 162, not 180. Examine the purity of a number of samples of commercial glycogen.

Remarks:

Scheduling: ------10th wk
Number of students: 60 @ 15 students/batch/day
Required chemicals: Anthrone reagent (2 g/liter in conc. H₂SO₄), Glucose (0.1 g/liter)
Glycogen (0.1g /liter), Any other carbohydrate
Required consumables-glass ware: 2litre beaker, beaker (100 ml ) nos 30 each day, test tube (75/day), glass pippetes 1, 5, 10 ml (3 nos each, 45 nos total)
Required consumables-plastic ware: Tips (5 ml), 60 nos, suckers for acid sucking (2 nos each=32 nos)
Required equipment: spectrophotometer (3+2) Autopipettes (5 ml) nos 15+2, water bath, marble, cuvettes (8), balance (1+1=2)
Others: gloves, graf papers, weighing boats, filter papers
Experiment 7: Quantization of proteins

Aim of the Experiment: Protein Estimation by Lowry Method

Principle: Proteins present in the sample reacts with Biuret and Folin – ciocalteau reagents to give a blue color complex. The blue color so formed is due to the i) the reaction of the peptide bonds of the protein with cupric copper under alkaline conditions as in biuret reaction and ii) the reduction of phosphomolybdate by tyrosine and tryptophan residues present in the protein.

Reagents:
1. Standard Protein solution: Bovine serum albumin (BSA) 1mg/ml of distilled water. Dilute five times for working standard solution.
2. Biuret Reagent: Mix 2% Na$_2$CO$_3$ in 0.1 N NaOH + 0.5% Cu SO$_4$ + 1% sodium potassium tartarate in 100:2:2 ratio.
3. Folin-ciocalteau reagent: Dilute the commercially available reagent three times before use.

Procedures:
1. Take different concentrations of working standard protein solutions in duplicates.
2. Make the volume to 0.5 ml with D.W.
3. In the blank test tubes take 0.5 ml of D.W. only.
4. Add 5 ml of Biuret reagent to all the tubes.
5. Mix thoroughly and allow them to stand for 10 min. at room temperature.
6. Then add 0.5 ml of Folin reagent, mix thoroughly and keep for 30 min. at room temperature.
7. Take absorbance at 700 nm and plot a standard curve taking different concentration of standard protein on X-axis and their respective absorbance on Y-axis.
Remarks:

Scheduling: ------11th wk
Number of students: 60@15 students/batch/day
Required chemicals: Folins Reagent, Biuret Reagent,
Required consumables-glass ware: Test tubes (100 pcs), beakers (30 nos)
Required consumables-plastic ware: tips (5 ml, 10 ml)
Required equipment: Spectrophotometer, cuvettes (8), balance (1+1=2), Millipore system
Others: gloves, graf papers, weighing boats, filter papers
Experiment: 8

Aim of the Experiment: Protein Estimation by Bradford Method

Principle:

The Bradford protein assay is one of the simple methods commonly used to determine the total protein content of a sample. The method is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change.

![CBB G-250](image)

Bradford reagent: Dissolve 100 mg Coomassie Brilliant Blue G-250 in 50 ml of 95% ethanol; add 100 ml of 85 % (w/v) orthophosphoric acid. Add distilled water to a final volume of 1 liter. Filter with Whatman No. 1 paper if any precipitate occurs.

Procedure:

1. Prepare a series of protein samples in test tubes with different concentration.
2. Add 5 ml of Coomassie Brilliant Blue G-250 solution to each test tube.
3. Mix well and allow the color to develop for at least 5 min. The brown-red dye turns blue when it binds protein.
4. Measure the absorbance at 595 nm.
5. Plot a standard curve using the standard protein absorbance verses concentration.
6. Calculate the protein in the experimental sample using standard curve.

Remarks:

Scheduling: -------12th wk
Number of students: 60@15 students/batch/day
Required chemicals: 100 mg Coomassie Brilliant Blue G-250 in 50 ml of 95% ethanol; 100 ml of 85 % (w/v) orthophosphoric acid, whatman filter paper
Required consumables-glass ware: Beakers, reagent bottles, glass pipettes
Required consumables-plastic ware: tips (5 ml), 10 ml, 200 microlitre, 10 microlitre nos 100/day/each
Required equipment: Spectrophotometer, cuvvetes (8), balance (1+1=2), Millipore system
Others: gloves, graf papers, weighing boats, filter papers
Experiment 9: Chromatography

Aim of the Experiment: Separation of Amino Acids by Paper Chromatography and their detection by Ninhydrin reagent.

Principle: Amino acids are separated based on their partition or distribution coefficients, between the liquid stationary phase (water held by the chromatographic paper) and the liquid mobile phase. Partition or distribution coefficient describes the way a compound (the analytes) distributes between two immiscible phases and at equilibrium the ratio of the concentrations of the substance in the solvents is constant. During the chromatographic separation the analytes continuously pass back and forth between the two phases so that differences in their distribution coefficients result in their separation. The amino acids separated on chromatographic paper are demonstrated by Ninhydrin reagent and identified by their retardation factor (Rf) value.

\[ Rf = \frac{\text{Distance moved by the compound from the origin}}{\text{Distance moved by the mobile phase from the origin}} \]

Rf value is more or less constant for a particular solvent system.

Procedure:
1. Take Whatman No.1 filter paper of appropriate size and handle the paper with disposable gloves.
2. Draw a pencil line across the paper denoting the point of origin.
3. Spot the amino acid solution with the help of a capillary tube or auto pipette (10µl) carefully so that it should not exceed 3-4 mm in diameter.
4. Place the paper in chromatographic chamber containing the mobile phase (n-butanol: acetic acid: water (3:1:1) in such a manner that sample spots are 2 cm above the solvent level.
5. After the run, the paper is removed and the solvent front is marked using a pencil.
6. Let the paper air dried at room temp. and then dipped in ninhydrin reagent (200 mg ninhydrin in 99 ml of acetone and 1 ml of acetic acid).
7. The chromatogram is allowed to develop in a hot air oven at 50 °C.
8. Alpha amino acids show purple color while imino acids give yellow color.
9. Outline the spots with a pencil and measure the distance in cm from the origin to the centre of the outlined spot.
10. Calculate the Rf value for the separated amino acids.

If time permits one can isolate chlorophyll from leaves and check their mobility

Remarks:

Scheduling: ------13th wk
Number of students: 60@15 students/batch/day
Required chemicals: Whatman chromatography paper, Butanol, acetic acid, water, Ninhydrin, acetone, amino acid standard kit, petroleum ether, acetone
Required consumables-glass ware: Beakers 500 ml: (15+1), Cover plates (15+1), glass rods (50/day), capillary tubes (50/day)
Required consumables: plastic ware: microcentrifuge tubes (100 nos)
Required equipment: autopippettes (20 and 100 microlitre, nos), oven (1), balance (1+1=2), Millipore system, Hot air oven
Others: gloves, graf papers, weighing boats, filter papers
Experiment 10: Study of DNA

Aim of the experiment: Isolation of genomic DNA and separation of DNA by Agarose Gel Electrophoresis.

Plants contain three types of DNA, i.e. nuclear, mitochondrial and chloroplast DNA. Most nucleic acid isolation protocol involves following four steps:

a) Cell lysis: Nucleic acids must be solubilized from cells. This solubilization is usually carried out under denaturing conditions. The denaturing conditions promote the removal of proteins during subsequent steps and inhibit the activity of nucleases that degrades the nucleic acids.

b) Enzymatic Treatments: Unwanted components like proteins and RNA are degraded by enzymes.

c) Differential solubility: Phenol is an organic solvent used to separate proteins from DNA. It is mixed in equal volumes to the DNA. The two phases are then separated by centrifugation and the upper aqueous phase that contains the nucleic acid is retained. Proteins are seen as flocculent material at the interface.

d) Precipitation: DNA is precipitated from dilute solutions with ethanol in presence of sodium acetate; pH 5.0-5.5, added to the final concentration of 0.3 M Sodium and acidic pH will neutralize the highly charged phosphate backbone and promote hydrophobic interactions. The precipitated DNA is collected by centrifugation. The pellet is rinsed with 70% ethanol to remove any excess salt, dried and dissolved in TE buffer.

Stock Preparations:

1. **1M Tris:** Dissolve 12.11 gm of Tris base (m.w.121.1) in 80 ml of distilled water. Adjust the pH to 8.0 with HCl. Then make up the volume up to 100 ml. Sterilize by autoclaving.  
2. **0.5M EDTA:** Dissolve 18.61 gm of EDTA (m.w. 372.2) in 80ml of distilled water. Adjust the pH to 8.0 by adding NaOH pellets to it. Finally make up the volume up to 100 ml. Sterilize by autoclaving.  
3. **2x CTAB:** Dissolve 2 gm of CTAB in 70 ml of distilled water, to it add 8.177 gm of NaCl. Then add 10 ml of 1 M Tris (pH 8.0) and 4 ml of 0.5 M EDTA (pH 8.0). Make up the vol. upto 100 ml. Sterilize by autoclaving. (Add 2% beta Mercaptoethanal to it before extraction on DNA.)  
4. **0.1X T.E.:** Add 0.02 ml of 1 Tris (pH 8.0) and 0.004 ml of 0.5 M EDTA (pH 8.0). Finally make up the volume up to 20 ml. Sterilize by autoclaving.  
5. **Chloroform: Isoamyl alcohol:** Add 24 ml of Chloroform and 1 ml of Isoamyl alcohol. Prepare it fresh and store in 4 degrees. 
6. **Gel loading dye:** Add 40 gm of sucrose to 100 ml distilled water, to it add 25 gm of Bromophenol blue. Mix it well. Store in 4 degrees.  
7. **TBE Buffer:** Take 300 ml of distilled water in a beaker. To it add 54 gm of Tris base (m.w.121.1) and after it dissolves, add 27.5 gm of Boric Acid (m.w.61.83). When the Boric
acid completely dissolves add 4.6 gm of EDTA (m.w. 372) and mix well. Adjust the pH to 8.2. Finally make up the volume to 500 ml. Sterilize by autoclaving.

Procedure:
1. Wash the leaf with sterile water and rinse it with alcohol. Take around 10 to 15mm of leaf disc and place it in 1.5ml sterile vial.
2. Cut the leaf into fine pieces and with the help of glass rod & porcelain plate grind them in presence of 200μl CTAB buffer. Then add 300μl more of CTAB buffer. And transfer them with the help of a cut tip into a 1.5ml centrifuge tube.
3. Add 500μl of pre-cooled solution of chloroform isoamyl alcohol 24:1
6. Centrifuge for 5 mins. at12,000 rpm for at room temperature.
7. Take the supernatant and add 1ml of cold alcohol. Mix gently by inverting the vial and incubate at -20°C for 30 minutes.
8. Centrifuge the vials at 12,000rpm for 20 minutes at 4°C temperature, drain out the supernatant and blot dry. Care should be taken not to dislodge the pellet. A white/ pigmented particulate matter sticking to the side of the vial indicates precipitated DNA.
9. Add 200μl of 70% alcohol and spin at 12,000 rpm for 4minutes. Drain out the supernatant completely and blot dry.
10. Suspend the DNA pellet in minimal amount of solution of 0.1X TE buffer 30 min.
11. Incubate the vial at 55°C for 3 minutes for complete solubilization.
12. Spin at 12,000 rpm for 10 minutes to remove any insoluble material and collect the supernatant in a sterile 1.5ml vial.
13. Add 3 microlit. of gel loading buffer to the DNA sample.
14. Load the samples along with 101-11 of control DNA on a 1 % agarose gel containing Ethidium Bromide (0.5g/ml).
15. Run the gel at 50-100 volt for 1-2 hours and observe under UV-Transilluminator.

Remarks:

Scheduling: -------14th week
Number of students: 60@15 students/batch/day
Required chemicals: ethidium bromide, agarose, Tris base, EDTa, Nacl, C TAB, Beta mercaptoethanol, ethanol, chloroform, isoamylalcohol, bromophenol blue, sucrose, buffer capsules.
Required consumables-glass ware: measuring cylinder, reagent bottles,
Required consumables-plastic ware: plastic racks, scissors, glass rods, porcelain plates, microcentrifuge tubes (1.5 ml), parafilm: tips (5 ml), 10 ml, 200 microlitre, 10 microlitre nos 100/day/each
Required equipment: cooling centrifuge, UV-Transilluminator, gel apparatus, power packs, accupippetes 920 microlitre, 10 microlitre, 200 microlitre, 1000 microlitre, 5 ml, 10 ml), balance (1+1=2), Millipore system, Gel doc system ,PH meter (2)
Others: gloves, weighing boats, filter papers

Experiment 11: Study of separation of proteins

Aim of the experiment: Protein Separation by SDS-PAGE

Principle: Electrophoresis is an important technique for separation of proteins/ nucleic acids based on the migration of charged micromolecules in an electric field. The force moving the macromolecule is the electrical potential $E$. The Electrophoretic mobility of the molecule $\mu$ is the ratio of the velocity of the particle $V$, to the electric potential. Electrophoretic mobility ($\mu$) is also equal to the net charge of the molecule $Z$, divided by the frictional coefficient $f$.

$$\mu = \frac{V}{E} = \frac{Z}{f}$$

Electrophoresis of proteins is generally carried out in gels made up of the cross-linked polymer poly acrylamide. The poly acrylamide gel acts as a molecular sieve, slowing the migration of proteins approximately in proportion to their charge-to-mass ratio. The detergent sodium dodecyl sulfate (SDS) is generally used for determination of molecular weight of proteins. One molecule of SDS binds with every two amino acid residues with hydrophobic interactions and contributes a large negative charge, rendering the intrinsic charge of the protein insignificant and conferring on each protein a similar charge-to mass ratio. In addition, the native conformation of a protein is altered when SDS is bound and most proteins assume a similar shape. After electrophoresis, the proteins are visualized by adding a dye Coomassie brilliant blue R-250, which binds to protein.

Reagents: 5x Sample buffer / gel loading buffer:
- 50 mM Tris buffer pH 6.8
- 2 % SDS
- 1 mg/ml bromophenol
- 10 % glycerol
- 5 % beta-mercaptoethanol

1x Gel running buffer (Tris Glycine buffer pH 8.3):
- 25 mMTRIS
- 250mM Glycine
- 0.1 % SDS

Stacking gel solution (pH 6.8, 5 % acrylamide) 5 ml:
- H2O- 3.4ml
Acrylamide; 0.83 ml 1 M
Tris-buffer pH 6.8: - O. 3 ml
10 % SDS:- 0.05 ml
10% APS: -0.05 ml
TEMED:- 0.005ml

**Resolving gel solution (pH 8.8, 8% Acrylamide)** 10 ml
H2O – 4.6ml
Acrylamide – 2.7 ml
1.5 mM Tris- buffer pH8.8, 2.5ml
10% SDS – 0.1ml
10%APS – 0.1ml
TEMED – 0.007ml

Staining solution 100ml: Methanol: Acetic acid: water(40: 7:53)
250mg CBB R-250
Destaining solution100ml ; Acetic acid: water(5: 7:88)

**Procedure:**

- Assemble the glass plate properly.
- Mix the components of resolving gel and pour the acrylamide solution into the gap between the glass plates and leave sufficient space or the stacking gel. Overlay the acrylamide solution with isobutanol distilled water.
- After polymerization is complete, pour off the overlay and wash the top of the ‘ei with deionized water to remove any unpolymerized acrylamide.
- Mix the components of stacking gel and pour the solution directly onto the surface or the polymerized resolving gel. Immediately insert a clean Teflon comb into the slacking gel. Place the gel in a vertical position at room temperature.
- While stacking gel is polymerizing, prepare the samples mixed with gel loading buffer and heat them to 100 °c for 3 min to denature the protein.
- After polymerization is complete, remove the comb carefully and wash the wells immediately with deionized water to remove the unpolymerized acrylamide.
- Mount the gel in the electrophoresis apparatus. Add Tris-glycine electrophoresis buffer to the top and bottom reservoirs.
- Load 20μl of each of the samples into the bottom of the wells and in one well load protein marker.
- Attach the electrophoresis apparatus to an electric supply and apply 8 Y/cm to the gel. After dye front has moved into the resolving gel, increase the voltage 15 Y/cm and run the gel until the bromophenol blue reaches the bottom of the resolving gel. Then turn off the power supply.
- Remove the glass plate from the electrophoresis apparatus and place them on a paper towel. Use all extra gel spacer to carefully pry the plates apart. Mark the orientation of the gels by cutting corner from the bottom of the gel that closest to the leftmost well.
- Immerse the gel in staining solution and place on a slowly rotating platform for 1 2 h at room temperature.
➢ Remove the stain and destain the gel in destaining solution for 2 h.
➢ Take the photograph of the stained gel and calculate the molecular weight of specific protein.

Remarks:

Scheduling: -------15th wk

Number of students: 60@15 students/batch/day

Required chemicals: Tris buffer, SDS, bromophenol blue, glycerol, beta-mercaptoethanol, Glycine, APS, Acrylamide, bis-Acrylamide, TEMED, Methanol, Acetic acid, buffer capsules

Required consumables-glass ware: measuring cylinder, reagent bottles, beakers 100ml, 50ml,250ml.

Required consumables-plastic ware: plastic racks, centrifuge tubes (1.5 ml), parafilm, centrifuge tubes 1.5ml, tips (5 ml), 10 ml, 200 microlitre, 10 microlitre nos 100/day/each

Required equipment: gel apparatus, power packs, accupippetes 20 microlitre, 10 microlitre, 200 microlitre, 1000 microlitre, 5 ml, 10 ml, 0.5-10microlit. Piettes, balance (1+1=2), Millipore system, Gel doc system, PH meter (2)

Others: gloves, weighing boats, filter papers
THIRD SEMESTER

BL301: MICROBIOLOGY LABORATORY
CREDITS -2
BL301: MICROBIOLOGY LABORATORY  CREDITS — 2

Laboratory Handout

Instructor — Dr. Harapriya Mohapatra

Projections:

- **Total no. of classes** @ 2/week = 7 classes (microbiology needs to have at least 2 consecutive classes in a week in order to make the students have a hands-on experience in microbiological technique. Total = 13 weeks for 2 laboratory courses of 2 credits each)
- **Total no. of students** = 14

<table>
<thead>
<tr>
<th>Exp.No.</th>
<th>Name of experiments</th>
<th>Tentative schedule</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Orientation &amp; General laboratory safety Guidelines</strong></td>
<td>1st week</td>
</tr>
<tr>
<td>1.</td>
<td>Culture media preparation, control of microbial growth disinfection &amp; sterilization.</td>
<td>1st week</td>
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</tbody>
</table>
| 2.      | Enrichment and isolation and characterization of pure culture of selective and differential media  
2.1 – spread plate technique  
2.2 – streak plate method | 2nd week |
| 3.      | Microscopic examination of fresh culture with different staining procedure  
3.1 – gram positive and gram negative staining  
3.2 – Endospore staining | 3rd week |
<table>
<thead>
<tr>
<th></th>
<th>Culture dependent analysis of microbial communities</th>
<th>4&lt;sup&gt;th&lt;/sup&gt; &amp; 5&lt;sup&gt;th&lt;/sup&gt; week</th>
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</thead>
<tbody>
<tr>
<td>4.1</td>
<td>use of differential, selective and enriched media</td>
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<tr>
<td>4.2</td>
<td>determination of extra cellular enzymatic activities of microorganisms by (a) starch hydriolysis (b) lipid hydrolysis (c) casein hydrolysis (d) Gelatin hydrolysis</td>
<td></td>
</tr>
<tr>
<td>4.3</td>
<td>use of representatives biochemical test</td>
<td></td>
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<tr>
<td>4.3.1</td>
<td>TSI</td>
<td></td>
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<tr>
<td>4.3.2</td>
<td>IMViC test</td>
<td></td>
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<tr>
<td>4.3.3</td>
<td>Nitrate reduction test</td>
<td></td>
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<tr>
<td>4.3.4</td>
<td>Urease, Catalase and Oxidae test</td>
<td></td>
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<tr>
<td>5.</td>
<td>Culture independent test analysis of microbial communities by 16srDNA sequencing method</td>
<td>6&lt;sup&gt;th&lt;/sup&gt; week</td>
</tr>
<tr>
<td>6.</td>
<td>Identification of genus of unknown bacterial cultures</td>
<td>6&lt;sup&gt;th&lt;/sup&gt; week</td>
</tr>
<tr>
<td>7.</td>
<td>Antibiotic susceptibility testing:</td>
<td>7&lt;sup&gt;th&lt;/sup&gt; week</td>
</tr>
<tr>
<td>7.1</td>
<td>Disk diffusion</td>
<td></td>
</tr>
<tr>
<td>7.2</td>
<td>MIC by tube dilution</td>
<td></td>
</tr>
</tbody>
</table>
Aim 3.2: To utilize differential staining for visualization of bacterial spore.

**Principle/Theory:** tinder unfavorable environmental conditions certain strains of bacteria undergo sporogenesis and give rise to endospore. Unlike the vegetative cells the spore cells due to their impervious coat does not accept primary stains easily by common procedures. The primary stain used for spore staining is malachite green and for its penetration moist heat is applied with replenishing the stain to prevent its evaporation. The deco lionizing agent used here is water, As the spore once accepts malachite green it cannot be colorized with water, instead the vegetative components which are stained with low affinity get decolorized. The counter stain used is safranin which colors the vegetative cells.

**Material & Reagents required:** End spore forming bacterial culture, Malachite green (5% solution in water), Bunsen burner, hot plate, water beaker, inoculating loop, staining tray, tissue paper, glass slides, and microscopes.

**Procedure:**

1. Prepare bacterial smear; air dry and heat fix them.
2. Flood smears with malachite green and place on top of a beaker of water sitting on a warm hot plate, allowing preparation to steam for 2-3 minutes. **Caution:** Do not allow stain to evaporate, replenish stain as needed. Prevent the stain from boiling by adjusting the temperature of the hot plate.
3. Remove slides from hot plate, cool and gently wash under running tap water.
4. Counter stain with safranin for 30 seconds
5. Wash with tap water.
6. Blot dry with tissue/blotting paper and observe under oil immersion.

**Observation:-**

(i) Make drawing of representative microscopie field
(ii) Describe the location of the endospore within the vegetative cell as being central, sub terminal or terminal
(iii) Indicate color of the spore and vegetative cell on each preparation.
Experiment 4: Culture dependent analysis of microbial communities

Aim 4.1: Enrichment and isolation and characterization of pure culture. Use of selective and differential media.

Objective: (i) to understand the use & function of specialized media for selection and differentiation of microorganisms, (ii) To observe how an enriched media can also function both as a selective and differential medium.

Principle:
Special purpose media are used for specific purposes such as isolation of bacteria from mixed population, differentiation among closely related groups and for characterization and identification of bacteria by their abilities to produce chemical changes in different media.

Selective media are used to select specific group of bacteria, they incorporate chemical substances that inhibit growth of one type of bacteria while permitting growth of another thus facilitating isolation. The selective media we would be using. Is 7.5% Sodium chloride. agar ‘N nich allows only halophilic organisms to grow inhibiting others.

Differential media incorporate chemical compounds that following incubation produce characteristic change in appearance of bacterial growth and or the medium surrounding the colonies. These are used to distinguish morphologically & biochemically related groups of organisms. We would be working with 2 different media

(i) MacConkey agar which contains crystal violet that inhibits growth of gram positive organism and allows growth of gram negative bacteria. Incorporation of lactose, bile salts and pH indicator neutral red permits differentiation of enteric bacteria on the basis of their ability to ferment lactose. Coliform bacilli such as Ecoli produce acid as a result of lactose fermentation. When this occurs the colonies colonies become red as the acid precipitates bile salts followed by absorption of neutral red.

(ii) Fosin Methylene Blue agar which utilizes lactose and the dyes eosin and methylene blue to permit differentiation between enteric lactose fermenters and non-fermenters as well. As identification of colon bacillus E. colt that produces blue-black colonies with a metallic green sheen caused by large quantities of acid produced which precipitates the dyes onto growth’s surface.

Enriched media are supplemented with highly nutritious material such as blood, serum or yeast extract to facilitate growth of Fastidious Organisms. We would be using blood agar.

Media preparation: per designated student one plate each of the following media
<table>
<thead>
<tr>
<th>7.5% sodium chloride agar (g/L)</th>
<th>MacConkey agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef extract 3.0</td>
<td>Dehydrated ready to use media will be</td>
</tr>
<tr>
<td>Peptone 5.0</td>
<td>prepared as per the manufacture’s instruction.</td>
</tr>
<tr>
<td>Sodium chloride 7.5</td>
<td></td>
</tr>
<tr>
<td>Agar 20</td>
<td></td>
</tr>
<tr>
<td>pH 7.0</td>
<td></td>
</tr>
</tbody>
</table>

| Eosin Methyl Blue Agar                             | Blood agar (g/L)                      |
| Dehydrated ready to use media will be prepared per the manufacturer’s instruction | Beef heart infusion 500              |
|                                                    | Tryptose 10.0                         |
|                                                    | Sodium chloride 5.0                   |
|                                                    | Agar 20                               |
|                                                    | pH 7.3                                |

Dissolve the above ingredients and autoclave. Cool the sterile blood agar base to 45°C, aseptically add 50 ml of sterile defibrinated blood, and mix thoroughly avoiding accumulation of air bubbles. Dispense in appropriate vessels.

Procedure:
1. Prepare media and sterilize it as per the directions given.
2. Divide bottom of each plate into 4 quadrants label with name of the organism.
3. Using sterile inoculation needle inoculate the plates as directed with one culture in each quadrant.
4. Incubate the plates overnight & record your observations.

Observation:
1. Note the amount of growth along the line of inoculation as follows: 0 = none, 1+ scant and 2+ = moderate to abundant.
2. Appearance of the growth: coloration and transparency.
3. Note the change in appearance of the medium surrounding growth: coloration, transparency etc.
Aim 4.2: Determination of extracellular enzymatic activities of microorganisms by (a) Starch hydrolysis (b) Lipid hydrolysis (iii) Casein hydrolysis (iv) Gelatin hydrolysis.

Principle:

(a) Starch hydrolysis: Its degradation requires an extracellular enzyme amylase which breaks down glycosidic bonds hydrolysing the starch to dextrins, that are finally hydrolyzed to maltose and then to glucose by the C1171C maltase. The starch agar plates are nutrient agar media supplemented with soluble starch upon which the microorganism is inoculated. Following incubation, the plate is flooded with iodine that imparts blue-black color to the medium indicating presence of starch. A clear zone indicates zone of hydrolysis and a positive result.

(b) Lipid hydrolysis: Degradation of lipids such as triglycerides is accomplished by extracellular enzyme lipase. Which cleaves ester bonds to form glycerol and fatty acids? The nutrient agar medium is supplemented with tributyrin that forms an emulsion; producing an opaque medium. Following inoculation and incubation, a positive organism develops a zone of lipolysis demonstrated by a clear area surrounding the bacterial growth.

(c) Casein hydrolysis: Casein is a major component of milk-protein. Its degradation involves breaking down of polypeptide bonds, in a process called proteolysis, which is mediated by enzyme protease. The medium contains nutrient agar supplemented with milk powder that gives medium its color and opacity. Lipon inoculation and incubation a positive culture will exhibit a clear zone of proteolysis due to hydrolysis of easeill to noncolloidal amino acids.

(d) Gelatin hydrolysis: Liquefaction of gelatin; is accomplished by enzyme gelatinase. Below temperature of 25°C gelatin will remain in gel state and above 25°C will remain in sol state. Upon degradation even at low temperatures it will remain in sol state. Nutrient agar medium supplemented with 12% gelatin in deep tubes is inoculated and incubated for 48 hours. Then the cultures are refrigerated at 4°C. For 30 minutes. A liquefied culture represents a positive reaction and rapid gelatin hydrolysis. Following incubation for longer period say 5 days & repeating the above process, a liquefaction denotes slow gelatin hydrolysis.
**Material, Reagents and methods:** Starch, lipid and casein agar plates (100 mL/group); gelatin deep tubes (251111/group); Gram’s iodine solution, bacterial cultures

### Preparation of Starch agar

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>5.0 g/L</td>
</tr>
<tr>
<td>Beef extract</td>
<td>3.0 g/L</td>
</tr>
<tr>
<td>Soluble starch</td>
<td>2.0 mL</td>
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<tr>
<td>Agar</td>
<td>20 g/L</td>
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<tr>
<td>pH</td>
<td>7.0 ± 0.2</td>
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</table>

### Preparation of milk-agar

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>Peptone</td>
<td>5.0 g/L</td>
</tr>
<tr>
<td>skim milk powder</td>
<td>100 g/L</td>
</tr>
<tr>
<td>agar</td>
<td>20 g/L</td>
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<tr>
<td>pH</td>
<td>7.2</td>
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### Preparation of Tributyrin agar

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Peptone</td>
<td>5.0 g/L</td>
</tr>
<tr>
<td>Beef extract</td>
<td>3.0 g/L</td>
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<tr>
<td>Tributyrin</td>
<td>10 g/L</td>
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<tr>
<td>Agar</td>
<td>20 g/L</td>
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<td>pH</td>
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</table>

### Preparation of Nutrient gelatin

<table>
<thead>
<tr>
<th>Component</th>
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<tr>
<td>Peptone</td>
<td>5.0 g/L</td>
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<tr>
<td>Beef extract</td>
<td>3.0 g/L</td>
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<tr>
<td>Gelatin</td>
<td>120 g/L</td>
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<tr>
<td>pH</td>
<td>6.8</td>
</tr>
</tbody>
</table>

### Procedure:

1. Prepare starch agar, tributyrin agar and milk agar plates. 2 plates per medium divide the bottom of each petri dish into 2 sections.
2. Using sterile technique make a single line streak inoculation of each test organism on agar surface of its appropriately labeled section.
3. Using sterile technique inoculate each experimental organism in its appropriately labeled gelatin deep tube by means of stab inoculation.
4. Incubate all plates in an inverted position for 24 to 48 hours at 37°C. Incubate the gelatin deep tube cultures for 48 hours.

### Observation:

1. After incubation flood the **starch agar plate**, with Gram's iodine solution, allow the iodine to remain in contact with medium for 1-2 minutes & pour off the excess. Examine the cultures for the presence or absence of blue-black color surrounding the growth of each test organism.
2. Examine the **tributyrin agar plate** cultures for the presence or absence of a clear area or

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zone of lipolysis surrounding the growth of each of the organisms.

(iii) Examine the milk agar cultures for the presence or-absence of a clear area or zone of proteolysis surrounding the growth of each of the bacterial test organisms.

(iv) Place all gelatin deep tube cultures into refrigerator at 4°C for 30 minutes. Examine all the cultures to determine whether the medium is solid or liquid. Based on the observation record gelatin hydrolysis ability and rate of hydrolysis of different organisms.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Starch hydrolysis</th>
<th>Tributyrin hydrolysis</th>
<th>Casein hydrolysis</th>
<th>Gelatin liquefaction (+) or (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Appearance of medium</td>
<td>Result</td>
<td>Appearance of medium</td>
<td>Result</td>
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BL301: MICROBIOLOGY LABORATORY

Laboratory Handout

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Instructor — Dr. Harapriya Mohapatra

Aim 4.3.2: To utilize IMViC test (Indole Methyl red, Voges-Proskauer and Citrate utilization) to distinguish between different groups of Enterobacteriaceae.

Principle: Principles of all the four tests are as follows:

**Indole Production test:** The SIM agar medium contains amino acid tryptophan. Tryptophan is hydrolyzed to indole by enzyme tryptophanase by selected microorganisms, which serves as a biochemical marker. The presence of indole is detectable by adding Kovacs reagent that produces cherry red reagent layer indicating a positive reaction. This color is produced by Kovac's reagent that is composed of p-dimethyaminobenzaldehyde, butanol and I ICI. Indole is extracted from the medium into the reagent layer by the acidified butyl alcohol component and forms complex with p-dimethyaminobenzaldehyde, yielding cherry red color.

SIM agar may also be used to detect motile of microorganisms. Motility is recognized when culture growth (turbidity) is not restricted to the line of inoculation. Growth of non-motile organisms is confined to the line of inoculation.

SIM agar is also used to detect HiS production. Ferrous ammonium sulfate in the medium combines with forming an insoluble black FeS that is seen along the stab inoculation, indicating positive reaction for ILS production.

(ii) **Methyl red test:** This is helpful in differentiating particularly between E. coli and E. aerogenes. The end product of glucose oxidation serves as the differentiating marker. Both these organisms initially produce organic acid and products during the early incubation period. The low acidic pH 4.0 is stabilized and maintained by E. coli at the end of incubation. But, during later incubation E. aerogenes enzymatically converts these acids to non-acidic end products such as 23-butanediol and acetoin (acetylmethylcarbinol) resulting in an elevated pH 6.0 (approx.). Methyl red indicator in acidic pH turns red indicating a positive test while at higher pH turns yellow indicating a negative test.

(iii) **Voges-Proskauer test:** Determines the capability of microorganisms to produce non-acidic or neutral end products, such as acetylmethylcarbinol, from organic acids that result from glucose metabolism. The Barrett’s reagent used in the test consists of alcoholic a-napthol and 40% potassium hydroxide solution. The acetaldehyde in presence of catalyst a-napthol and guanidine group of peptone from the medium, form a diacetyl compound which gives the pink coloration, 15 minutes after the addition of Barrett's reagent. This indicates a positive reaction.

(iv) **Citrate utilization test:** The ability of microorganisms to utilize citrate as carbon source depends upon presence of enzyme citrate permease that facilitates transport of citrate into the cell. It is a major product of Kreb's cycle. The enzyme citrase degrades citric acid to oxaloacetic acid and acetate; these are finally converted to pyruvic acid and CO2, due to which the medium becomes alkaline (CO, combines with sodium and water to form sodium bicarbonate). Indicator bromothymol blue under alkaline conditions changes from green to deep prussian blue accompanied by growth of organisms on surface of slants. This reaction indicates a citrate positive colony.
Material, Reagents and methods: Per group 35mL of media, dispensed into tubes fit) 5ml tube = 7 tubes, 7 110.5 of clean sterile autoclaved test tubes, test tube rack, inoculating needle, Kovac's reagent, Methyl red indicator, Baritt's reagents A & B.

<table>
<thead>
<tr>
<th>(i) For indole production test: SIM agar deep tubes</th>
<th>(ii) Methyl red and VP test: MR-VP broth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>Peptone</td>
</tr>
<tr>
<td>30.0g/L</td>
<td>7.0g/L</td>
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<tr>
<td>Ferrous ammonium sulphate</td>
<td>Dextrose</td>
</tr>
<tr>
<td>3.0 g/L</td>
<td>5.0 g/L</td>
</tr>
<tr>
<td>Sodium thiosulphate</td>
<td>Potassium phosphate</td>
</tr>
<tr>
<td>0.2 g/L</td>
<td>5.2 g/L</td>
</tr>
<tr>
<td>Agar</td>
<td>Adjust pH to 6.9</td>
</tr>
<tr>
<td>3.0 g/L</td>
<td></td>
</tr>
<tr>
<td>Adjust pH to 7.3</td>
<td></td>
</tr>
<tr>
<td>Autoclave at 110°C for 15 min.</td>
<td></td>
</tr>
</tbody>
</table>

Following inoculation, aliquots (2.5 ml) has to taken out into set of clean, dry, autoclaved tubes for VP test & rest proceeded for MR test

<table>
<thead>
<tr>
<th>(iii) Citrate utilization test: Simmon’s citrate agar</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium dihydrogen phosphate</td>
<td>1.0g/L</td>
</tr>
<tr>
<td>Dipottasium phosphate</td>
<td>1.0 g/L</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0 g/L</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>0.2 g/L</td>
</tr>
<tr>
<td>Adjust pH to 7.3</td>
<td></td>
</tr>
<tr>
<td>Agar</td>
<td>3.0 g/L</td>
</tr>
<tr>
<td>Bromothymol blue</td>
<td>0.08 g/L</td>
</tr>
</tbody>
</table>

Procedure:
1. Using sterile technique inoculate each experimental organism into its appropriately labeled tube as directed. The last tube will serve as control.

2. Incubate for IS to 24 hours at 37°C.

Observation:

(i) For indole production test:

a. Observe for the motility which iSH-ecognized when culture growth of flagellated organisms is not restricted to the line of iiuilation. Growth of non-motile organisms is confined to the line of inoculation.

b. SIM medium contains peptone & sodium thiosulfate as sulfur substrates resulting in the production of 1-1,S gas. Ferrous ammonium sulfate in the medium serves as an indicator
by combining with the gas forming an insoluble black ferrous sulfide precipitate seen along
the line of the stab.

c. Add 10 drops of Kovac's reagent to all deep tubes & agitate the cultures gently. Examine
the colour of the reagent layer in each culture. Determine and record whether or not each
organism was capable of hydrolyzing tryptophan.

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Motility (+) or (-)</th>
<th>H₂S production (+) or (-)</th>
<th>Color of reagent layer</th>
<th>Tryptophan hydrolysis (+) or (-)</th>
</tr>
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(ii) Methyl red and VP test:

a. Transfer approximately 2.5ml of each culture into labeled empty test-tube and set these
tubes aside for VP test.

b. For MR test Add 5 drops of methyl red indicator to the remaining aliquot of each culture.
Examine the color of these cultures & record whether each of the cultures as capable of
fermenting glucose with the production & maintenance of high concentration of acid.

c. For VP test to the separated aliquots add 1 0 drops of Barritt's reagent A and shake the
culture. Immediately add 10 drops of Barritt's reagent B and shake, Reshake every 3-4
minutes. Examine the color of the cultures after addition of Barritt's reagent. Based on your
observation determine whether or not each organism was capable of fermenting glucose
with ultimate production of acetylmethylcarbinol.

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Methyl red test</th>
<th>Voges-Proskauer test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Color of medium (+) or (-)</td>
<td>Color of medium (+) or (-)</td>
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</table>

(iii) Citrate utilization test:
Examine all agar slant cultures for the presence or absence of growth and coloration of the
medium. Based on your observation record whether or not each organism was capable of using
citrate as its sole source of carbon.

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Presence or Absence of growth (+) or (-)</th>
<th>Color of medium</th>
<th>Citrate utilization (+) or (-)</th>
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BL301: MICROBIOLOGY LABORATORY CREDITS — 2
Aim 4.3.3: To determine the ability of some microorganisms to reduce nitrates (NO₃⁻) to nitrites (NO₂⁻) or beyond nitrite stage (Nitrate reduction test)

Principle: The reduction of nitrates can occur under aerobic or anaerobic conditions. In these anaerobic organisms the cell uses inorganic substances such as NO₃ to supply O₂ that is subsequently utilized as a final H acceptor during energy formation. The biochemical transformation is as follows:

\[ \text{NO}_3^- + 2\text{H}^+ + 2e^- \rightarrow \text{NO}_2^- + \text{H}_2\text{O} \]

Some organisms further can enzymatically convert nitrates to NH₃* or N₃

Following incubation of the cultures an organism's ability to reduce nitrates to nitrites is determined by addition of 2 reagents:

Solution A - sulfanilic acid followed by Solution 13 - α-napthylamine, which will produce an immediate cherry red compound due to formation of sulfobenzene azo-a-napthy lamine.

\[ \text{NO}_3^- \text{ nitrate reductase} \quad \text{NO}_2^- \text{(red color on addition of Solutions A & B)} \]

No change in color suggests 2 things: (i) nitrates were not reduced or (ii) the organism had the potential to rapidly convert nitrates to NH₃ or N₃. To determine whether or not nitrates were reduced past the nitrite stage small amount of Zn powder is added to the colorless solution already containing Solutions A & B. Zn reduces NO to NO⁻-. If a red color develops then it signifies that nitrates were not reduced to nitrites, indicating a negative nitrate reduction test. If no color change has occurred it signifies that nitrates in the medium were reduced beyond nitrites to ammonia or nitrogen gas, indicating a positive nitrate reduction test.

Material, Reagents and methods: Nitrate broth per group 25mL of media dispensed into tubes @ 3mL/tube = approx. 7 tubes; Solution A (Sulfonilic acid), Solution B (α —napthylamine) and Zn powder, Bunsen burner, inoculating loop, te.4 tube rack. Nitrate broth composition (g/L):

- Peptone — 5.0
- Beef extract 3.0
- Potassium nitrate — 0.5%
- pH 7.2

Procedure
1. Using sterile technique inoculate each experimental organism into its appropriately labeled tube as directed. The last tube will serve as control.

2. Incubate lot. 24 to 48 hours at 37°C.

Observations:
- Add 5 drops of Solution A and then 5 drops of Solution B to ad nitrate broth cultures. Observe and record whether or not red coloration has developed.
- Add a pinch of Zn powder to the cultures which did not show any red coloration. Observe the color change if any.
- Based on your observations determine and record whether or not each organism was capable of nitrate reduction and to which extent.

<table>
<thead>
<tr>
<th>Bacterial Species</th>
<th>Red coloration with soln. A &amp; B (+) or (-)</th>
<th>Red coloration with Zn (+) or (-)</th>
<th>Nitrite reductions (+) or (-)</th>
<th>End products</th>
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</thead>
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Aim 4.3.4: Detection of enzymes urease, catalase and cytochrome oxidase activity in groups of bacteria

Principle: Urease enzyme produced by some micro-organisms attacks the N₂ and C bond in amide compounds such as urea and forms alkaline end product NH₃. The presence of urease is detectable when organisms are grown in urea broth medium containing pH indicator phenol red. As the substrate is split into its products, the alkaline environment created due to NH₃ is detected as phenol red turns deep pink. This indicates a positive reaction. This is especially useful in identification of *Proteus vulgaris*.

During aerobic respiration microorganisms produce H₂O₂ which in some cases is an extremely toxic compound. Organisms capable of producing catalase rapidly degrade H₂O₂ to H₂O and O₂.

**Catalase** production can be determined by adding the substrate H₂O₂ to an appropriately incubated slant culture. If catalase is present bubbles of O₂ will be observed indicating a positive reaction. Cytochrome oxidase catalyzes the oxidation of reduced cytochrome by O₂ resulting in the formation of H₂O or H₂O₂. The ability of bacteria to produce cytochrome oxidase can be determined by addition of test reagent p-aminodimethylaniline oxalate to colonies grown on a plate medium. This light pink reagent serves as an artificial substrate donating electrons and thereby becoming oxidized to a blackish compound in presence of oxidase and free O₂. Following addition of the test reagent the development of purple and then dark coloration is indicative of Cytochrome oxidase production and represents a positive test. No color change or light pink coloration indicates a negative test.

Material, Reagents and methods:

For urease test: Urea broth - 21 tubes @ 3ml/tube, add filter sterilized urea broth concentrate @ 10% to sterilized distilled water: Bunsen burner, inoculating loop, test tube rack, methyl red solution. For catalase test: Fresh culture slants, 3% F170, solution, Bunsen burner, inoculating loop, test tube rack, For oxidase test: Fresh culture slants, p-aminodimethylaniline oxalate, Bunsen burner, inoculating loop, test tube rack.

Procedure: For urease test:
- Using sterile technique inoculate each experimental organism into its appropriately labeled tube as directed. The last tube will serve as control,
- Incubate for 24 to 48 hours at 37°C.
For catalase test:
- Using sterile technique, inoculate each experimental organism into its appropriately labeled tube by means of a streak inoculation. The last tube will serve as control.

For oxidase test:
- Prepare appropriate media plate for inoculation by dividing bottom of the plates into appropriate number of sectors.
- Inoculate the test organisms into each sector by a single line streak inoculation.
- Incubate for 24 to 48 hours at 37°C.

Observations:

For urease test: Examine the urea broth cultures for color & record your observations.

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Color of medium</th>
<th>Urea hydrolysis (+ or -)</th>
</tr>
</thead>
</table>

For catalase test: Allow 3-4 drops of 3% 11202 to flow over the entire surface of each slant culture. Examine for the presence or absence of bubbling or foaming. Record your observations.

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Presence or Absence of bubbling</th>
<th>Catalase production (+ or -)</th>
</tr>
</thead>
</table>

For oxidase test: Add 2 or 3 drops of p-aminodimethylaniline oxalate to the surface of growth each test organism. Observe the color change from pink to deep purple within 5-10 seconds for +ve reaction. Record whether the colonies are oxidase +ve or —ve.

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Color of colonies</th>
<th>Oxidase production (+ or -)</th>
</tr>
</thead>
</table>

BL301: MICROBIOLOGY LABORATORY
Laboratory Handout
Instructor — Dr. Harapriya Mohapatra

Experiment 5: Culture independent analysis of microbial communities by 16srDNA sequencing method

Principle: The culture independent method utilizes PCR to amplify the DNA encoding 16srRNA gene and then subsequently sequence the purified amplification product to obtain the sequence.
The DNA sequence thus, obtained is compared for identity using BLAST tool for identifying the species till the genus level.

Material, Reagents & equipment used: Test-tubes, 0.2ml PCR tubes, 1.5ml MCTs 1-200µl 1 - 1000µl 1 tips, autopipettes, scalpels, gloves, tissue papers. Ice-bucket, 96 well optical clear plates for sequencing machine.

1 X PCR buffer, forward & reverse primers for target gene, cINIP (2.5m4), MgCl7 (25µM), Taq polymerase, template DNA lysate, nuclease free water, ready reaction mix with dNTP’s 8,7, co-factors, sequencing PCR dilution buffer. Sterile MQ water, Hi-Di formamide, gel band PCR purification kits, ethanol (70%) freshly prepared

PCR machine, water bath & dry bath, electrophoretic gel apparatus, Gel-Doc system & UV-transilluminator, DNA genetic analyzer.

Procedure:

1. PCR reaction is set up to amplify the gene of interest as per the protocol given by the instructor.

2. Following it the bands are separated by agarose gel electrophoresis using horizontal DNA gel electrophoretic apparatus

3. The PCR bands are eluted using PCR gel elution kit.

4. Sequencing PCR is set up using the purified PCR DNA as template and the 16srRNA as forward and reverse primers.

5. After this the sequencing PCR reaction mixture is cleaned up using standard protocol as per the instructions given by the instructor.

6. The purified amplified products are loaded onto the 3130XL Genetic analyzer and run as per the standard protocol.

7. Following completion of the run the sequence obtained is taken for alignment with the NCBT database and local alignment is done using BLAST tool. The genus shown highest identity is selected.

Note: For complete confirmation of say a new genus/species in addition to 16srDNA sequencing other analytical methods such as fatty acid profile. DNA-DNA hybridization etc. also needs to be done.

Observations:

Copy & paste your sequence data. Note & write down your BLAST results along with alignment details.
Experiment 6: Identification of the genus of unknown bacterial cultures

Principle: Utilizing the diagnostic biochemical tests such as done in experiments 1 to 4 and using the Bergey's manual the organism’s arc assigned into different genus/groups. The results thus obtained is corroborated with the sequencing (expt. 5) data to finally assign a given isolate to the genus level.

Procedure:
Using identification flow charts as in Bergey's manual of determinative Bacteriology, assign the unknown bacterial isolates upto the genus level. Co-relate your observations with those reported.

Observations:

Tabulate your observations as follows:

<table>
<thead>
<tr>
<th>Biochemical test</th>
<th>Test Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Isolate no.1</td>
</tr>
<tr>
<td></td>
<td></td>
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</table>
Principle: Antibiotic resistance is the acquired or inherent ability of a bacterial isolate to resist effects of the therapeutic agent. This is validated by creating a lawn of bacterial culture on a non-selective culture medium such as Muller-Hinton broth/agar. Followed by inoculating a standardized antibiotic disk above the lawn. Following overnight incubation the antibiotics loaded on the disk diffuses into the agar medium and affects the bacterial growth. If the strain is resistance towards the given antibiotics, then its growth will not be inhibited and it will not show a zone of growth inhibition surrounding the disk. Lithe strain is sensitive towards the antibiotics then a zone of inhibition would be observed around the disk. As per the CLSI standards which are followed universally, specific diameters of zone or inhibition has been allotted against resistance, intermediate or sensitive for a particular potency of the antibiotic loaded on the disk.

Materials and reagents required: Muller-Hinton broth, Muller-Hinton agar plates, blunt ended forceps, different antibiotic disks, sterile cotton swab, burner, ethanol (70%).

Procedure
1. Raise a log phase of bacterial culture in Muller-Hinton broth.
2. Using sterile cotton swab make a lawn of the culture on Muller-Hinton agar plate.
3. On top of the lawn place carefully an antibiotic disk carefully so as not to dig into the agar or allow the disk to jump over the plate. Using a sterile blunt ended forceps slightly press the disk against agar, to make it stay.
4. Incubate the plates overnight in inverted position inside an incubator at 37°C for growth and observe the plates the following day.

Observations:
Tabulate our observations as follows:

<table>
<thead>
<tr>
<th>Antibiotic used</th>
<th>Abbreviation</th>
<th>Potency</th>
<th>Diameter of clearance</th>
<th>Resistance/ Intermediate/ Sensitive</th>
</tr>
</thead>
</table>

Aim 7.2: Determining the Minimum Inhibitory Concentration (MEC) of a given antibiotic towards a bacterial isolate.
Principle: The MIC is defined as the concentration at which the bacterial ceases to grow. The MIC level of a resistant isolate is important criteria for determining the level/extent of resistance exhibited by the bacteria for a particular antimicrobial agent. This in turn will help determine whether a particular antibiotic can still be effectively used at higher concentrations as a therapeutic agent.

Materials and reagents required: Muller-Hinton broth, different antibiotic stock solution, sterile 1- 200u1 and 1 00-1 000u1 sterile rips, test-tubes, burner, ethanol (70%), sterile 0.22micron syringe filters, 5m1 disposable syringes, 0.22micron filter membranes for syringe litters, autopippettes, sterile saline.

Procedure:
1. A pure culture of a single microorganism is grown in Muller-Hinton broth, or other broth as appropriate.
2. The culture is standardized using standard microbiological techniques to have a concentration of very near I million cells per milliliter. The more standard the microbial culture, the more reproducible the test results.
3. The antimicrobial agent is diluted a number of times, 1:1, through sterile diluents (usually Mueller- Hinton broth).
4. After the antimicrobial agent has been diluted, a volume of the standardized inoculums equal to the volume of the diluted antimicrobial agent is added to each dilution vessel, bringing the microbial concentration to approximately 500,000 cells per milliliter.
5. The inoculated, serially diluted antimicrobial agent is incubated at an appropriate temperature for the test organism for a pre-set period, usually .18 hours. The more standard the incubation period, the more reproducible the test results.
6. After incubation, the series of dilution Vessels is observed for microbial growth, usually indicated by turbidity and/or a pellet of microorganisms in the bottom of the vessel. The last tube in the dilution series that does not demonstrate growth corresponds with the minimum inhibitory concentration (M1C) of the antimicrobial agent.

Observations:
Observe the series of dilution for microbial growth and note the antibiotic concentration of the tube that does not show any bacterial growth.
STANDARD OPERATING PROCEDURE FOR MICROBIOLOGY LAB

1. Handling Microscopic slide: Holding the slide through its side, clean commercially available microscopic slides to remove finger marks, dust particles.

2. Handling culture vessels: Organize all experimental culture media & sterile vessels at the beginning of each experiment. Label culture vessels with non-water soluble marker directly below the cap of the culture tube. Label should always contain name of the test organism, name of the medium dilution if nay, date and your initials. Use leak proof containers in sealed plastic bags for transportation of the sample. In case of petri plates organism name should be written on the bottom periphery to prevent obstruction for viewing the results.

3. Handling media & reheating media: While weighing media for preparation make sure you switch off the ceiling fans, use a clean, dry spatula & use a clean butter paper or any other material for dispensing the dehydrated materials. After use close tight the respective bottles with their lid. Make sure NOT to S W A P the covers. Add required amount of water, cover with plug put in the autoclave for sterilization. Make sure there is sufficient water in the autoclave before switching it on. Take out the media from the autoclave only when the pressure in the autoclave has come down and materials/glassware’s cool enough to handle. Always use gloves. DO "NO' swirl vigorously hot agar/solidified media. The plug might pop-up due to steam pressure and spills the hot media on hand.

4. Handling inoculation loops & needles: It is essential that you incinerate entire wire to ensure absolute sterilization. Briefly also pass the shaft through the flame to remove dust or possible contaminants. Cool the inoculation loop b slowly and gently tapping the inner surface of culture tube or petri dish cover before taking out inoculums. While transferring from an agar culture touch only a single area of growth, NEVER drag the loop or needle over entire surface. DO NOT dig into the solid medium. In case of broth culture, DO NOT lap the tubes vigorously as this may lead to frothing and denaturation of proteins in the medium.

5. Using Laminar Air Flows/Biosafety cabinets: Perform all work on isolates in Laminar Airflows or Biosafety safety cabinets. DO NOT open culture plate/tubes outside the cabinets. Clean the hoods/cabinets before and after use with 70% ethyl alcohol. DO NOT leave used tips, swabs, loops unsterilized inside the cabinet. Sterilize and dispose of them aseptically immediately after you have finished working. DO NOT crowd up the cabinet with items which are not in regular use such as test-tubes, unopened petridishes. Beakers with tips, swabs etc.

6. Place test-tubes containing culture medium in racks for incubation. Always incubate petri-dishes in inverted position to prevent water condensation from dropping onto surface of culture medium. Excess moisture serves as vehicle for producing content rather than discrete colonies.

7. Meticulously record all observed data, wherever necessary illustrate your observations/techniques by drawing and label the diagram.
8. At the end of the session disposes off contaminated materials such as swabs, disposable pipettes and paper towels in autoclavable bags/biohazard receptacles.
9. Neatly place all the reagents, supplies and chemicals t their original locations. Place all capped test tube cultures and closed petri-dishes in designated incubation/disposal area as appropriate. Wipe the working table clean with disinfectant & wash your hand before leaving the laboratory.

Experiment 1: CULTURE media preparation, Control of microbial growth disinfection & sterilization

Aim: To get to know basic laboratory techniques involved in microbiology such as:
   (i) Media preparation (ii) sterilization (iii) disinfection

**Theory/Principle:** A solution containing soluble low molecular weight substances often derived from enzymatic degradation of complex nutrients that supports growth of microorganisms is called a culture medium. Culture medium may be liquid i.e. lacks a solidifying agent (called broth) or solid i.e. supplemented with a solidifying agent such as agar.
Agar serves as solidifying agent because it liquefies at 100°C and solidifies at 40°C. A completely solid medium requires an agar concentration of 1.5-2%. Solidified medium help in isolation of discrete colonies of unadulterated species of cells - termed as pure culture, from a mixed microbial culture. The solidified culture media when in gel state can be placed in test-tubes which subsequently are allowed to harden in slanted position referred to as slants or hardened in upright position called deep tubes or poured into petri dishes producing agar plates. In addition to nutritional need it is essential to maintain the cultures at appropriate environmental conditions such as pH, temperature and gaseous requirements.

Diagrams

In order to successfully work with only a single microbial species all the equipments needs to be sterilized. Sterilization refers to the process by which an article, surface or medium is freed from all living microorganisms either in vegetative or spore state, whereas, disinfection means destruction or removal of all pathogenic organisms. Various physical and chemical agents can be used for the purposes. Most commonly used methods include dry heating - flaming, incineration, hot air; moist heating boiling, steam under pressure; filtration - bacterial membranes; radiation - UV, y-irradiation. Most common chemicals used include -ethyl alcohol, formaldehyde, gluteraldehyde, halogens such as I2, surface active agents as found in wetting agents, detergents and emulsifiers. In day to day laboratory practices UV irradiation, flaming & steam under pressure are commonly used methods of physical sterilization while chemical agents used include ethyl alcohol, formaldehyde etc. Steam under pressure is carried out in specialized equipment called as 'autoclave' which essentially works on the principles used in pressure cooked in our homes. For efficient sterilization it is recommended to use a temperature of 121°C (15lb pressure) for 15 minutes.

Material, Reagents and methods: Per group/student: Test-tubes - 16 no's; Petri dishes - 5 no’s; Marker pen 1 no’s; Pipette for dispensing 5ml - 1 no’s Conical flask 250ml - 2 no’s, 100 ml - 1 no’s; Test-tube rack - 2 no’s; Peptone, Beef extract, 0.0 1 N NaOH I 0.0 1N HCl for pH adjustment, Agar-agar.

Procedure:

- Per group/student will be preparing nutrient broth tubes - 5 no’s @ 3ml/tube, nutrient agar deep tubes - 5 110.S & Nutrient agar slants - 5 no’s @ 3ml/tube, and plates - 5 no’s @ 18-20 ml/plate
- Accordingly calculate the quantity of each type of media to be prepared.
- Preparation of Nutrient Broth: In a 100ml conical flask prepare 25ml of nutrient broth using the following composition (g/L):
  - Peptone - 5.0
  - Beef extract - 3.0
Using 0.01N NaOH/ HCl adjust pH to 7.0.

- In another 250ml conical flask prepare 125ml Nutrient broth, adjust H and then add agar-agar @ 2%.

- Dispense the media into respective tubes but not into petri plates. Keep the media for the petri plates in the flask only.

- Plug the tubes with non-absorbent cotton, wrap with brown paper and put them in a basket, keep ready for autoclaving.

- Set the autoclave at required temperature and sterilize the media.

- Remove from the autoclave after cooling it down, keep the nutrient agar tubes in slanting position 8t 30-45° angle for slant preparation , keep others upright standing for deep tube, pour the rest of the media into petri-dishes inside laminar flow for plate preparation.

- Keep the media overnight for solidification & checking for contamination.

Observation:
- Observe and draw the slant deep to be and plate formation 0f the media
- See if there is any turbidity in the liquid media or any colonies on the solid media.

Conclusion:

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Expedment 2: isolation and culture and characterization of pure culture; enumeration & use of selective and differential media.

Aim 2.1 Isolation of single colony by streak plate method and determining cultural characteristics of organisms,

Theory/Principle: Isolation of discrete colonies requires that the number of organisms in the inoculum be reduced, The resulting diminishing population size ensures that after
inoculation, individual cells will be sufficiently far apart on the surface of agar medium to allow the separation of different species/colonies present. Streak plate technique involves spreading a loopful of culture over the surface of an agar plate. The four-way quadrant streaking technique is most commonly used the purpose. (a) A loopful of inoculums is placed on the surface of agar (area I), subsequently the I OR is flamed, cooled by touching the unused part of agar surface close to the periphery of the plate, dragged rapidly over area I (b) reflamed, petri dish turned 90°C, loop touched to the corner of the culture area I, dragged several times in area 2 (c) & (d) the process repeated two more times as depicted in the figure.

When grown on a variety of media, microorganisms exhibit differences in macroscopic appearance of their growth. These characteristics aid in separating the microorganisms into taxonomic groups and (Ire determined by culturing them on nutrient agar, slants, broths and plates.

(i) Nutrient agar slants: (a) Abundance of growth: Amount of growth is designated as none, slight, moderate or large; (b) Pigmentation: Many of the chromogenic microorganisms produce either intracellular pigment responsible for coloration of the colonies while others may produce diffusible extracellular soluble pigments. Majority of the microorganisms however appear white or gray. (c) Optical diffusive characteristics: Colonies may be opaque, translucent or transparent; (d) Form: Appearance of colony on agar plates.

(ii) Nutrient agar plates: (a) Form (b) Margin (c) Elevation

(iii) Nutrient broth cultures: (a) Uniform fine turbidity -finely dispersed growth throughout; (b) Flocculent -flaky aggregates dispersed throughout; (c) pellicle -thick, padlike growth on surface; (d) sediment -concentration of growth at the bottom of broth culture may be granular, flaky or flocculent

Material, Reagents and methods: Per group/student: nutrient agar slants tubes -2 no’s each; Petri dishes -2 no’s; nutrient broth -2 no’s. Marker pen I no.; Test-tube rack -I no’s; Bunsen burner, laminar’ air hood, revived cultures, inoculating needles.

Procedure:
1. Using sterile transfer technique, inoculate each of the appropriately labeled media as follows:

   (a) Nutrient agar plates: With a sterile loop prepare a streak plate inoculation of the cultures provided for isolation of discrete colonies. Inoculate the plates in inverted position.

   (b) On nutrient agar slants make a single line streak of the culture provided, drawing the needle from the centre to the outer edge
(c) On nutrient broth using a sterile loop inoculate each organism into a tube of nutrient broth. Shake the loop well to dislodge the inoculums.

2. Incubate the cultures at 37°C for 24 hours & make your observation.

Observation:

(a) From nutrient agar plates draw distribution of colonies & their morphology
(b) Draw the distribution of growth on the slant surface
(c) Observe and draw the growth pattern in nutrient broth.

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Aim 2.2 Isolation of single colony by spread plate method and determining bacterial cell count by CFU method.

Principle: This technique requires spreading and separating out individual bacterial cells from a mixed population using previously diluted bacteria cultures (this can be a mixed population or single population). In case of a mixed population the technique helps isolate distinct colonies while in a single culture would enable indirect determination of the bacterial cells in a given population,
The dilution technique followed for spread plate is mainly the 10-fold dilution technique depicted in the figure. The number of bacterial cells in the parent is given by:
Number of bacteria I cells/ml = number of colonies on plate x reciprocal of dilution of sample used

Materials & reagents required: Bent glass rod, beaker, turn table, 95% ethyl alcohol, nutrient agar plate, auto pipette, 1ml tips. Phosphate buffer saline (sterile), test-tubes, Bunsen burner, bacterial inoculums.

Procedure:

1. Place bent glass rod into a beaker to which sufficient amount of 95% EtOH is added.
2. With a sterile pipette onto agar plate place 50 μl of diluted culture mixture.
3. Remove glass rod from ethyl alcohol, flame, cool for 10-15 seconds, remove the petri dish cover and spread the mixture with moving glass rod to and fro over the plate.
4. Observation: Draw the colonies as they appear on agar plate

Experiment 3: Microscopic examination of fresh culture with differential staining producers
Aim 3.1: To differentiate the given bacterial strains into two principal groups of bacteria: Gram negative and Gram positive

Principle/Theory): Differential staining requires the use of at least three chemical reagents that are applied to a heat fixed smear. First is the primary stain which colors all cells. Second the decolorizing agent which depending upon the chemical composition of the cell may or may not remove the primary stains from the entire cell. Final one is the counter stain which gets absorbed only if the primary stain is washed out. In addition sometimes certain chemical compounds
may be used which increases the cells affinity for a stain. In Gram staining procedure Crystal violet is used as the primary stain, Gram's Iodine is used as a mordant which forms a complex with crystal violet and intensifies the color. The decolorizing agent is 95% ethyl alcohol and the counter stain is safranin.

Material and reagents required: 24 hours old bacterial cultures, crystal violet, Gram's iodine, 95% ethyl alcohol and safranin, Bunsen burner, inoculating loop, staining tray, tissue paper, glass slides, and microscopes.

Procedure:

1. Using sterile technique prepare smear of each of the organisms given on the glass slides. Allow it to air dry & then heat fix.
2. Gently flood smears with crystal violet and let stand 1 minute.
3. Gently wash off the excess dye with tap water.
4. Gently flood smears with Gram's iodine morden and let stand for 1 minute.
5. Gently wash with tap water
6. Decolorize with 95% ethyl alcohol Caution: Care has to be taken so a~ not to over decolorize the primary stain. Add reagent drop by drop until alcohol runs almost clear, showing only a blue tinge.
7. Gently wash with tap water.
8. Counter stain with safranin for 45 seconds.
9. Gently wash with tap water.
10. Blot dry with tissue/blotting paper and observe under oil immersion

Observation:

(ii) Make drawing of a representative microscopic field
(iii) Describe cells according to morphology and arrangement
(iv) (iii) Describe the color of the stained cells
Experiment: Determination of proteins by Bradford method

Principle

This is a rapid, simple and sensitive method for estimation of proteins in a sample extract. The color development is virtually complete in 2 min and the color is stable for about 1h. Unlike Lowry method, metal ions such as NH4, Na, K+, phenols and carbohydrates such as source do not interfere in this assay. The procedure is based on interaction of a dye, Coomassie Brilliant Blue, with proteins. The unbound dye has an absorbance maximum at 465 nm. However, on interaction with proteins the dye turns blue and their absorbance maximum is displaced to 595 nm. Thus from the absorbance at 595 nm the amount of protein in a sample solution can be quantitatively
estimated. However, as in Lowry procedure, detergents such as SDS, Triton X-100 etc. interfere in estimation of proteins by this method.

Materials and Reagents
1. Colorimeter
2. Sample extract: prepare as given in step 1 of experiment 4.4.2
3. Bradford reagent: dissolve 100 mg of Coomassie Brilliant Blue G 250 in 50 ml of ethanol, add 100 ml of 85% of phosphoric acid and make the volume to 1 L with water.
4. 0.1 M phosphate buffer (pH 7.5): see section 1.3.8 for preparation of this buffer.
5. Standard protein solution: Dissolve 5 mg of bovine serum albumin in 50 ml of 0.1 M phosphate buffer. This solution contains 100 μg protein/ml.

Procedure:
1. Take 0.1 ml of sample solution (see step 1, EXPERIMENT 4.4.2) and make the volume of 1 ml with 0.1 M phosphate buffer (pH 7.5).
2. Pipette appropriate aliquots of bovine serum albumin solutions containing 0-100 μg protein. Make the volume to 1 ml with 0.1 M phosphate buffer (pH 7.5) in all the tubes.
3. Add 5 ml of Bradford reagent to all the tubes and mix thoroughly.
4. Record the absorbance at 595 nm against the reagent blank.
5. Plot a standard curve of A_{595} versus μg of proteins in the standards (step 2).
6. Determine the protein content in the sample extract from the standard curve.
7. Calculate the amount of protein per ml of the sample preparation.

Experiment: Estimation of DNA by diphenylamine reaction

Principle
This is a general reaction given by deoxypentoses. The 2-deoxyribose of DNA, in the presence of acid, is converted to ω-hydroxylevulinic aldehyde which reacts with diphenylamine to form a blue colored complex with absorbance maxima at 600 nm. Compounds such as furfuryl alcohol and arabinal, which can be converted in to ω-hydroxylevulinic aldehyde will also give this reaction. In DNA, since only deoxyribose of purine nucleotides is released, the value obtained represents half of the total deoxyribose in the sample. The reacting leading to the formation of the colored complex are as follows:
Materials and Reagents
1. Colorimeter or spectrophotometer.

2. Standard DNA solution: Dissolve calf thymus DNA (100 μg/ml) in 1N HClO₄ by heating at 70°C for 15 min. make different dilutions of this stock solution ranging from 20-100 μg DNA/ml using 0.5 N HClO₄.

3. 1.6% (w/v) acetaldehyde: prepare by dissolving 1 ml of ice cold acetaldehyde in 50 ml of distilled water.

4. Diphenylamine solution: Dissolve 1.5 g of diphenylamine in 100 ml of glacial acetic acid and 1.5 ml of conc. H₂SO₄.

5. Diphenylamine reagent: prepare by mixing 0.5 ml of 1.6% acetaldehyde and 100 ml of diphenylamine solutions. This solution must be prepared fresh.

Procedure:
1. Take 2.0 ml aliquot of the sample in which DNA has to be estimated in a test tube.

2. In another set of test tubes, pipette 2.0 ml of standarad DNA solution of different dilutions. In one of the test tube 2.0 ml of 0.5N HClO₄ as a reagent blank.

3. Add 4.0 ml diphenylamine reagent (Reagent no. 5) to all the tubes, mix the contents properly and keep at room temperature in dark for 16-18h or overnight. Alternatively, keep the tubes in boiling water for 10 min and cool them under running tuning tap water.

4. Record the absorbance at 600 nm.

5. Draw a standard curve of A₆₀₀ vs DNA concentration. From absorbance of the sample, determine the amount of DNA in it. Express the results as mg of DNA/g fresh weight of tissue.

Experiment: Determine of RNA by Orcinol method.

Principle
Orcinol, in the presence of ferric chloride as a catalyst, react with furfural producing a green colored compound with absorbance maxima at 665 nm. DNA gives a limited positive reaction with orcinol test. The reactions leading to the formation of a green colored complex are as follows:

Materials and Reagents
Colorimeter or spectrophotometer.
1. Boiling water bath.
2. 5% HClO$_4$


4. Orcinol reagent: Dissolve 100 mg of ferric chloride (FeCl$_3$.6H$_2$O) in 100 ml of conc. HCl and then add 3.5 ml of 6% solution of orcinol prepared in alcohol.

Procedure
1. Take 2.0 ml solution of each of the dilutions of RNA standard solutions, test sample and 2.0 ml of 5% HClO$_4$, as a blank, in different test tubes.

2. Add 3.0 ml orcinol reagent to all the tubes and mix properly.

3. Keep the test tubes in a boiling water bath for 20 min.

4. After cooling them, add 7.0 ml of n-butanol to each tube and measure the $A_{665}$ against blank.

5. Plot a graph between $A_{665}$ vs amount of RNA and from this standard curve determine the amount of RNA in the provided sample.

Experiment: Quantitative determination of DNA and RNA by spectrophotometric method.

Principle
Spectrophotometric method can also be employed for judging purity of DNA and RNA extract and these have absorption maximum at 280 nm. The ratio of absorbance at 260 and 280 nm, hence, provides a rough idea about the extent of contamination in the preparations. A ratio between 1.8-2.0 is indicative of fairly pure DNA and RNA preparations but values less than 1.8 signify presence of proteins as impurities.

Materials and Reagents
1. UV Spectrophotometer.
2. Saline sodium citrate (SSC) solution: Prepare 0.015M solution of sodium citrate (pH 7.0) and dissolve NaCl so that its final concentration in solution is 0.15M.

Procedure
1. Switch ON Spectrophotometer and allow it to warm up for about 10 min. Adjust the wavelength at 260 nm and put ON the UV-lamp.

2. Set the instrument at zero absorbance with SSC solution.

3. Read absorbance of solution of the provided sample. If O.D. is too high appropriately dilute the sample solution with SSC and again take the reading.

4. Calculate the concentration of DNA and RNA in the sample from following formulae:

   For double standard DNA:
   \[
   \text{Concentration of DNA in sample solution (\(\mu g/ml\))} = 50 \times A_{260} \times \text{Dilution factor}
   \]

   For RNA:
   \[
   \text{Concentration of RNA in sample solution (\(\mu g/ml\))} = 40 \times A_{260} \times \text{Dilution factor}
   \]
Experiment: determination of melting temperature and base composition of DNA from thermal denaturation characteristics

Principle
When a dilute aqueous solution of double stranded DNA is heated, the two strands get separated due to disruption of H-bonds between the complementary bases. Such DNA is referred to as denatured DNA and the process is called denaturation. Denaturation is a reversible process. If the above solution is allowed to cool slowly to room temperature, the complementary strands of DNA resemble to give a duplex DNA. DNA is now said to be reannealed and process is called reannealing of renaturation of DNA. Upon denaturation, the absorbance of DNA at 260 nm increase by 30-40% due to exposure of bases. This is called hyperchromic effect. Heat treatment at temperatures of upto 80°C causes only a very slight increase in absorbance at 260nm.

Materials and Reagents
1. UV-spectrophotometer with thermoprogrammer.
2. Saline sodium citrate (SSC) solution: Prepare 0.015M solution of sodium citrate (pH 7.0) and dissolve NaCl so that its final concentration in solution is 0.15M.
3. DNA: Dissolve 50 μg DNA/ml of SSC.

Procedure A
1. Switch ON Spectrophotometer and after allowing sufficient period for warming up, set it zero absorbance at 260 nm with SSC.
2. Measure A260 of the DNA samples at p-nitophenol.
3. Heat the DNA solution at a rate of 1°C rise /min upto 100°C with the help of a thermoprogrammer. Record the absorbance values manually or with the primer.
4. Calculate A260 (T°C)/A260 at 25°C for each of the following temperatures: 25, 35, 50, 70, 75, 80, 90, 95 and 100°C and plot the absorbance ratio against the temperature.
5. Determine the midpoint of increase in absorbance and by extrapolation find the corresponding temperature which represents Tm for the DNA sample.
6. Calculate % (G+C) content of the DNA using the following equation:

   \[
   \% \text{(G+C)} = (\text{Tm}-69.3) \times 2.44
   \]

Procedure B
If UV- Spectrophotometer with thermoprogrammer is not available, then the following procedure can be adopted.

1. Arrange a series of constant temperature water baths maintained at 25, 50, 75, 80, 85, 90, 95 and 100°C respectively.

2. Record absorbance at 260 nm of DNA sample solution 50 μg/ml kept at 25°C.

3. Distribute the above solution into eight test tubes and put one test tube each in the water baths maintained at different temperatures. Allow the tubes to stand for 15 min. after incubation, quickly cool all the tubes (except one at 25°C) by placing them in ice bath for 10 min.

4. Record the $A_{260}$ of these samples.

5. Then proceed as in steps 4 to 6 of the procedure A described earlier.
**Experiment: To study time course of the reaction catalysed by alkaline phosphatase (EC 3.1.3.1)**

**Principle**
Phosphatase is a broad term used for non-specific phosphomonoeserases which hydrolyze organic phosphatase esters liberating in alcohol derivative of the substrate and inorganic phosphate (Pi). These enzyme catalyze the following reaction:

Orthophosphoric monoester + H₂O → alcohol + Pi

Depending on their pH optima, phosphatases have been classified into two groups’ viz. acid phosphatases and alkaline phosphatases. Acid phosphatases function optimally at acidic pH (4.0 – 5.5) whereas alkaline phosphates give maximum activity at alkaline pH (8-10). For assaying phosphatases, p-nitophenyl phosphatase can be used as substrate which is hydrolysed to p-nitophenyl and Pi. p-nitophenyl is colorless at acidic or neutral pH but at alkaline pH of 11 it gives yellowish colour with absorbance maxima at 410 nm. Hence the activity of alkaline or acid phosphatases can be conveniently determined calorimetrically by determining the amount of p-nitophenyl produced. The reaction catalysed by alkaline phosphatases:

**Materials and Reagents**
1. Water bath at 35°C
2. Calorimeter
3. Refrigerated centrifuge
4. Glycine-NaOH buffer (0.005 M, pH 10.5): Dissolve the 375 mg glycine in small volume of water, add 42 ml of 0.1N NaOH solution and adjust the pH 10.5
5. NaOH solution (0.085N): prepare by dissolving 340 mg of NaOH in 100 ml of distilled water.
6. MgCl₂ solution (10.5mM): weigh 10 mg of MgCl₂ and dissolve it in 10 ml of distilled water. 0.1 ml of this solution is used in 3.5 ml of reaction mixture so that the final conc. Of Mg²⁺ during the assay is 0.3mM.
7. p-nitophenyl phosphatase (35mM): take 38.8 mg of p-nitophenyl phosphatase and dissolve it in 5 ml of 0.05 M glycine –NaOH buffer pH 10.5.
8. Standard solution of p-nitophenyl (100mM): weigh 69.75 mg of p-nitophenol and dissolve it in 5 ml of distilled water. This solution contains 100 μ moles p-nitophenol/ml.
9. Plant material: use 5 days old germinating pea seeds which have been germinated at 25°C in petri plates lined with two layers of whatman no. 1 filter paper. Ensure that the petri plates contain sufficient amount of water throughout this period.
Procedure

1. All the operations for preparation of the tissue extract have to be carried out in cold at 0-4°C. Weigh 1 g of the germinating seeds and grind them in chilled pestle and mortar in presence of 10 ml of glycine-NaOH buffer (0.05 m, pH 10.5). A small amount of acid washed river sand may be used as an abrasive to facilitate complete breakage of the cells.

2. Centrifuge the homogenate in a refrigerated centrifuge at 10,000 x g for 20 min. Decant the supernatant and use it as the enzyme preparation.

3. Take nine numbered test tubes and add 3.0 ml of glycine-NaOH buffer, 0.1 ml of MgCl2 and 0.3 ml of the enzyme preparation into each of them.

4. Transfer these tubes to a water bath maintained at 37°C. After 3 min start the reaction in seven of the above tubes by adding 0.1 ml p-nitophenyl phosphate. Note down the time of starting the reaction for each tube.

5. Exactly after 5, 10, 15, 20, 25, 30 and 45 min stop the reaction by adding 9.5 ml of 0.085 N NaOH to each tube. In the eighth tube add NaOH followed by 0.1 ml p-nitophenyl phosphate. This represents 0 min control. In the ninth tube, instead of p-nitophenyl phosphatase add 0.1 ml of 0.05 M glycine –NaOH buffer (pH 10.5) and this serves as the reagent blank.

6. To prepare a standard curve, take 0-1 ml (0-100) μ moles of p-nitophenyl. Add 3 ml of 0.05 M glycine–NaOH buffer (pH 10.5) to all the tubes and make the final volume to 3.5 ml with distilled water. Pipette 9.5 ml of 0.085N NaOH into each tube, mix the contents and record the absorbance at 410 nm using the tube without p-nitophenol for setting the instrument to 100% transmission or zero absorbance.

7. Plot a graph of A410 vs μ moles of p-nitophenol to obtain a standard curve.

8. Determine the amount of μ moles of p-nitophenol in the tubes 1-8 of step 5.

9. Draw a graph of μ moles of p-nitophenol produced vs reaction time.

10. From this graph note down the maximum assay period upto which the production of p-nitophenol is linear. In all the subsequent experiments the duration of reaction should be well within this time limit.

11. Using the experimental data plot another graph of velocity of reaction vs reaction time.
**Experiment: To determine the temperature optima for alkaline phosphatase**

**Principle**
All the enzymes have a narrow temperature range for their efficient functioning. The reasons for decline in velocity at temperature beyond optimum temperature have been discussed in section 7.3.5.4. For determining enzyme activity in the sample, assay should be carried out at a optimum temperature. So before undertaking further investigations on an enzyme, temperature optima for its activity ought to be determined in a preliminary experiment. While carrying out any enzyme assay it is essential that appropriate controls must be run simultaneously.

**Materials and Reagents**
1. All the requirements listed in experiment 7.8.1
2. In addition several water baths maintained at the chosen temperatures (say 20, 25, 30, 35, 40, 45 and 50°C) will be needed.

**Procedure**
1. Prepare the enzymes extract as outline in experiment 7.8.1 (steps 1 and 2)
2. Take two sets of tubes (one set for zero minute control and the other set for actual enzyme assays) and pipette 3.0 ml of 0.05M glycine NaOH (PH 10.5) and 0.1ml of Mgcl2 into each tube.
3. Add 0.3 ml of the enzyme extract to the assay tubes. Keep one assay tube and one control tube at the temperature at which the enzyme activity has to be determined (20, 25, 30, 35, 40, 45 and 50°C) and after 3 min add 0.1 ml of p-nitophenol phosphate to all the tubes.
4. Allow the reaction to proceed for 30 min or any other suitable time period which falls within linear region of curve of the graph constructed in experiment 7.8.1 and stop the reaction with 9.5 ml of 0.085N NaOH. After this add 0.3 ml of the enzyme preparation to control tubes.
5. Record the absorbance of all the tubes at 410 nm.
6. Deduct the obtained A410 for the controls from the A410 of the corresponding assay tubes and from the standard curve determine the amount of p-nitophenol formed in the assay tubes.
7. Draw a graph of amount of p-nitophenol produced at different temperatures.
8. From the graph determine the optimum temperature for the activity of alkaline phosphatase.
Experiment: To examine the effect of pH on activity of alkaline phosphatase.

Principle

The influence of pH on activity of enzymes has been discussed in details in section 7.3.5.3 for measurement of enzyme activity in biological sample; the assay ought to be conducted at its optimum pH. For determining the pH optima the enzyme activity is measured at varying pH of the reaction mixture. Each buffer has a limited range over which it can effectively fulfill the buffering role. In many instances two different buffers may have to be tried to ascertain effect over a broader range of pH. In such cases it is advisable to select at least one common pH for both these buffers so that from this overlapping pH the variation in enzyme activity due to nature of the buffer can be distinguish from the actual effect of pH.

Materials and Reagents

1. All the requirements as in experiment 7.8.1 except that 0.05 M glycine NaOH of different pH (8.6, 9.0, 9.6, 10, 10.6, 11, 11.5) by adjusting the pH to the desired level with increasingly higher volumes of 0.1M NaOH to 50 ml 0.1 M solution of glycine and then making the final volume to 100 ml with water.

2. 0.05M Tris-HCl buffer: prepare 0.05M Tris-HCl of pH 7.2, 7.6, 8.0, 8.6, and 9.0 as described in section 1.3.8.

Procedure

1. Prepare the enzyme extract accordingly to the method given in steps 1 and 2 of experiment 7.8.1.

2. Arrange two sets of tubes (one for assay and the other for zero min control). Pipette 3.0 ml of different buffers in succession to control and assay tubes followed by 0.1 ml MgCl2 to all tubes.

3. Add 0.3 ml of enzyme preparation in the assay tubes and incubate them at 37°C (or more appropriately at the optimum temperature as determined in experiment 7.8.3). Start the reaction by pipetting 0.1 ml of p-nitophenol to all the tubes.

4. After 30 min, stop the reaction with 9.5 ml of 0.085N NaOH in all the tubes. Now add 0.3 ml of the enzyme extract in the control tubes.

5. Note the absorbance at 410 nm against distill water.

6. Deduct absorbance of the controls from the corresponding assay tubes and using the standard curve (see experiment 7.8.1, step 7). Determine the amount of p-nitophenol formed in the assay tubes.

7. Since the enzyme extract was prepared in a buffer of pH 10.5, the actual pH of the reaction mixture might deviate from that of the assay buffer. Hence prepare a mixture containing
extraction buffer and assay buffer in the same ratio as used for measuring enzyme activity and using pH meter determine its pH which will represents the actual pH of the reaction mixture.

**Experiment: to study the effect of substrate concentration on activity of alkaline phosphatase and determine the Km and Vmax of the reaction.**

**Principle**
The enzyme activity is measured at varying concentrations of the substrate under optimal conditions on the basis of the information obtained in experiment 7.8.1-7.8.4. The Km and Vmax of the reaction are then determined by processing the data and drawing Lineweaver-Burk($1/v$ vs $1/[S]$), Eadie-hostee ($v$ vs $v/[S]$), Hanes ($[S]/v$ vs $v$) or eisenthal and Cornish-Bowden ($v$ vs $[s]$) plots.

**Materials and Reagents**
All the requirements as mentioned in experiment 7.8.1 except for reagents 7. For this experiment prepare 5mM solution by dissolving 38.8 mg p-nitophenyl phosphate in 35 ml of 0.05 M glycine-NaOH buffer (pH 10.5).

**Procedure**
1. Prepare the enzyme extract from the germinating pea seeds as outlined in experiment 7.8.1 (step 1 and 2).

2. Take eight numbered test tubes and add 0.0, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.40 and 0.50 ml of p-nitophenol phosphate in successive tubes.

3. Add calculate amount of 0.05M glycine-NaOH buffer (pH 10.5). So that the total volume of p-nitophenol phosphate and the buffer in each tube is 3.0 ml. pipette 0.1 ml of 10 mM MgCl2 to the tubes and keep them in water bath at 370C.

4. After letting them to warm up for 3 min, start the reaction with 0.3 ml of the enzyme extract in each tube.

5. Stop the reaction after 30 min with 9.5 ml of 0.085N NaOH.

6. Record the absorbance of the color formed at 410 nm against distilled water.
Experiment: to prepare various sub-cellular fractions of rat liver cells.

Principle
Liver is one of the vital organ in an animal body and its function is to process the incoming nutrients from alimentary canal and maintain them at steady level in the blood. In addition it performs excretory functions by catabolizing the amino acids and the other nitrogen containing compounds via urea cycle and degradation of protoporphyrin IX to metal devoid open chain tetrapyrroles. The bile pigment such as bilirubin. It also serves as the principal storage organ for glycogen which may account for about 10% fresh weight of the liver in normal adult.

Materials and Reagents
1. Experimental animal: a young adult rat
2. Scissors
3. Potter-Elvejhem Homogenizer
4. Cheese cloth
5. Wide mouthed canula attached to a syringe.
6. Refrigerated high speed centrifuge.

Procedure
1. Anaesthetize the rat by injecting pentabarbitone sodium (6mg/ml) intraperitoneally using 1 ml for 100 g of the rat weight. Decapitate to kill the rat and let it bleed from the neck to drain out the blood.

2. Dissect the rat and open the abdomen with amid line incision, displace the intestine to right and excise the liver and transfer it to pre weight chilled beaker containing 20 ml of the homogenate media. After weighing the liver, drain out the media and slices in to small pieces with scissors. Add calculated volume of ice cold homogenization media (4ml/g liver). Preparation of the liver slices and all the subsequent step should be carried out at 0-4°C.

3. Transfer the liver slice along with homogenization media into chilled Potter-Elvemjem Homogenizer. Operate the Homogenizer at about 500 rpm and push the glass tube and down 6-8 times to ensure proper breakage of the cells. Filter the homogenate through 3-4 layer of cheese cloth which has been wetted with homogenization media. Stir the homogenate with glass rod to facilitate quicker filtration.

4. Pour the filtrate into 50 ml centrifuge tubes and centrifuge at 1.000 x g for 10 min to sediment the heaviest material (Pi). Since the pellet formed is not well packed and is fluffy. Remove the supernatant carefully with the help of a wide bore cannula attached to a syringe. Centrifuge this supernatant at 3,000 x g for 10 min to obtain P2 which is again a
loose packed one. Recover the pellet by withdrawing the supernatant with a syringe as described above.

5. Subject the supernatant from the preceding step to centrifugation at 10,000 x g for 30 min. gently recover the pellet and label it as P3.

6. Finally centrifuge the remaining supernatant from step 5 at 10,000 x g for 40 min to obtain pellet (P4). Decant the supernatant into chilled beaker and store it on ice.

7. Suspend the pellet (P1+P4) in original volume of the homogenization buffer and store them in cold on ice.
**Experiment: Elecroblotting (western blotting) of proteins from SDS-polyacrylamide gel.**

Procedure
When an electric field is applied, proteins migrate from cathode to anode and as they come in contact with nitrocellulose sheet.

Materials and Reagents
1. Western blot apparatus consisting of gel holder, sponge and transfer tank.
2. Power pack and electrical leads.
3. Slab gel containing separated proteins
4. Nitrocellulose sheet cut to the size of the gel.
5. Whatman 3mm paper cut to the size of the gel.
6. Transfer buffer: it consist of
   a. Tris 3.0 g
   b. Glycine 14.7 g
   c. Metanol 200 ml

After mixing the above components, adjust the pH of the solution to 8.3 and make the final volume to 1L with distilled water.

Procedure
1. Take the gel obtained after electrophoresis in experiment 10.4.3. Do not stain it and mark it by cutting of the one corner. Place the gel form 30 min in transfer buffer for equilibration.

2. Take a nitrocellulose sheet, cut to the size of the gel and dip it in the transfer buffer by carefully wetting one edge and then slowly lowering the sheet into the buffer. Leave it under the buffer for 30 min.

3. Soak the sponge in transfer buffer and place the wet sponge on the gel holder. Now keep a sheet of Whatman 3mm paper on the sponge.

4. Place the equilibrated gel carefully on the filter paper avoiding trapping of any air bubbles.

5. Now lay down carefully the Nitrocellulose membrane with shining side towards the gel on top of the gel. Gently roll a sterile 10 ml pipette over the membrane to remove air bubbles for ensuring a good contact between the membrane and the gel.

6. Complete the sandwich by placing wet Whatman 3mm filters paper and a second sponge on the filter paper. Close the gel holder and place it in the transfer tank containing sufficient transfer buffer to completely cover the blot.
7. Connect to the power supply and run for 5 h at 60v or at 30v over night.

8. When the transfer is complete, lift the membrane from the gel. Stain and destain it as in experiment 10.4.1 using Coomassie Brilliant Blue R-250 stain. Examine the nitrocellulose sheet for the presence of blue bands of the transferred proteins.

**Experiment: To precipitate proteins from protein solution, using ammonium sulphate.**

**Principle**
Solubility of proteins is lowered at high salt concentration and the protein is precipitated out. This phenomenon is called salting out. Hydrophobic region generally are protected from hydrophilic region which interact with water when salting take place. Water is generally attacked by thr higher salt concentration this generally increase protein–protein interaction and result in coagulation with each other.

**Requirements**
Protein solution, suspension buffer (20 mM tris + 100 mM NaCl pH 7.5) ammonium sulphate, centrifuge, stirrer, etc.

**Procedure**
Protein solution, is taken out from 4°C and suspension buffer is added to dissolve the pellet and thaw it. Make the solution upto 20 ml and then add 2.2 g of ammonium sulphate so as to make the solution 20% saturation. The solution is stirred continuously in the presence of ice to dissolve the salt in solution. The solution is centrifuged at 15 k rpm for 15 min at 4°C. the pellet id collected and stored at 4°C. the supernatant is agin mixed with suspension buffer to make the volume upto 20 ml than again add 2.2 g of ammonium sulphate to make solution 40% saturation. The salt is added very slowly keeping the solution continuously in ice. The solution is again centrifuged at 15 k rpm for 15 min at 4 °c. pellet is collected and dissolved in suspension buffer while supernatant is processed same way but with 60% ammonium sulphate saturation. This process is repeated with 80% ammonium sulphate saturation. Pellet and supernatant are stored at -80°C.

**GTPase Assay**

Expression and purification of GTPase (dynamin):
10 grow tetrahymena (harboring TAP tagged GTPase under MTT1 promotor) culture in CHELAX treated SPP media upto cell density 3x10^5
1. Prepare SPP

1. Make SPP
   a. Proteose petone-1%
   b. Glucose-0.2%
   c. Yeast extract- 0.1%
   d. EDTA (ferric sodium salt) 0.003%

2. Autocalve

2. Prepare SPPC (chelax-100 treated SPP)
   1. Chelax-100 (sodium form) biorad # 143-2832
   2. Add 50 g of Chelax-100 to 1000 ml of SPP
   3. Shake the stir for 2 hours
   4. Decant or spin down (230xg, 4 min)

3. Prepare SPPCT (SPPC + 3 essential trace metals)
   make a 100 x solution containing 3 trace metals
   1. FeCl3.6H2O --- 1 mg/ml
   2. Co(No3)2.6H2O --- 0.05mg/ml
   3. MnSo4.4H2o --- 0.16 mg/ml

Filter sterilize
Add the trace metals solution at a 1:100 dilution to SPPC
   2. Add 2μg/ml CdCl2 to the culture media and incubate at 30°C for 3 h
   3. Pellet down the cells in appropriate bottle at 1100 x g for 10 min
   4. Add 20 ml of 20mM Tris-cl pH7.5, 200 mM NaCl, 1.05NP40, protease inhibitor cocktail and incubate for 30 min on ice.
   5. Centrifuge at 15000 rpm for 30 min
   6. Collect the supernatant and incubate with 1 ml of rabbit IgG Agarose resin for 1 h
   7. Centrifuge at 3000 rpm for 5 min
   8. Wash the resin with 10 ml with lysis buffer for 5 times
9. Incubate with TEV protease in TEV cleavage buffer for 1 h at room temp.

10. Centrifuge at 3000 rpm for 5 min and collect the supernatant that contains purified dynamain protein.

11. Use this protein for GTPase assay or further purify by calmodulin-sepharose column.
Experiment 1: DETERMINATION OF ARTERIAL BLOOD PRESSURE IN HUMANS BY SPHYGMOMANOMETER

Principle: Blood pressure is defined as the lateral pressure exerted on the walls of blood vessels
by the circulating blood. The systolic pressure is the maximum pressure on the arteries at the height of systole of the heart and diastolic pressure is the lowest pressure during systole.

**Requirements:**
1. The apparatus for measurement of blood pressure is known as “Sphygmomanometer”. It consists of an inflatable cloth-covered rubber bag, which is fitted around the upper arm and is held in position by wrapping an extension of cloth covering the bag like a bandage. The air of the bag directly communicates with a mercury manometer through rubber connecting tubes. The bag can be inflated by a pressure bulb. There is a valve arrangement on the tube leaving from the bulb of the bag, which can be opened gradually to allow air to escape and the pressure in the bag to fall.

2. A stethoscope.

**Procedure:** Two methods are in use for measurement of blood pressure- the palpatory method and the auscultatory method. The second one will be applied in our experiment.

The subject is directed to lie or to sit with her forearm comfortably resting on a table in a supine position and at the level of the heart. This procedure renders the brachial artery very prominent - which can be felt on the inner side of the bicep tendon with the fingers. The radial pulse is also felt. The artery is gently compressed till the radial pulse is no longer felt.

**Auscultatory method:** The sphygmanometer is kept at the level of heart and the cuff is tied around the upper arm. Pressure is raised to 200 mm Hg and then gradually released. Variations of sound are heard with a stethoscope by placing its chest piece on the brachial artery, a little below the cuff. The sound is heard due to occurrence of turbulence in the blood flow through the narrowed blood vessels, when the manometric pressure in the cuff just coincides with the systolic blood pressure. By giving air pressure in the cuff, the vessel is pressed and blood flow is obliterated. But while releasing air pressure, gradually, blood just begins to flow through the narrowed blood vessels and the pattern of the flow is changed from streamline flow (silent) to turbulent (noisy). When the pressure is further released, normal streamline flow is established and the sound is no longer heard. At this point, manometric pressure coincides with the diastolic pressure. So as the pressure is released following variations of sound are heard.

Phase I: Sudden appearance of a clear tapping sound which persisted while the pressure fell through 15 mm of Hg. This indicates systolic pressure.

Phase II: The tapping of sound is replaced by a murmer persisting for another 15 mm of Hg.

Phase III: The murmer is replaced by a clear sound lasting for next 20 mm of Hg.

Phase IV: The loud sound suddenly became muffled and rapidly began to fade. This point indicates diastolic pressure.

Phase V: Absence of all sounds.
**Observation:**

Subject:
Age:
Sex:
Height:
Weight:

<table>
<thead>
<tr>
<th>Systolic pressure</th>
<th>Diastolic pressure</th>
<th>Pulse pressure = Systolic pressure - Diastolic pressure</th>
<th>Mean pressure = (Systolic pressure + Diastolic pressure) / 2</th>
</tr>
</thead>
</table>

**Remarks:**

**Required chemicals:** N/A
**Required consumables-glass ware:** N/A
**Required consumables-plastic ware:** N/A
**Required equipment:** Sphygmomanometer (Required 3), stethoscope (Required 5), height and weight measurement scale (Required 1)
Experiment 2: ESTIMATION OF HEMOGLOBIN CONTENT OF BLOOD.

**Principle:** For clinical analysis, Sahli’s haemoglobinometer is widely used. The principle of estimation is based on treatment of the whole blood with diluted acid to produce a brown acid hematin color followed by gradual dilution until the color exactly matches a standard brown glass rod. From the dilution, hemoglobin (Hb) content is obtained in percentage.

Estimation of Hb percentage has a great clinical importance. Hb is that portion of the blood which carries oxygen. One gram of Hb is capable of combining with 1.36 ml of oxygen under optimal conditions. The normal value of Hb as reported for human blood is 14.7 gm percent. The normal values depend upon a number of factors, viz., age, sex, climate, altitude and environmental factors. It is also influenced by the red blood count.

**Requirements:** Sahli’s hemoglobinometer, sterile needle, N/10 HCl, distilled water, freshly drawn human blood or goat’s blood, glass rod (stirrer), Na-citrate, glass pipettes.

**Procedure:** The apparatus consists of two vertical sealed tubes carrying standard brown color. Between the two tubes is a compartment to hold a graduated tube to be used to place the blood sample for dilution and matching with the standard glass tubes. On one side this is graduated in g % and on other side in % only. The instrument is supplied with a Pasteur pipette for sucking blood which is graduated upto 20 cm mark. Clean the tubes and the pipettes wall. Take N/10 HCl in the measuring tube upto 20 mark on the % side. Prick the left hand index finger and draw out blood in the Pasteur pipette upto 20 cm mark. Suck out the blood in the tube containing N/10 HCl and suck in and suck out 2 or 3 times to clean the pipette. Stir the blood with a glass rod. Add HCl drop by drop occasionally and shake well until the color of the diluted blood matches the standard glass tubes. Read the Hb % against a white background (supplied). Repeat the process three times and take the average.

The same experiment can be done with goat’s blood (mixed with Na-citrate) and result can be compared with human Hb content.

**Observation:**

**Remarks:**

**Required chemicals:** HCl (Required 1 bottle), Na-citrate (Required 1 bottle- minimum size available) **Required consumables-glassware:** Glass rod (stirrer), glass pipettes

**Required consumables-plasticware:** Sterile needle (14 pieces), 50 ml centrifuge tube to save goat’s blood.

**Required equipment:** Sahli’s hemoglobinometer (Required 7 piece, but as these are breakable we should have extra)

Absorbent cotton (2 packs) and ethanol to wipe hands
Experiment 3: DETERMINATION OF BLOOD CLOTTING TIME.

Principle: When the blood is exposed to air it undergoes coagulation or clotting. The blood plasma contains protein fibrinogen which is converted into fibrin when the blood undergoes clotting. This is aided by calcium ion. The clotting time is the time required for blood to form a solid clot under well defined conditions. It takes about 5 to 10 minutes for 1 ml of whole blood to clot in test tube at 37 °C. Variations in clotting time are possible which are due to various factors. If the anticoagulant heparin is added to the blood, clotting may not take place.

Requirements: Sterile needle, stop watch, capillary tube

Procedure: Obtain fresh blood. It is always better to take out blood from one’s own finger. Clean your index finger with spirit and prick it by a sterilized needle. Wipe away the first drop of blood. Hold one end of the capillary tube close to the second drop and allow the blood to run through it. As soon as capillary tube is filled with blood, start the stop watch. Hold the capillary tube in your palm to keep it warm. After two minutes, break a small piece of the capillary tube. If the blood has not solidified, allow more time. Check every 30 seconds after breaking off a small piece of the capillary until clotting has taken place. Stop the watch and note the time.

Clotting time may vary from the blood of one individual to another and also from the blood of one individual to that of another.

Observation:

Remarks:

Required chemicals:

Required consumables-glass ware: Capillary tube (1 pack= 100 pcs)
Required consumables-plastic ware: Sterile needle (Required 14)
Required equipment: Stopwatch (Required 7),
Absorbent cotton ( 2 packs) and ethanol for cleaning hands
Experiment 4: DETERMINATION OF DIFFERENTIAL BLOOD COUNT (ERYTHROCYTES AND LEUCOCYTES) IN THE HUMAN BLOOD BY HEMOCYTOMETER.

Requirements: Compound microscope, hemocytometer with white blood cell and red blood cell pipettes, cover slips, needle, absorbent cotton, ethyl alcohol 70%; RBC diluting solution, WBC diluting solution, glacial acetic acid (if WBC diluting fluid is not available).

Procedure:

1. Red blood corpuscles: collect a drop of blood in the manner that follows: clean the surface of the ring finger with 70% alcohol and allow it to dry. Take a sterile needle. Puncture the skin of the finger tip with a quick and firm stab. Discard the first drop of blood and wipe clean the finger tip. Without applying any pressure allow the drop of blood to form on the finger tip. Take the RBC pipette and suck the blood upto mark 1 carefully. Dry the tip of the pipette. If mark 1 is passed, touch the tip of the pipette with a filter paper until mark 1 is reached. Now quickly suck RBC diluting solution until the mixture reaches 101 mark. Close the mouth piece of the rubber with your tongue and hold the pipette in horizontal position in your hand. In case 101 mark is passed clean the tube and repeat the exercise to reach 101 mark accurately. Do not allow the blood to coagulate in the pipette. Detach the rubber tubing of the pipette and close the ends of the pipette with thumb and finger. Rotate for 23 minutes to allow mixing of the blood and RBC diluting solution. Again attach the rubber tubing and blow out first drop, wipe the end of the pipette and transfer a small drop on the disc of the counting chamber. The chamber should be completely filled but not flooded over the edges. Cover the disc with a cover-slip by pressing it down gently. Allow the cells to settle down for about 2/3 minutes and place the slide on the microscope stage.

Observe the counting slide under the microscope and find the cross line. Under high power you will find sixteen small squares enclosed within double lines. If the distribution of corpuscles is uneven, dry the slide and use another drop of mixture. Count the corpuscles under high power in at least 40 squares by including those lying on the lines at the top and the left hand side of the squares under consideration. Count the number of corpuscles in each of the 40 squares and calculate the average. The small square measures 1/20 by 1/20 mm and the distance between cover glass and the rules surface is 0.40 mm. determine the number of red blood corpuscles in 1 c mm of blood keeping in view of the dilution factor.

Counting of RBC: Counting is to be done under high power (45x) of the microscope objective. The RBCs are counted in the central chamber as explained above and it is customary to count cells in five chambers; four corner squares and the central one.
Counting can be done visually or by a hand tally counter.

If the total number of RBC in five squares is 450 (80 small squares), the number of RBCs in one small square will be $= \frac{450}{80} = 5.62$. Since the blood is diluted 200 times, number of RBC in cm $= 5.62 \times 4000 \times 200 = 4496000$. 
1. **White blood corpuscles**: Use a WBC pipette for collecting blood and follow the direction given in case of RBC. Draw blood in the collecting pipette to exactly mark. Draw WBC diluting fluid to 11.0 mark (1:10) dilution. The diluting fluid makes RBC invisible while WBCs are stained. Shake the pipette for about two minutes to distribute the cells uniformly. Place a drop on the counting slide and count the corpuscles in five of the large squares each of which is formed by 16 or 25 smaller squares. Calculate the average of the larger squares and determine the number per cubic millimeter.

Counting of WBC: Apply a drop of diluted blood on the corner of the counting slide and count four corner squares of the ruled area. Each corner square is further subdivided in small squares having an area 1/16 sq. mm. The depth of the counting chamber is 1/10 mm and hence the volume of the diluted blood in each small square is 1/16x 1/10 = 1/16 c mm. volume of blood in each corner square is 16x 1/60 = 1/10 c mm. Blood is diluted 1:20. The volume of each corner square is 1/10 x 1/20 = 1/200 c mm. Count the number of WBC in four corner squares of both counting chambers and take the average multiplied by 200 will give WBC/c mm of the blood.

Total number from 5 square x 5 = total number in 25 squares (i.e., total number in 1/10,000 cc). The figure multiplied by 10,000 will give the number present in 1 cc suspension taken. Each of the double line squares (0.04 sq mm) is further divided into small squares with an area of 0.0025 sq mm. Count at least two or three sets of 16 squares and calculate as follows:

Total number in one set of 15 squares x 25= number present in 0.1 c mm or 1/10,000 cc. The figure multiplied by 10,000 will give the number present in 1 cc of the suspension.

**Observation:**

**Remarks:**

**Required chemicals**: ethyl alcohol 70%, RBC diluting solution (Required 5 bottles), WBC diluting solution (Required 5 bottles)

**Required consumables-glass ware**: cover slips (Required 50 at least)

**Required consumables-plastic ware**: needle (Required 50 at least)

**Required equipment**: Compound microscopes (an inverted microscope would be better) hemocytometer with white blood cell and red blood cell pipettes (Required 12)

Absorbent cotton (2 packs)
Experiment 5: ASSAY OF AMYLASE ACTIVITY IN HUMAN SALIVA

**Materials:** Test tubes, 15 ml centrifuge tubes, beaker 50 ml, thermometer, glass rods, pipette, petri plates, microfuge tubes, whatman filter paper, 1% starch solution, burner, buffer solution pH 6.8, iodine solution 0.02 N, 1% NaCl solution, paraffin or cotton thread.

**Procedure:** Preparation of salivary amylase: Wash mouth with distilled water and then chew a piece of paraffin for 2-3 minutes. Collect the fluid in a clean beaker and repeat the procedure once more according to the amount of material required. The fluid thus collected should be transferred to a flask and filtered. Take 1 ml of this salivary extract and dilute to 10 ml by adding distilled water.

Another saliva collection method is to use plain (non-citric acid) cotton. Samples may also be collected using cotton ropes, or by passive drool. For accurate results collection devices should be completely saturated before removal. **Do not** add sodium azide to saliva samples as a preservative. Samples visibly contaminated with blood should be recollected. Freeze at -20°C or lower for long-term storage. Saliva samples should be frozen prior to assay to precipitate the mucins. On day of assay, thaw completely, vortex, and centrifuge at 1500 x g (@3000 rpm) for 15 minutes. Pipette clear sample into appropriate wells. Particulate matter may interfere with the reaction, leading to inaccurate results.

**Method:** Prepare fresh 1% starch solution (make a paste of the weighed amount with distilled water and then add warm distilled water to make it 1% and then cool). Take 3 ml of starch solution and add 2 ml of buffer at pH 6.8 and two drops of NaCl solution. Mix well and place the tube in a water bath adjusted to 37 deg C. Place a series of iodine drops on the petri plate. Add 2 ml of salivary extract to the tube containing starch solution and buffer and mix well. This is the digest. With the help of a glass rod take digest drop by drop to the iodine drops on the patri plate, mix with another glass rod and note the color change, if any. Iodine inhibits the enzyme reaction. From time to time allow a drop of digest to fall on the iodine drops. A blue color is produced at first followed by blue-violet, red-violet, red-brown, light-brown and finally no change in iodine color. Note the time taken by the digest when it does not produce any change in color of iodine. This is the end point representing digestion of starch by the enzyme.

**Observation:**

**Remarks:**

**Required chemicals:** soluble starch (2 bottles), buffer solution pH 6.8 (5 bottles), iodine solution 0.02 N (3 bottles), NaCl (2 bottles)

**Required consumables-glassware:** Test tubes (40 test tubes), beaker 50 ml (10 each
of 50 ml and 25 ml), Thermometer (Hg free) (required 6), glass rods (Required 30), pipette (2, 5, 10, 25 ml pipettes 25 pieces each), petri plates (25 pieces),

**Required consumables-plasticware:** 15 ml centrifuge tubes (50 pieces), 1.5 ml centrifuge tubes (Required 50)

**Required equipment:** water bath, bench top centrifuge machine

Also needed whatman filter paper, burner, paraffin, cotton or cotton thread.
Experiment 6: IDENTIFICATION OF HISTOLOGICAL SLIDES OF MOUSE TISSUES:

Requirements: 19 histological slides as mentioned in the excel file in duplicates i.e. total 38 slides and 4 compound microscopes

Student will also be trained to process the tissue sections and prepare permanent slides. The protocol for the same is described below.
HEMATOXYLIN AND EOSIN STAINING

Objective: Preparation of permanent histology slides.

Materials: Slides, coverslips, DPX, Xylene, ethanol, distilled water, staining jars, forceps, Hematoxylin and eosin stain, ammonia, phloxine,

Procedure:

Section will be cut on a cryotome or regular microtome and collected on slides. Re-hydrate in 2 changes of D. water 5 minutes each.
Stain in Harris hematoxylin solution for 8 minutes.
Wash in running tap water for 5 minutes and differentiate in 1% acid alcohol for 30 seconds.
Wash in running tap water for 1 minute and bluing in 0.2% ammonia water for 30 seconds to 1 minute. Wash in running tap water for 5 minutes and rinse in 95% alcohol, 10 dips.
Counterstain in eosin-phloxine solution for 30 seconds to 1 minute.
Dehydrate through 95% alcohol, 2 changes of absolute alcohol, 5 minutes each. Rinse in xylene 2 times, 5 minutes each.
Mount with DPX mounting medium using a coverslip. Observe under the microscope.

Observations:

Hematoxylin will stain nuclei and appear blue, whereas eosin stains cytoplasm and appears red.

Required chemicals: DPX (2 bottles), Xylene (4 bottles), ethanol (4 bottles), distilled water, Hematoxylin and eosin stain (2 bottles each), phloxine,

Required consumables-glassware: staining jars (15), forceps (6), beakers (5), measuring cylinders (4), glass bottles (5), Pasteur pipettes with rubber bulb (20), Slides (10 box), coverslips (4 box),

Required consumables-plasticware: Required

Required equipment: Microscope
Experiment 7: PREPARATION OF HAEMIN CRYSTALS

Principle:

The red cells make up about 45% by volume of the blood. Haemoglobin is the red colouring matter of the blood which is about 14.7 g per 100 ml of the whole blood. Haemoglobin is readily separated into its protein and prosthetic components by treatment with acetic acid. The globin is usually denatured in the process, and the iron containing portion is in the form of insoluble crytaline compound haemin. The formation of haemin crystals is used as a test for detecting presence of blood and not any other agent.

Requirements:

Microscope; test tube; slide and cover slips, glacial acetic acid, distilled water

Procedure

Take a drop of fresh blood on a slide and evaporate it to dryness by gentle heating on flame. Add 2-3 drops of glacial acetic acid to the dried blood and put a cover slip over it. Group the slide over a low flame until the material is dried up. Cool the slide in air and observe in microscope. You will notice haemin crystal as shining prism-like or star shaped bodies.

Observations:

Remarks:

Required chemicals: Glacial acetic acid
Required consumables-glassware: Slides (2 box), coverslips (2box), glass bottles (8), Pasteur pipettes with rubber bulb (20)
Required consumables-plasticware:
Required equipment:
Microscope
Other consumables: cotton, disposable sterile needles (100)
Experiment 8: COMPARISION OF RESPIRATORY ORGANS IN *LABEO ROHITA* AND *CLARIAS GARIEPINUS*:

**Principle:**
The animals have adapted to the environment and the respiratory organs have evolved as per the need. While fish is an aquatic animal which has specialized respiratory structures called as gills for aquatic mode of respiration, there are some fish species which has both aquatic as well as aerial modes of respiration. The positions, organization, and structure-function relationship of these respiratory organs will be studied.

**Requirements:**
Fish, *Labeo rohita* and the catfish, *Clarias gariepinus* will be used. Dissection instruments like forceps, scissors, and dissection tray are required.

**Procedure:**
Fish will be anaesthetized using 2-phenoxy ethanol. The respiratory organs, gills and the air breathing organ in the fish will dissected out and compared for their organization and structure function relationship.

**Observations:**

**Remarks:**

**Required chemicals:** 2-phenoxy ethanol  
**Required consumables-glassware:** petri dishes (15)  
**Required consumables-plasticware:**  
**Required equipment:** Binocular/Dissecting microscope  
**Other consumables:** fish from local market, dissecting instruments
FIFTH SEMESTER
PLANT PHYSIOLOGY
Experiment: 1 Isolation and quantification of anthocyanin in plants

Principle:
Anthocyanins are water soluble vacular pigment that may appear red, purple, or blue according to pH. They belong to a parent class of molecules called flavonoid synthesized via the Phenyl propionic acid pathway; they are odorless and nearly flavorless, contributing to taste as a moderately astringent sensation. Anthocyanins occur in all tissues of higher plants, including leaves, stems, roots, flowers and fruits. Anthocyanins are derivatives of anthocyanidins which include pendant sugars. In addition to their role as light-attenuators; anthocyanins also act as powerful antioxidant.

Procedure:
Thirty grams of each sample were grounded with pestles and mortars after frozen with liquid nitrogen. Anthocyanins were extract after grounded samples were soaked in 30 ml hexane for 24 hr to remove fats and oils. Three grams of the ground materials were extracted twice by mixing with 30 mL of methanol acidified with 1.0 N HCl (85:15, v/v) and shaking on a shaker at 4 for 24 hr. The crude extracts were filtered with Whatman No. 1 paper. Anthocyanins in the partially purified extracts were separated and quantified with reverse phase HPLC equipped with a Xterra MS C18 column (3.5 μm, 4.6 × 250 mm), Tosoh TSK-6040 UV-Vis detector (Tokyo, Japan). The column was eluted with a mobile phase consisting of H2O: methanol: formic acid (75:20:5, v/v/v) with flow rate of 0.5 ml/min. The separated anthocyanins were detected and measured at 530 nm, and the identity of anthocyanins was based on the congruence of retention times and UV-Vis spectra with those of pure authentic standards.
Experiment: 2 PLANT DNA ISOLATION

PRINCIPLE

Plant cells possess’ rigid cellulose cell wall and a cell membrane. Breaking the cell, layer by layer releases the cellular constituents. At first, the plant cell wall has to be damaged. It is accomplished by grinding the plant tissues and homogenizing in the extraction buffer. The components of the extraction buffer cause cell lysis.

Tris (Tris-hydroxymethyl aminomethane) in the extraction buffer, buffers the pH of the cells at 8.0. EDTA (Ethylene diamine tetraacetic acid) deters the DNase activity, chelates the metal ions which are cofactors for them. It also weakens the cell membrane stability. Sodium chloride also helps in maintaining the osmoticum of the cells. Chloride component enter into the cells by ionic transport along with water by diffusion. This influx expands the cells, prevents cell aggregation and weakens the membrane integrity.

Mercaptoethanol cleaves the disulphide bridges of proteins and helps in denaturation of membrane proteins and cytosolic proteins. SDS is an anionic detergent, which also denatures the membrane proteins and disrupts the cell membrane. This also results in the breakdown of the nuclear envelope and thereby releases the nucleoplasm. SDS also helps in inhibition of nucleases.

DNA in the nucleoplasm is neutralized by Potassium acetate. K+ ions bind with negative phosphate backbone of the DNA and shield them. This favors DNA precipitation from ethanol in cold condition. Upon centrifugation, the neutralized DNA with impurities goes into the solution this time. When alcohol is added in cold condition to the solution, DNA strands come close together and coalesce. But other impurities are soluble in ethanol and are removed in the supernatant. DNA precipitate is settled down as a pellet by centrifugation, purified by 70 % ethanol wash, hydrated in TE buffer and analyzed on agarose gel.
Procedure

1. For disruption using the Tissue Ruptor, follow step 2; for disruption using the Tissue Lyser, follow steps 3–6. Alternatively, plant or fungal tissue can be ground to a fine powder under liquid nitrogen using a mortar and pestle. Transfer the tissue powder and liquid nitrogen to an appropriately sized tube and allow the liquid nitrogen to evaporate. Do not allow the sample to thaw. Proceed immediately to step 7.

2. Tissue Ruptor procedure: Place the sample material (100 mg wet weight or 20 mg lyophilized tissue) into a 2 ml micro centrifuge tube. Add liquid nitrogen to the tube, and freeze the sample for 30 s. Keep the sample submerged in liquid nitrogen, and disrupt for approximately 30 s at full speed. Allow the liquid nitrogen to evaporate, and proceed immediately to step 7.

Alternatively, fresh or lyophilized material can be directly disrupted in lysis buffer (after step 7) without using liquid nitrogen, but this may cause shearing of highmolecular-weight DNA. We do not recommend disrupting frozen material in lysis buffer as this can result in low yields and degraded DNA.

3. Tissue Lyser procedure: Place the sample material (100 mg wet weight 20 mg lyophilized tissue) into a 2 ml safe-lock microcentrifuge tube, together with a tungsten carbide bead. Freeze the tubes in liquid nitrogen for 30 s. When using lyophilized tissue, the tubes do not need to be frozen in liquid nitrogen.

4. Place the tubes into the TissueLyser Adapter Set 2 x 24, and fix into the clamps of the TissueLyser. Immediately grind the samples for 1 min at 30 Hz.

5. Disassemble the adaptor set, remove the tubes, and refreeze in liquid nitrogen for 30 s. When using lyophilized tissue, the tubes do not need to be frozen in liquid nitrogen.

6. Repeat step 4, reversing the position of the tubes within the adaptor set. Proceed immediately to step 7.
To prevent variation in sample homogenization, the adaptor sets should be removed from the Tissue Lyser and disassembled after the first disruption step. For the second disruption step, the adaptor sets should be reassembled so that the tube order is reversed. Rotating the racks of tubes in this way ensures that all samples are thoroughly and equally disrupted.

Note: The majority of plant tissue is ground to a fine powder after 2 disruptions Steps, however, for some materials one disruption step may be sufficient. Other Tissues, such as seeds and roots, may require disruption steps. Optimization of the disruption procedure may be required for some plant material.

7. Add 400 μl Buffer AP1 and 4 μl RNase a stock solution (100 mg/ml) to a maximum of 100 mg (wet weight) or 20 mg (dried) disrupted plant or fungal tissue and vortex vigorously. No tissue clumps should be visible. Vortex or pipet further to remove any clumps. Clumps of tissue will not lyse properly and will therefore result in a lower yield of DNA. In rare cases, where clumps cannot be removed by pipetting and vortexing, a disposable micropette may be used.

Note: Do not mix Buffer AP1 and RNase A before use.

8. Incubate the mixture for 10 min at 65°C. Mix 2 or 3 times during incubation by inverting tube. This step lyses the cells.

9. Add 130 μl Buffer AP2 to the lysate, mix, and incubate for 5 min on ice. This step precipitates detergent, proteins, and polysaccharides.

10. Recommended: Centrifuge the lysate for 5 min at 20,000 x g (14,000 rpm). Some plant materials can generate very viscous lysates and large amounts of precipitates during this step. This can result in shearing of the DNA in the next step. In this case, optimal results are obtained if the majority of these precipitates are removed by centrifugation for 5 min at 20,000 x g (14,000 rpm). After centrifugation, apply supernatant to QIAshredder Mini spin column and continue with step 11.
11. Pipet the lysate into the QIAshredder Mini spin column (lilac) placed in a 2 ml.

12. Collection tube, and centrifuge for 2 min at 20,000 x g (14,000 rpm). It may be necessary to cut the end off the pipet tip to apply the lysate to the QIA shredder Mini spin column. The QIA shredder Mini spin column removes most precipitates and cell debris, but a small amount will pass through and form a Pellet in the collection tube. Be careful not to disturb this pellet in step 12. Transfer the flow-through fraction from step 11 into a new tube (not supplied) without disturbing the cell-debris pellet Typically 450 μl of lysate is recovered. For some plant species less lysate is recovered. In this case, determine the volume for the next step.

13. Add 1.5 volumes of Buffer AP3/E to the cleared lysate, and mix by pipetting. For example, to 450 μl lysate, add 675 μl Buffer AP3/E. Reduce the amount of Buffer AP3/E accordingly if the volume of lysate is smaller. A precipitate may form after the addition of Buffer AP3/E, but this will not affect the DNeasy procedure.
Note: Ensure that ethanol has been added to Buffer AP3/E.
Note: It is important to pipet Buffer AP3/E directly onto the cleared lysate and to mix immediately.

14. Pipet 650 μl of the mixture from step 13, including any precipitate that may have formed, into the DNeasy Mini spin column placed in a 2 ml collection tube (supplied). Centrifuge for 1 min at 6000 x g (corresponds to 8000 rpm for most microcentrifuges), and discard the flow-through.* Reuse the collection tube in step 15

15. Repeat step 14 with remaining sample. Discard flow-through* and collection tube.

16. Place the DNeasy Mini spin column into a new 2 ml collection tube (supplied), add 500 μl Buffer AW, and centrifuge for 1 min at 6000 x g  8000 rpm). Discard the flow-through and reuse the collection tube in step 17.
Note: Ensure that ethanol is added to Buffer AW.
17. Add 500 μl Buffer AW to the DNeasy Mini spin column, and centrifuge for 2 min at 20,000 x g (14,000 rpm) to dry the membrane. It is important to dry the membrane of the DNeasy Mini spin column since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during elution. Discard flow-through and collection tube. After washing with Buffer AW, the DNeasy Mini spin column membrane is usually only slightly colored. In the rare case that the membrane remains significantly colored after washing with Buffer AW, refer to “Darkly colored membrane or green/yellow eluate after washing with Buffer AW”

Note: Following the centrifugation, remove the DNeasy Mini spin column from the collection tube carefully so the column does not come into contact with the flow through, as this will result in carryover of ethanol.

18. Transfer the DNeasy Mini spin column to a 1.5 ml or 2 ml micro centrifuge tube (not supplied), and pipet 100 μl Buffer AE directly onto the DNeasy membrane. Incubate for 5 min at room temperature (15–25°C), and then centrifuge for 1 min at 6000 x g 8000 rpm) to elute. Elution with 50 μl (instead of 100 μl) increases the final DNA concentration in the elute significantly, but also reduces overall DNA yield. If larger amounts of DNA (>20 μg) are loaded, eluting with 200 μl (instead of 100 μl) increases yield.

19. Repeat step 18 once. A new micro centrifuge tube can be used for the second elution step to prevent dilution of the first eluate. Alternatively, the micro centrifuge tube can be reused for the second elution step to combine the eluates.

Note: More than 200 μl should not be eluted into a 1.5 ml micro centrifuge tube because the DNeasy Mini spin column will come into contact with the eluate.
**Experiment: 3 Osmotic effects on imbibition of pea seeds**

**Principle:** Imbibition in pure water and in solution are not alike. Free water concentration is more in pure water which decreases with increase in concentration of solution. The rate of imbibitions is inversely proportional to the concentration of solutes in a solution and shows a diminishing trends with increase in concentration of solution.

**Procedure:**
1. Weight about 2.5gm of pea seeds and put in sucrose solution of different molarity (0.10, 0.20, 0.30, 0.40 and 0.50 M).
2. After 30 minute take the final weight of the imbibed seeds.
3. Before taking the final weight blot the solution adhered to the surface of the seeds.
4. After getting the difference between the final and initial weights, calculate the present imbibitions in each case. Prepare histogram on graph paper and give your conclusion.
Experiment: 4 Liberation of heat during imbibitions

Principle:
Kinetic energy of water molecule loosed when adsorbed to the colloidal surface of the seeds. This energy is immediately converted into the heat energy to the surroundings which is measured by means of thermometer and the amount of heat released is thus calculated.

Procedure:
1. Weight about 50gm of pea seeds and put inside the thermos bottle.
2. Add approximately 50 ml of water to it.
3. Record the temperature of water plus pea seeds at zero time.
4. Keep constant watch on the thermometer and note the temperature change.
5. The highest record temperature is used for the purpose and calculate the amount of heat liberated during imbibition using differential reading in temperature.
Experiment: 5 Effect of temperature on Imbibition

**Principle:** It is known that increase in temperature of the system increase the kinetic energy of the water molecule. Thus there is a rapid rate of imbibitions of water molecule to the colloidal surface with in a stipulated time

**Procedure:**
1. Take 5 sets of petri dishes and in each take water with varying temperature. (0°C, 30°C, 40°C, 50°C and 60°C).
2. Put approximately 5gm of pea seeds separately to each petridish.
3. Leave this for thirty minutes
4. After expirey of time soak the adhering water molecules on seed.
5. Take the final weight.
6. The difference in weight indicates the amount of water imbibed by the seed material.
7. Calculate the % of Imbibition by the following formula

\[
\text{% of imbibition} = \frac{\text{final weight - initial weight}}{\text{Initial weight}}
\]
Experiment: 6 Effect of seed coat on the rate of imbibition of water

Principle:

Imbibition is considered a special type of diffusion since the net movement of water is along a diffusion gradient. Two conditions appear to be the requisite for imbibition to occur.

(A) A diffusion pressure gradient between the imbibant and the substance imbibed.
(B) The existence of certain affinity between the component of the imbibant and the imbibed substance.

When the seeds are puts in soil water first enter into the seeds through imbibation and thus helps to start the process of germination. Hence water entered into the seed through the varying seed coats which are responsible to determine the rate / speed of imbibation.

Procedure:

1. Keep equal amount of sun flower, gram, rice and pea seeds inequal amount of water in the beaker separately for 60 minutes.
2. Take this out after 60 minutes
3. Remove the water particles adhered to the external surface of the seed coat with the help of bloating paper.
4. Take the weight of the seeds separately and record the observation in a tabular form by calculating the % of imbibition.
Experiment: 7
Demonstration of imbibition in pea seeds

Principle:
An index of potential maximum pressure which can be developed in a solution as a result of osmosis is called imbibition pressure. It is a colligative property of solution, i.e. It is directly proportional to the number of solute molecule in a given solvent.

Procedure:
1. Take about 50gm of healthy and well dried pea seeds in the conical flask.
2. Fill the flask with water. Close the mouth of the flask with rubber cork which has a glass capillary tube in its centre.
3. Make the whole apparatus air tight by applying thin layer of grease to the adjoining sites.
4. Keep the apparatus undisturbed at least for 45 minutes and take the observation (Record at least 20 observations).
5. Plot the observation on a graph paper against time intervals.
Experiment: 8

Purification of plant Nuclei and observation of phytochrome nuclear complexes

Principle:
Nuclear import of phytochrome is light dependent. Here we will investigate and demonstrate using microscopy and biochemistry the subcellular phytochrome domain in different light condition.

Procedure:
All procedures were performed at 4 °C unless stated otherwise. 100 ml of BY-2-mGFP5-ER cells in exponential phase (3 days after inoculation) were collected by filtering through a nylon mesh (mesh diameter, 20 m) and subsequently resuspended in 10 volumes of chilled buffer A (10 mM Tris-HCl, pH 6.8; complemented with 5 mM MgCl2, 10 mM - mercaptoethanol, 0.15 M sucrose, 50% [v/v] glycerol, 1 mM phenylmethylsulfonyl fluoride, 50 M N-p-tosyl-L-phenylalanine chloromethyl ketone, and 0.6% [v/v] Triton X-100). Cells were homogenized in a Waring blender for 30 s and filtered through Miracloth (Calbiochem, Merck Biosciences Ltd., Nottingham, U.K.). Cell material retained by the filter was collected and resuspended in 10 volumes of buffer A, homogenized once again for 30 s, and filtered through Miracloth. Filtrates were mixed and filtered through a nylon mesh (mesh diameter, 20 m). The filtrate was centrifuged at 500 g for 5 min at 2 °C, and the resulting supernatant was centrifuged again at 2000 g for 10 min at 2 °C. An aliquot of 100 l was collected from the supernatant and stored at 20 °C as fraction I. The sediment containing nuclei was resuspended carefully in 1 ml of cold buffer A, and again an aliquot of 100 l was collected as fraction II. Nuclei were further purified by centrifugation through a 25% to 50% Percoll gradient prepared in buffer A at 7000 g for 30 min at 2 °C. The interface which contained the nuclei was collected, resuspended in 15 ml of buffer A, and centrifuged at 4000 g for 10 min at 2 °C. Sedimented nuclei were washed with buffer A and centrifuged at 2000 g for 10 min at 2 °C. Again, an aliquot of 100 μl was collected from the supernatant as fraction III. The sedimented nuclei (fraction IV) were assayed under the
microscope and proteins were extracted and separated electrophoretically. For protein purification nuclei are lysed in lysis buffer with various parameter.

**Experiment: 9**

**Determination of the osmotic potential of cell saps of Rhoeo discolor leaves by plasmolytic method.**

**Principle:** When a living plant cell is placed in an solution with an osmotic potential higher than its own cellsap, there will be a net movement of water out of the cell vacuole into the external solution. This results in loss of turgor, shrinking of the vacuole and pulling away of the cell membrane from the cell wall. The cell in this condition is called plasmolysed and the process is called plasmolysis.

**Procedure:**
Prepare 1M sucrose solution by dissolving 171.0 gm of sucrose in water and make upto the volume of 500 ml. Out of the 1 m sucrose stock solution prepare a series of solution with strength of 0.05 to 0.30M concentration in separate watch glasses. Take sucrose solution of 0.05M to 0.3M concentration in separate watch glasses. Cut small strips from the lower epidermis, peeling from just above the midrib of the leaves (the red coloured pigment is anthocyanin). Immerse one such strip in each solution and cover the watch glass. After 30 minute mount one of the strip on a slide in the solution in which it has been immersed and examine under the microscope for plasmolysis.
**Experiment: 10**

**POLYMERASE CHAIN REACTION OF PLANT DNA**

**Principle**

Polymerase Chain Reaction (PCR), invented by Kary B. Mullis, at the Cetus Corporation, who was awarded the 1993 Nobel Prize for chemistry for PCR, is a technique to exponentially amplify in vitro a small quantity of a specific nucleotide sequence in the presence of template sequence, two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA, a thermostable (taq) DNA polymerase. The reaction is cycled involving template denaturation, primer annealing, and the extension of the annealed primers by DNA polymerase until enough copies are made for further analysis.

**Steps for Standard PCR Reaction**

Design primers. In general, primers should have the following properties:

• Length of 18-24 bases
• 40-60% G/C content
• Start and end with 1-2 G/C pairs
• Melting temperature (Tm) of 50-60°C
• Primer pairs should have a Tm within 5°C of each other
• Primer pairs should not have complementary regions

Tip: Primer3 is an excellent resource for choosing primers.

Tip:

2. Set up PCR tubes.

If you will be including a restriction site at the 5' end of your primer, note that a 3-6 base pair spacer should be added in order for the enzyme to cleave efficiently.

• Place thin-walled PCR tubes on ice.
• For a 50 µL reaction, add:
2 μL Template DNA (10 ng-500 ng) 5 μl 10X Taq buffer with MgCl2 1 μl dNTP mix (10 mM each nt) 2.5 μL Forward Primer (10 μM stock) 2.5 μL Reverse Primer (10 μM stock) 0.2 μL Taq DNA Polymerase (5 units/μL) 32.8 μL Sterile deionized water (variable)

Tip: If you are doing multiple PCR reactions, save time by creating a "master mix."

3. PCR: The following is a typical PCR program. The annealing temperature should be 5oC below the primer Tm. The extension step should be 1-2 minutes per kilobase of product, depending on whether you are using a polymerase with proofreading capabilities. See manufacturer's instructions.

Step 1: Initial Denaturation for 2 minutes at 95oC
Step 2: Denature for 1 minute at 95oC
Step 3: Anneal primers for 30 seconds at 55oC (or 5oC below Tm)
Step 4: Extend DNA for 2 minutes at 72oC
Step 5: Repeat steps 2-4 for 25-30 cycles
Step 6: Final Extension for 10 minutes at 72oC

4. Run 2 μL on a 1.5% agarose gel to check size and concentration of PCR product.
Experiment: 11
Measurement of rate of photosynthesis by wilmott Bubbler

**Principle:** It is known that photosynthesis, in green tissues, takes place in the presence of CO2 water and light. In the process there is synthesis of carbohydrate and O2 comes out as end product. Light 2n H2O + nCO2 -------------- (CH2O) n + nH2O + O2 chlorophyll
\[ \uparrow \]
The striking photochemical reaction is photolysis, which involved splitting of water, accompanied by evolution of O2 and the release of proton which is captured by NADP.

**Procedure:**
Cut several healthy actively growing twigs of hydrilla, about 15 cm length from the growing apex under water. Insert the cut end through a fine jet glass tubing of wilmott bubbler and cover it in water under an inverted test tube. Before that the jar of the bubbler is filled in with water and add about 1-2 gm NaHCO3/KHCO3. Keep the apparatus under sun. Bubbles start coming out of the glass tube. Determine the relative number of bubbles coming out per unit time under different light condition (Placing electrical bulb of different wattage and varying distance) also the wilmott bubbler mat be kept under fan and shade to determine the number of bubbles coming out as a result of photosynthesis and compare the result with that of light.
Experiment: 12

PHOTOTROPISM

Principle
Phototropism is directional growth in which the direction of growth is determined by the direction of the light source. The cells on the plant that are farthest from the light have a chemical called auxin that reacts when phototropism occurs. This causes the plant to have elongated cells on the farthest side from the light. Phototropism is one of the many plant tropisms or movements which respond to external stimuli. Growth towards a light source is a positive phototropism, while growth away from light is called negative phototropism (or Skototropism). Most plant shoots exhibit positive phototropism, while roots usually exhibit negative phototropism, although gravitropism may play a larger role in root behavior and growth. Some vine shoot tips exhibit negative phototropism, which allows them to grow towards dark, solid objects and climb them.

Procedure
Seeds of different genetic background such as phy B, phyA, phyAB, etc will be demonstrated under different light condition such as RL, FR, BL, WL and dark in light chamber and growth cabinet. Different fluorescence will be tested and fluorescence and light microscopy will be done.
**Experiment: 13**

**Estimation of chlorophyll pigments from the plant samples by acetone, extraction method.**

**Introduction:**
The green pigments of chloroplast are the most important photosynthesis plant pigments. Chlorophyll a, b, c, d and e, bacteriochlorophyll and bacterioviriden are chlorophyll pigments. Chlorophyll a and b are abundant in higher plants. Chlorophylls are green pigments which are lipophillic in nature hence are soluble in polar solvents like, acetone, alcohol, benzene and so on. The extent/ intensity of green color is quantified by measuring the absorbance (optical density) in spectrocolourimeter.

**Procedure:**
Weigh exactly 250 mg of fresh leaf, macerate it in a mortor and pestle by adding small amount of 80% acetone. Filter the ground material by using whatman No. 1 filter paper and collect the filtrate in to 25 ml volumetric flask. The mortar is rinsed twice with 80% acetone and the residue is filtered. Make up the volume of the filtrate to 25 ml mark by adding extar amount of 80% acetone. Measure the absorbance of the extract at 645 and 663 nm in spectrocolourimeter using 80% acetone as reference solution. Calculate the amount of chlorophyll as outlined by Arnon, 1949.

\[
\text{Chl 'a'} = [12.7 (D \text{ 663}) - 2.69 (D \text{ 645})] \times V \times 1000 \times W \times 1 \text{ mg g}^{-1} \text{ fresh tissue.}
\]

\[
\text{Chl 'b'} = [22.9 (D \text{ 645}) - 4.68 (D \text{ 663})] \times V \times 1000 \times W \times 1 \text{ mg g}^{-1} \text{ fresh tissue.}
\]

\[
\text{Total Chlorophyll} = [20.2 (D \text{ 645}) + 8.02(D \text{ 663})] \times V \times 1000 \times W \times 1 \text{ mg g}^{-1} \text{ fresh tissue.}
\]

where, \(D \text{ 645} = \text{Absorbance at 645 nm}\)

\(D \text{ 663} = \text{Absorbance at 663 nm}\)

\(V = \text{Volume of the filtrate}\)

\(W = \text{weight of the tissue in grams}.\)
**Experiment: 14**

**Test of starch in photosynthesizing tissue:**

**Introduction:**
Photosynthesis is building-up process with the help of light energy. This primary process helps in the conversion of light energy to chemical energy which occurs by photophosphorylation. Chemical energy is stored in the tissue of plant in the form of sugar, carbohydrates and starch molecules. Starch when combines with I₂ KI produces blue colour which indicates the occurrence of photosynthesis in intact plants tissues.

**Procedure:**
Take an intact fresh leaf and dip it in a containing 80% acetone for a least one hour. Take it out after expiry of the time and test it for starch with I₂ KI solution in fact during one hour, chlorophyll pigments will be bleached to acetone leaving whitish surface on leaf. To this bleached leaf, put one/ two drops of I₂ KI which will produce blue color indicating the presence of starch synthesized upon Photosynthesis during light hour. The above test may be compared with the leaf of a plant grown in dark. Discuss the results with your salient comments.
Experiment: 15

QuantiTect Reverse Transcription

Introduction
The QuantiTect Reverse Transcription is a convenient procedure for efficient reverse transcription and effective genomic DNA elimination. This method is used for cDNA yields for sensitive quantification of even low-abundance transcripts.

Principle and procedure The QuantiTect Reverse Transcription procedure takes only 20 minutes and comprises 2 main steps: elimination of genomic DNA and reverse transcription.

Elimination of genomic DNA The purified RNA sample is briefly incubated in gDNA Wipeout Buffer at 42°C for 2 minutes to effectively remove contaminating genomic DNA. In contrast to other methods, the RNA sample is then used directly in reverse transcription. Accurate results in real-time RT-PCR depend on the use of primers or probes designed to eliminate or minimize detection of genomic DNA. If such primers or probes are not available, then genomic DNA contamination in RNA samples must be eliminated. Reverse transcription After genomic DNA elimination, the RNA sample is ready for reverse transcription using a master mix prepared from Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, and RT Primer Mix. The entire reaction takes place at 42°C and is then inactivated at 95°C. In contrast to other methods, additional steps for RNA denaturation, primer annealing, and RNase H digestion are not necessary. Quantiscript Reverse Transcriptase has a high affinity for RNA and is optimized for efficient and sensitive cDNA synthesis from 10 pg to 1 μg of RNA. This high RNA affinity, in combination with Quantiscript RT Buffer, enables high cDNA yields, even from templates with high GC-content or complex secondary structure. RT Primer Mix ensures cDNA synthesis from all regions of RNA transcripts, even from 5' regions. This allows high yields of cDNA template for real-time PCR analysis regardless of where the target region is located on the transcript.
Experiment: 16

**QuantiTect Reverse Transcription Procedure**

Mix RNA, gDNA Wipeout Buffer, and RNase-free water. Incubate at 42°C for 2 min. Add Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, and RT Primer Mix, and mix. Incubate at 42°C for 15 min. Incubate at 95°C for 3 min to inactivate Quantiscript Reverse Transcriptase. Add cDNA to real-time PCR mix and distribute Quantitative, real-time PCR.
Experiment: 17

**RNA purification using RNeasy technology**

**Principle**
The RNeasy procedure represents a well-established technology for RNA purification. This technology combines the selective binding properties of a silica-based membrane with the speed of microspin technology. A specialized high-salt buffer system allows up to 100 μg of RNA longer than 200 bases to bind to the RNeasy silica membrane. Biological samples are first lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate–containing buffer, which immediately inactivates RNases to ensure purification of intact RNA. Ethanol is added to provide appropriate binding conditions, and the sample is then applied to an RNeasy Mini spin column, where the total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA is then eluted in 30–100 μl water. In addition to this RNA stabilization is an absolute prerequisite for reliable gene expression analysis. Immediate stabilization of RNA in biological samples is necessary because, directly after harvesting the samples, changes in the gene expression pattern occur due to specific and nonspecific RNA degradation as well as to transcriptional induction. Such changes need to be avoided for all reliable quantitative gene expression analyses, such as microarray analyses, quantitative RT-PCR, such as TaqMan® and LightCycler® technology, and other nucleic acid-based technologies.

**Procedure**
1. Determine the amount of plant material. Do not use more than 100 mg. Weighing tissue is the most accurate way to determine the amount.

2. Immediately place the weighed tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into an RNase-free, liquid-nitrogen–cooled, 2 ml microcentrifuge tube (not supplied). Allow the liquid Plants nitrogen to evaporate, but do not allow the tissue to thaw. Proceed immediately to step 3. RNA in plant tissues is not protected
until the tissues are flash-frozen in liquid nitrogen. Frozen tissues should not be allowed to thaw during handling relevant procedures should be carried out as quickly as possible.

3. Add 450 μl Buffer RLT or Buffer RLC (see “Important points before starting”) to a maximum of 100 mg tissue powder. Vortex vigorously. A short 1–3 min incubation at 56°C may help to disrupt the tissue. However, do not incubate samples with a high starch content at elevated temperatures, otherwise swelling of the sample will occur.
Note: Ensure that β-ME is added to Buffer RLT or Buffer RLC before use (see “Things to do before starting”).

4. Transfer the lysate to a QIAshredder spin column (lilac) placed in a 2 ml collection tube, and centrifuge for 2 min at full speed. Carefully transfer the supernatant of the flow-through to a new microcentrifuge tube (not supplied) without disturbing the cell-debris pellet in the collection tube. Use only this supernatant in subsequent steps. It may be necessary to cut off the end of the pipet tip to facilitate pipetting of the lysate into the QIAshredder spin column. Centrifugation through the QIAshredder spin column removes cell debris and simultaneously homogenizes the lysate. While most of the cell debris is retained on the QIAshredder spin column, a very small amount of cell debris will pass through and form a pellet in the collection tube. Be careful not to disturb this pellet when transferring the lysate to the new microcentrifuge tube.

5. Add 0.5 volume of ethanol (96–100%) to the cleared lysate, and mix immediately by pipetting. Do not centrifuge. Proceed immediately to step.
Note: The volume of lysate may be less than 450 μl due to loss during homogenization.
Note: Precipitates may be visible after addition of ethanol. This does not affect the procedure.

6. Transfer the sample (usually 650 μl), including any precipitate that may have formed, to an RNeasy pin column (pink) placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at 8000 x g (10,000 rpm). Discard the flow through. Reuse the collection tube in step 7. If the sample volume exceeds 700μl, centrifuge successive aliquots in the same RNeasy spin column. Discard the flow-through after each centrifugation.*
Optional: If performing optional on-column DNase digestion (see “Eliminating
Optional: If performing optional on-column DNase digestion follow steps D1–D4 after performing this step.

7. Add 700 μl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 8000 x g (10,000 rpm) to wash the spin column membrane. Discard the flow-through.* Reuse the collection tube in step 8.
 Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely. Skip this step if performing optional on-column DNase digestion

8. Add 500 μl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 8000 x g (10,000 rpm) to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in step 9.
 Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use (see “Things to do before starting”).

9. Add 500 μl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at 8000 x g (10,000 rpm) to wash the spin column membrane. The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.
 Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

10. Optional: Place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min. Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column after step 9.

11. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 μl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at 8000 x g (10,000 rpm) to elute the RNA.
12. If the expected RNA yield is >30 μg, repeat step 11 using another 30–50 μl Rnase free water, or using the eluate from step 11 (if high RNA concentration is required). Reuse the collection tube from step 11. If using the eluate from step 11, the RNA yield will be 15–30% less than that obtained using a second volume of RNase-free water, but the final RNA concentration will be higher.

**RNA Cleanup Procedure**

1. Adjust the sample to a volume of 100 μl with RNase-free water. Add 350 μl Buffer RLT, and mix well.
2. Add 250 μl ethanol (96–100%) to the diluted RNA, and mix well by pipetting. Do not centrifuge. Proceed immediately to step 3.
3. Transfer the sample (700 μl) to an RNeasy Mini spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at 8000 x g (10,000 rpm). Discard the flow-through.* Reuse the collection tube in step 4.
   Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Be sure to RNA Cleanup empty the collection tube completely. Optional: If performing optional on-column DNase digestion follow steps D1–D4 after performing this step.

4. Add 500 μl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 8000 x g (10,000 rpm) to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in step 5.
   Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use (see “Things to do before starting”).

5. Add 500 μl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at 8000 x g (10,000 rpm) to wash the spin column membrane. The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.
   Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.
6. Optional: Place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min. Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column after step 5.

7. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 μl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at 8000 x g (10,000 rpm) to elute the RNA.

8. If the expected RNA yield is >30 μg, repeat step 7 using another 30–50 μl RNase free water, or using the eluate from step 7 (if high RNA concentration is required). Reuse the collection tube from step 7. If using the eluate from step 7, the RNA yield will be 15–30% less than that obtained using a second volume of RNase-free water, but the final RNA concentration will be higher.
Experiment: 18

Isolation of chloroplast and observation of absorption spectra

**Principle:** The cell organelles, depending upon their size and weight, sediment at different centrifugal fields. Normal absorption spectrum of chloroplast show blue peak at 438 nm. Red peak at 678 nm and carotenoids shoulder band at 475 nm.

**Procedure:**
1. Take about 25 leaves and homogenize in a pre-chilled mortar and pestle with ice-cold isolation medium containing 0.4 M sucrose, 0.01 M EDTA-Na and 0.1 m phosphate buffer (pH 7.8).
2. Squeeze the homogenate through cheese cloth and centrifuge the filtrate at 1000 rpm for 5 min.
3. Take the supernatant and again centrifuge at 3000 rpm for 10 min.
4. Discard the supernatant and add 300ul of homogenizing medium and make a chloroplast suspension.
5. Take 20 µl of chloroplast suspension in 3 ml of homogenizing medium and record the absorbance from 380 nm to 760 nm.
Experiment: 19

Measurement of chlorophyll a and phaeopigments by fluorometric analysis

1.0 Scope and field application
Chlorophyll a measurements have historically provided a useful estimate of algal biomass and its spatial and temporal variability. The fluorometric method is extensively used for the quantitative and analysis of chlorophyll a and phaeopigments. However, errors can be introduced into the results when chlorophyll b and/or chlorophyll c are present. Chlorophyll b is the main source of error in this method. While generally not abundant in surface waters, chlorophyll b can be as high as 0.5 times the chlorophyll a concentration in the deep chlorophyll maximum, causing slight underestimations of the chlorophyll a concentration, and drastic overestimations of the phaeopigment concentrations. Divinyl chlorophyll a also interferes and is taken as chlorophyll a by this method. The procedure described here is appropriate for all levels of chlorophyll a concentration in the marine environment. Filtration volumes should be modified for the different environments. Scientist who employ this or other method to measure pigments should make themselves aware of the current and historical issues that surround these techniques and make appropriate decisions about specific methodologies for their application based on the scientific requirements and constraints of their individual programs.

2.0 Definition
The concentrations of chlorophyll a and phaeopigments in seawater are given as µg kg.

3.0 Principle of analysis
Algal pigments, particularly chlorophyll a, fluorescence in the red wavelength after extraction in acetone when they are excited by blue wavelength of light. The fluorometer excites the extracted sample with a broadband blue light and the resulting fluorescence in the red is detected by a photo multiplier. The significant fluorescence by aphaeopigments is corrected for by acidifying the sample which converts all of the chlorophyll a to phaeopigments. By applying a measured conversion for the relative strength of chlorophyll and phaeopigment fluorescence, the two values can be used to calculate both the chlorophyll a and phaeopigments concentrations.

4.0 Appratus

4.1 Filtration system and what man GF/F filters
4.2 Liquid nitrogen and freezers for storage and extraction.
4.3 Glass centrifuge tubes for extraction, 15 ml
4.4 Turner fluorometer fitted with a red sensitive photomultiplier, a blue lamp 5-60 blue filters and 2-64 red filters.

5.0 Reagents:
5.1 100% acetone
5.2 90% acetone
5.3 1.2 M HCL (100 ml HCL in 900 ml de-ionised water)

6.0 Sample Collection and storage
Water samples are collected from niskins into clean polyethylene bottles with tygon tubing. Samples are immediately filtered through 47 mm GF/F filters using polycarbonate in-line filters (Gelman) and a vacuum of less than 100 mm Hg. Filters are folded in half twice and wrapped in aluminium foil, labelled and stored in liquid nitrogen (to avoid formation of degradation products) until shore analysis. Alternatively, filters can be placed immediately in acetone for pigment extraction if analysis is to be carried out onboard ship.

In oligotrophic waters, for this measurement coupled with HPLC determined pigments, 4 litres are filtered. For fluorometric analysis alone, a smaller volume (0.5-1.0) may be sufficient. In coastal regions, a volume of 0.1-0.51 may be adequate. In this case, use of 25 mm GF/F filteres may be appropriate.

7.0 Procedure

7.1 After removal from liquid nitrogen or freezer, the pigments are extracted by placing the filters in 0.5 ml 100% acetone. For 47 mm GF/F filters, 0.8 ml of water is retained adjusting the final extraction solution to 86% acetone and the final extraction volume to 5.8 ml. The samples are covered with Para film to reduce evaporation, sonicated (0°C, subdued light) and allowed to extract for 4 hours in the dark at -20°C. Following extraction, samples are vortexed, filters are pressed to the bottom of the tube with a stainless steel spatula and spun down in a centrifuge for 5 minutes to remove cellular debris. For fluorometric analysis (not HPLC), decantation can replace centrifuging.

7.1.1 The addition of 5.0 ml acetone for pigment extraction is necessary to completely submerge 47 mm GF/F filters in 15 ml centrifuge tubes. This volume may be altered depending on the size of the filter and volume of the extraction tube.

7.2 The fluorometer is allowed to warm up and stabilize for 30 minutes prior to use.
7.3 The fluorometer is zeroed with 90% acetone.

7.4 1.0 ml of pigment extract is mixed with 4.0 ml 90% acetone in a cuvette and read on the appropriate door to give a reading between 30 and 100. The samples is then acidified with 2 drops of 1.2 M HCl. Further dilutions may be necessary for higher chlorophyll a concentrations.

7.5 Standardization

7.5.1 For laboratory use, the fluorometer is calibrated every 6 months with a commercially available chlorophyll a standard (Anacystis nidulans, Sigma chemical Company). If the fluorometer is taken to sea, it is recommended that the fluorometer be calibrated before and after each cruise.

7.5.2 The standard is dissolved in 90% acetone for at least 2 hours and it’s concentration (mg 1\(^{-1}\)) is calculated spectrophotometrically as follows:

\[
\text{Chl a} = \frac{\text{A}_{\text{max}} - \text{A}_{750\text{nm}}}{\text{Ex}1} \times 1000 \text{ mg 1 gram}
\]

Where:

\[
\text{A}_{\text{max}} = \text{absorption maximum (664 nm)}
\]

\[
\text{A}_{750\text{nm}} = \text{absorbance at 750 nm to correct for light scattering}
\]

\[
\text{E} = \text{extinction coefficient for chl a in 90% acetone at 664 nm (87.67 L g}^{-1} \text{ cm}^{-1})
\]

\[
\text{I} = \text{cuvette path length (cm)}
\]

7.5.3 From the standard, a minimum of five dilutions are prepared for each door. Fluorometer readings are taken before and after acidification with 2 drops 1.2 M HCl.

7.5.4 Linear calibration factor (Kx) are calculated for each door (x) as the slope of the unacidified fluorometer reading vs. chlorophyll a concentration calculated spectrophotometrically.

7.5.5 The acidification coefficient (Fm) is calculated by averaging the ratio of the unacidified and acidified readings (Fo/Fa) of pure chlorophyll a.

7.5.6 Samples are read using a door setting that produces a dial reading between 30 and 100. The fluorometer is zeroed with 90% acetone each time the door setting is changed.

8.0 Calculation and expression of results
The concentrations of chlorophyll a and phaeopigments in the sample are calculated using the following equations:

\[
\text{Chl (µg/l)} = \frac{(F_m - F_a) \times K_x \times V_{\text{ex}}}{F_m - 1 \times V_{\text{fil}}}
\]

\[
\text{Phaeo (chl equiv. weight)} = \frac{(F_m - [(F_m - F_a) - F_o] \times K_x \times V_{\text{ex}})}{F_m - 1}
\]

Where:

- \( F_m \) = acidification coefficient (Fo-Fa) for pure Chl a (usually 2.2)
- \( F_o \) = reading before acidification
- \( F_a \) = reading after acidification
- \( K_x \) = door factor from calibration calculations
- \( V_{\text{ex}} \) = extraction volume
- \( V_{\text{fil}} \) = sample volume

9.0 References

Experiment: 20

Growth and differentiation of physcomitrella patens

Principle:
During last decade the moss physcomitrella patens has emerged as a model system for basic and applied plant science due to several characteristics which make it unique among lower and higher plant. It is the only plant known to date showing high rate of homologous recombination in its nuclear DNA, allowing reverse genetics by gene targeting. Compared to higher plant genetic and phenotypic analysis is most straight forward due to the haploid status of the moss gametophytes. Physcomitrella is also a model organism for physiological analysis because of its clearly separated and well defined developmental stages.

Procedure:
1. Media and supplements

1.1 Minimal medium (‘BCD’)
Calcium is not added to this medium until after autoclaving

Stock solution B 10 ml
Stock solution C 10 ml
Stock solution D 10 ml
Trace element solution 1 ml
Agar 8 gm
D.W. 1L

Sterilised by autoclaving
Calcium (as sterile 500 mM CaCl2 stock solution) is added before use, as required. 1 mM CaCl2 is used routinely. Except for protoplast generation.

1.2 Protoplast regeneration media
Bottom layer (PRMB)
Liquid BCD medium 1L
D-mannitol 60 g
di-ammonium (+) tartrate 920 mg (=5mM)
agar 8 g

Top layer (PRMT)
Liquid BCD medium 1 L
D-mannitol 80 g
di-ammonium (+) tartrate 920 mg (=5mM)
agar 5 g

Sterilised by autoclaving. CaCl2 is added to a final concentration of 10 mM before use.

1.3 Agar

It is probably that any high grade agar can be used to gel moss media. We have used Oxoid number 1 in the past, but now use Sigma High Gel Strength Agar (cat # A9799). This can be used at a lower strength than Oxoid number 1. The quantities in the recipes below refer to sigma Agar # A9799, and may need to be altered for other agars. Some agars may affect medium pH.

1.4 Growth supplements

Many supplements have been used but the following are sufficient for almost all work

<table>
<thead>
<tr>
<th>Substance</th>
<th>concentration</th>
<th>weight/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-aminobenzoic acid</td>
<td>1.8 µM</td>
<td>247 µg</td>
</tr>
<tr>
<td>di-ammonium (+) tartrate</td>
<td>5 mM</td>
<td>920 mg</td>
</tr>
<tr>
<td>nicotinic acid</td>
<td>8 µM</td>
<td>1 mg</td>
</tr>
<tr>
<td>thiamine HCl</td>
<td>1.5 µM</td>
<td>0.5 mg</td>
</tr>
</tbody>
</table>

Supplements may be kept as aqueous 100 x concentrated stock solutions, sterilised by autoclaving, and added to growth media as required to give the concentrations listed above. All of the supplements listed may be added to growth medium prior to autoclaving.

2. CULTURE METHODS

2.1 Temperature

Physcomitrella patens and Ceratodon purpureus grow on solid media in temperatures up to about 28°C, possibly somewhat higher in liquid medium. Little difference in growth rate is observed in the temperature range 20°C to 26°C. Temperatures between 24°C to 26°C are used for routine culture. Growth is slower

Selection of hybrids is speeded by transferring to appropriate BCD selective medium after 4-5 days, when protoplast regeneration is completed. Selection of hybrids using vitamin auxotrophies usually takes about 3 weeks using transgenic antibiotic resistances, hybrids can usually be identified after only 5 days.

Calcium protoplasts wash (CPW)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-mannitol</td>
<td>80 g</td>
</tr>
<tr>
<td>50 mM aqueous CaCl2</td>
<td>1L</td>
</tr>
</tbody>
</table>
Sterilise by autoclaving.

Protoplast fusion solution

Polyethyleneglycol (PEG) M.Wt. 6000 500 g
50 mM aqueous CaCl2 solution

Dissolve PEG by streaming or heating, mix, dispense into appropriate aliquot, e.g. 2, 5 or 10 ml and sterilise by autoclaving.

50 mM CaCl2 solution

\[
\text{CaCl}_2 \cdot 6\text{H}_2\text{O} \quad 10.95 \text{ g}
\]

Distilled water 1 L

Sterilise by autoclaving

6.3 Transformation.
Transformation using protoplast and PEG (Schaefer et al., 1991)

1. After the second wash following protoplast isolation in the procedure described in section 3 above, resuspend protoplast (from four seven day old cellophane-overlay plates) in 10 ml 8% (w/v) d-mannitol solution (see session 3). Estimate protoplast density using a haemocytometer. Centrifuge (100 to 200 x g for 5 min) and resuspend in sufficient D-mannitol /MgCl2/MES solution to give a final protoplast density of 1.6 x 10^6/ml. this should yield about 2 to 4 ml of protoplast suspension.

2. Meanwhile, prepare DNA to be used in transformation by dispensing 10 to 50 µg of DNA (volume no more than 30 µl, but otherwise not critical) into sterile 10 ml tubes. Centrifuge gently to bring DNA to bottom of tube.

3. Add 300 µl protoplast suspensions from 1 to DNA from 2. Add 300 µl PEGT solutions.

4. Heat for 5 min at 45^0. Return to room temperature (20^0) for 5 min.

5. Add 300 µl 8% D-mannitol solutions. Invert gently to mix. Wait 3 minutes.

6. Repeat step 5, four more times.

7. Add 1 ml 8% D-mannitol solution. Invert gently to mix. Wait 3 minutes.

8. Repeat step 7, four more times.

9. Centrifuge (700-800 rpm x g for 5 min). Remove supernatant.

10. Resuspend in 5 ml liquid BCD + 8% mannitol +10 mM CaCl2 + 0.5% D-glucose.

11. Incubate for 24 h at 25^0 in darkness

12. Centrifuge, remove supernatant and resuspend in 500 µl 8% mannitol solution.

13. Add 2.5 ml molten PRMT medium (kept molten at 42^0)

14. Dispense at rate of 1 ml per 90 mm diameter plate of PRMB plates overlayed with cellophane.

15. Incubate in light for 5 days at 25^0, transfer protoplasts on top layer to selective medium if appropriate.
**D-mannitol/Mgcl2/MES solution**

D-mannitol | 910 mg
Distilled water | 8.85 ml

Sterilise by autoclaving. Store at room temperature if necessary.

On day of use, add:
- 1M Mgcl2 solution | 150 µl
- 1% MES pH 5.6 | 1 ml

Filter sterilise.

**1 M MgCl2**

MgCl2.6H2o | 203.3 g
Distilled water | 1L

**1% MES pH 5.6**

Use 1% (w/v) 2-[N-morpholino] ethanesulphonic acid) in distilled water.
Adjust to pH 5.6 with 0.1 M KOH. Sterilise by autoclaving. Store at 4°C.

**PEG solution for transformation (PEGT)**

PEG 6000 | 2 g

Autoclave in a glass universal bottle or equivalent vessel.
On day of transformation, melt PEG in microwave. Add 5 ml D-mannitol/Ca(NO3)2 solution and mix well. Leave at room temperature for 2 to 3 h before use.

**D-mannitol/ Ca(NO3)2 solution**

Make up fresh, on day of use
- 8% (w/v) D-mannitol solution | 9 ml
- 1 M / Ca(NO3)2 solution | 1 ml
- 1 M tris buffer, pH 8.0 | 100 µl

Filter sterilise

**1 M Ca(NO3)2 solution**

Ca(NO3)2 4.H2o | 236.1 g
Distilled water | 1L

Sterilise by autoclaving. Store at 4°C.
Transformation using micro-projectile bombardment (Sawahel et al., 1992)

1. This method uses 6 day old protonemal tissue grown by the standard method on cellophone overlay plates (see section 2.3), containing BCD medium with ammonium as nitrogen source and supplemented as appropriate.

2. 50 mg of dry tungsten powder (M17 grade, obtainable from Sylvania, Towanda, PA 18848, USA) is suspended in 300 µl of absolute ethanol in a 1.5 ml eppendorf tube, mixed vigorously, and then centrifuged at 10,000 x g for 5 min. Withdraw the ethanol carefully.

3. Wash the tungsten by the addition of 1.5 ml of sterile distilled water. Resuspended and centrifuge. Repeat twice more (three washes in sterile distilled water in toto) and finally resuspended the tungsten in 1 ml of 50% (w/v) sterile glycerol.

4. 25 µl of the tungsten suspension prepared in 2 is mixed, preferably in a 500 µl Eppendorf tube, with:
   - 5 µl of CsCl purified supercoiled plasmid DNA (1 µg DNA / µl SDW)
   - 25 µl sterile 2.5 M CaCl2 solution
   - 10 µl sterile 0.1 M Speridine (free base) solution

   Mix gently and allow to stand for 10 min. carefully withdraw 35 µl of the supernatant and discard. Transfer to ice until used for bombardment.

5. The vacuum chamber of the particle gun and its components are thoroughly cleaned with SDW and then with ethanol.

6. The addition of CaCl2 and spermidine causes DNA-tungsten complexes to form. These must be dispersed immediately before use. Flicking the tube with your finger is effective. Immediately after dispersal, 3 µl of suspension is placed in the centre of the front surface of a plastic macro projectile, which is then put into the barrel of the gun. The tissue is placed in the chamber without removal from the cellophane overlay plate. The lid of the Petri dish is removed and a sterile stainless steel mesh (aperture size 1 mm x 1 mm) is placed over the open plate. The chamber is evacuated to 28 chambers before discharge.

7. Our present gun was made to the design of Lonsdale et al., 1990, J. Exp. Bot. 41, 1161-1165, by Sheerline Precision Engineering Ltd. Cambridge road, Milton, Cambridge CB4 4AT, England, and uses macro projectiles of the polycarbonate obtained from Turner-Richards, Birmingham, England and 0.22” Blank launcher cartridges from Winchester Group, East Alton, 1162024, USA. Our gun gives best result with a distance of 150 mm between the stopper plate and the tissue, and with two discharges per Petri dish of tissue (the position of the dish is moved between discharges). Presumably each gun will need some calibration.

8. Following bombardment, the tissue is incubated under standard conditions for 48 h.

9. After 48 h incubation, the tissue is harvested and either blended and plated onto selective medium (as described for the preparation of cultures for cellophane overlay plates—see
section 2.3.2), or, if single cell clones are required, protoplasted before plating onto osmotically–buffered selective medium.

Notes on transformation

1. It is convenient to carry out a number of transformations at the same time (10 is not difficult). Make sure a minus DNA control is included, to assess protoplast viability.

2. The following concentrations of antibiotic are routinely used for selection:
   - G418: 50 µg/ml
   - Hygromycin: 30 µg/ml
   - Sulfadiazine: 150 µg/ml

3. We can define three classes of antibiotic–biotic resistance regenerants.
   - Transient: do not retain resistance upon sub-culture.
Experiment: 21

Chlorophyll and carotenoids in plant material

Introduction
The contents of pigments in the leaves of plants are important to evaluate the photosynthetic apparatus of plants. This protocol describes how the contents of chlorophyll (Chl) a, Chl b, and total carotenoids can be determined in a whole-pigment extract of green plant tissue by UV-VIS spectroscopy (Lichtenthaler, 1987).

Principle
Pigments are extracted from the plant tissue by a solvent (96% ethanol). The concentrations of the pigments are quantified by light spectroscopy.

Protocol
1. Freeze the leaf-sample in liquid nitrogen and lyophilise in a freeze drier.
2. Cut the tissue into small pieces by scissor.
3. Take approx. 5 mg of the homogenised freeze-dried tissue in a test-tube (extract weight must be known, DW (mg)2 decimals.
4. Add 100 µl distilled water and wait until all the material is hydrated (approx. 10 min)
5. Add 8.0 ml of 96% ethanol and vortex.
6. Wrap the test tubes in aluminium foil and let them incubate at room temperature in an exhaust hood over night.
7. The next day, vortex the samples and wait until the particulates have fallen to the bottom (or centrifuge).
8. Measure absorbance of the extract at 470.0 nm, 648.6 nm and 664.2 nm (eventually also at 750 nm to be able to correct for impurities). The absorbance should be in the range 0.2 to 0.8.
1. Prepare tissue by culturing a well blended colony from an established spot culture onto 2 starting BCD plates. Grow for 5 days, split each onto 2 plates, grow a further 5 days split half onto 2 plates; grow for 3 days, split onto 12 plates and grow for a further 3 days before use.

2. The day before you start prepare 500ml BCD Ammonium tartrate with 4g agar, 2.5g glucose and 33g mannitol added and 100ml BCD Ammonium tartrate with 0.7g agar, 0.5g glucose and 8.5g mannitol (plating medium) and leave to autoclave first thing.

3. Collect protonema from all 12 plates and transfer to a petri dish containing 25 ml 8.5% mannitol with 1% driselase.

4. Leave for 30 min with occasional gentle mixing.

5. Filter through a stainless steel sieve into a clean petri dish and leave for a further 15 min.

6. Filter again and transfer to a 50ml Falcon.

7. Spin at 600rpm for 5 min to pellet cells.

8. Resuspend the pellet in 10ml 8.5% mannitol.

9. Repeat spin and resuspension.

10. Count the protoplasts.

11. Spin again and resuspend to the required concentration in 8.5% mannitol.

12. Mix protoplasts with protoplast plating medium in a 1:1 concentration and plate out 2 ml per plate (use about 10-30,000 per plate).
EVEN SEMESTER:
SECOND SEMESTER
BIOLOGY LABORATORY
**EXPERIMENT: 1. Use of compound microscope**

**Aim of the experiment:** Staining of mitochondria in human cheek epithelial cells with Janus Green

**Objective:** A compound microscope achieves higher levels of magnification than a stereo or low power microscope. It is used to view smaller specimens such as cell structures which cannot be seen at lower levels of magnification.

A compound microscope consists of structural and optical components. These key microscope parts are explained below.

**Structural components**

The three basic structural components of a compound microscope are the head, base and arm.

- Head/Body houses the optical parts in the upper part of the microscope
- Base of the microscope supports the microscope and houses the illuminator
- Arm connects to the base and supports the microscope head. It is also used to carry the microscope.

Always carry a compound microscope always by holding both the arm and base, simultaneously.
Optical components

There are two optical systems in a compound microscope: eyepiece lenses and objective lenses:

**Eyepiece** or Ocular is what you look through at the top of the microscope. Typically, standard eyepieces have a magnifying power of 10x. Optional eyepieces of varying powers are available, typically from 5x-30x.

**Eyepiece tube** holds the eyepieces in place above the objective lens. Binocular microscope heads typically incorporate a diopter adjustment ring that allows for the possible inconsistencies of our eyesight in one or both eyes. The monocular (single eye usage) microscope does not need a diopter. Binocular microscopes also swivel (Interpupillary Adjustment) to allow for different distances between the eyes of different individuals.

**Objective Lenses** are the primary optical lenses on a microscope. They range from 4x-100x and typically, include, three, four or five on lens on most microscopes. Objectives can be forward or rear-facing.

**Nosepiece** houses the objectives. The objectives are exposed and are mounted on a rotating turret so that different objectives can be conveniently selected. Standard objectives include 4x, 10x, 40x and 100x although different power objectives are available.

Coarse and Fine Focus knobs are used to focus the microscope. Increasingly, they are coaxial knobs - that is to say they are built on the same axis with the fine focus knob on the outside. Coaxial focus knobs are more convenient since the viewer does not have to grope for a different knob.

**Stage** is where the specimen to be viewed is placed. A mechanical stage is used when working at higher magnifications where delicate movements of the specimen slide are required.

**Stage Clips** are used when there is no mechanical stage. The viewer is required to move the slide manually to view different sections of the specimen.

**Aperture** is the hole in the stage through which the base (transmitted) light reaches the stage.
Illuminator is the light source for a microscope, typically located in the base of the microscope. Most light microscopes use low voltage, halogen bulbs with continuous variable lighting control located within the base.

Condenser is used to collect and focus the light from the illuminator on to the specimen. It is located under the stage often in conjunction with an iris diaphragm.

Iris Diaphragm controls the amount of light reaching the specimen. It is located above the condenser and below the stage. Most high quality microscopes include an Abbe condenser with an iris diaphragm. Combined, they control both the focus and quantity of light applied to the specimen.

Condenser Focus Knob moves the condenser up or down to control the lighting focus on the specimen.

**Principle of compound microscope:**
Principle of the compound microscope which uses two lenses for the greater strengthening. The upper lens is the eye piece and the lower lens is at the opposite end of the tube which is called the objective. The image will be seen upside down, and then the upper lens called the eyepiece takes over and enlarges the image once again. As a result of this we always see the image reversed. The compound microscope works by first the first the magnified image has been formed by the objective lens and has been projected up into the tube.

The beam passes through the stage window and through the specimen, and then the light brightens the area through the specimen. The compound microscope consists of convex lenses fitted at either end of the tube. The light source can be a mirror or a lamp in the microscope base. The level of the contrast is controlled by controlling the amount of the illumination.

**Working principle of lenses:**
When an object is in focus the lower lens called the objective lens form a real inverted image of the object. The upper lens called the eyepiece treat the inverted image as an object and produces an image of that object. This image is the enlarged image that has been seen by the observer.

**REQUIREMENTS:**

1. Compound Microscopes
2. Permanent Slides

**Study of permanent slides**
1. Stages of mitosis

2. MEIOSIS STAGES

3. GRAM POSITIVE and GRAM NEGATIVE BACTERIA
4. SECTION OF LIVER

5. SECTION OF HUMAN TESTIS

6. SECTION OF HUMAN KIDNEY
7. SECTION OF HUMAN INTESTINE

8. SECTION OF HUMAN SPLEEN
EXPERIMENT: 2. Study of mitochondria

Aim of the experiment: Staining of mitochondria in human cheek epithelial cells with Janus Green

Principle: Mitochondria are considered as power houses of a cell as it produces ATP by a process called oxidative phosphorylation. Each cell contains large number mitochondria and they can be observed under a light microscope if stained with Janus green. This stain is bluish green in colour when oxidized and colourless when reduced. When a dilute solution of the stain is applied to stain the cells, it enters in the cytoplasm as well as in mitochondria. Since mitochondrial inner membrane contains cytochrome oxidase enzyme, which can keep the stain in oxidized state, the mitochondria appear stained while in rest of the cytoplasm the stain gets reduced and thus appears colourless.

Materials Required
Ethanol soaked tooth pick, slide, cover glass, 0.01% Janus green B stain in normal saline

Procedure
1. Dry the ethanol soaked tooth pick in air and scrape gently the inner side of cheek. A large number of cells will come on the tooth pick.
2. Gently rub the tooth pick on slide in one direction to make a spread of cells. Dry the cells on slide so that the cells will not get washed away while staining.
3. Put a few drops of Janus green stain and leave for 5-10 min for staining.
4. After 5 min of staining, rinse cells once with distilled water so that complete stain is not gone and a diluted stain remains. Mount the cells in a drop of distilled water with a cover glass and observe under the bright field students’ microscope. The cells can alternatively be mounted in the stain itself. A few air bubbles remaining inside the cover glass give a background stain that makes the viewing easy. The slide can be observed under the high magnification of a student microscope.

Observation
Each cell is seen to contain a large number of tiny round or elongated bacteria like bodies in the cytoplasm mainly around the nucleus. Generally they are not strongly stained thus appear like pimples on a face. Mitochondrion can be easily distinguished from a bacterium as bacterial cells become more prominently stained and appear sharper than mitochondria. Also as bacteria are on the surface of cells, they will be focused at a slightly different level than mitochondria and can be distinguished.

HUMAN CHEEK CELLS
EXPERIMENT: 3. Study of mitosis in plant

**Aim of the experiment:** To observe different stages of Mitotic cell division in onion root tip cells.

**Principle:**

Multicellular organisms grow and develop by increasing their number of somatic cells by a process of cellular division known as mitosis. Mitosis is one of the phases of cell cycle and organisms control their growth by regulating different phases of the cell cycle.

In plants, the roots continue to grow as they search for water and nutrients. Cell division is especially rapid in the growing root tip; therefore, it is easier to observe each stage of mitosis in root tip tissues than in slowly growing tissues. To observe mitosis in root tip tissues, young tips are fixed in Carnoy’s solution (a mixture of glacial acetic acid and ethyl alcohol in the ratio of 1:3) where glacial acetic acid helps to precipitate the nucleoproteins and to dissolve cytoplasmic organelles whereas ethyl alcohol hardens the tissue and facilitates the penetration of acetic acid inside the tissue. Acetocarmine is used to stain the chromosome for its high binding affinity to the chromosome so that the chromosomes can be easily visible.

**Requirements:**

1. Carnoy’s solution (a mixture of glacial acetic acid and ethyl alcohol in the ratio of 1:3) where glacial acetic acid
2. Slides
3. Onion to get root tips from fresh sprouts

**Procedure:**

1. Cut the onion root tips in around 5 mm length.
2. Fix the tips in Carnoy’s solution for 1 hour.
3. Transfer the tips to a clean slide and put a drop of acetocarmine stain.
4. Cover the tip with a cover slip and leave for 4-5 mins.
5. Warm the slide intermittently over a spirit lamp.
6. Press or squash the root tip with thumb.
7. Observe under the microscope for different stages of mitotic cell division.

**Observation:**

Draw the stage of mitosis which you have observed in your preparation
PROPHASE

METAPHASE

ANAPHASE

TELOPHASE

CYTOKINESIS
EXPERIMENT 4: Study of meiosis in plants

Aim of the experiment: Study of different stages of meiosis in onion anthers.

Principle: Meiosis is a reduction division where two successive division of the nucleus resulted into ‘n’ number of chromosomes and occur only in germ cells. Meiosis results in the formation of either gametes (in animals) or spores (in plants). Meiosis I is the reduction division that reduces the chromosome number from diploid to haploid and separate the homologous pair of chromosomes while in meiosis II, sister chromatids separate. As a result four haploid gametes are formed.

MATERIALS REQUIRED
Aceto-ethanol fixative (1:3), 2% aceto-carmine stain, slide, cover glass, sealing wax or nail polish, onion anthers.

PROCEDURE

Staining and making squash preparation:

1. Fix the anthers in aceto-ethanol fixative for 1 hr.
2. Stain the fixed anthers in aceto-carmine for 30 min.
3. Take a drop of aceto- carmine on slide; place an anther on it, heat it for 1-2 min.
4. Place a cover glass on the anther and squash using the thumb pressure.
5. The slide is ready for observation under a microscope.
6. If your slide is good, seal the cover glass with sealing wax or nail polish.
EXPERIMENT 5. Differential staining of bacteria

Aim of the experiment: To perform differential staining in order to group the microorganisms as Gram positive and Gram negative bacteria.

Principle: Gram staining (or Gram’s method) is an empirical method of differentiating bacterial species into two large groups (Gram-positive and Gram-negative) based on the chemical and physical properties of their cell walls. The method is named after its inventor, the Danish scientist Hans Christian Gram. The concept of stain retention basically depends upon the chemical compositions of cellular components, i.e., mainly peptidoglycans and lipid concentrations. Gram positive bacteria have a thick mesh-like cell wall made of peptidoglycan (50-90% of cell wall), which stains purple while Gram-negative bacteria have a thinner layer (10% of cell wall), which stains pink. Gram-negative bacteria also have an additional outer membrane which contains lipids, and is separated from the cell wall by the periplasmic space. There are four basic steps of the Gram stain, which include applying a primary stain (crystal violet) to a heat-fixed smear of a bacterial culture, followed by the addition of a mordant (Gram’s iodine), rapid decolorization with alcohol or acetone, and counterstaining with safranin or basic fuchsin.

Crystal violet (CV) dissociates in aqueous solutions into CV⁺ and chloride (Cl⁻) ions. These ions penetrate through the cell wall and cell membrane of both Gram-positive and Gram-negative cells. The CV⁺ ion interacts with negatively charged components of bacterial cells and stains the cells purple. Iodine (I⁻ or I₃⁻) interacts with CV⁺ and forms large complexes of crystal violet and iodine (CV–I) within the inner and outer layers of the cell. When a decolorizer such as alcohol or acetone is added, it interacts with the lipids of the cell membrane. A Gram-negative cell will lose its outer membrane and the peptidoglycan layer if left exposed. The CV–I complexes are washed from the Gram-negative cell along with the outer membrane. In contrast, a Gram-positive cell becomes dehydrated from an ethanol treatment. The large CV–I complexes become trapped within the Gram-positive cell due to the multilayered nature of its
peptidoglycan. After decolorization, the Gram-positive cell remains purple and the Gram-negative cell loses its purple color. Counterstain, which is usually positively-charged safranin or basic fuchsin, is applied last to give decolorized Gram-negative bacteria a pink or red color.

**Materials:** Sterile test tube containing 10 ml sterile water, 5 gms of overnight kept curd, wire loop, a grease-free slide, a low flame, crystal violet solution, Gram’s iodine solution, alcohol-acetone solution, safranin solution, gloves

**Procedure:**

1. Mix 5 gms of ON-kept curd with 10 ml sterile water
2. Take a loopful of suspension on a clean, grease-free slide, smear and heat fix
3. Cover the film with crystal violet solution and keep for 1 min
4. Wash off excess stain with running tap water and cover with Gram’s iodine solution for 1 min
5. Wash the slide with alcohol-acetone solution till the violet color is drained off
6. Wash the slide thoroughly with tap water followed by covering with safranin solution for 30 sec
7. Wash finally with tap water, followed by distilled water and blot dry and then observe under oil-immersion microscope
EXPERIMENT 6. Study of blood cells

Aim of the experiment: Preparation and staining of thin blood film

Objective: After staining, blood films are used for
   a) Determining leukocyte type
   b) For detecting abnormal red cells
   c) For identifying certain blood parasites.

Principle: Fixation is the process by which blood cells are made to adhere to the slide and staining is the process by which the cells (cytoplasm and nuclei) are stained. The blood cells are fixed by methanol. Leishman stain is used to stain the blood film which is a mixture of methylene blue and eosin in methyl alcohol. Methylene blue (basic dye) stains the acidic part of the cell i.e. the nuclei (DNA) and cytoplasm (RNA) of WBCs and granules of basophiles. Eosin (acidic dye) stains the basic part of the cell, i.e., eosinophilic granules and hemoglobin of red cells. Methyl alcohol fixes the smear to the slide. It should be acetone free because acetone causes lysis of the cell.

Materials Required:
Slides
Methylene blue
Leishmans stain
Eosin
Methyl alcohol
Acetone
Sterile syringes
Wash bottles

Procedure:
1. A thin smear is prepared by spreading a small drop of blood evenly on a clean slide so that there is only one layer of cells
2. Dry the film by waving it rapidly about 5 cm away from the flame of a spirit lamp
3. Mark the dry film
4. Pour Leishman stain on the slide so that it just covers the smear
5. Leave it for 2 minutes. During this time, the alcohol in the stain fixes the cells
6. Add double the amount of distilled water, then mix well with the stain. A metallic shiny layer should form on the top of the mixture
7. Leave it for 7-10 minutes
8. Pour off the stain and wash the slide gently and thoroughly with tap water. Make sure that the stream of water does not fall directly on the smear. While pouring off the stain it should be ensured that the greenish scum does not stick to the surface of the smear
9. Drain off all water adhering to the slide and allow drying by setting the slide in an upright position

**Precaution:**
The smear should dry completely before staining; otherwise the smear will be removed while washing.
Excess stain should not be applied; it should just cover the smear

**Observation:**
- RBC - orange to red
- Neutrophils - dark purple nuclei, pink cytoplasm, reddish granules
- Eosinophils - blue nuclei, pink cytoplasm, large red granules
- Basophils - dark blue/purple nucleus, blackish large granules
- Lymphocytes - dark purple to deep bluish purple nuclei, sky blue cytoplasm
- Platelets - violet to purple granules

**BLOOD SMEAR**
EXPERIMENT. 7. Study of Plant Tissue

Aim of the Experiment: To study the arrangement of vascular bundle in a Dicot stem and Monocot Stem.

Principle: Vascular tissue is made up of different types of plant cells which transport water and organic and inorganic molecules. These transport tissues are grouped together to form vascular bundles. The arrangement of the vascular tissue in a plant stem differs in dicotyledonous and monocotyledonous plants. The two main transport tissues in a vascular bundle are phloem and xylem and between these is a very important layer of cells, the cambium, which is able to divide. The xylem differentiates from the part of the vascular bundle nearest the centre of the stem and then progressively towards the cambium in the middle of the bundle. Protoxylem is the xylem which differentiates first and may consist of a different combination of xylem cells when compared to the metaxylem which differentiates later and lies closer to the cambium. The xylem is responsible for transporting water and dissolved nutrients. The phloem starts differentiating on the side of the vascular bundle orientated towards the outside of the stem and then progressively towards the middle, towards the cambium in the middle of the bundle. The phloem transports organic substances through the stem.

Requirements

1. Eosin stain
2. Blades
3. Watch glass
4. Compound microscopes.
5. Plant tissue

Procedure:

1. Keep the whole plant with root dipped in dilute solution (100X) of Eosin stain for about four hours.
2. Cut very thin sections of the stem with the help of a new razor blade carefully and suspend the sections in a watch glass with dist. water.
3. Put two or three sections separately in a drop of dist. water over the slide and observe in 4X and 10 X resolution of the compound microscope.
4. Identify the tissues, write your observations and make a labeled diagram.

DICOT STEM

MONOCOT STEM
EXPERIMENT 8: Detection of Cell Viability

Aim of the experiment: Cell viability assay by trypan blue exclusion assay

Principle: Live cells have intact cell membranes which exclude certain dye such as trypan blue (also eosin, or propidium) whereas dead cells uptake those dyes. In this test, a cell suspension is mixed with trypan blue and then examined under microscope. A viable cell will have a clear cytoplasm whereas a nonviable/dead cell will have a blue cytoplasm.

Materials: 0.4% trypan blue solution (visible ppt should be filtered), haemocytometer, coverslips, compound microscopes

Procedure:

1. Centrifuge (5 min at 100xg) an aliquot of cell suspension and discard the supernatant

2. Cells should be suspended in a microfuge tube in PBS/ FBS-free cell culture media (serum proteins stain with trypan blue) and then diluted 1:1 by mixing with 0.4% trypan blue (mix 10 ul of cell suspension with 10 µl of 0.4% trypan blue solution)

3. Allow mixture to stand 3 min at room temperature. Cells should be counted within 3 to 5 min of mixing with trypan blue, as longer incubation periods will lead to cell death and reduced viability counts

4. Take a hemocytometer and place a coverslip on that. Place 10 ul mix under the coverslip. Vol that can remain under the coverslip and the 16 small squares of hemocytometer is 0.1 ul.

5. Place the hemocytometer on the stage of a binocular microscope and focus on the cells. Blue cells are dead cells

6. Total number of viable cells/ml of aliquot= total number of viable cells x 2 (dilution factor) x 10^4. Total number of cells/ml of aliquot= total number of viable and nonviable cells x 2 (dilution factor) x 10^4.
Hemocytometer

LIVE CELLS

DEAD CELLS
EXPERIMENT. 9. Blood Grouping

Aim of the experiment: Determination of ABO blood groups and Rh factor.

Principle: The ABO blood group system is the most important of all blood group systems and was first discovered by Karl Landsteiner in 1900. In this system, the red blood cell membrane contains two different types of antigens namely A and B and the corresponding plasma contains the natural antibodies namely anti B and anti A respectively. Based on the presence or absence of these antigens, four blood groups are classified:
- Group A: Antigen A is present.
- Group B: Antigen B is present.
- Group AB: Both A and B antigens are present.
- Group O: Neither A nor B antigen is present.

When the antigens in one’s blood (say antigen A) comes in contact with corresponding antibody (anti A), the blood coagulates and the blood group is identified. Besides ABO system another system called Rhesus system which is of importance for the hemolytic disease in the new born and transfusion reactions. Rh system has two blood groups: Rh positive: antigen D present and Rh negative: antigen D absent but there is no naturally occurring antibodies. The antibody in this system is called anti D antibody and produced only when an Rh negative individual receives the Rh positive blood. This can be occurring in case of blood transfusion or in case of pregnancy.

Requirements: Sterile needles, Blood group detection kits, slides

Procedure:
1. Prick the finger with sterilized needle.
2. Put three drops of blood separately on a clean slide.
3. Put the antisera A and antisera B on the first and second drop of blood and antisera D on the third drop of blood.
4. The blood should be mixed thoroughly with respective antiserum.
5. Leave the slide undisturbed for two minutes to allow the reaction to take place.
6. Record the presence or absence of agglutination in each slide and interpret your result.

<table>
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<th>Genotype</th>
<th>Antibodies made by body</th>
<th>Reaction to added antibodies</th>
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<tr>
<td>A</td>
<td>i^A i^A or i^B i^B</td>
<td>Anti-B</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>i^B i^B or i^B i^B</td>
<td>Anti-A</td>
<td></td>
</tr>
<tr>
<td>AB</td>
<td>i^A i^B</td>
<td>Neither anti-A nor anti-B</td>
<td></td>
</tr>
<tr>
<td>O</td>
<td>i^O i^O</td>
<td>Both anti-A and anti-B</td>
<td></td>
</tr>
</tbody>
</table>
EXPERIMENT 10: Plant Physiology

Aim of the Experiment: To examine water and solute transmembrane transport in a typical plant cell.

Principle: The movement of water (osmosis) and solutes (diffusion, facilitated diffusion, active transport) across plasma membranes is of fundamental importance in the life of a cell. Red onion is useful for studies of water movement since it has large, translucent cells, each surrounded by a cell wall. When a red onion is placed in any solution that is hypotonic with respect to the contents of the vacuoles within its cells, the vacuoles become turgid, since water tends to flow into areas of higher solute concentrations by osmosis, and the tonoplast (the membrane surrounding the vacuole) becomes tightly pressed against the cell membrane (which lines the inside of the cell wall). If a red onion is placed in a hypertonic solution, the vacuoles lose water and are seen to “shrink” and pull away from the cell wall. This is called plasmolysis. If the red onion is then returned to a hypotonic solution (or isotonic solution), the vacuoles swell up again to full turgidity. This is called deplasmolysis.

In this lab, you will observe the process of plasmolysis in red onion cell vacuoles by placing cells in solutions of different sucrose concentrations in part A. In part B, you will examine the same process by immersing epidermal cells in different concentrations of a salt solution.

Prepare a wet mount of a small section of red onion epidermis. Use a razor blade to shave off a very thin section of the red epidermis of the onion, or snap a piece of onion to remove the epidermis. The section must be only the epidermis to ensure the experiment will work. Place the section on a slide and add two drops of water and a cover slip. Place the slide on the microscope and observe.

Requirements:
Fresh Onion
Water
Sucrose
NaCl
Procedure:

A. DETERMINATION OF ISO-OSMOTIC THRESHOLD OF RED ONION CELLS USING A NONELECTROLYTE

1. Prepare a small section of red onion epidermis and place it on a slide. Add several drops of 0.6M sucrose to cover the epidermis and allow the slide to sit for at least 5 minutes. Remove the excess solution and add a coverslip. Place the slide on the microscope and determine if the cells have undergone plasmolysis. Once plasmolysis has been observed, discard the slide in the appropriate container.

2. Prepare additional slides of red onion epidermis and observe the cells when placed in 0.5M, 0.4M, 0.3M, and 0.2M sucrose solutions, following the same procedures as explained above. Allow each preparation to incubate for at least 5 minutes. For each preparation, note whether plasmolysis has occurred and, if so, the extent of plasmolysis.

3. Determine the solution that most closely approximates the iso-osmotic threshold of the epidermal cells.

B. DETERMINATION OF ISO-OSMOTIC THRESHOLD OF RED ONION CELLS USING AN ELECTROLYTE

Follow the same procedure as in part A using solutions of NaCl instead of sucrose. First, make a 1 M solution of NaCl. Use the 1 M solution to make 0.05, 0.1, 0.2, 0.3, and 0.4 M NaCl solutions.

Remember, you only need a small volume of each concentration. Is the iso-osmotic threshold for a solution of NaCl the same as a sucrose solution?
PLASMOLYSIS
EVEN SEMESTER
FOURTH SEMESTER
CELL BIOLOGY
B243: Cell Biology Laboratory, Credits – 2

Jan-April 2012 session

Syllabus:

2. Culture and growing of different type of cells
3. Staining and visualization of different cellular organelles
4. Analysis of cellular functions like endocytosis, exocytosis, cell migration, attachment, cell division, cell death

Experiments planned

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</table>
**Experiment 1:** Cell splitting and growing of mammalian immune cells (RAW cells as a model system)

**Equipments:** Cell culture incubator, laminar hood, Microscope, Pipette-man, Liquid N<sub>2</sub>-tank, Autoclave

**Consumables:** Cell culture flasks, Cryo-vials, RAW cells, DMEM (Dulbecco’s modified eagles medium, FBS, Trypsin-EDTA, Penicillin-streptomycin (PS), gloves, Ethanol, dish for cell culture, pipette tips, eppendorf tubes, serological pipettes, cell scraper, Haemocytometer, cell scraper

**Methodology:**
2. The cells were grown in a T25 flask with 7ml of DMEM (with 10% FBS and PS).
3. After the cells get confluent (48hrs) cells can be splitted. RAW cells are adherent cells; cells can be splitted either by using cell scraper or by Trypsinization.

**Cell splitting by Trypsinization**
1) Take the tissue culture flask containing cells.
2) Discard the media without disturbing the monolayer and keep that media in another tube for further use.
3) Wash the cells with 1X PBS once gently.
4) Discard the PBS and add Trypsin-EDTA (5 ml for T-175, 3 ml for T-75, 1-2 ml for T-25)
5) Keep it for 5 minutes at 37°C (as required)
6) Cells will come out from the flask surface and will be in the suspension. Tap the flask gently few times.
7) Add equal amount of old media to neutralize the Trypsin.
8) Centrifuge it at 1500 RPM for 5 minutes.
9) Discard the supernatant and re-suspend the pellet by gently tapping it.
10) Add required amount of media into the cells.
11) Distribute the culture in T-flasks for further use.

**Cell splitting by cell scraper**
1) T-flask containing culture was taken.
2) Discard the media without disturbing the monolayer and keep that media in another tube for further use.
3) Wash the cells with 1X PBS.
4) Add 2 to 3 ml media in the flask and scrap the cells gently, from corners also.
5) Transfer the scrapped cells in to 15 ml falcon tube; centrifuge it at 900 RPM for 10 Minutes, at 23°C.
6) Re-suspend the pellet in the media and distribute it into 2 different flasks.

**Precautions:**
a) Trypsin should be added in the recommended volume otherwise cells can die.
b) Trypsinization is time sensitive as Trypsin treatment for long duration can lead to cell death and for short duration may lead to inefficient cell detachment.
c) Wipe your hands thoroughly with alcohol before handling cells (Sterilization is needed).
**Experiment 2: Culturing of plant cells and visualization of chloroplast (moss cells as a model system)**

**Materials required:**

- **Equipments:** Autoclave, Incubator shaker, Centrifuge, Microscope, Computer lab

- **Consumable:** BCD media, glass slides, cover slips, conical flask, moss culture, Nuclease and ion-free water, Moss culture, Sterile flasks (20x 500 ml), pipette man,

**Procedure:**

1) BCD media is prepared (solution A 0.1M MgSO$_4$. 7H$_2$O, 2.7m KH$_2$PO$_4$, 1KNO$_3$, 0.008M FeSO$_4$, 1mM CaCl$_2$)

2) Moss culture was grown for 7 days.

3) Take 1ml of moss culture from the flask into eppendroff tube.

4) Centrifuge the cells at 13000g for 10 min to obtain pellets of moss colonies,

5) Washed with non ionised distilled water and again centrifuged at 13000g for 8 minutes.

6) Take the pellets (this is called washing).

7) Re-suspend the moss cells in 200ul of non ionised distilled water.

8) Place a small droplet of moss cells on glass-slides and covered by cover slips.

9) Visualize the cells by using a microscope and capture the images.

10) Use Adobes Photoshop for further editing the images.

**Additional:**

 Mitotracker Red and DAPI can be added and incubated for different times to the moss suspension in order to visualize the mitochondria and nucleus.
Experiment 3: Image analysis and extracting information

Materials required: Computers with monitor, LSM Image analysis software and Adobe Photoshop, different confocal images. (Need computer lab)

Description: Demonstration of different imaging and image analysis techniques

Methodology: Based on computer application
Experiment 4: Counting of cells by using haemocytometer (RAW cells and *Tetrahymnea* as model systems)

**Materials required:** Haemocytometer, microscope, cell suspension, media for cells, formaldehyde, Cell culture incubator, gloves, dish for cell culture, pipette-man, pipette tips, eppendorff tubes

**Procedure:**

Take 10 µl of cell suspension and introduce this into the space between cover slip and haemocytometer. Count the number of cells in four large corners (each corner with 16 large squares). Average the cells per large corner and multiply it by $10^4$. This would give the number of cell per ml. If the sample is diluted before counting, then the final count is multiplied by the appropriate dilution factor.

Note: *Tetrhymena* moves/swims very fast in the media. Therefore, fix the cells by adding 4ul of formaldehyde solution to 1ml culture before using it for counting.

**Precautions:**

1. A common mistake is to add sample to counting chamber before adding cover-slips. In this case it is possible that the cells would sediment to the glass and thereby cause the over estimation.
2. Do not use the paper to wipe of the extra liquid. The same capillary action may cause drying of the sample in the chamber.
3. The haemocytometer is thicker than normal microscope slides. Thus attempt to focus it too closely may cause damage to the objective lens.
**Experiment 5: Observation of micro- and macro-nucleus by fluorescence microscopy (Tetrahymnea as model system)**

**Equipments:** Incubator for *Tetrahymnea* cell culture, Laminar hood, Microscope, Pipette man, Liquid N<sub>2</sub>-tank, Autoclave, Centrifuge

**Consumable:** Cell culture flasks, *Tetrahymnea*, SPP media, gloves, Ethanol, dish for cell culture, pipette tips, eppendorf tubes, serological pipette, Haemocytometer, ice box

**Materials required:** 4% PFA in HEPES buffer (dissolve 0.4 gm of PFA in 10ml solution of 50 mM of HEPES pH 7.3 and incubate at 60°C for 15 min with some mixing), 0.1% triton X in 50mM HEPES pH 7.3 solution in 10ml, DAPI 5-10ug/ml in 50mM HEPES buffer.

**Procedure:**

a) *Tetrahymnea* cells are spun down at 5x10<sup>5</sup> RPM and fix the cells by adding 2.5 ml of 4% PFA solution. Incubate for 40min.

b) Wash the cells with 50mM HEPES (pH 7.3)

c) Permeablize the cells by incubating with 3ml of 0.1% Triton X in 50mM HEPES (pH 7.3) for 8 minutes

d) Wash the cells twice with the 50 mM HEPES buffer (pH 7.3) and incubated within100ul of 1ug/ml DAPI for 10min.

e) Wash the cells 3 times with 50 mM HEPES (pH 7.3) and re-suspend in the mounting solution

f) Put a drop of cells in glass slides and cover it with the glass cover-slips.

g) Visualise the cells under a fluorescence microscope using the blue filter.
**Experiment 6**: Visualisation of haploid nucleus by DAPI staining (using bovine sperm cells as model system)

**Chemicals required**: 4% PFA, Triton X 100, DAPI, PBS solution, 300ml of HBS, Fluoromount-G (separates egg cell from the egg yolk when they are in liquid nitrogen)

**Equipments needed**: Microscope, Pipette man, Liquid N₂-tank, centrifuge, Water bath at 37°C

**Materials**: centrifuge ice box, *Bovine sperm cells vial suspended in egg yolk stored in liquid nitrogen, fluorescence microscope, glass slides, tissue paper.

* Bovine sperm cells are generally collected from Frozen Semen Bank (Khapuria, Cuttack with prior appointment).

**Procedure:**

1) Collect the fresh sperm cells with or without diluents from Frozen Semen Bank (Khapuria, Cuttack. Store the vials with sperm cells (with egg yolk as diluents) at liquid N₂ at -196°C.
2) In case the cells were taken from liquid N₂, thaw the cells directly by putting them at 37°C water bath.
3) Aliquot 30ul of sample (stored in egg yolk) in microfuge tube
4) Add 300ml of HBS and centrifuge at 200g
5) Sperm cells appear in the pellet faction and debris is in the supernatant,
6) Discard the supernatant
7) Take a drop of pellet and put it on clean glass slide.
8) Smear the drop along the glass slide by using another glass slide and air dry it.
9) Add PFA and incubate the slides for 5 minutes at RT.
10) Add 0.1% Triton X-100 along the slides and wash off it very gently
11) Allow the slides to dry in air
12) Wash the slides 3 times gently with PBS solution
13) Add DAPI dye to the slide and incubate the slides with DAPI for 15 minutes in RT
14) Wash the slides gently once again with PBS
15) Observe the slides by using blue filter of the fluorescence microscope.
Experiment 7: Visualisation of diploid nucleus by DAPI staining (using mammalian_cells as model system)

Materials required: HeLa cell lines, glass slides, 12 well plates, DMEM, FBS, DAPI, PBS, buffer, pipette-man, forceps, parafilm, Cell culture flasks, Cryo-vials, HeLa cells, DMEM, Trypsin-EDTA, Penicillin-streptomycin, gloves, Ethanol, dish for cell culture, pipette tips, eppendorf tubes, serological pipettes, Haemocytometer, Fluoromount-G, PFA, glass slides, cover slips,

Equipments: Cell culture incubator, laminar hood, Microscope, Pipette man, Liquid N2-tank, Autoclave, Water bath, inverted microscope

Procedure:

a) Grow HeLa cells in DMEM media (+10% FBS and PS) for 24 H at 5% CO₂ incubator.
b) Detach cells by adding Trypsin-EDTA (2 to 3 ml) for T75, and kept for 10min in incubator
c) Count the number of cells using haemocytometer for equal splitting of cells.
d) Distribute the cells in 6 well plates over cover-slips. The cover-slips are previously washed with alcohol and dried and then placed in 6 well plates.
e) Cells are allowed to grow for 24 hours.
f) Fix the cells by adding 4% PFA, incubate 10 minutes. Wash with PBS.
g) Add DAPI (0.1ug/ml final concentration) and incubated for 20 minutes.
h) Wash the cells gently with phosphate buffer saline pH 7.4.
i) Mount the cover-slips on glass slides with Fluoromount-G.
j) Let the slides to dry up and image the slide subsequently by using a fluorescence microscope.
Experiment 8: Labelling and visualisation of lysosomes by Lysotracker-Red in mammalian cells

Equipments: Cell culture incubator, laminar hood, fluorescence microscope, Pipette-man, Liquid N₂-tank, Autoclave

Materials required: HeLa cell lines, glass slides, 6well plates, DMEM, FBS, Penicillin and streptomycin, Trypsin-EDTA, Lysotracker-Red, DAPI, PBS buffer, pipette man, forceps, paraflim, glass slides, 12 well plates, Cell culture flasks, Cryo vials, gloves, Ethanol, dish for cell culture, pipette tips, eppendorf tubes, serological pipettes, Haemocytometer,

Procedure:

a) HeLa cells are cultured in DMEM media (+10% FBS, PS) at 5%CO₂ and 37°C.
b) Once the cells become confluent, split the cells and grow them over sterilised cover slips for 24 hours.
c) Dilute the Lysotracker dye in the growth medium only (final working conc. is 50-75 nM).
d) Add Lysotracker containing media.
e) Incubated the cells with the dye for 1hour.
f) Discarded the media and fix the cells with 4% PFA. Wash with PBS pH 7.4 for 3 times.
g) Mount the cover-slips on glass slide with the Fluoromount-G.
h) Image the slides under fluorescence microscope.
Experiment 9: Visualisation of mitochondria and nuclei by Mitotracker Red and DAPI in eukaryotic cells (Double labelling)

**Equipments:** Cell culture incubator, laminar hood, fluorescence microscope, Pipette man, Liquid N\textsubscript{2}-tank, Autoclave

**Material required:** Cover-slips, 12 well plate, cover-slips, T-flask, DMEM media, FBS, Mito tracker red, DAPI, Para formaldehyde (PFA), PBS (phosphate buffer saline) pH 7.4, Trypsin-EDTA, mammalian cell lines, forceps, parafilm, serological pipette, Fluoromount-G

**Protocol:**

a) Grown cells in T25 flask.

b) After the cells become confluent, split them and distribute equally in 12 well plates (cell counting should be done with haemocytometer for equal distribution of cells).

c) Keep round cover-slips (previously washed in ethanol and air dried and sterilized under UV) ready. Place these cover-slips inside the 12 well plates.

d) After splitting the cells into 12 well plate, allow the cells to grow on cover-slips for 24 hours inside the incubator maintained at 5% CO\textsubscript{2}, 90% humidity and temperature 37°C.

e) After 24 hours add Mitotracker (final conc. 1µM) and incubate for half an hour.

f) Fix the cells by adding 4% PFA (same volume to the media), incubate for 10 minutes.

g) Discard the media and wash 3times in phosphate buffer saline (pH 7.4).

h) The cover slips are than taken out carefully and placed on a hard surface (e.g. glass plate) already covered with parafilm.

i) Wash the cells with PBS (pH 7.4) and add DAPI (final conc. 0.1µg/ml) and incubated for 20 minutes.

j) Wash the cells with phosphate buffer saline 3times.

k) Mount the cover-slips by using Fluoromount-G

l) Keep the slides for drying for overnight.

m) The slides are imaged under fluorescence microscope.
Experiment 10: Visualisation of actin cytoskeleton by Phalloidin dye in eukaryotic cells

Equipments required: Cell culture incubator, laminar hood, Microscope, Pipette-man, Liquid N₂-tank, Autoclave

Materials required: Mammalian cell lines, glass slides, cover-slips, 12 well plates, DMEM, FBS, Penicillin and streptomycin, Trypsin-EDTA, Alexa-dye labelled Phalloidin, DAPI, PBS buffer, pipette man, forceps, paraflim, glass slides, 12 well plates, Cell culture flasks, Cryo vials, gloves, Ethanol, dish for cell culture, pipette tips, eppendroff tubes, serological pipettes, Haemocytometer, 0.1% Triton X-100 BSA stock solution (5% in PBS), Triton-X-100, Tween 20, PFA (4% in PBS buffer)

Procedure:

a) Split the cultured mammalian cells. Discard the old media and add fresh media to the cells to make it a cell suspension
b) Add the cells to the sterile cover-slips placed on a 12 well plate and allow the cells to adhere on the glass cover-slips
c) Allow the cells to grow for next 24 hours.
d) Fix the cells by adding 4% PFA slowly to the medium without disturbing the cells.
e) Incubate the cells with PFA for 10 minutes in 37°C.
f) Discard the liquid and wash the cover-slips (with adhered cells) with PBS for three times and decant/aspirate the PBS from the bottom
g) Incubate the cells with PBS supplemented with 0.1% Triton X-100 (2 minutes) and remove the liquid.
h) Wash the cells with PBS slowly
i) Block the cells with 5% BSA in a PBS for 30 min
j) DAPI and Alexa dye labelled Phalloidin (1:1000 ratio) are added carefully to the slides. Make sure that whole cover-slip is covered with the liquid and it is not dry.
k) Wash the cover-slips carefully with PBS for 3 times
l) Mount the cover-slips on glass slides and mark it.
m) Visualise the slides by using fluorescence microscope.
**Experiment 11: Analysis of cell cycle and cell division by indirect immune-fluorescence analysis and/or by FACS methods**

**Material required:** HeLa, T75 flasks, RPMI media, FBS, L-glutamine, Penicillin – streptomycin, Propidium Iodide, 12x75mm snap cap tubes (Falcon 2054), PBS, alpha tubulin antibody, Alexa-Red labelled secondary antibody, 0.1% triton X-100, 5% BSA

**Instrument needed:** CO₂ incubator, laminar hood, fluorescence microscope, inverted microscope.

**Methodology**

1) HeLa cells are grown in T75 flask in DMEM media, 5% FBS, at 5% CO₂ at 37°C.

2) After the cell get confluent, they are splitted and grown over cover slips in 6 well plate.

3) The cells are allowed to grow for 24 hours.

4) Fix the cells with 4% PFA, than wash with PBS pH 7.4 for 3 times. The cover slips are taken out carefully (without breaking) and mounted on hard surface already covered with parafilm such as on glass plate.

5) Add 0.1% TritonX-100 and incubate for 2 minutes, wash with PBS pH 7.4, than add 5% BSA for blocking and incubated for half an hour.

6) Wash with PBS 3 times, (each wash for 10minutes) and add primary α-tubulin antibody (1:1000) dilution and incubate for 2 hours at room temperature.

7) Wash with PBS (pH 7.4), 3 times for 10 minutes. Secondary antibody (1:1000) dilution is added and incubated for 1 hour.
8) Wash 3 times with PBS (pH 7.4), each time for 10 minutes,

9) Add DAPI (0.1µg/ml) and incubate for half an hour and then wash with PBS (pH 7.4).

10) Mount the cover slips on glass slides with Fluoromount-G and image under fluorescence microscope.

Optional: The same can be done by using FACS as a method also. For that the reagents are same as mentioned in Experiment 12
Experiment 12: Analysis of cell death by a) FACS analysis
b) Western blot analysis (Caspase-9 activation) c) DNA smear test

a) Analysis of cell death by FACS

Material required: Jurkat cells (non adherent), T75 flasks, RPMI media, FBS, L-glutamine, Penicillin–streptomycin, 7-Amino-actinomycin stock (7AAD), Annexin-V-PE, Annexin binding buffer (0.1 M HEPES/NaOH (pH 7.4), 1.4 M NaCl, 25 mM CaCl₂) 7-AAD stock (1µg/ml), PBS (pH 7.4)

Instrument required: Inverted microscope, laminar hood, CO₂ incubator, FACS machine.

Procedure:
1) Grow the cells in a T75 flask, with 13-15ml of media (with 10% FBS, 1% glutamine, and 1% pen strep). Grow the cells for 24 hours
2) Re-suspend the cells (at least 0.5 x 10⁶ cells) in 12x75mm tubes at a concentration of 10⁶ cells/ml.
3) Add 2ml of cold PBS to tubes.
4) Centrifuge for 8 minutes at 1800 RPM.
5) Resuspend pellet in 2ml of cold PBS.
6) Centrifuge for 8 minutes at 1800rpm.
7) Resuspend cells in 0.1ml of 1x binding buffer (0.1 M HEPES/NaOH, pH 7.4, 1.4 M NaCl, 25 mM CaCl₂)
8.) Aliquot cells in microfuge tubes and add Annexin V-PE and 7-AAD (1µg/ml).
9) Gently vortex.
10) Incubate at room temperature for 15 minutes in the dark.
11) Add 400µl of 1x binding buffer in each tube
12) Analyze samples within 1 hour of staining.

Note: The analysis need controls: For that treat cells with Annexin V-PE only, Treated cells with 7-AAD only, Untreated cells with both stains, cell only (no stain, no treatment)
FACS setup:

a) **Primary laser:** 488nm (visible), 250mW.

b) **Annexin V detection-PE:** 530/20 BP filter, Fl-1 detector.

c) **7-AAD detection:** 660/20 BP filter, Fl-3 detector.
**b) Cell death by western blotting (Caspase 9 activation):**

**Material required:** Cells, lysis buffer (20mM TRIS pH 8, 130mM 1% Triton X-100, 10mM sodium fluoride, 10mM, RPMI media, FBS, Jurkat cells (non adherent), T75, RPMI media, FBS, L-glutamine, Penicillin–streptomycin, sodium docecyl sulphate (SDS), protein marker, Bradford reagent, acrylamide, TEMED, bovine serum albumin, skimmed milk, Caspase-9 antibody, HRP-labelled secondary antibody (species specific), PVDF membrane, Whattmann papers. TBS-T, Western blot transfer buffer, PBS (pH 7.4), Ionomycin.

**Instruments:** SDS –PAGE, semi dry western blot transfer, SDS –PAGE casting apparatus, Gel documentation unit, Computer

**Procedure:**
1) Grow Jurkat cells in RPMI media (+10% FBS, PS) at 37 °C, CO₂ incubator for 24 hours.
2) The cells are treated with Ionomycin (1 to 100 nm working concentration) and keep a parallel sample as non-treated control sample.
3) Centrifuge the cells at 800 RPM for 10 minutes and discard the media. Wash the pellet with PBS pH 7.4
4) Add Lysis buffer (100 µl for 1x10⁶ cells) and mix by tapping. Incubate on ice for half an hour.
5) Centrifuge the cells at 12000 RPM for 15 minutes.
6) Discard the pellet.
7) Estimate the protein content of the cell extract by Bradford method
8) Cast 10% SDS gels and perform electrophoresis of cell extracts at 20mA
9) Transfer the protein is from gel to PVDF membrane 50mA for one mini-gel for 90 minutes. (Activate the PVDF membranes with methanol before using them for western blot analysis),
10) Wash the membrane is washed with 3 times with TBS-T
11) Blocked with 5% skimmed milk for overnight or for one an hour
12) Wash 3 times in TBS-T and than Caspase-9 primary antibody is added (1:1000)
dilution and incubated for 2 hours, again washed with TBS-T for 3 times for 15 minutes.

13) Secondary antibody and incubated for one hour, washed with TBS-T.

14) The blot is developed using luminal and at suitable exposure time is observed in Chemi-doc.
c) Cell death by DNA-smear formation

Material required: Jurkat cells, RPMI media, FBS, Jurkat cells (non adherent), T75 flask, FBS, L-glutamine, Penicillin –streptomycin, Agarose, DNA ladder, DNA loading buffer. TE buffer, DNA-staining dye, Ionomycin.

Note: This experiment also needs all the reagents needed for genomic DNA preparation (refer to all the chemicals related to genomic DNA isolation).

Instrument required: Agarose gel electrophoresis apparatus, Power pack, CO₂ incubator, laminar hood, inverted microscope. Gel doc

Procedure:

a) Jurkat cell are grown in RPMI media, 10% FBS, 37°C, CO₂ incubator and grown for 24 hours.

b) The cells are treated with ionomycin (1to 100 nm working concentration). Keep another sample as non-treated control. Grow cells for 24 hours in these conditions.

c) The cells are centrifuged at 800rpm for 10 minutes, the media is discarded and then the pellet is washed with PBS pH 7.4

d) Isolate the genomic DNA from Jurkat cells by manual method

e) Run the genomic DNA in 0.7% Agarose gel.

f) If the cells have undergone apoptosis the DNA band will appear as a smear.
Experiment 13: Analysis of endocytosis and exocytosis in mammalian cells (Using Alexa-labelled Transferrin incorporation assay)

Equipments: Cell culture incubator, laminar hood, fluorescence microscope, Pipette man, Liquid N_2-tank, Autoclave

Material required: Cover slips, 12 well plate, cover slips, T-flask, DMEM media, FBS, Alexa dye labelled Transferin, DAPI, Para formaldehyde (PFA), PBS (phosphate buffer saline) pH 7.4, Trypsin-EDTA, mammalian cell lines, forceps, parafilm, serological pipette, Fluoromount-G, Ionomycin

Protocol:

a) Grown cells in T25 flask.

b) After the cells become confluent, split them and distribute equally in 12 well plates (cell counting should be done with haemocytometer for equal distribution of cells).

c) Keep round cover slips (previously washed in ethanol and air dried and sterilized under UV) ready. Place these cover slips inside the 12 well plates.

d) After splitting the cells into 12 well plates, allow the cells to grow on cover slips for 24hours inside the incubator maintained at 5% CO_2, 90% humidity and temperature 37˚C.

e) After 24 hours add Alexa dyes labelled Transferin (final concentration 1x) and incubate for half an hour. Repeat the same with a cell culture that has been activated by Ionomycin (will induce more Ca^{2+}-driven exocytosis).

f) Fix the cells by adding 4% PFA (same volume to the media), incubate for 10 minutes.

g) Discard the media and wash 3times in phosphate buffer saline (pH 7.4).

h) The cover slips are than taken out carefully and placed on a hard surface (e.g. glass plate) already covered with parafilm.

i) Wash the cells with PBS (pH 7.4) and add DAPI (final conc. 0.1µg/ml) and incubated for 20 minutes.

j) Wash the cells with phosphate buffer saline 3times.

k) Mount the cover slips by using fluoromount-G
l) Keep the slides for drying for overnight.

m) The slides are imaged under fluorescence microscope.
Experiment 14: Analysis of cell adhesion using RAW cells

Material required: RAW cells, DMEM, FBS, L-glutamine, Penicillin-streptomycin, Trypsin EDTA, paraformaldehyde, cover slips, glass slides, DAPI, 6 well plate, Fluoromount-G, Cytochalasin B.

Instrument needed: fluorescence microscope, inverted microscope, CO\textsubscript{2} incubator, laminar hood

Protocol:

a) Grow the cells in a T25 flask (7ml media) at 37°C and 5% CO\textsubscript{2}.

b) Split the cells by using Trypsin –EDTA and count the cells.

c) Place sterile cover slips in 12 well plates.

d) Add media with 1000 cells per cover slips and allow them to adhere for different time periods (1 min, 5 min, 15 min, and 45 min). Repeat the same with cells in the same media but supplemented with actin disrupting drug (Cytochalasin B).

e) Fix the cells with 4% PFA

f) Wash the cell with PBS (pH 7.4) and transfer the cover slips on hard surface (such as glass plated wrapped with parafilm).

g) Label the cells with 0.1ug/ml of DAPI (incubated for 20-30min).

h) Wash the cells with PBS (pH 7.4) three times and mount on glass slide with Fluoromount-G.

i) Image the slides under fluorescence microscope and count the number if nuclei

J) Prepare the graphs.

Precaution:

1) The cells should not be overgrown.

2) Care must be taken while splitting the cells using Trypsin-EDTA, as excess if it and treatment for longer time can lead to cell death.

3) Concentration of DAPI should not be high.

4) Equal number of the cells should be added in each well.
**Experiment 15: Cell migration assay using mammalian cells**

**Instrument needed:** Inverted microscope, CO₂ incubator, laminar hood,

**Material required:** RAW cells, DMEM, FBS, L-glutamine, Penicillin-streptomycin, Trypsin EDTA, cover-slips, glass slides, 1ml sterile tip, 6 well plates, Taxol and Nocodazole

**Protocol:**

a) The cells are grown T25 flask in DMEM, 5% FBS, under 5% CO₂, 37°C.

b) Once the cells become confluent, split the cells by using Trypsin-EDTA and distributed equally in 6 well plates.

c) Add equal number of cells in each well (10,000cell/ml) (should be counted using haemocytometer).

d) Allow the cells to grow till the wells are not confluent. Grow in CO₂ at 37°C.

e) After 24 hours the cells are scratched with 1ml sterile tip gently twice, making “+” symbol.

f) The cell migration is done under two conditions one is treated with low dose of Taxol (0.1uM working conc.). The other one is in low dose of Nocodazole (0.1 uM working conc.). Keep one as untreated control. Each condition should have at least 3 replicates.

g) After the “scratching”, allow the cells to grow for 2, 4, 6, 8, 10, 12, 16 and 24 hours. Observed the scratched “+” area under inverted microscope at regular intervals and capture images.

h) Count the number of cells present in the scratched area. Plot the graphs accordingly with X-axis denoting the time and Y-axis representing the number of cells in the scratched area.

**PRECAUTION:**

1) The cells should be fully confluent before making the “+” sign.

2) Care must be taken while splitting the cells using Trypsin-EDTA, as excess if it and treatment for longer time can lead to cell death.

3) Equal no of the cells should be there in each well.

4) The cells should be in healthy condition.
EVEN SEMESTER: FOURTH SEMESTER

GENETICS LABORATORY
Experiment 1: LRPAP 1 Insertion/Deletion Polymorphism

The polymorphism arises due to 37 bp insertion. Insertion allele is larger than the deletion allele. This difference in size is detected by gel electrophoresis. Small fragment containing this polymorphism can be amplified using the primer system.

Materials and Methods:

Primer Sequence:
Forward: 5' GGTGTTTCTGGACACAAAGGA 3'
Reverse: 5' AGTGTGCCTGGAGCCTATG 3'

PCR Reaction Parameters

<table>
<thead>
<tr>
<th>Reaction mixtures (Total Volume 25µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock Conc.</td>
</tr>
<tr>
<td>10X PCR Buffer</td>
</tr>
<tr>
<td>25 mM dNTP</td>
</tr>
<tr>
<td>Forward Primer (10 µl)</td>
</tr>
<tr>
<td>Reverse Primer (10 µl)</td>
</tr>
<tr>
<td>Taq DNA Polymerase (5U/µl)</td>
</tr>
<tr>
<td>Genomic DNA (50 ng)</td>
</tr>
<tr>
<td>Water added to a final volume of 25 µl</td>
</tr>
</tbody>
</table>

Temperature Cycling conditions

Initial Denaturation 94°C...2-3 mins
(Anneaturation 94°C...........30 secs
Annealing temperature 55°C...30 secs
Extension 75°C...............45 secs) 30 cycles
Final Extension 72°C.......5-7 mins
4°C forever
Procedure

1. Thaw 10X buffer, dNTPs, and primers and keep on ice.
2. NOTE INCLUDE A REACTION TUBE BLANK WHICH WILL HAVE ALL THE INGREDIENTS EXCEPT DNA. THIS IS AN ESSENTIAL CONTROL FROM EACH PCR REACTION.
3. Prepare Master mix.
4. Use 0.2 ml thin walled centrifuge tubes for reaction. Label top of the tubes with samples #.
5. Pippete mix reagent (except template) into each tube.
6. Pippete DNA samples into appropriate tubes using a new tip sample.
7. Mix tubes well. Spin for 5 secs on microfuge to remove liquid from side of the tube.
8. Place tubes in PCR machine, programme machine and begin PCR reaction.
9. Analyse the PCR gel products by 2 agarose gel electrophoresis of a 5 µl aliquot from a total reaction.
10. Visualise by UV transillumination of the ethidium bromide stained gel.
11. Take pictures and analyse results.
   Depending on the size 222 allele will be designated the l allele and 185 bp band as the D allele.
12. Store reaction product at -20°C until needed.
Experiment 2: DNA isolation from mammalian cells and tissues

1. Lysis Cells
   For 10 ml of lysis buffer for 1 ml cell suspension, or blood sample, incubate 1 hour at 37°C.

2. Add 10 vol of lysis buffer in a tube and proteinase K (20 mg/ml) to a final lysis buffer, incubate 3 hours at 50°C. Swirl tube from time to time.

3. Treatment with phenol
   Add equal volume of phenol, gently mix for 10 mins and centrifuge 10 mins at 5000g at RT. Separate 2 phases.

4. Transfer aqueous phase to anew tube, add 0.2 vol of 10 M acetate, and add 2.5 volume of 100% ethanol at RT. Mix well. See DNA fragments, centrifuge at 5000g. If DNA is not seen then freeze tube at -20°C overnight. Centrifuge later on.

5. Wash with 70% ethanol twice.

6. Dissolve DNA with TE buffer.

7. Lysis Buffer

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM Tris HCl</td>
<td>pH 8.0</td>
<td></td>
</tr>
<tr>
<td>0.1 M EDTA</td>
<td>pH 8.0</td>
<td></td>
</tr>
<tr>
<td>0.5% w/v SDS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 µl DNAase free pancreatic RNAase</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Experiment 3: Protocols for SNP detection from human blood samples

It includes the following methods

A. DNA isolation from human blood samples
B. Standardization of DNA amplification by PCR
C. Restriction digestion for detection of specific SNP
D. Agarose gel preparation to confirm the genotype.

A. DNA isolation from human blood samples

1. Take 500µl to 1000µl of human blood sample in 1.5 ml eppendorf tube from EDTA vaccume tubes
2. Centrifuge at 11000 rpm for 5 mins to pellet down the cells.
3. Discard the supernatant having serum only and add 1000µl of RBC lysis buffer.

Composition of the RBC lysis solution:

A. 320 mM Sucrose
B. 1% triton X 100
C. 1 mM MgCl$_2$
D. 12 mM Tris –Cl PH 8

Procedure of making RBC Lysis Solution:

a. Take a 500 ml bottle and label as RBC lysis Solution.
b. Pour 300 ml of MilliQ water.
c. Weigh 57.27 g sucrose (FW 104.54 g) and mix it in the water in the bottle.
d. Add 50 ml of 1 M MgCl$_2$(FW 203.3) as follows. Weigh 10.165 g of MgCl$_2$ powder and put it in a 30 ml of MilliQ water and dissolve it completely. Then volume make upto 50 ml with MilliQ water.
e. Add 0.5ml of 1 M MgCl2 to buffer solution at step b.
f. Add 5 ml of Triton X 100 to the Buffer solution at step d. This is a viscous solution like glycerol and is not well studied. Therefore handle with care and use 10 ml measuring cylinder to make 5 ml out of it.
g. Add 500 ml of 1 M Tris HCl (FW 157.6 pH 8) solution as follows. Take 300 ml of MilliQ water in 500 ml of beaker. Weigh 78.8 g of Trizma hydrochloride and add to it. Now keep this solution in the magnetic stirrer and put both the electrodes of the pH meter into the solution. See the reading. A pH less than 7.00 indicates acidic pH. Now add NaOH pellets one by one and wait after each addition of pellet for its complete dissolution. After addition of 35 pellets the pH will be 8. (After leaving Overnight pH might need to be adjusted to 8).

h. Add 6 ml of 1 M Tris HCl pH 8 to the RBC buffer solution of step e

i. Now pour the RBC lysis solution of step g in a 500 ml measuring cylinder, make the volume to 500 ml with milliQ water. Now put it back in RBC lysis buffer bottle.

j. Do not autoclave this buffer as sucrose is a carbohydrate and can blacken after heating. Even if it is required to autoclave then do it for not more than 15 mins for 1.5 kg/cm²/1.5 psi.

4. Break the pellet by inverting the eppendorf tube for several times for not more than 1 min and centrifuge at 11000 rpm for 5 mins.

5. Discard the supernatant, again add 200µ of RBC lysis buffer to it, mix and centrifuge at 11,000 rpm for 5 mins.

6. Discard the supernatant having lysed RBC and take the pellet which is WBC only (few reddish colour of deposited haemoglobin may appear on the pellet) let it be there which will be removed in the following steps.

7. Dissolve the pellet in 200 ml of MilliQ water by 2-3 times pippeting and centrifuge at 13500 rpm for 5 mins.

8. Discard the supernatant and to this pellet add 80 µl of proteinase K buffer and 10 µl of 10% SDS, now frothing is done by several times pippeting.

**COMPOSITION OF PROTEINASE K BUFFER**

1. 375mM NaCl
2. 120 mM EDTA pH 8

**Procedure of making proteinase K Buffer**

a. Take a 500 ml bottle and label as proteinase K.
b. Pour 300 ml of MilliQ water in this bottle.

c. Make 500 ml of 5 M NaCl (FW 58.44 g) Take 400 ml of MQ water in a beaker with calibration of volume upto 500 ml and add 146.1g of NaCl to it and do its magnetic stirring after 15-20 mins. If it is not dissolved then heat the beaker for 60 seconds in oven and make up the volume upto 500 ml till a clear solution is obtained.

d. Add 37.5 ml of 5 M NaCl solution to the bottle of step a.

e. Make 500 ml of 0.5 M EDTA solution pH 8.0: Weigh 93.75 gram of Sodium EDTA (FW 186.1g).
   Put it in a beaker of 400 ml of MilliQ. Place this beaker for magnetic stirring and simultaneously put both the electrodes of the pH meter in a beaker. See the reading. Now add 20 g NaOH pellets one by one and wait after each addition of pellet for its complete dissolution till pH turns 8.
   Make up the volume to 500ml of milliQ water. Until pH is 8 EDTA will not dissolve.

f. Add 120 ml of 0.5 M EDTA solution to the bottle of step c and make up the volume upto 500 ml with MilliQ water.

g. Autoclave it at 1.5 psi for 20 mins.

**Composition of 10% SDS, Sodium dodecyl sulfate/Sodium Lauryl sulphate:**

A. 10% w/v in MilliQ water

B. Take 400 ml of MilliQ water in a beaker add 50g of SDS, to it. Dissolve it on magnetic stirrer (If does not dissolve add few drops of conc HCl to get pH 7.2).

Do not autoclave this solution as nothing can grow here. It is a detergent which makes froth on boiling and has a property of removing protein from DNA strand.

9. Add 100 µl of prechilled 5M NaCl solution to eppendorf tube and mix by tapping.

10. Add 200 µl of milliQ water in the above solution and 400 µl of company made/self made. (Tris Saturated Phenol: Chloroform: isoamyl alcohol=25:24:1) is added.

11. Tubes are mixed by inverting until they turn milky, centrifuge at 12,000 rpm for
10 mins.
12. The aqueous layer is taken out in fresh tubes and 1000µl of prechilled absolute alcohol is added for its precipitation. And then mixing is done gently by inverting the tubes. Now tubes are centrifuged at 13,000 rpm for 10 mins.
13. Discard supernatant by draining off. Pellets may not be visible in a few samples. Add 200µl of 70% ethanol, then centrifugate at 13000 rpm for 5 mins.
14. Discard the supernatant by draining off and the pellet is dried at 56°C for at least 3-4 hours.
15. Finally the pellets of DNA strands are dissolved in a 30µl of Tris-EDTA buffer pH 8.0 and leave the solution at 56°C for 1 hour to completely dissolve the DNA. TE buffer keeps DNAase inactive if it is present in solution and keeps DNA strands away from other ionic interactions.

**Composition of Tris-EDTA buffer pH 8**

10 mM Tris-C pH 8.0, 1 mM EDTA solution

**Procedure for making Tris-EDTA Buffer pH 8**

a) 300 ml MilliQ water

b) 5 ml of 1 M Tris-HCl pH 8.0 and 1 ml of 0.5 M EDTA pH 8 to it.

c) Volume make up to 500 ml with MilliQ water to 500 ml

d) Autoclave it.

16. Take the measurement of conc in the Nanodrop which is a modified version of the classical spectrophotometer which gives the value. Absorbance ratio at two different wavelengths of light 260 nm /280 nm and 260nm /230 nm and the conc of DNA in the solution as ……..ng/µl on putting 1 µl of DNA sample at the specified point in the nanodrop machine. Note if the conc of DNA is higher than 100ng/µl then dilute it to 100ng/µl and then take the reading in the Nanodrop.

**260/280 ratio**

The ratio of absorbance at 260 nm and 280 nm is used to access the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as pure for DNA. A ratio ~2 is accepted for “pure” RNA. If the ratio is lower in either case it may indicate the presence of protein, phenol, or other contaminants that absorb
strongly at or near 280 nm.

B. Standardisation of DNA amplification by PCR

<table>
<thead>
<tr>
<th>SL NO</th>
<th>Material</th>
<th>Stock</th>
<th>Concentration</th>
<th>Volume to be taken</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MQ Water</td>
<td></td>
<td></td>
<td>19.5 µl</td>
</tr>
<tr>
<td>2</td>
<td>TAQ Buffer A</td>
<td>10X</td>
<td>1X</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>3</td>
<td>dNTP mixture</td>
<td>2.5mM</td>
<td>0.1mM</td>
<td>1.0µl</td>
</tr>
<tr>
<td>4</td>
<td>Forward Primer</td>
<td>50µM</td>
<td>0.5µM</td>
<td>0.25µl</td>
</tr>
<tr>
<td>5</td>
<td>Reverse primer</td>
<td>50µM</td>
<td>0.5µM</td>
<td>0.25µl</td>
</tr>
<tr>
<td>6</td>
<td>Template</td>
<td>100 ng/µl</td>
<td>100 ng in 25µl</td>
<td>1.0µl</td>
</tr>
<tr>
<td>7</td>
<td>Taq DNA Polymerase</td>
<td>3 unit /µl</td>
<td>0.5 U in 25µl</td>
<td>0.5 µl here</td>
</tr>
</tbody>
</table>

PCR Reaction Mixture

1. Make a receipe of the reaction mixture in the same order as mentioned above.
2. Make master mix of all reactions to be set at a time.
3. To set 1 or 2 PCR reactions at a time make 50µ reaction for each.
4. Stock of primers: add 200µl of MQ water or as written in the primer tubes. Mix it with pipettes to dissolve the pellets of the primers completely. By using M1V1=M2V2 formula make a dilution to 50µM.
5. When dNTPs are not available and all 4 DNTPS are in separate tubes then make each stock of concentration 10mM and take 0.25µl from each to get 0.1µM conc of each dNTP in the final reaction volume.
6. Use ice bucket to keep chemicals when addition is being performed.
7. Set thermal cycle in the PCR machine as follows 94 for 5 mins then 35 cycles of 994 for 45 secs, Tm to be standardize for the first use of primers for 45 secs and 72 for 45 secs) and then 72 for 10 mins and finally 4 for infinity time period i.e., end of the reaction.
8. Standardisation of the set of primers to get specific bands of expected size for a particular DNA fragment.

To obtain desired specific bands of DNA fragment 10 PCR reaction 25µl is set a time with different Tm values in a PCR program. To set a particular temperature in columns 1-12 of the PCR, thermal cycle is set as the gradient. The range of temperature for the gradient depends upon +/- 5 degree Celsius of average of Tm of both the sets of primers.

9. Products are run at 3% gel to know that at which specific temperature the specific band is formed and there are no non-specific bands.

LOADING DETAILS

<table>
<thead>
<tr>
<th>Loading details:</th>
<th>12ul in each well</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 100bp Ladder</td>
<td>10ul + 6x Loading Dye = 2ul {Total 12ul}</td>
</tr>
<tr>
<td>2. PCR product 1st</td>
<td>[around 1ug] = 5ul + 6x Loading Dye = 2ul + MQ water = 5ul {Total = 12ul}</td>
</tr>
<tr>
<td>3. PCR product 2nd</td>
<td>[around 1ug] = 5ul + 6x Loading Dye = 2ul + MQ water = 5ul {Total = 12ul}</td>
</tr>
<tr>
<td>4. PCR product 3rd</td>
<td>[around 1ug] = 5ul + 6x Loading Dye = 2ul + MQ water = 5ul {Total = 12ul}</td>
</tr>
<tr>
<td>5. PCR product 4th</td>
<td>[around 1ug] = 5ul + 6x Loading Dye = 2ul + MQ water = 5ul {Total = 12ul}</td>
</tr>
<tr>
<td>6. PCR product 5th</td>
<td>[around 1ug] = 5ul + 6x Loading Dye = 2ul + MQ water = 5ul {Total = 12ul}</td>
</tr>
<tr>
<td>7. PCR product 6th</td>
<td>[around 1ug] = 5ul + 6x Loading Dye = 2ul + MQ water = 5ul {Total = 12ul}</td>
</tr>
<tr>
<td>8. PCR product 7th</td>
<td>[around 1ug] = 5ul + 6x Loading Dye = 2ul + MQ water = 5ul {Total = 12ul}</td>
</tr>
<tr>
<td>9. PCR product 8th</td>
<td>[around 1ug] = 5ul + 6x Loading Dye = 2ul + MQ water = 5ul {Total = 12ul}</td>
</tr>
<tr>
<td>10. PCR product 9th</td>
<td>[around 1ug] = 5ul + 6x Loading Dye = 2ul + MQ water = 5ul {Total = 12ul}</td>
</tr>
</tbody>
</table>

10. Now see the gel. The sample that gives the band of desired size and has no other specific bands. The temperature is considered for further analysis and this Tm is fixed for all future successive PCR reactions for those sets of primers of a gene.

11. Even if the specific band does not appear increase the temperature or add 10% DMSO organic solvent.
C: RESTRICTION DIGESTION FOR DETECTION OF SPECIFIC SNP

1. The restriction digestion was performed in the following reaction mixture of 20µl in 0.5 ml tube.
   - MQ water: 10.1µl
   - 10X PCR Buffer: 2µl
   - PCR Product: 7.5 µl (around 3µg)
   - Enzyme: 0.4µl around 4-9 units

   Buffers are specific for a particular enzyme so a particular buffer is used.

2. Now incubation is performed at 370°C for 1 hour in thermomixer having 0.5 ml blocks without any rotation of mixture.

3. Now heat inactivation was performed for NEB enzymes and Bangalore Genei enzymes for 80°C for 20 mins or at 65°C for 20 mins respectively.

4. Now loading is done immediately in 3% agarose gel or stored in -20°C.

**Reaction Volume 200µl (Sambruk)**

<table>
<thead>
<tr>
<th></th>
<th>Make upto 200 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>MQ water</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>10X Buffer</td>
<td>170µl (around 25-50µg)</td>
</tr>
<tr>
<td>PCR product</td>
<td>75-150 units (around 3 fold)</td>
</tr>
</tbody>
</table>

**DNA AGAROSE GEL PREPARATION TO CONFIRM THE GENOTYPES**

To confirm the particular mutation in the digested PCR products the loading of the sample are done in 3% agarose.

Take 50µl of 1X TAE buffer in a conical flask add 1.5 g of agarose powder in this and heat it to melt it completely. After heating there shouldn’t be any solid particle laid in the flask.

**COMPOSITION OF 1X TAE (Tris Acetate EDTA) BUFFER**

- 40 mM Tris Acetate
- 1mM EDTA pH 8.0

**How to make 1 X TAE buffer**
1000 ml of stock of 50X TAE buffer as follows

1. 242 g (Fw = 121.14) of Tris base
2. Dissolve in 750 ml of deionised water
3. Add 57.1 ml of glacial acetic acid
4. 100 ml of 0.5 M EDTA (pH 8.0) and adjust the solution to a final volume of 1 L.
5. This stock solution can be stored at room temperature.
6. pH of this buffer is not adjusted and should be 8.5

To make 1000 ml of 1X TAE from 50X TAE stock solution, 20 ml of stock of 50X is taken and 480 ml of MQ water is added to it.

7. Loading of the samples is done in the following composition and series, DNA is loaded in the well kept towards the negative electrode.
   a. 100 bp ladder = 10 µl + 6X Dye + 4 µl + MQ water = 10 µl
   b. PCR product 1\textsuperscript{st} = 5 µl + 6X Dye = 4 µl + MQ water = 15 µl
   c. Digested PCR product 1\textsuperscript{st} = 20 µl (whole reaction mixture) + 6X Dye = 4 µl
   d. PCR product 2\textsuperscript{nd} = 5 µl + 6X dye = 4 µl + MQ water = 15 µl
   e. Digested PCR product = 20 µl (whole reaction mixture) + 6X dye = 4 µl
EXPERIMENT: GENOTYPING OF MSPI POLYMORPHISMS PRESENT IN APO A1(-75) AND APO A1 (+83) INTRON

Background:
Apolipoprotein A1 is a major lipoprotein of high density lipoprotein and is involved in reverse cholesterol transport. Variation in the apolipoprotein in the A1 gene (APOA1) might influence the function of the protein and thus braidn cholesterol metabolism, leading to an increased risk for Alzheimer’s disease or coronary artery disease. Two polymorphisms of APOA1, a G/A substitution at position -75 bp and a C/T and a G/A base substitution at position +83 bp or +84 bp or both in the APOA1 promoter, have been described which have been associated with the disease.

SETTING UP OF THE PCR REACTION:

Day 1

1. For a Single 25 µl PCR Reaction:

<table>
<thead>
<tr>
<th>Component</th>
<th>µl (Final 1X)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x PCR buffer</td>
<td></td>
</tr>
<tr>
<td>dNTP mixture (10 mM each dNTP)</td>
<td></td>
</tr>
<tr>
<td>5' primer (20 µM)</td>
<td>µl (final concentration = 1.0 µM)</td>
</tr>
<tr>
<td>3' primer (20 µM)</td>
<td>µl (final concentration = 1.0 µM)</td>
</tr>
<tr>
<td>Taq DNA polymerase (5 U/µl stock)</td>
<td>µl (1-2 units)</td>
</tr>
<tr>
<td>DNA template</td>
<td>(0.1 to 1.0 µg for genomic DNA; 1 ng or less for cloned or amplified DNA)</td>
</tr>
<tr>
<td>Sterile Water</td>
<td>to 25 µl</td>
</tr>
<tr>
<td>25mM MgCl₂ (optional)</td>
<td></td>
</tr>
</tbody>
</table>

2. Use various annealing temperatures and/or MgCl₂ conc. To optomise the PCR condition.

3. Report final result (reaction conditions and reaction component conc.) in record.
Procedure:

1. Thaw 10X buffer, dNTPs and primer. Keep on ice.
2. Note: Include a reaction tube “blank” which will have all ingredients except DNA. This is an essential control for each PCR Reaction.
3. Prepare the master mix.
4. Use 0.2 ml thin walled centrifuge tubes for reaction. Label top of the tubes with sample #.
5. Pipete mix reagent (except template) into each tube.
6. Pipete DNA samples into each tube, using a new tip for each sample.
7. Mix tubes well. Spin for 5 secs in microfuge to remove liquid from the sides.
8. Place tubes in PCR machine, programme machine and begin PCR reaction.
9. Analyse the PCR gel reaction products by agarose gel electrophoresis of a 5 µl aliquot from a total reaction. The products should be readily visible by UV transillumination of the ethidium bromide stained gel.
10. Store reaction products at -20°C until needed.
11. Take pictures and analyse results.

Day 2

PCR product size 433 bp

Digest with 1 unit MspI at 37°C O/N at 12-16 hours.

Take 10 µl of the PCR product

10X buffer   1X buffer   1ul in this case
1U MspI      calculate volume after looking at the vial
Mix gently
Keep in water bath at 37°C over night

Next day morning keep sample back in 4°C.
Prepare 3% good quality agarose gel.
Load 10µl on the gel and run full length (smaller bands should not run out).
Use 50 bp/smaller DNA ladder.
Experiment: Genotyping for Variable Numbers of Tandem Repeats

**Background:** Variability at the D1S80 locus is caused by the presence of variable number of Tandem Repeats or (VNTR). VNTRs are polymorphic DNA sequences composed of different number of repeated core sequences arranged sequentially. The size of the core sequences can vary from 8-100 bp in different VNTRs and the number of repeats in the VNTR locus also varies widely. The D1S80 locus located on chromosome 1, the largest human chromosome and the repeating sequence at D1S80 is 16 bp in length. It shows high heterozygosity.

**Materials and Methods:**

The primer sequences for the D1S80-locus

- primer 1: 5’-GAAACTGCGCTCCAAACACTGCCCCGCG-3’ (28mer)
- primer 2: 5’-GTCTTGTGGAGATGCACGTGCCCTTGC-3’ (29mer)

**PCR reaction parameters.**

<table>
<thead>
<tr>
<th>Stock conc.</th>
<th>Final conc.</th>
<th>Vol added</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 × PCR buffer</td>
<td>1X</td>
<td>2.5 ul</td>
</tr>
<tr>
<td>2.5mM dNTP’s</td>
<td>200uM</td>
<td>2 ul</td>
</tr>
<tr>
<td>F primer (10μM)</td>
<td>1uM</td>
<td>2.5ul</td>
</tr>
<tr>
<td>R primer (10μM)</td>
<td>1uM</td>
<td>2.5ul</td>
</tr>
<tr>
<td>Taq DNA Polymerase (5U/ul)</td>
<td>1U</td>
<td>0.2ul</td>
</tr>
<tr>
<td>genomic DNA (50 ng)</td>
<td>50ng/ul</td>
<td>1 ul</td>
</tr>
<tr>
<td>H2O added to a final volume of 25ul</td>
<td></td>
<td>14.3ul</td>
</tr>
</tbody>
</table>

**Temperature Cycling conditions**

- Initial Denaturation 94°C...2-3 mins
- (Denaturation 94°C...........30 secs
- Annealing temperature 55°C...30 secs
- Extension 75°C..................45 secs) 30 cycles
- Final Extension  72°C........5-7 mins
- 4°C forever
Procedure:

1. Thaw 10X buffer, dNTPs and primer. Keep on ice.
2. Note: Include a reaction tube "blank" which will have all ingredients except DNA. This is an essential control for each PCR Reaction.
3. Prepare the master mix.
4. Use 0.2 ml thin walled centrifuge tubes for reaction. Label top of the tubes with sample #.
5. Pipete mix reagent (except template) into each tube.
6. Pipete DNA samples into each tube, using a new tip for each sample.
7. Mix tubes well. Spin for 5 secs in microfuge to remove liquid from the sides.
8. Place tubes in PCR machine, programme machine and begin PCR reaction.
9. Analyse the PCR gel reaction products by 12-15% agarose gel electrophoresis of a 5 µl aliquot from a total reaction.
10. The products should be readily visible by UV transillumination of the ethidium bromide stained gel.
11. Store reaction products at -20°C until needed.
12. Take pictures and analyse results.
Experiment: Drawing pedigree and identifying mode of inheritance

1. Draw your own family pedigree

2. Riya is 35 years old. She has a brother Suresh who is 32. Riya and Suresh are the only children of Ramesh, who dies at 61 from Cancer and Nina who is alive and well at 57 years old. Riya is married to Dinesh who is 36 and they have identical 6 year old twins boys, Luv and Kush. Suresh and Gita have 5 year old daughter Sarah and a 2 year old son Milind. Suresh and Gita are recently divorced. Draw the pedigree.

3. Maya is 28 years old (9.1.80). She has an appointment on a wound on her left leg that isn’t healing well. Maya has a younger brother Nick and her little sister Sharong. Mayas parents are George and Mourin who is obese. George’s brother Edward is obese. George’s sister Rachael has type II diabetes. George’s parents are both diseased and his parents died of a heart attack. On his mother’s side she has three aunts who is all alive and well. Maya’s maternal grandparents are Ralph and Beatrice who turned 75 in 2007 (so were born in 1932).

4. Find out the mode of inheritance in the given pedigree.

5. Find out the mode of inheritance in the given pedigree. Do you think incomplete penetrance may be involved? If yes explain why?
EXPERIMENT: DNA fingerprinting using Amplified Fragment Length Polymorphism (AFLP)

Principal:

The Amplified Fragment length polymorphism technique (AFLP) Technique of DNA finger printing takes advantage of polymerase chain Reaction (PCR) to amplify limited set of DNA fragments from a specific DNA sample.

AFLP from Complex genomes involves 5 steps:

1. **RESTRICTION OF GENOMIC DNA**: Restriction fragments of the genomic DNA are produced by using 2 different restriction enzymes a frequent cutter—the 4 base restriction enzyme (Taq I/MseI) and a rare cutter (6 base restriction enzyme Eco RI/ Pst I). Three types of restriction fragments are generated. Ones with Eco RI cuts at both ends, ones with EcoRI cuts at one end and the Taq I cut at the other end and done with Taq I cuts at both ends.

2. **LIGATION OF OLIGONUCLEOTIDE PRIMERS**: Double stranded adapters consist of a core sequence and an enzyme specific sequence. They are specific for either the EcoRI or Taq I specific site. Ligation of the adapter to the restricted DNA alters the restriction site so as to prevent a second restriction from taking place after ligation has occurred.

3. **PRE-SELECTIVE AMPLIFICATION**: Primers used in the step consist of the core sequence an enzyme specific sequence and a selective single base extension at the 3’ end. Sequences for the adapters and the restriction sites serves as primer binding sites for the “pre selective PCR amplification”. Each preselective primer has a selective nucleotide that will recognize the subset of restriction fragments having the matching nucleotide downstream from the matching nucleotide. The primary products of the preselective PCR are those fragments having 1 TAQ 1 cut and 1 Eco R1 cut and also having the matching nucleotide.

Example of an EcoRI Adapter sequence is as follows:

5’ATG TTA GAG TGC GTA CCA ATT C-3’:

Core sequence: ATG TTA GAG TGC GTA CC

Enzyme Specific sequence: A ATT

Selective single-base extension: C
IV. SELECTIVE AMPLIFICATION: The selective identification step consists of an identical sequence to the pre selection primers plus two additional selective nucleotides at the 3’ end (i.e., a total of three selective nucleotides). These two additional nucleotides can be any of the sixteen possible combinations of the four nucleotides. From the huge number of fragments generated from the two restriction enzymes, only that subset of fragments having matching nucleotides at all three positions will be amplifies at this stage (50-200 fragments). This step reduces the complexity of the PCR product mixture. Different primer combinations will generate different sets of fragments. Preliminary screening is used to choose primer pairs that generate suitable level of variation of the taxa being studied.

V. SCORING AFLP PRODUCTS: AFLP PCR products can be scored with a variety of techniques, ranging from simple gel electrophoresis to automated genotyping. Polyacrylamide gel electrophoresis provide maximum resolution of AFLP banding pattern to the level of single nucleotide length differences.

**Procedure:**

Day 1: Restriction Digestion:

1. Add following components for digestion.
   - Yeast genomic DNA 2μl
   - EcoR I 1μl
   - Taq I 1μl
   - 10X Buffer E 2.5μl
   Make final volume to 25μl with nuclease free water.

2. Mix properly and give it a short spin. Incubate it at
   - 37°C 2 hours
   - 65°C 2 hours
   - 70°C 20 minutes
   - 4°C hold

Adapter Ligation
Add the following components for adapter ligation

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRI adapter</td>
<td>2µl</td>
</tr>
<tr>
<td>TaqI adapter</td>
<td>5µl</td>
</tr>
<tr>
<td>10X Ligase Buffer</td>
<td>5µl</td>
</tr>
<tr>
<td>Instant Ligase</td>
<td>2µl</td>
</tr>
<tr>
<td>Restriction Digested sample</td>
<td>25µl</td>
</tr>
</tbody>
</table>

Make the volume to 50µl with nuclease free water.

4. Mix properly and then give it a short spin. Incubate it at 37°C for 30 mins.
5. Dilute the ligated sample 1:10 with nuclease free water as follows i.e., mix 10µl of ligated sample with 90µ of nuclease free water in a 1.5 ml vial. Store at 4°C.

6. Day 2:

Pre-Selective Amplification:

6. Set up the reaction as follows

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Restriction/ligation diluted sample</td>
<td>5µl</td>
</tr>
<tr>
<td>Primer P1</td>
<td>2µl</td>
</tr>
<tr>
<td>Primer P2</td>
<td>2µl</td>
</tr>
<tr>
<td>10mM dNTP mix</td>
<td>1µl</td>
</tr>
<tr>
<td>Taq DNA Polymerase</td>
<td>1µl</td>
</tr>
<tr>
<td>10X Taq Buffer A</td>
<td>5µl</td>
</tr>
</tbody>
</table>

Make the volume to 50µl with nuclease free water, mix properly. Give it a short spin.

Conduct a PCR.
7. Make 1:10 dilution with nuclease free water i.e., 10µl of pre selection amplification product +90 µl of nuclease free water in a 1.5 ml vial.

8. Store at 4°C/continue with next step.

**Note: When diluting the sample use separate tips.**

Selective Amplification

9. Set up the selective amplification reactions with 2 different sets of primers.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Components</th>
<th>Tube I</th>
<th>Tube II</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nuclease free water</td>
<td>34.0 µl</td>
<td>34.0 µl</td>
</tr>
<tr>
<td>2</td>
<td>10X Taq Buffer A</td>
<td>5.0 µl</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>3</td>
<td>10mM dNTP mix</td>
<td>1.0 µl</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>4</td>
<td>Primer P3</td>
<td>2.0 µl</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Primer P4</td>
<td>2.0 µl</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Primer P5</td>
<td>-</td>
<td>2.0 µl</td>
</tr>
<tr>
<td></td>
<td>Primer P6</td>
<td>-</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>6</td>
<td>Sample (pre-selective; 1:10)</td>
<td>5.0 µl</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>7</td>
<td>Taq DNA Polymerase</td>
<td>1.0 µl</td>
<td>1.0 µl</td>
</tr>
<tr>
<td></td>
<td><strong>Total Volume</strong></td>
<td><strong>50.0 µl</strong></td>
<td><strong>50.0 µl</strong></td>
</tr>
</tbody>
</table>
GEL ANALYSIS:

1. Prepare 8% native PAGE mini gel.
2. Mix 10µl of each PCR product with 5µl of gel loading dye in a 1.5 ml vial and load.
3. Mix 5µl of 20 bp DNA ladder with a 5µl of gel loading dye in a 1.5 ml vial and load.
4. Run the gel at a constant voltage 100 volts for 1-2 hours.
EXPERIMENT : CULTURE AND HANDLING

OF Drosophila melanogaster.

Introduction: Drosophila melanogaster has been used as a model system for genetic and developmental studies for the following reasons:

1. It has a short generation time and progeny size is large.
2. Can be reared easily in the laboratory.
3. Small body size requires little space and can be handled easily.
4. Sharply defined body phenotypes facilitates the identification of numerous mutants.
5. Presence of polytene chromosomes facilitating easy localization of genes and their activity.
6. Easily identifiable developmental stages and numerous structures.

Drosophila is a holometabolous insect. The different stages of life cycle are egg, larva, pupae and adult. The duration of each stage varies with temperature. The average extent of egg to larval period at 20°C is 8 days at 25°C it is reduced to 5 days. At 20°C the pupal life is about 6.3 days whereas at 25°C it is about 4.2 days. At 25°C therefore the life cycle is completed in about 10 days and at 20°C about 15 days are required.

Identification of Sex:

Male and female flies can be distinguished from each other. The tip of the abdomen is elongated in the female and somewhat rounded in the male. As the female ages and the abdomen become distended with maturing eggs, flies of this sex are recognizable at a glance. The abdomen of the female has 7 visible segments whereas that of the male has 5. The patterns of darker markings of the abdominal segments is sufficiently distinct in the two sexes. In males of several species of Drosophila the abdominal tip appears black due to fusion of last 3 segments. The males also possess sex comb as a fringe of about ten stout black bristles on the first tarsal segment of the first leg (both sides).

Collection of virgins:
The flies start mating about 6 hours of emergence at 25°C. If a genetic cross is to be set with flies of different genotypes then virgin females are collected (female flies are separated from males within two hours of emergences) and they are mated with the males of desired genotypes.

**DEVELOPMENTAL STAGES:**

The egg of the *Drosophila melanogaster* is white in colour and is about 0.5 mm in length. The outer membrane, the chorion is composed of cells is hexagonal in outline. Extending from the anterodorsal surface is a pair of filaments which keeps the egg from sinking into the soft food in which it is laid. Beneath the chorion is a membrane called the vitteline membrane that covers the egg proper.

**LARVA:**

The larva after hatching from the egg undergoes two molts so that the larval period consist of three stages (instars), first instar, second instar, third instar. The egg hatches in 24 hour time at 25°C into a tiny first instar larva. These larva crawl on the food. After a period of 24 hours it sheds its cuticle and thus enters into the second instar stage. This stage lasts for another 24 hours. Larva enters into the food and feed actively. After shedding the cuticle for the second time it enters into the third instar stage. The duration of the third instar stage is longer (3 days at 25°C) and larvae grow to the full size. The different larval stages can also be identified by seeing the serration of the mandible. At first instar stage they have only one serration, in second instar stage they have two and in third instar stage they have multiple serrations.
PREPARATION OF *Drosophila* Food

*Drosophila* food Ingredients (1 unit)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar-agar</td>
<td>13 gms</td>
</tr>
<tr>
<td>Maize powder</td>
<td>17 gms</td>
</tr>
<tr>
<td>Sugar</td>
<td>15 gms</td>
</tr>
<tr>
<td>Yeast (dried)</td>
<td>6 gms</td>
</tr>
<tr>
<td>Nepagin</td>
<td>1 gm (dissolve in 1-2 ml of 90% alcohol)</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>1 ml</td>
</tr>
<tr>
<td>Tap water</td>
<td>360 ml</td>
</tr>
</tbody>
</table>

To approximately 100 ml of warmed tap water, agar agar is added and boiled. A paste of maize powder and sugar is made using approximately using 150 ml of water and the same is added to boiling agar agar solution. The mixture is allowed to boil again and a paste of yeast is added. It is further boiled till the good cooking smell stars coming. Nepagin dissolved in alcohol and propionic acid is now added after putting off the heater. Food can now be poured in bottles and or vials and allowed to solidify by covering them with a cloth. After 4-5 hours the vials and bottles are plugged with non absorbant cotton and stored at 4°C for further use.

**Requirements:**

Culture bottle, culture vial, bottle plugs, vial plugs, non absorbant cotton, agar agar, maize powder, sugar, yeast, nepagin, 90% alcohol, propionic acid, cheese cloth.

**Procedure for Fly handling:**

In order to examine and count the flies, they are anaesthesised with a light dose of ether. The bottle or vial is inverted over the etherizer and the flies are allowed to fall into the etherizer by gentle tapping. After the flies stop movement they are
transferred into the white glass plate for observation. They are now well etherized but still alive. Over-etherized flies extend their wings to the right angles to the body and can be considered as dead. If the flies begin to revive after the observation is completed, they can be re-etherized. The re-etherizer consists of a small petridish with a strip of blotting paper fastened inside. A few drops of ether are placed on the paper and the flies are covered with the re-etherizer until they stop movement. After observation flies are either discarded into the morgue or they are transferred into a fresh food vial/bottle.

Students observe the various stages of development under stereomicroscope and also prepare the food and step us Morgues and Fly trap in the lab. A brief introduction of the *Drosophila* life cycle is provided by the instructor.
EXPERIMENT: Demonstration of Basic laws of Genetics through genetic crosses

The desired strains (mutants or wild types) can be done by taking ~ 10 males of one type and ~ 10 females of other types in a bottle. In a week the females lay sufficient eggs for obtaining a sizeable number of F1 progeny. The parents are then discarded and allowed the F1 progeny to emerge. The progeny flies are used to set up another cross for obtaining F2. Schemes of some representative crosses are done below.

![Diagram of Linkage and Crossing Over]

Cross 1:

\[
\begin{align*}
+ & \quad + \\
+ & \quad + \\
\text{wild type virgin} & \quad \text{se} & \quad \text{e} \\
\text{♀} \times & \quad \text{♀} \\
\text{♀} & \quad \text{se} & \quad \text{e} \\
\downarrow & \quad \text{se} & \quad \text{e} \\
\text{F1} & \quad + & \quad + \\
\text{All flies heterozygous and phenotypically wild type}
\end{align*}
\]

sepia eye, ebony body colour
(On 3rd chromosome)
Since in F2 the recombinants are more than the parental types this cross shows linkage and crossing over. % of recombination will reflect the distance between the two loci in the centi Morgan Unit.
Test Cross 2 (Reciprocal cross)

\[ + \quad + \]
\[ \quad \quad \quad se \quad e \quad \hat{\sigma} \]
\[ se \quad e \] (Recessive)

\[ \downarrow \]

\[ F2 \quad + \quad + \]
\[ \quad \quad \quad se \quad e \quad \hat{\sigma} \]
\[ se \quad e \] (Recessive)

\[ X \quad \quad se \quad e \]
\[ se \quad e \] (Virgin)

\[ se \quad e \] (Homozygous)

Since recombination does not occur in males of *D. melanogaster*, the parental types are only expected in F2.
**Sex linkage** (i.e. the given gene is linked to X-chromosome)

Cross 1

+  
---
♀ X  
+  
Wild type virgin  
↓  
F1  
+  
---
♀  
w  
Wild type ♀  
+w+  
---
Wild type ♂

F1 female and F1 males are crossed to obtain F2 progeny

F1  
+  
---
♀ X  
+w  
---
♂  
↓  
F2  
+  
---
♂  
+  
---
♀ wild  
w  
♀ wild  
+w  
---
♂ white  

Wild type ♀  Wild type ♂  White ♂  
2 : 1 : 1
Cross 2

\[ \begin{array}{cc}
  w & + \\
  \_ & \_ \\
  \_ & \_ \\
  w & + \\
  \_ & \_ \\
  \_ & \_ \\
  w & + \\
  \_ & \_ \\
  \_ & \_ \\
  w & + \\
  \_ & \_ \\
  \_ & \_ \\
\end{array} \]

White eyed virgin \quad \text{Wild type}

\rightarrow 

F1

\[ \begin{array}{cc}
  w & + \\
  \_ & \_ \\
  \_ & \_ \\
  w & + \\
  \_ & \_ \\
  \_ & \_ \\
  w & + \\
  \_ & \_ \\
  \_ & \_ \\
  w & + \\
  \_ & \_ \\
  \_ & \_ \\
\end{array} \]

wild type \quad \text{white eyed}

F1 female and F1 males are crossed to obtain F2 progeny

\[ \begin{array}{cc}
  w & + \\
  \_ & \_ \\
  \_ & \_ \\
  w & + \\
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\end{array} \]

wild type \quad \text{white eyed}

F2

This cross also shows cis-cross inheritance

wild $\varnothing$ : wild $\sigma$ : white $\varnothing$ : white $\sigma$

1 : 1 : 1 : 1
EXPERIMENT: STUDY OF HOMEOTIC MUTANTS OF *Drosophila*

INTRODUCTION:
Those genes whose abnormal function lead to transformation of one body structure into another are called homeotic genes. After the segmental boundaries have been established, the characteristics structures of each segments are specified. This specification is accomplished by the homeotic genes. There are two regions of *Drosophila* Chromosome 3 that contain these homeotic genes. One region the Antennapedia complex contain the homeotic genes the Libial, The Proboisipidia, Deformed, Sex Comb reduced and Antennapedia. The labial, probosipidia and Deformed genes specify the head segments while sex comb reduced and Antennapedia contribute to giving the thoracic segments and their identity. The second region of the homeotic genes is the Bithorax Complex and there are protein coding regions in this complex namely ultrabithorax, Abdominal A and Abdominal B. Ultrabithorax is required for the identity of the third thoracic segment while Abdominal A and Abdominal B are responsible for the segmental identities of the abdominal segments.

The Antennepedia genes specify the identity of the second thoracic segment. In the dominant mutation of Antennipedia, this gene is expressed in the gene and the thorax, and the imaginal disc of the head region is specified as thoracic. The antenna gets transformed into leg like structure. Likewise when the ultra bithorax is deleted, the third thoracic segment becomes transformed into another second thoracic segment and the result is the fly with four wings.

MATERIALS REQUIRED

Mutant flies, 5% KOH, spirit lamp, glass test tube, test tube holder, ascending grades of alcohol, Xylene, DPX mountant
PROCEDURE

1. Etherize flies, transfer them in a test tube containing 5% KOH and boil gently till the soft tissues are dissolved and only clean cuticular structures remaining. Boiling should be done with constant shaking so that KOH does not bump out. Do not overboil.

2. Wash the flies in tap water, dehydrate through ascending grades of alcohol, pass through Xylene and mount in DPX.

OBSERVATION

The transformation of antennae in Antennapedia mutants or transformation of haltere to wing will be clearly visible.
EXPERIMENT: SHORT TERM CULTURE OF WHOLE BLOOD AND PREPARATION OF METAPHASE CHROMOSOME

INTRODUCTION:
One of the most vital limitations in understanding many physiological, genetic and other functions of the body is the difficulty in devising suitable experiments directly on the organism. The worst sufferer of the human system has been the human system. Tissue culture techniques long as well as short term have therefore been developed to stimulate *in vitro* and the *in vivo* conditions so that the various molecular, cellular and organic function could be better understood. Short time lymphocyte culture is the simplest form of tissue culture in which genetically inert lymphocytes are stimulated to proliferate by using a lectin as a mitogen, phytohemagglutinin, concanavalin A, pokeweed mitogen. Its most obvious application is diagnostic with regards to chromosomal system in man and other systems. Uses of various banding techniques have made it possible to identify small fragments of chromosome which allow detection of even minor chromosomal rearrangements. It is also possible to address several cell biological and immunological questions through lymphocyte culture. These cells can also be used in somatic cell fusion and hybridization for genetic mapping and studies in differentiation.
MATERIALS REQUIRED:

Sterilized glass ware: Pipettes (10ml, 5ml, 1ml), culture vials (universal containers), conical flask 100ml, Syringes (5ml, 1ml), needles (#22, #26), Millipore filter assembly

Chemicals: Tissue culture media (RPMI 1640 + L-glutamine to be added at the time of setting up the culture), fetal calf serum (FCS) (alternatively, heat inactivated human AB serum), phytohaemagglutinin-m (PHA), heparin, 0.2µg/ml colcemid, 1N HCl, 90% alcohol, 0.56% KCl (Hypotonic), Acetic acid methanol (1:3-Fixative), Acid-cleaned slides (maintained in 70% alcohol), Giemsa stain, Incubator, centrifuge, clean-sterile work bench, Giemsa stain

Stock soln - Giemsa Powder
         Methanol
         Glycerol

Leave overnight at 37°C. Filter the stain and store.

Working soln - Stock soln
         Methanol
         Giemsa water

Giemsa water - 0.2M Na₂HPO₄
         Distilled water

Adjust pH to 6.8 with 0.1M Citric acid (roughly 24ml) and then make up the volume to 1 liter
PROCEDURE:
Setting the culture

1. About 5 ml of blood is collected in a sterile, heparinised syringe in a suitable clean environment. Blood is kept in the refrigerator until used.

2. Arrange TC Medium FCS PHA, L-Glutamine, Pipettes, beakers, and flask etc on an alcohol swabbed work bench before setting up the culture.

3. Prepare the TC medium by adding antibiotics, L-Glutamine, and 10% BSA, (10 ml of serum added to 100 ml of culture, serum is added separately to the culture). Since commercial media has phenol red as an indicator, colour of the medium indicates its pH which must be 7.2 (colour light pink to orangish). If the pH is alkaline, indigo, use CO2 or a few drops of 1 N HCl to bring it to the proper range.

4. Prepare a working solution of PHA (5 ml in sterile distilled water). This can be stored under sterile conditions at 1-2°C months at 4 degree.

5. Before using pippetmans, containers, culture vial etc, flame them gently but use only after cooling.

6. For each culture add the following in the order given below:
   TC medium
   FCS
   Blood PHA

7. In order to buffer the pH blow CO2 from a CO2 cylinder or bubble exhaled air orally through a cotton plugged pipette.

8. Culture is kept in an incubator at 37°C for 48 to 72 hours.

9. Culture must be inspected every morning and evening for change in the pH and infection and shaken to break the clumps of RBC.

10. About 2-3 hours prior to harvesting, the culture for chromosome preparation colcemid (working conc. 0.02 µg/ml is added)
**Chromosome Preparation**

1. Transfer the culture to a centrifuge tube and centrifuge at 1000 to 1200rpm for 5min.
2. Decant the supernatant and make a fresh suspension of cells in prewarmed 0.56% KCl (hypotonic). Initially add slowly and agitate the sediment. Once the cells come in suspension, make up the volume to 8-10ml. Keep in incubator (37°C) for 18-20min.
3. Immediately before centrifugation, add 3-4 drops of fixative to the tube and mix. Spin (1000 - 1200 rpm) the tubes and decant the hypotonic completely. Add the fixative drop-by-drop to fix the cells and to keep them in suspension. Make up the volume to ~8-10ml. Keep for 15min.
4. Recentrifuge (1000-1200 rpm) for 5min, decant the supernatant and resuspend the pellet in fresh fixative (~ 8-10ml). Keep for 10min.
5. Recentrifuge (as in #4) and discard the supernatant. Add only about 0.2-0.4ml. Resuspend the cells well with a Pasteur pipette.
6. Take out a slide from 70% alcohol and wipe it with a clean piece of cloth. Add 2-3 drops of the cell suspension on the slide and either blow it dry (air-drying) or expose to a flame for instant drying (flame drying).
7. Stain the slide with Giemsa stain for 3-4min and rinse in 2 changes of distilled water (pH 6.8-7.2) or clean tap water. Dry it fully and mount with DPX mountant using a 24x60mm coverglass.
EVEN SEMESTER:
6<sup>th</sup> SEMESTER

IMMUNOLOGY LABORATORY
Lab techniques and experiments to be followed:

Experiment 1: Cell culture and in-vitro T cell and non T cell activation:

Cell lines (e.g. Raw, Jurkat, etc) and primary cells (mouse spleen cells, human PBMC, etc) will be grown in tissue culture flasks at 37ºC in 5% carbon dioxide containing humified incubator. Cells will be cultured in 10% fetal Bovine serum (FBS) containing complete IMDM or DMEM media in a T-25/75 culture flask. ConA (5 μg/ml) or LPS (1 μg/ml) driven in vitro immune-activation will be performed for 48 h in 24 multi well plates.

Experiment(s):
  5. To study the microscopic nature of immune cells and cell lines
  6. To study the effect of mitogen (e.g. ConA) for T cell activation assay on mouse spleen cells.
Experiment 2: Flow Cytometry:

Single cell suspensions will be prepared and used for flow cytometry on a FACS Calibur instrument (BD Biosciences) and Cell Quest Pro software program (BD Biosciences). In brief, the cells will be incubated with fluorochrome-labeled antibodies (for specific cell-surface markers and control antibodies) in staining buffer (1% fetal calf serum, 0.1% sodium azide in PBS) for half an hour. The labeled cells will be washed, detected by Flow Cytometry using a FACS Calibur and analyzed by Cell Quest Pro software (BD Biosciences). A total of more than 10,000 cells will be analyzed sample. Cells were further gated for respective live cell populations. We will study different immuno-markers (e.g. CD3, CD25, CD68, CD14, CD19, etc) using fluorochorme conjugated monoclonal antibodies (mAb) of mouse and human cells and cell lines by Flow cytometry.
Experiment(s):

4. To study the phenotypes of immune cells and cell lines (e.g. T cells, B cells, Macrophages and other subsets)

5. To study the effect of mitogen (e.g. ConA) for T cell activation assay on mouse spleen cells by changes in phenotypes.

6. To study the effect of LPS on immune cells phenotypes especially on macrophages.

7. To analyze the flow cytometric data using Cell Quest Pro software program.
Experiment 3: Sandwich ELISA to study functional changes of host immune cells

This ELISA is fast and accurate, and if a purified antigen standard is available, the assay can determine the absolute amounts of antigen in unknown samples. The sensitivity of the Sandwich ELISA is dependent on the number of molecules of the first antibody that are bound to the solid phase, the avidity of the first antibody for the antigen, the avidity of the second antibody for the antigen and the specific activity of the enzyme labeled secondary antibody. Cytokine sandwich ELISA is sensitive enzyme immunoassays that can specifically detect and quantify the concentration of soluble cytokine and chemokine proteins. The basic cytokine sandwich ELISA method makes use of highly-purified anti-cytokine antibodies (capture antibodies) which are noncovalently adsorbed ("coated"—primarily as a result of hydrophobic interactions) onto plastic microwell plates. After plate washings, the immobilized antibodies serve to specifically capture soluble cytokine proteins present in samples which were applied to the plate. After washing away unbound material, the captured cytokine proteins are detected by biotin-conjugated anti-cytokine antibodies (detection antibodies) followed by an enzyme-labeled avidin or streptavidin stage. Following the addition of a chromogenic substrate, the level of colored product generated by the bound, enzyme-linked detection reagents can be conveniently measured spectrophotometrically using an ELISA-plate reader at an appropriate optical density (OD). To study the immune-functional outcome of immune cells we will perform. Here we will perform a basic Sandwich ELISA with quantization of an unknown antigen to know the basic technique we will perform a basic Sandwich ELISA with quantization of an unknown antigen to know the basic technique.
Experiments:

1. To measure the amount of unknown antigen by Sandwich ELISA technique.

Experiment 4: Immuno-diffusion Assay:

Radial immuno-diffusion is a reliable quantitative method and is particularly useful for difficult samples, e.g., that are turbid and for which other methods are inappropriate. This technique detects the quantity of antigen by measuring the radius surrounding samples of the antigen, marking the boundary between it and antibody. As the antigen diffuses into the gel, it reacts with the antibody and when the equivalence point is reached a ring of precipitation is formed. This test is commonly used in the clinical laboratory for the determination of immunoglobulin levels in patient samples.

The immune system is a remarkably versatile defense system that has evolved to protect animals from invading pathogenic microorganisms and cancer. It is able to generate an enormous variety of cells and molecules (antibody) capable of specifically recognizing and eliminating a variety of foreign invaders (antigen). Antigen and antibody interaction is the fundamental reaction of immunology. These interactions are useful in the defense of the body against bacterial and viral infections and toxins. The defense capabilities are dependent upon the recognition of antigens by humoral components of the immune system. Specific antibodies are then produced in response to exposure to the antigen. The binding of an antibody with an antigen results in the formation of large macromolecular complex. These complexes form precipitates, which are useful for laboratory and diagnostic tests. Precipitation reactions of antibodies and antigens in agarose gels provide a method of analyzing the various antibody-antigen reactions in a system. When antibodies and antigens are inserted into different areas of an agarose gel, they diffuse toward each other and form opaque bands of precipitate at the interface of their diffusion fronts.
Experiment 5: Radial Immunodiffusion (RID) Assay

RID is a specialized form of immunodiffusion in which antibody is incorporated into molten agarose, which is poured into a petridish and allowed to solidify. Small wells are cut into the agarose gel and are filled with known concentrations of antigen, which corresponds to the antibody in the agarose. Samples of unknown concentrations are placed in similar wells. The antigens in solution then diffuse outwards from the well in a circular precipitate ring surrounding the well. Generally it takes 24 to 48 hours for optimal diffusion to occur and precipitation to become apparent. The diameter of the precipitin ring is proportional to the concentration of the antigen present in the test sample. By comparing the diameter of the test specimen precipitin ring to known standards, a relatively less sensitive estimation of the concentration of specific antigen can be achieved.

Experiment:
1. To quantify the level of an unknown antigen by Radial Immuno-diffusion assay.
SIXTH SEMESTER
MOLECULAR BIOLOGY LABORATORY
Aim:

To clone a gene of interest in a bacterial vector and screen for the clones.

Material required
Vector and insert containing plasmids
Agarose
Gel elution kit DNA Liagse
Competent cells
LB
LB amp plates Solution I

50 mM glucose
25 mM Tris-cl pH-8
10 mM EDTA Solution II
0.2 N NaOH 1 SDS

Solution III
5M potassium acetate (60 ml) Glacial acetic acid (11.5 ml) Water (28.5 ml)

Procedure
- Digestion of vector and insert containing plasmids

<table>
<thead>
<tr>
<th>Components</th>
<th>vector</th>
<th>Insert</th>
</tr>
</thead>
<tbody>
<tr>
<td>MilliQ</td>
<td>1.5 IA</td>
<td>2.5 pi</td>
</tr>
<tr>
<td>10X buffer</td>
<td>2.5 pl</td>
<td>1.5 pi</td>
</tr>
<tr>
<td>plasmid</td>
<td>20 III</td>
<td>10 pi</td>
</tr>
<tr>
<td>Enzyme 1 (20U/μl)</td>
<td>0.5 [il</td>
<td>0.5 pl</td>
</tr>
<tr>
<td>Enzyme 2 (20U/111)</td>
<td>0.5 ill</td>
<td>0.5 111</td>
</tr>
<tr>
<td>Total</td>
<td>25 pil</td>
<td>15 pl</td>
</tr>
</tbody>
</table>

- Digested product is electrophoresed in 0.8 % agarose gel
Gel purified using Qiagen gel elution kit

Ligation reaction is setup as following

<table>
<thead>
<tr>
<th>Components</th>
<th>Vector-insert</th>
<th>pTYB2</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X quick ligase buffer</td>
<td>101A1</td>
<td>10111</td>
</tr>
<tr>
<td>Vector</td>
<td>5 pi</td>
<td>4 Ill</td>
</tr>
<tr>
<td>Insert</td>
<td>3 Ell</td>
<td></td>
</tr>
<tr>
<td>Quick DNA Ligase</td>
<td>1 IA</td>
<td>1 1.1.1</td>
</tr>
<tr>
<td>MilliQ</td>
<td>1</td>
<td>5 iil</td>
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<tr>
<td>Total</td>
<td>2010</td>
<td>20 iil</td>
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</tbody>
</table>

Transformation

Preparation of chemically competent cells

- Have the following solutions at 0-4 °C:
  - 100 mM MgCl2
  - 100 mM CaCl2-15% glycerol
  - sterile GSA bottles and pre-cooled rotor
  - Grow a 5 mL overnight culture of bacteria.
  - Dilute 1:100 and shake at 37°C (5 mL into 500 mL).
  - After 1.5-2 hours, $\lambda_{600} = 0.5-0.6$ (0.4 also works well).
  - When cells reach proper density, transfer to GSA bottles and spin down 5000 rpm (JA-10), 10 minutes at 4 °C.
  - Pour off supernatant and keep the cells on ice.
  - Resuspend in 100 mL of 100 mM MgCl2 (ice-cold) by pipetting up and down.
  - Incubate on ice 20-30 minutes.
  - Spin down cells at 4000 rpm for 10 minutes at 4 °C. Discard supernatant and keep cells on ice.
  - Cool small eppendorf tubes on ice.
  - Resuspend in 10 mL of 100 mM CaCl2-15% glycerol (ice-cold).
  - Aliquot into tubes (170 glitube) and put at -80 °C (quick freezing not necessary).
**Transformation of frozen competent cells**

Thaw frozen cells on ice, 10-15 minutes.

- Add 80 μl cells to the 20 μl ligation reaction.
- Incubate on ice for 5 minutes.
- Heat shock cells for 10 minutes at 37 °C.
- Bring up in 1 mL LB and shake gently for 1 hour at 37 °C.
- Plate 200 μl in LB plate containing appropriate antibiotic.

**Screening of transformants**

**Day 1**

1. Inoculate 5 ml of LBM medium containing 50 μg /ml ampicillin with a single bacterial colony containing the desired plasmid or cosmid. Incubate at 37 degrees C overnight on a roller drum.

**Day 2**

1. Spin down cells at 2500 rpm in Beckman low speed centrifuge (e.g. the J-6) for 15 minutes. Discard supernatant.
2. Resuspend each pellet in 200 ul solution I. Transfer samples to labeled eppendorf tubes. Incubate at room temperature for 5 minutes.
3. Add 400 ul of a freshly prepared solution of lysis solution (solution II). Mix gently. Place on ice for 5 minutes.
4. Add 300 ul of an ice-cold solution III. Mix gently. Place on ice for 5 minutes.
5. Centrifuge for 1 minute at 4 degrees C. Transfer the supernatant to a clean tube.
6. Add 0.6 volume (540 ul) isopropanol to the supernatant mix and incubate at room temperature for 2 minutes. Pellet the nucleic acid (a 1 minute microcentrifuge spin top speed). Discard the supernatant.
7. Wash the pellet twice with 1 ml 70% ethanol. Spin for 1 minute and discard the supernatant.
8. Dry the pellet for 10 minutes in the lyophilizer or allow to air dry. Resuspend in 50 ul TE pH 7.5. Add 1 ul of a 10 mg/ml solution of RNAase and incubate for 30 minutes at room temperature.
9. Assay the DNA on a minigel with appropriate concentration standards before restricting the DNA. The expected yield is 5-10 ug plasmid DNA

Digestion of plasmid with the appropriate enzyme as done earlier (protocol used to prepare vector and insert)
Aim:
To identify the gene of interest in the genomic DNA by Southern blotting.

Introduction

Localization of particular sequences within genomic DNA is usually accomplished by the transfer techniques described by Southern (1975). Genomic DNA is digested with one or more restriction enzymes, and the resulting fragments are separated according to size by electrophoresis through an agarose gel. The DNA is then denatured in situ and transferred from the gel to a solid support (usually nitrocellulose filter or nylon membrane). The relative positions of the DNA fragments are preserved during their transfer to the filter. The DNA attached to the filter is hybridized to radiolabeled DNA or RNA, and autoradiography is used to locate the positions of bands complementary to the probe. A sequence of 1000 bp that occurs only once in the mammalian genome can be detected in an overnight exposure if 10 tig of genomic DNA is transferred to the filter and hybridized to a probe several hundred nucleotides in length.

Steps involved
1. Restriction digestion of genomic DNA (overnight)
2. Agarose gel electrophoresis (6-16 hours)
3. Southern transfer to Nylon membrane (overnight)
4. Prehybridization (2 hours)
5. Radioactive labeling of probe (1 hour)
6. Hybridization (overnight)
7. Wash (2 hours)
8. Detection (1 to 6 days)
9. Stripping / Reprobing (1 hour)
10. Supplies and Equipment

Materials required
- Microfuge
- Waterbath 37, 100°C
- Agarose gel electrophoresis supply
- S&S Nytran Membranes
- Whatman 3MM paper
- paper towels
- UV-crosslinker
- hybridization bottles
Procedure

**Restriction digestion of genomic DNA**

1. Cut off and autoclave pipette tips for handling high-molecular-weight genomic DNA.

2. Digest 10 lig genomic DNA in 400 ill total volume with 3 units restriction enzyme per p.g DNA at 37°C for overnight. (For random primer labeling you will also need to cut out the cDNA probe from vector.) *(To ensure homogeneous dispersion of the genomic DNA: (a) allow the DNA to stand at 4°C for several hours after dilution and addition of 10 x restriction enzyme buffer, (b) gently stir the DNA solution from time to time, (c) after adding restriction enzyme, gently stir the solution for 2-3 minutes at 4°C before warming up to 37°C, (d) after digestion for 30 minutes, add a second aliquot of restriction enzyme and stir as described above)*

3. Add 0.1 volume (10g1) 3 M sodium acetate, mix well. Add 2.5 volumes ice-cold 100% ethanol. Precipitate at -80°C for 1 hour. Spin in microfuge at high speed for 20 minutes. Discard supernatant, air-dry for 5 minutes. Resuspend in 15 tl 10mM Tris pH-7.5. *(Residual ethanol can cause the DNA sample to 'crawl' out of the well when loaded onto the gel. Heating the solution of redissolved DNA to 65-70°C in an open tube for 10 minutes can usually drive off most of the ethanol.)*

4. Add 3 tl 6 x DNA-loading buffer (total volume 18 p.l).

**Agarose gel electrophoresis (6-16 hours)**

1. Pour a 0.8% agarose gel.

2. Prepare DNA size markers.

3. Submerge gel in 2000 ml 1X TAE with 0.514/ml ethidium bromide.
(i.e. 40 ml of 50 x TAE, 100 ,ul of 1% ethidium bromide stock solution, dH2O ad 2 liter)

4. Load samples, markers and controls.
   (If DNA does not sink to the bottom of the well, make sure that all the ethanol was removed. Load samples very slowl. After loading, allow the gel to stand for a few minutes so that the DNA can diffuse evenly throughout the wells.)

5. Electrophorese at 40 Volts, 35 mAmps for 6-16 hours.

6. Photograph with fluorescent ruler 1/8th sec with ruler and an additional 1 sec on gel alone. Cut off the bottom left-hand corner of the gel helping orientation

7. For partial fragmentation of large DNA fragments (>10kb) prior to the transfer, leave on UV light box for 10 minutes.
   (Alternatively, partial fragmentation can be achieved by acid depurination: Invert gel, place into 0.25M HCl for 10 min, rinse briefly in dH20.)

3. Southern transfer to Nylon membrane - overnight

1. Just prior to the transfer, float the Nylon membrane (S&S Maximum Strength Nytran) on top of distilled water to wet thoroughly. Let stand in water or transfer buffer until use. Cut off lower left-hand corner for orientation.

2. Denaturation: Soak gel in at least two gel volumes (500 ml) of 1.5 M NaCl / 0.5N NaOH for 2 x 15 minutes.
   If gel floats to the surface of the liquid, weigh it down with several pasteur pipets.

3. Set up transfer in large electrophoresis tray: Fill transfer buffer (10 x SSC) into the two side-trays. Place the following items (gel-sized, saturated with transfer buffer) on the middle support. All air-bubbles should be carefully smoothed-out with a glass pipet or gloved fingertip in each layer seperately.
   - Wick (3mm paper); this should be the same width as your gel and long enough to drape well into the transfer solution.
   - 3 pieces of Whatman 3MM paper
   - Gel (upside down)
   - Nylon membrane (Always handle with clean gloves and blunt-ended forceps. Do not adjust the Nylon membrane once it is placed on the gel)
   - 3 pieces of Whatman 3MM paper
   - dry 5 cm stack of paper towels
   - glass plate with weight (ca. 500 g)
Surround gel with plastic wrap or parafilm to prevent the paper towels to come in contact with the wet paper below the gel. Prevent evaporation of the transfer solution by sealing with plastic wrap or parafilm on either end of the tray. Let transfer overnight.

4. Next day, take off blotting material and mark the position of the wells with very-soft-lead pencil. Soak the gel in 5 x SSC for 5 minutes to remove bits of gel or particles from membrane.

5. Place wet membrane on a paper towel of equal size. Immobilize the DNA by UV-crosslinking (100 mJ/cm², push "optimal cross-linking"). Dry membrane thoroughly at room temperature before placing in prehybridization solution.

4. Prehybridization (2 hours)

Place membrane in hybridization bottle with 20 ml (min. 100 g cm⁻²) of prehybridization buffer (use nylon mesh as a spacer before rolling). Incubate in hybridization oven at 42°C for 1-2 hours. Alternatively, without formamide, incubate at 68°C. Some workers leave out Denhardt's in hybridization and /or prehybridization solution.

Prehybridization Buffer (20 ml)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Conc.</th>
<th>Volume</th>
<th>Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>deionized formamide</td>
<td>50%</td>
<td>10 ml</td>
<td></td>
</tr>
<tr>
<td>20 x SSPE stock solution</td>
<td>6 x</td>
<td>6 ml</td>
<td></td>
</tr>
<tr>
<td>50 x Denhardt's reagent</td>
<td>5 x</td>
<td>2 ml</td>
<td></td>
</tr>
<tr>
<td>20% SDS stock solution</td>
<td>0.5%</td>
<td>5001A</td>
<td></td>
</tr>
<tr>
<td>Salmon Testes DNA (Sigma D-9156), 10.4 mg/ml, boil 5 min. before use</td>
<td>100 jig/ml</td>
<td>200 gAl</td>
<td></td>
</tr>
<tr>
<td>optional: dextrane sulfate</td>
<td>10%</td>
<td>2 g</td>
<td></td>
</tr>
<tr>
<td>dH₂O ad 20 ml</td>
<td></td>
<td>13ml</td>
<td></td>
</tr>
</tbody>
</table>
Radioactive labeling of probe (1 hour)

1. Isolate 1-10 tg target cDNA from vector with restriction digestion, agarose gel electrophoresis, gel extraction of appropriate band. Resuspend in 10 mM Tris.

2. Label 25-50 ng cDNA-probe with $^{32}$P using the Prime It® II Random Primer Labeling Kit

**Supplies & Equipment:**

- Prime It® II Random Primer Labeling Kit (Stratagene #300385)
- Waterbath 37°C
- Waterbath 100°C
- Microfuge

**Reagents:**

- Random 9-mer primers or specific primers from PCR (10 gM) or sequencing reaction (10 ng/gl)
- 5 x dATP buffer (for use with [$a^{-32}$]dATP) containing dGTP, dCTP, dTTP, or 5 x *dCTP buffer (for use with [$a^{-32}$]dCTP) containing dGTP, dATP, dTTP
- [$a^{-32}$]dATP or [$a^{-32}$]dCTP - not provided by the kit
- Exo(-) Klenow fragment of DNA polymerase I (5 U/gl)
- Stop mix (0.5M EDTA, pH 8.0)

**Procedure:**

1. Add to the bottom of a microfuge tube:
   - 25-50 ng (1-23 gl) of template DNA
   - 0-23 gl dH20
   - 10il primer (either random oligonucleotide primers from kit or specific primers (diluted: 10 11,M or 10 ng / gl) from PCR or sequencing)

2. Boil reaction tube in water bath for 5 minutes, then centrifuge briefly at room temperature to collect condensed liquid from cap of tube.

3. Add the following reagents to the reaction tube:
   - 10il of 5 x primer buffer (either 5 x *dATP or 5 x *dCTP buffer)
   - 5 pa of labeled nucleotide (either [$a^{-32}$]dATP) or [$a^{-32}$]dCTP)
1. 1 µl Exo(-) Klenow enzyme (5 U/µl)

Mix the reaction components thoroughly with pipet tip.

4. Incubate the reaction at 37 - 40°C for 2 - 10 minutes.

5. Stop the reaction by adding 2 µl of stop mix.

6. Purify probe with either one of the following kits:
   - NucTrap® Probe Purification Push Columns (Stratagene #400701), for DNA- or RNA-probes 17 bp - 50 kb
   - Qiaquick PCR Purification Kit (Qiagen #2804) for DNA-probes 100 bp - 10 kb

7. Count radioactivity of probe. Take 1 µl probe, dilute with appropriate amount of scintillation fluid, use same amount of scintillation fluid as negative control. Count. You should have 1-5 x 10^6 cpm per ml hybridization reaction (20-100 x 10^6 cpm).

8. Denature probe by boiling 5 min. and chill on ice immediately before use.

6. Hybridization (overnight)

1. Add denatured probe to prehybridization solution.

(If Denhardes was in the prehybridization solution and should not be in the hybridization solution, prepare fresh prehybridization solution without Denhardes for hybridization.) Incubate overnight (12-24 hours) at 42°C (or at 68°C without formamide) in hybridization oven. The hybridization solution can be reused.

(Pour into a 50 ml plastic disposable centrifuge tube. The probe is good for a couple of weeks, and must be boiled 5 min and chilled on ice before reuse.)

7. Wash (2 hours)

1. Soak membrane twice for at least 15 minutes each with 100 ml of 7 x SSPE / 0.1-0.5% SDS at room temperature. Perform this step in hybridization oven rotating at very low speed.

(for 500 ml: 175 ml 20 x SSPE, 2.5 - 12.5 ml 20% SDS, 322.5 - 312.5 ml dH20)

2. Then soak the membrane twice for at least 15 minutes each in 100 ml of 1 x SSPE / 0.5-1% SDS at 37°C. Perform this step in hybridization oven rotating at very low speed. (for 500 ml: 25 ml 20 x SSPE, 12.5 - 25 ml 20% SDS, 462.5 - 450 ml dH20)

3. Finally soak for 1 hour in 100 ml of 0.1 x SSPE / 1% SDS at 68°C.

(for 500 ml: 2.5 ml 20 x SSPE, 25 ml 20% SDS, 472.5 ml dH20)

For detection of poorly matched hybrids use a lower temperature in the last wash. Check
radioactivity with Geiger-counter. Do not expect a signal from single copy genes.

8. Detection (1 to 6 days)

After washing, blot the membrane with filter paper (Whatman 3MM or S&S GB003) to remove most of the excess moisture. Wrap moist blots in plastic wrap prior to autoradiography. Expose to X-ray film with an intensifying screen at -80°C for 1 to 6 days.

9. Stripping / Reprobing (1 hour)

Do not allow blot to dry at any time prior to removing the probe, as drying will cause the probe to bind irreversibly. To remove probe from blot for reuse, incubate one of the following solutions:

- Wash blots in 50% formamide, 6 x SSPE for 30 minutes at 65°C.
  (For 50 ml: 25 ml deionized formamide, 15 ml 20 x SSPE, 10 ml dH2O)
Rinse in 2 x SSPE, remove excess liquid by dabbing the blot on 3MM paper. Wrap in Saran wrap and store. Expose overnight to check for the absence of radioactivity. After stripping probe, start again with prehybridizing step.
Aim:

To determine the interaction between DNA and protein using electrophoretic mobility shift assay.

Materials required:

- Biotin 3' or 5' end-labeled DNA target. Use existing end-biotinylated DNA targets or prepare them using a biotin end-labeling kit (see Related Thermo Scientific Products). Do not use probes with internal biotin labels (i.e., targets biotinylated at sites other than the 3' or 5' end, such results from random prime labeling methods) because the internal labels may inhibit binding of the DNA binding protein.
- Positively charged nylon membrane (see Related Thermo Scientific Products)
- 5X TBE (450mM Tris, 450mM boric acid, 10mM EDTA, pH 8.3)
- X-ray film (see Related Thermo Scientific Products) or CCD camera
- UV lamp or crosslinking device equipped with 254nm bulbs or 312nm transilluminator
- Electrophoresis apparatus
- Electroblotter or capillary transfer apparatus
- High-quality blotting paper
- Circulating waterbath
- Plastic forceps
- Polyacrylamide gel in 0.5X TBE
### Procedure

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Contents of Reaction</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>Biotin-EBNA Control DNA</td>
<td>No protein extract for DNA to bind; therefore, no shift is observed. Establishes the position of an unshifted probe band.</td>
</tr>
<tr>
<td>#2</td>
<td>Biotin-EBNA Control DNA + EBNA extract</td>
<td>Contains sufficient target protein to effect binding and shift of the Biotin-EBNA DNA. Shift detected by comparison to band position in #1.</td>
</tr>
<tr>
<td>#3</td>
<td>Biotin-EBNA Control DNA + EBNA extract + 200-fold molar excess of unlabeled EBNA DNA</td>
<td>Demonstrates that the signal shift observed in #2 can be prevented by competition from excess non-labeled DNA, i.e., the shift results from specific protein:DNA interaction.</td>
</tr>
</tbody>
</table>

#### A. Plan Binding Reactions
Binding reactions for Control EBNA System

<table>
<thead>
<tr>
<th>Component</th>
<th>Final Amount</th>
<th>Control Reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>#1</td>
<td>#2</td>
</tr>
<tr>
<td>Ultrapure Water</td>
<td>12AL</td>
<td>11 ILL</td>
</tr>
<tr>
<td>10X Binding Buffer (20148A)</td>
<td>IX</td>
<td>2pL</td>
</tr>
<tr>
<td>50% Glycerol (20148F)</td>
<td>2.5%</td>
<td>1 tiL</td>
</tr>
<tr>
<td>100mM MgCl₂ (201481)</td>
<td>5mM</td>
<td>1 pL</td>
</tr>
<tr>
<td>1 pg/pL Poly (d1’dC) (20148E)</td>
<td>50 ng/pL</td>
<td>1 pL</td>
</tr>
<tr>
<td>1% NP-40 (20148G)</td>
<td>0.05%</td>
<td>1 A</td>
</tr>
<tr>
<td>Unlabeled EBNA DNA (20148C)</td>
<td>4 pmol</td>
<td></td>
</tr>
<tr>
<td>EBNA Extract (20148D)</td>
<td>1 Unit</td>
<td></td>
</tr>
<tr>
<td>Biotin—EBNA Control DNA</td>
<td>20 fmol</td>
<td>2pL</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Binding reactions for the Test System

<table>
<thead>
<tr>
<th>Component</th>
<th>Final Amount</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>#1</td>
<td>#2</td>
</tr>
<tr>
<td>Ultrapure Water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10X Binding Buffer (20148A)</td>
<td>1X</td>
<td>2111,</td>
</tr>
<tr>
<td>Ittg/pL Poly (c1’c1C) (20148E)</td>
<td>50 ng/pL</td>
<td>1pL</td>
</tr>
<tr>
<td>Optional: 50% Glycerol (20148F)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Optional: 1% NP-40 (20148G)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Optional: 1M KCl (20148H)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Optional:</strong> 100mM MgCl₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Optional: 200mM EDTA (201481)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unlabeled Target DNA</td>
<td>4 pmol</td>
<td>—</td>
</tr>
<tr>
<td>Protein Extract (e.g., 2-3 pi NE- system)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biotin End-Labeled Target DNA</td>
<td>20 fmol</td>
<td></td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td></td>
<td>20pL</td>
</tr>
</tbody>
</table>
B. Prepare and Pre-Run Gel

1. Prepare a native polyacrylamide gel in 0.5X TBE or use a pre-cast DNA retardation gel. The appropriate polyacrylamide percent depends on the size of the target DNA and the binding protein. Most systems use a 4-6% polyacrylamide gel in 0.5X TBE.

2. Place the gel in the electrophoresis unit, and clamp it to obtain a seal. Fill the inner chamber with 0.5X TBE to a height several millimeters above the top of the wells. Fill the outside of the tank with 0.5X TBE to just above the bottom of the wells, which reduces heat during electrophoresis. Flush wells and pre-electrophorese the gel for 30-60 minutes. Apply 100 V for an 8 x 8 x 0.1cm gel.

C. Prepare and Perform Binding Reactions

1. Thaw all binding reaction components, EBNA Control System components and Test System samples, and place them on ice. Avoid excessive warming of DNA probes. Do not thaw the EBNA Extract until immediately before use.

2. Prepare complete sets of 20 gl binding reactions for the Control EBNA System and/or the Test System according to Procedure Section A, Tables 2 and 3.

3. Incubate binding reactions at room temperature for 20 minutes.

4. Add 5 IA of 5X Loading Buffer to each 20 gl binding reaction, pipetting up and down several times to mix. Do not vortex or mix vigorously.

D. Electrophorese Binding Reactions

1. Switch off current to the electrophoresis gel.

2. Flush the wells and then load 204 of each sample onto the polyacrylamide gel.

3. Switch on current (set to 100 V for 8 x 8 x 0.1cm gel) and electrophorese samples until the bromophenol blue dye has migrated approximately 2/3 to 3/4 down the length of the gel. The free biotin-EBNA Control DNA duplex migrates just behind the bromophenol blue in a 6% polyacrylamide gel.

E. Electrophoretic Transfer of Binding Reactions to Nylon Membrane

1. Soak nylon membrane in 0.5X TBE for at least 10 minutes.

2. Sandwich the gel and nylon membrane in a clean electrophoretic transfer unit according the manufacturer's instructions. Use 0.5X TBE cooled to —10°C with a circulating waterbath. Use very clean forceps and powder-free gloves, and handle the membrane only at the corners.
3. Transfer at 380 mA (-100V) for 30 minutes. Typical transfer times are 30-60 minutes at 380mA using a standard tank transfer apparatus for mini gels (8 x 8 x 0.1cm).

4. When the transfer is complete, place the membrane with the bromophenol blue side up on a dry paper towel. (There should be no dye remaining in the gel.) Allow buffer on the membrane surface to absorb into the membrane. This will only take a minute. Do not let the membrane dry. Immediately proceed to Section F.

F. Crosslink Transferred DNA to Membrane

Three options are available for crosslinking:

- **Option 1**: Crosslink at 120 mJ/cm² using a commercial UV-light crosslinking instrument equipped with 254nm bulbs (45-60 second exposure using the auto crosslink function).

- **Option 2**: Crosslink at a distance of approximately 0.5 cm from the membrane for 5-10 minutes with a hand-held UV lamp equipped with 254nm bulbs.

- **Option 3**: Crosslink for 10-15 minutes with the membrane face down on a transilluminator equipped with 312nm bulbs.

After the membrane is crosslinked, proceed directly to Section G. Alternatively, the membrane may be stored dry at room temperature for several days. Do not allow the membrane to get wet again until ready to proceed with detection.

G. Detect Biotin-labeled DNA by Chemiluminescence

The recommended volumes are for an 8 x 10cm membrane. If larger gels are used, adjust volumes in Section G accordingly. Perform all blocking and detection incubations in clean trays or in plastic weigh boats on an orbital shaker.

1. Gently warm the Blocking Buffer and the 4X Wash Buffer to 37-50°C in a water bath until all particulate is dissolved. These buffers may be used between room temperature and 50°C as long as all particulate remains in solution. The Substrate Equilibration Buffer may be used between 4°C and room temperature.

2. To block the membrane add 20mL of Blocking Buffer and incubate for 15 minutes with gentle shaking.

3. Prepare conjugate/blocking buffer solution by adding 66.711L Stabilized Streptavidin-
Horseradish Peroxidase Conjugate to 20mL Blocking Buffer (1:300 dilution).

4. Decant blocking buffer from the membrane and replace it with the conjugate/blocking solution. Incubate membrane in the conjugate/blocking buffer solution for 15 minutes with gentle shaking.

5. Prepare 1X wash solution by adding 40mL of 4X Wash Buffer to 120mL ultrapure water.

6. Transfer membrane to a new container and rinse it briefly with 20mL of 1X wash solution.

7. Wash membrane four times for 5 minutes each in 20mL of 1X wash solution with gentle shaking.

8. Transfer membrane to a new container and add 30mL of Substrate Equilibration Buffer. Incubate membrane for 5 minutes with gentle shaking.

9. Prepare Substrate Working Solution by adding 6mL Luminol/Enhancer Solution to 6mL Stable Peroxide Solution.

10. Remove membrane from the Substrate Equilibration Buffer, carefully blotting an edge of the membrane on a paper towel to remove excess buffer. Place membrane in a clean container or onto a clean sheet of plastic wrap placed on a flat surface.

11. Pour the Substrate Working Solution onto the membrane so that it completely covers the surface. Alternatively, the membrane may be placed DNA side down onto a puddle of the Working Solution. Incubate membrane in the substrate solution for 5 minutes without shaking.

12. Remove membrane from the Working Solution and blot an edge of the membrane on a paper towel for 2-5 seconds to remove excess buffer. Do not allow the membrane to become dry.

13. Wrap the moist membrane in plastic wrap, avoiding bubbles and wrinkles.

14. Expose membrane to an appropriately equipped CCD camera, or place the membrane in a film cassette and expose to X-ray film for 2-5 minutes. Develop the film according to manufacturer's instructions. Exposure time may be adjusted to obtain the desired signal.

**AIM: To determine the interaction between two known proteins by using Yeast two hybrid systems**

**Strategy:**
Generating Bait and Prey Piasmids

Tailor-made entry clone or Ultimate" ORF

or

Retransformation assay

– for Pro Quest

Screen

st- Reverse Two-Hybrid Screen

LR recombination reaction

LR recombination reaction

with pOEST'32

with pOEST''22

Bait

Plasmid Prey Pismid

Transform yeast strain MaV203

Testing Specific 2-Hybrid Interaction

Test Reporter Genes

14183, UR43 and As a
**Generation of bait and prey plasmids**

The gene of interest for the bait is cloned into pDEST\textsuperscript{Tm}32, and the gene of interest for the prey is cloned into pDEST\textsuperscript{Tm}22. Gateway Technology is a universal cloning method that takes advantage of the site-specific recombination properties of bacteriophage lambda to provide a rapid and highly efficient way to move DNA sequence of interest into multiple vector systems.

**Steps**

To construct your bait or prey plasmids, perform the following steps:
1. Identify or generate a suitable entry clone.
2. Perform an LR recombination reaction between entry clone and pDEST\textsuperscript{Tm}32 or pDEST\textsuperscript{Tm}22.
3. Transform competent cells.
4. Select the proper expression clone
Tailor-made entry clone  Ultimate" ORF

LR recombination reaction with pDEST"32  LR recombination reaction with pDEST"22

Transform Competent Cells  Transform Competent Cells

pEXP"12 expression clone  Select pEXP"22 expression clone

Bait Plasmid  Prey Plasmid

Materials required:

- Purified plasmid DNA of your entry clone for the bait and/or your entry clone for the prey (50-150 ng in TE, pH 8.0)
- pDEST™32 and/or pDEST™22 (both 150 ng/pd in TE, pH 8.0)
- LR Clonase™ II enzyme mix (keep at -20°C until immediately before use)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- 2 pg/ill Proteinase K solution (supplied with the LR Clonase\textsuperscript{Tm} II enzyme mix; thaw and keep on ice until use)
- Positive Recombination Control pENTR-gus, if desired

Procedure

1. Add the following components to 1.5 ml microcentrifuge tubes at room temperature and mix.

<table>
<thead>
<tr>
<th>Component</th>
<th>Forming Bait Plasmid</th>
<th>Forming Prey Plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sample</td>
<td>Negative Control</td>
</tr>
<tr>
<td>Entry clone for bait (50-150 ng/reaction)</td>
<td>1-7 pl</td>
<td>1-7111</td>
</tr>
<tr>
<td>Entry clone for prey (50-150 ng/reaction)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>pDES1\textsuperscript{32} (150 ng/pl)</td>
<td>1 pl</td>
<td>1 pl</td>
</tr>
<tr>
<td>pDEST\textsuperscript{22} (150 ng/p1)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>pENTIr-gus (50 ng/p1)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>TE Buffer, pH 8.0</td>
<td>to 8 pl</td>
<td>to 10 pl</td>
</tr>
</tbody>
</table>

2. Remove the LR Clonase\textsuperscript{Tm} II enzyme mix from -20°C and thaw on ice (— 2 minutes).
3. Vortex the LR Clonase\textsuperscript{Tm} II enzyme mix briefly twice (2 seconds each time).
4. Add 2 pl of LR Clonase\textsuperscript{Tm} II enzyme mix to the sample vial. Do not add LR Clonase\textsuperscript{Tm} H enzyme mix to the negative control vial. Mix well by vortexing briefly twice (2 seconds each time).
5. Incubate reactions at 25°C for 1 hour.
6. Add 1 pl Proteinase K solution to each reaction. Incubate 10 minutes at 37°C.
7. Proceed to transform (with 1 pl) a suitable E. coli host and select for expression clones.
8. Transformed colonies plated on 10 pg/m1 gentamicin (for bait plasmids) or 100 pg/m1 ampicillin (for prey plasmids).
9. Isolate the plasmid form E. coil.

Testing Specific Two-Hybrid Interaction

- Transform your bait and prey plasmid into MaV203 cells, and test activation of the three reporter gene.
Materials required:

- YPAD
- 1X TE
- 1X LiAc (100mM Lithium Acetate/0.5X TE)
- Denatured sheared salmon sperm DNA.
- Plasmid DNA to be transformed
- 1X LiAc/40% PEG-3350/1X TE
- DMSO. For best results, use fresh DMSO from an unopened bottle. DMSO that has been stored at -20°C also works well.
- SC-Leu-Trp plates, for selection of yeast cells transformed with both the bait and prey plasmid, or LEU2 and TRP1 plasmid of the controls.
- Bait plasmid
- Prey plasmid known to interact with bait in yeast two-hybrid (if available)

pDEST\textsuperscript{Tm}32, pDEST\textsuperscript{Tm}22, pEXPTm32/Krevl, pEXPTm22/RalGDS-wt, pEXP\textsuperscript{Tm}22/RalGDS-m1, pEXPTm22/RalGDS-m2.

Yeast competent cell preparation:

1. Inoculate 10 ml of YPAD with a colony of MaV203 and shake overnight at 30°C.
2. Determine the 0D600 of your overnight culture. Dilute culture to an 0D600 of 0.4 in 50 ml of YPAD and grow an additional 2-4 hours.
3. Pellet the cells at 2500 rpm and resuspend the pellet in 40 ml 1X TE.
4. Pellet the cells at 2500 rpm and resuspend pellet in 2 ml of 1X LiAc/0.5X TE.
5. Incubate the cells at room temperature for 10 minutes
6. Proceed immediately to transform the competent MaV203 cells
Transformation of Competent MaV203 Cells

1. For each transformation, mix together 1 gg plasmid DNA and 100 gg denatured sheared salmon sperm DNA with 100 gl of the yeast suspension from Step 5, above.
   a. Add 700 gl of 1X LiAc/40% PEG-3350/1X TE and mix well.
2. Incubate solution at 30°C for 30 minutes.
3. Add 88 gl DMSO, mix well, and heat shock at 42°C for 7 minutes.
4. Centrifuge in a micro centrifuge for 10 seconds and remove supernatant.
5. Resuspend the cell pellet in 1 ml 1X TE and re-pellet.
6. Resuspend the pellet in 50-100 p1 TE and plate on a selective plate.
Characterization of Transformants:

MaV203 cells that contain bait and prey proteins that strongly interact will induce all three reporter genes present in this system (HIS3, URA3, lacZ). Identify these colonies by a series of patching and replica plating steps onto the selection/screen plates.

Mansformants on SC-Lau-Tip

to a single 9C-Leu-lip plate patch:
Four isolated colonies/pDEST*32 + prow to test
Four isolated coked’s/sat + prey to test
Aso patches of Controls 2-8
Incubate 30°C, h

Note: you can also perform the quantitative ONPG or CPRG assay, described in the appendix
Plate preparation

<table>
<thead>
<tr>
<th>Test</th>
<th>HIS induction</th>
<th>URA3 induction</th>
<th>LIRA3 induction</th>
<th>rt-Galactosidase induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay</td>
<td>His auxotrophy</td>
<td>5F0A sensitivity</td>
<td>Uracil am:atrophy</td>
<td>X-gal assay</td>
</tr>
<tr>
<td>Plates used</td>
<td>SC-Leu-Trp-His+3AT</td>
<td>SC-Leu-Trp+SF0A 0.2% 5F0A</td>
<td>SC-Leu-Trp-Ura</td>
<td>YPAD</td>
</tr>
<tr>
<td>Concentrations</td>
<td>10 m114 3AT</td>
<td>25 inhil 3AT</td>
<td>No Uradl</td>
<td>Not applicable</td>
</tr>
<tr>
<td></td>
<td>50 mM 3AT</td>
<td>100 mM 3AT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Materials Needed

- Fresh plates:
  - SC-Leu-Trp plates, to grow yeast containing the 2 plasmids to be tested
  - YPAD plates, for X-gal assays to test lacZ induction
  - SC-Leu-Trp-Ura plates to test URA3 induction
  - SC-Leu-Trp-His+3AT plates, to test HIS3 induction
  - SC-Leu-Trp+SF0A plates, to select yeast cells that do not induce URA3
- nitrocellulose or nylon membrane
- 30°C incubator
- Autoclaved velvets for replica plating/cleaning
- X-gal (5-bromo-5-chloro-3-indolyl-β-d-galactoside)
- N,N-dimethyl formamide (DMF)
- Z buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, pH 7.0)
- 2-mercaptopethanol
- 125-mm Whatman 541 filter papers
- 15-cm petri dishes
- Forceps
- Liquid nitrogen

Generating master plate

1. Using an autoclaved toothpick or loop, patch onto a single SC-Leu-Trp plate the following:
   - Two isolated colonies of yeast controls 2-8 (see table, page 25)
   - Four isolated colonies of transformants containing pDEST™32 and prey for an interaction you want to test (up to two clones can be analyzed per plate)
• Four isolated colonies of transformants containing bait plasmid and prey for an interaction you want to test (up to two clones can be analyzed per plate)

2. If more than 2 preys need to be tested, use additional plates. On each plate include the yeast controls 2-8. Put transformants containing the same prey and pDEST\textsuperscript{Tm}32 or bait plasmid on one plate.

3. Incubate plates for 18 hours at 30°C.

**Testing reporter genes**

1. Replica plate onto the following plates, in the order listed. Be sure to make asymmetric marks on the plates and membrane to allow for realignment with the master plate. Replica clean where indicated.
   - YPAD containing a nitrocellulose or nylon membrane for an X-gal Assay
   - SC-Leu-Trp-Ura
   - SC-Leu-Trp-His+3AT; replica clean
   - SC-Leu-Trp+5F0A; replica clean

2. Incubate all plates for —24 hours at 30°C.

3. After 18 to 24 hours incubation of the YPAD plates containing a membrane, perform an X-gal Assay on the membrane as described below.

4. After incubation of the selection plates for 24 h, replica clean the following plates:
   - SC-Leu-Trp-Ura
   - SC-Leu-Trp+5F0A
   - SC-Leu-Trp-His+3AT

5. Incubate for 2 additional days at 30°C.

6. Compare the phenotypes of the transformants to yeast control and to the phenotype exhibited in the original screen. Weak phenotypic differences should be considered. A particular prey that activates target genes in the presence of DEST\textsuperscript{Tm}32 is likely a false positive.

**X-gal Assay**

1. For each membrane, dissolve 10 mg X-gal in 100 ill DMF. Combine 100 111 X-gal in DMF, 60 pi 2-mercaptoethanol and 10 ml Z buffer.

2. Stack two round 125-mm Whatman 541 filter papers in a 15-cm petri dish. Saturate with —8 ml of the X-gal solution. Remove any air bubbles.

3. Using forceps, carefully remove the membrane from the surface of the YPAD plate. Completely immerse the membrane in liquid nitrogen for 20-30 seconds. Place the frozen membrane on top of the soaked Whatman filters colony side up. Remove any air bubbles. Tip the plates slightly and remove excess buffer.

4. Cover the plates and incubate at 37°C. Tip the plates at a slight angle so excess X-gal solution does not accumulate on the membrane. Monitor the appearance of blue color over a 24-h period. Score final results at 24 hours.
• Add 500 of the luciferase S30 control reaction to the first tube, mix and pipet 501.11 from first tube to second tube. Mix, and continue the series of twofold dilutions in the remaining two tubes.
6. Place 10-20111 of each dilution into a microcentrifuge tube or the well of a white 96-well plate.
7. Measure luminescence by luminometry, scintillation counting, photography or visual detection (see Sections 5.B, 5.0 and 5.D of TB092).
Polysome profiling by sucrose gradient

Aim of the experiment - to study the effect of inhibitor like cyclohexamide, edeine A on protein synthesis by analysis polysome profiling obtained by sucrose gradient.

Material required —
Sucrose (68.46%)
Magnesium Chloride
Potassim Chloride (KCl)
DTT
Protease inhibitor
Clean Ultracentrifuge tubes 6

Buffers composition-

Buffer B (10X)- composition

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Concentration needed</th>
<th>Stock conc.</th>
<th>Volume of stock For 150m1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris pH 7.5</td>
<td>200mM</td>
<td>1M</td>
<td>30m1</td>
</tr>
<tr>
<td>KC1</td>
<td>500mM</td>
<td>2M</td>
<td>37.5m1</td>
</tr>
<tr>
<td>MgC12</td>
<td>100mM</td>
<td>2M</td>
<td>7.5m1</td>
</tr>
<tr>
<td>Distilled water</td>
<td></td>
<td></td>
<td>Make 150m1 volume</td>
</tr>
</tbody>
</table>

Filter the buffer with 0.45 gm filter and store in clean bottle.

Sucrose solution for gradient

<table>
<thead>
<tr>
<th>R Reagents</th>
<th>5%</th>
<th>45%</th>
<th>15%</th>
</tr>
</thead>
<tbody>
<tr>
<td>StStock sucrose Solution</td>
<td>3.65ml</td>
<td>32.86ml</td>
<td>10.95ml</td>
</tr>
<tr>
<td>10X stock buffer B</td>
<td>5m1</td>
<td>5m1</td>
<td>5m1</td>
</tr>
<tr>
<td>Distilled water</td>
<td>41m1</td>
<td>12.14m1</td>
<td>34m1</td>
</tr>
<tr>
<td>DTT (1M)</td>
<td>50g1</td>
<td>501.t1</td>
<td>50g1</td>
</tr>
<tr>
<td>Total volume</td>
<td>50m1</td>
<td>50m1</td>
<td>50m1</td>
</tr>
</tbody>
</table>

Mix it well in rotator for 10 min
Sample preparation-
1. Harvest the yeast culture (250m1) at OD 1.5-1.7 and wash the cell twice with distilled water.
2. Resuspend the pellet in 0.5m1 of breaking buffer B +protease inhibitor
3. Break the cell by adding glass beads and vortex it for 20 sec (3 times) in between 1 min on ice.
4. Extract the supernatant by centrifuge at 14000 rpm/4°C/10minute.
5. Collect in fresh tube.
6. Take the absorbance of the crude extract at wavelength 260nm.

GRADIENT FORMATION
1. Clean all the six tube used for ribosome profiling (tube should fit in SW41 rotor
2. First add 5% of sucrose solution till marked tube with the help of syringe.
3. Then add 45% of sucrose solution to the bottom of the tube with the help of syringe. So the high density sucrose solution displaces the low density and gradient formation occurs.
4. Then level the gradient formation platform, verify its level.
5. Place the six tubes with stand in the exact middle of the platform.
6. Program the machine for 5-45% gradient formation
   a). Angle - 86.6
   b). Time 10 min
   c). Rotation speed - 17
7. After this carefully kept it at 4°C for overnight or for 2-3 hours.

SAMPLE LOADING ON THE GRADIENT TUBE
1. Measure the weight of all the tubes (along with cap) and balance the opposite tubes (like 1-4, 2-5 and 3-6 tube numbers).
2. Load the protein sample by overlaying on to the top of the tube.
3. Again measure the weight and check all tubes are balance.
4. Close the tubes with cap.

ULTRACENTRIFUGATION
1. After balancing all 6 tubes, place them in rotor (in cold condition) by clinging to the hook.
2. In ultracentrifuge machine, first set the 4°C temperature and press vacuum to maintain inside temperature 4°C.
3. Place the rotor properly
4. Set the condition - 39000rpm/2hrs 30 min/4°C.
5. Again press vacuum, when vacuum is created from 750 to 200. Press "start" button.

OBTAINING POLYSOME PROFILE IN FRACTIONATION COLLECTOR
After completion of ultracentrifuge, release the pressure of vacuum and carefully take out the rotor and then tubes.
Switch on fractionation collector; follow the instruction display on machine.
Then set speed of piston that is 0.09 and set UV monitor at 2.0
Wash the machine and then fix the tube in fractionation collector and start the fractionator at sample rate 2 in channel 1.
Whiling the piston is going through tube, observe polysome profile on monitor. Save the data.
Similar repeat for other tube if necessary.
SOUTHERN HYBRIDIZATION (added 31.3.14)

**Introduction**

It is one of the standard techniques to detect DNA of the gene of interest in a given samples. It involves the separation of digested DNA fragments on agarose gel followed by transfer to nylon membrane on which the DNA is often cross linked by UV radiation. DNA of interest is hybridized to a probe, which is complementary to the DNA and detected by either radioactive or chemiluminescent manner. This technique is invented by British biologist Edwin Southern in 1975.

**Material and reagents**

- pPRS313-SU15 (A619), pYcPlac33 (A309), and pYcPlac33-SU15 (A703).
- Eco RI-HF
- Sal I-HF
- 10X buffer
- milliQ- 5lit
- 1X TAE-1 lit
- Agarose -0.8 g
- Etbr (10mg/ml)
- DNA gel apparatus
- Falcon tubes-15 and 50 ml (10 each)
- Magnetic stirrer-2
- Millipore blotting apparatus
- Random biotin labeling kit
- North to south hybridization and detection kit
- Rocker
- Hybridization oven
- Roller bottle small-2
- Tissue roll-1
- Tips-each box one
- Pipettes- 2 sets
- Staining tray

**Material and reagents**

- **Denaturing solution (1L)**
  - 1.5 M NaCl-87.55 g
  - 0.5 M NaOH-20 g

- **Neutralizing solution (1L)**
  - 3 M NaCl-175.5 g
  - 6.7 g Tris base
  - 70.2 g Tris-Cl
  
  \[
  0.5 \text{ M Tris}
  \]

- **SSC (20X)-1L**
  - 3 M NaCl-175 g
  - 0.3 M Na3 citrate.2H2O-88 g
  - Adjust the pH to 7.0 with 1M Hcl

- Nylon membrane
- 100 % ethanol (1 ml)
- 70 % ethanol (1ml)
- Tris-cl (pH-7.5)-0.5 ml

**Procedure**

**Restriction Digestion**

- Set up the following reaction

<table>
<thead>
<tr>
<th>Components</th>
<th>pPRS313-SUI5 (A619) rxn-1</th>
<th>pYcPlac33 (A309) rxn-2</th>
<th>pYcPlac33-SUI5 rxn-3(A703)</th>
<th>pPRS313-SUI5 (A619) rxn-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>MilliQ</td>
<td>10 µl</td>
<td>10 µl</td>
<td>7 µl</td>
<td>7 µl</td>
</tr>
<tr>
<td></td>
<td>1.5 µl</td>
<td>1.5 µl</td>
<td>1.5 µl</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>--------------------------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td><strong>10X buffer 4</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Plasmid DNA (300 ng/µl)</strong></td>
<td>3 µl</td>
<td>3 µl</td>
<td>3 µl</td>
<td>6 µl</td>
</tr>
<tr>
<td><strong>Eco RI-HF (20U/µl)</strong></td>
<td>0.25 µl</td>
<td>0.25 µl</td>
<td>0.25 µl</td>
<td>0.25 µl</td>
</tr>
<tr>
<td><strong>Sal I-HF (20U/µl)</strong></td>
<td>0.25 µl</td>
<td>0.25 µl</td>
<td>0.25 µl</td>
<td>0.25 µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>15 µl</td>
<td>15 µl</td>
<td>15 µl</td>
<td>15 µl</td>
</tr>
</tbody>
</table>

- Incubate the reaction vial at 37 °C for 2-3 hours.
- Electrophorese the undigested and digested product in 0.8 % agarose gel.
- Note: Gel purify the SUI5 gene form reaction number 4 and proceed to probe preparation.

**Blotting**
- Place the gel in the tray containing 500 ml of 0.2 N HCl rock the tray gently for 10 min.
- Decant the acid and rinse the gel with water (several times) [bromophenol blue will change the color].
- Add 500 ml denaturing solution to tray. After 15 min of rocking decant the solution and add fresh denaturing solution. Rock gently the tray for 15 min [bromophenol blue once aging change back to blue color].
- Decant denaturing solution and add 500 ml of neutralization solution.
- Rock gently for 30 min.
- Measure the gel size (length and width).
- Put the gel into neutralizing solution.
- Take the nylon membrane (cut in such a way that it is 3 mm smaller in both dimension than the gel).
- Wet the filter paper in water for 1 min and place it into the tray of 20X SSC for 5-10 min.
- Cut the whatmann (less than 7 mm in both dimension than nylon membrane-2cmX40cm)
- Wet the whatmann paper wick into the 20X SSC.
- Place one glass plate on the tray and place the wick (let both ends of the wick hang) to touch the 20X SSC.
- Remove the air bubbles between wick and glass plate.
- Take the gel from the neutralization solution and place it on the wick. Remove bubbles between gel and wick.
- Take the nylon membrane form 20 X SSC tray and place it on the gel (make sure that membrane does not overhang the gel).
- Wet stack of whatmann in 20X SSC.
- Place it on the membrane.
Place stack of paper towels.
Place a glass plate and over it weight.
Allow the transfer for overnight.
Mark the membrane

**Biotin random primer labeling**
1. In a microcentrifuge tube, dilute ~100ng of linear DNA for labeling (template DNA) to a final volume of 24μL in nuclease-free water. For the positive control, dilute 0.5μL of control DNA (125ng) to 24μL.
2. Add 10μL of heptanucleotide mix and denature the DNA template by boiling the tube for 5 minutes.
3. Quickly freeze the denatured DNA solution by placing the tube in a dry ice/ethanol bath for 5 minutes.
4. Thaw DNA solution at 4°C and briefly centrifuge to collect liquid in the bottom of the tube. Place the tube on ice.
5. In the order stated, add the following components to the prepared sample solution on ice:
   - 10μL of 5X dNTP mix (dNTP+ biotinylated UTP)
   - 5μL of 10X Reaction Buffer
   - 1μL Klenow fragment
   (Final volume = 50μL)
6. Mix contents by flicking or briefly vortexing the tube. Centrifuge briefly to collect liquid in the bottom of the tube.
7. Incubate labeling reaction for 60 minutes at 37°C.
8. Inactivate the enzyme by adding 2μL of 500mM EDTA, pH 8.0.

**Ethanol Precipitation to Remove Unincorporated Nucleotides**
1. Adjust the reaction to contain 0.5M ammonium acetate. For example, add 5μL of 5M NH₄OAc to a 50μL labeling reaction and mix well.
2. Add 2 volumes of 100% ethanol and mix well. For example, add 110μL to the 55μL volume from step 1.
3. Chill tube at -20°C for 15 minutes.
4. Centrifuge tube at 4°C for 30-60 minutes at maximum speed (> 10,000 X g).
5. Carefully aspirate to remove (and discard) the supernatant.
6. Wash the DNA pellet once by adding ice-cold 70% ethanol and centrifuging for 30-60 minutes at maximum speed. Carefully remove and discard the supernatant.
7. Dissolve the DNA pellet in 100μL of 1X TE or nuclease-free water and store at -20°C. For long-term storage, prepare single-use aliquots and store at -70°C.

**Hybridization:**
1. Equilibrate kit buffers to room temperature before use. If there is a precipitate in any of the kit buffers, heat buffer in a 37oC water bath until precipitate disappears.
2. Heat incubator to appropriate temperature (e.g., 55°C for DNA hybrids or 65°C for RNA:RNA hybrids).
3. Quantify probe using a nano drop
4. Equilibrate the North2South® Hybridization Buffer to room temperature (RT).
5. Place blot in a container such as a 50 ml centrifuge tube and add sufficient Hybridization Buffer to completely cover the blot. Use at least 0.1 ml per cm² of membrane.
6. Seal the container and pre-hybridize the membrane with shaking or rotating for at least 30 minutes. For DNA hybrids incubate at 55°C; for RNA:RNA hybrids incubate at 65°C.
7. While pre-hybridizing, denature the biotinylated DNA probe. Heat DNA probe at 100°C for 10 minutes and place on ice for 5 minutes.
8. After pre-hybridization, add the denatured biotinylated probe. For biotinylated RNA probes, add 3-5 ng of probe per milliliter of hybridization buffer; for biotinylated DNA probes, add ~30 ng of probe per milliliter of hybridization buffer.
9. Incubate overnight with shaking or rotating at 55°C for DNA hybrids or 65°C for RNA:RNA hybrids.

Stringency Washes
10. On the next day, equilibrate the North2South® Hybridization Stringency Wash Buffer (2X) to RT. Once the Wash Buffer is fully in solution, add an equal volume of sterile ultrapure water. The resulting 1X buffer contains 2X SSC/0.1% SDS.
11. Note: If required use other wash conditions to increase or decrease stringency. In general, stringency increases with increasing temperature and decreasing ionic strength.
12. Wash blot three times for 15-20 minutes per wash with agitation. Use 0.2 ml of 1X Stringency Wash Buffer per cm² of membrane and perform washes at 55°C for DNA hybrids or 65°C for RNA:RNA hybrids.

Probe Detection
13. Note: Use clean forceps to handle only the corners of the membrane. For optimal results, rinse forceps with ethanol and allow them to dry between steps.
14. Decant the Stringency Wash Buffer and add sufficient Blocking Buffer to generously cover the membrane. Use at least 0.25 ml/cm² of membrane. Incubate with shaking or rotating for 15 minutes at RT.
15. Determine the amount of Streptavidin-HRP to add to obtain a 1:300 final dilution in the tube with the blot but do not add it yet. Decant a portion of the Blocking Buffer solution from the tube containing the membrane into a separate tube.
16. Add the Streptavidin-HRP conjugate from step 2 to the separated buffer. Add the buffer/Streptavidin-HRP mix to the tube containing the membrane and incubate for 15 minutes at RT with agitation.
17. Note: Performing steps 2 and 3 as described prevents the undiluted conjugate from coming in direct contact with the membrane, which would produce undesirable results.

18. Dilute Wash Buffer (4X) to 1X with sterile ultrapure water. Wash the membrane four times for 5 minutes each with 1X Wash Buffer at RT with agitation. Place membrane into a clean wash container for the next step.

19. Add Substrate Equilibration Buffer to container with the blot. Use 0.25 ml/cm² of membrane. Incubate blot for 5 minutes at RT with agitation.

**Substrate Development**

20. Prepare the Substrate Working Solution by mixing equal volumes of the Luminol/Enhancer Solution and Stable Peroxide Solution. Prepare enough solution to completely cover the membrane (i.e., ~0.1 ml/cm²).

21. Note: The Working Solution is stable for 6 hours at room temperature. Exposure to the sun or any other intense light can harm the Working Solution. For best results keep the Working Solution in an amber bottle and avoid prolonged exposure to any intense light. Short-term exposure to typical laboratory lighting will not harm the Working Solution.

22. Place the moist membrane on a tray or a piece of plastic wrap and cover with the Substrate Working Solution. Incubate for 5 minutes at RT. Make sure the membrane is fully covered with substrate.

23. Drain substrate from the membrane surface and transfer the moist membrane to a sheet protector or wrap in clear plastic wrap. Remove any trapped air bubbles or wrinkles within the plastic wrap and blot any substrate that may have leaked at the edges.

24. Expose blot to film for 1 minute. Adjust the exposure time as needed to obtain the desired signal.

25. Develop the film according to the manufacturer’s instructions.

26. **Note:** For images that have been overexposed, or that show high background and/or speckling, use the Erase-It® Background Eliminator Kit (Product No. 21065).

27. **Note:** For best results, use a new membrane for each hybridization procedure. If stripping and reuse of the membrane is required, test the stripping protocol by incubating the stripped membrane in Substrate Working Solution, placing it in a sheet protector and exposing it to film for at least 30 minutes. If no bands are visible upon developing the film, rinse the membrane with Wash Buffer and re-probe.

**Detection:**

1. Slowly warm the Blocking Buffer and the 4X Wash Buffer to 37-50°C in a water bath until all particulates are dissolved. These buffers may be used between room temperature and 50°C provided all particulate remains in solution. The Substrate Equilibration Buffer may be used between 4°C and room temperature.

2. To block membrane, add 16mL Blocking Buffer and incubate for 15 minutes with gentle shaking.

3. Prepare conjugate/blocking buffer solution by adding 50μL of the Stabilized Streptavidin-Horseradish Peroxidase Conjugate to 16mL Blocking Buffer (1:300 dilution).
**Note:** This conjugate/blocking buffer solution has been optimized for the Chemiluminescent Nucleic Acid Detection Module and should not be modified.

4. Decant blocking buffer from the membrane and add 16mL of the conjugate/blocking solution. Incubate membrane in the conjugate/blocking buffer solution for 15 minutes with gentle shaking.

5. Prepare 1X wash solution by adding 40mL of 4X Wash Buffer to 120mL ultrapure water.

6. Transfer membrane to a new container and rinse briefly with 20mL of 1X wash solution.

7. Wash membrane four times for 5 minutes each in 20mL of 1X wash solution with gentle shaking.

8. Transfer membrane to a new container and add 30mL of Substrate Equilibration Buffer. Incubate membrane for 5 minutes with gentle shaking.

9. Prepare Chemiluminescent Substrate Working Solution by adding 6mL Luminol/Enhancer Solution to 6mL Stable Peroxide Solution.

**Note:** Exposure to the sun or any other intense light can harm the Working Solution. For best results keep the Working Solution in an amber bottle and avoid prolonged exposure to any intense light. Short-term exposure to typical laboratory lighting will not harm the Working Solution.

10. Remove membrane from the Substrate Equilibration Buffer and carefully blot an edge of the membrane on a paper towel to remove excess buffer. Place membrane in a clean container or onto a clean sheet of plastic wrap placed on a flat surface.

11. Pour the Substrate Working Solution onto the membrane so that it completely covers the surface. Alternatively, the membrane may be placed nucleic acid side down onto a puddle of the Working Solution. Incubate membrane in the substrate solution for 5 minutes without shaking.

12. Remove membrane from the Working Solution and blot an edge of the membrane on a paper towel for 2-5 seconds to remove excess buffer. Do not allow the membrane to become dry.

13. Wrap the moist membrane in plastic wrap, avoiding bubbles and wrinkles.

14. Expose membrane to an appropriately equipped CCD camera, or place the membrane in a film cassette and expose to X-ray film for 2-5 minutes. Develop the film according to manufacturer’s instructions. Shorter or longer exposures may be used to obtain the desired signal.
Yeast two hybrid (added 31.3.2014)

Aim
To determine the interaction between two known proteins by using Yeast two hybrid system.

Principle
Yeast two hybrid system is used to detect the protein-protein interaction. Transcription factor is involved in recruiting RNA polymerase to promoter for transcription initiation. They have 2 domains (DNA binding domain (DBD) and activation domain (AD)). During transcription, DBD binds to promoter and AD recruits RNA polymerase to the promoter for transcription initiation of most of the genes. This principle is employed in two hybrid system, where DBD is fused to one protein (bait) and AD is fused to the other protein (prey). Upon interaction of these two protein brings both DBD and AD close to recruit the RNA polymerase to transcribe the reporter genes (LacZ, HIS3, URA3).

Materials required:
- YPD broth (250 ml)-yeast extract-2.5g, peptone-5 g, after autoclave add 2% sterile dextrose solution
- Sterile test tubes (2)
- 10x TE-50 ml
- 1 X TE-50 ml
- 1M LiOAc-50 ml
- 44% PEG-50 ml
- 40% PEG PEG-50 ml
- Control and test plasmids (pDEST32, pDEST™22, pEXP32/Krev1, pEXP22/RalGDS-wt, pEXP22/RalGDS-m1, pEXP22/RalGDS-m2)
- SCD+Ura+His plates-10
- SCD+Ura+50mM 3AT-1
- SCD +His-2
- Denatured sheared salmon sperm DNA-200 µl (10 mg/ml)
- Sterile MilliQ-100 ml

**Strategy**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Bait (TRP1 marker)</th>
<th>Prey (LEU2 marker)</th>
<th>Role</th>
<th>Interaction with pEXP32-Krev1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pDEST32</td>
<td>pDEST22</td>
<td>Negative control</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>pDEST32</td>
<td>pDSET22-wt Ral GDS</td>
<td>Negative control</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>pDEST32-wt Krev1</td>
<td>pDEST22</td>
<td>Negative control</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>pDEST32-wt Krev1</td>
<td>pDSET22-wt Ral GDS</td>
<td>Test</td>
<td>+++</td>
</tr>
<tr>
<td>5</td>
<td>pDEST32-wt Krev1</td>
<td>pDSET22-m1 Ral GDS</td>
<td>Test</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>pDEST32-wt Krev1</td>
<td>pDSET22-m2 Ral GDS</td>
<td>Test</td>
<td>-</td>
</tr>
</tbody>
</table>

Transform your bait and prey plasmid into MaV203 cells, and test activation of the three reporter genes

**Yeast competent cell preparation and transformation:**
1. Inoculate 4 ml of YPD with a colony of MaV203 and shake overnight at 30 °C.
2. Subculture in 20 ml (OD<sub>600</sub> -0.4) YPD and grow an additional 2-4 hours at 30 °C.
3. Pellet the cells at 5000 rpm for 4 min and resuspend the pellet in 1 ml of 1X TE.
4. Pellet the cells at 5000 rpm for 4 min and resuspend pellet in 1 ml of 100 mM LiOAc.
5. Pellet the cells at 5000 rpm for 4 min and resuspend pellet in 0.5 ml of 100 mM LiOAc.
6. Incubate the cells at 30 °C for 30 minutes.
7. 60 µl of comp cells+1.5 µg of plasmid each+10 µl calf thymus DNA+300 µl of 40 % PEG.
8. Incubate the cells at 30 °C for 30 minutes.
10. Spin at 5000 rpm for 4 min.
11. Resuspend the pellet in 200 µl and plate it on SCD+Ura+His.

12. Incubate the plates at 30 °C for 2 days.

Characterization of Transformants
MaV203 cells that contain bait and prey proteins that strongly interact will induce all three reporter genes present in this system (*HIS3, URA3, lacZ*). Identify these colonies by a series of patching and replica plating steps onto the selection/screen plates.

Generating master plate
- Using an autoclaved toothpick, patch all 6 different transformants (4 each) onto a SC+Ura+His plate.
- Incubate all plates for ~24 hours at 30°C.
- Replica plate it on
  1. SCD+Ura+His plates
  2. SCD+Ura+50mM 3AT
  3. SCD+His

5. Incubate for 2 days at 30°C.
6. Compare the phenotypes of the control and test transformants.
DEVELOPMENTAL BIOLOGY
EXPERIMENT 1

TARGETING TISSUE SPECIFIC EXPRESSION OF A GENE USING THE GAL4 - UAS SYSTEM IN DROSOPHILA

INTRODUCTION

Targeted expression of a gene (reporter or any other gene) in a tissue-specific manner is a very powerful technique for analyzing the regulation and function of the given gene as also for studying the normal development and function of the specific group of cells. The development of the P-element based germline transformation and the enhancer-trap systems have allowed such studies to be undertaken. As mentioned in the experimental procedure for P-insertion mutagenesis, the P-element system permits synthesis of chimeric genes comprising a well defined promoter (with conditional or tissue-specific expressivity) and the desired gene of the same or different organism. However, this strategy has certain limitations.

The conditional promoter (like the heat shock promoter) may cause the given gene to express globally, rather than in a tissue-specific manner or the experimental expression of the gene in a particular cell type may be grossly deleterious so that such lines cannot be established or maintained. Therefore, to avoid such situations, a conditional or tissue-specific promoter may be used in a "binary" system such that the expression of the gene of interest is dependent upon the simultaneous presence of two genetic components that are combined in one individual only for the purpose of the experiment but are otherwise maintained in separate transgenic lines. This ensures that the gene of interest is expressed only in the progeny of the cross between the two lines.

Gal4 is a transcription factor of baker's yeast which stimulates transcription of genes that carry Gal4-response elements, called the UASG (upstream activating sequence). No gene in Drosophila is known to be activated by Gal4 but the GAL4 can activate transcription in fruit flies if a transgene carrying UASG is present. Thus if a cross is made between a transgenic line carrying Gal4 gene under a Drosophila gene promoter and another transgenic line carrying the gene of interest under UASG promoter, the progeny shows activity in tissues.

Using this strategy, more versatile "enhancer traps", so-called "second generation enhancer traps" have been designed by Drosophila geneticists (see Kaiser, 1993).

Restricted expression of Gal4 in certain cells of Drosophila can be achieved either by fusing the Gal4 gene to a well characterized Drosophila promoter or by randomly integrating an enhancerless Gal4 gene in Drosophila genome. This is done through P-insertional mutagenesis scheme using a P-Gal4 transposon, analogous to the P-lacZ transposon. Such random integration of the P-Gal4 transposon brings the enhancerless Gal4 gene under the control of diverse genomic enhancers causing expression of Gal4 in different cell types in different lines. To monitor the Gal4 expression in a given line, this line is crossed to the transgenic line carrying the UASG fused to the specific gene of interest. Once the lines that express Gal4 in the specific cells of one's interest are identified, it is possible to express any desired gene in that tissue by making germline transformants that carries the desired gene under the UASG promoter. Appropriate P-vector (pUAST) carrying the Gal4 UASG is used for such transformation. The gene that is put under the UASG can be any gene that one wants to express ectopically to monitor its effects or it can be a cell lethal (or a toxin) gene that kills the cells that express so that one may study the consequence of death of specific cells. Using such strategies, a large number of Gal4 lines have been generated in different laboratories.
around the world and many of them have been characterized with respect to the cell types that express Gal4 at certain stages of development.

In the experiment, we will use such Gal4 lines in conjunction with the UAS-gene line to monitor the cell types that express Gal4.

**MATERIALS REQUIRED**

1. Fly lines carrying Gal4 insertion at diverse locations in the genome
2. UASG-gene transgenic fly line

**PROCEDURE**

1. Cross the Gal4 and UASG-gene line to obtain progeny embryos, larvae, pupae and adult flies.
2. The different developmental stages are processed for observing phenotypes and adults are subjected to behavioral assays.

**OBSERVATIONS**

Phenotypes associated to be noted.

**DISCUSSION**

This "second generation enhancer trap" strategy is used in two broad applications:

1. a given Drosophila gene is forced to be expressed in a different cell type or at a different stage of development than its normal pattern and the consequences of its ectopic (spatial and/or temporal) expression are monitored with a view to understand the given gene's effects/functions;

2. a gene that may cause death of cells (e.g., a toxin gene) is made to express in specific cell types to kill those cells very specifically: the consequence of such specific killing of cells allows one to examine the roles that those cells play in normal development

**REFERENCES**

EXPERIMENT: 2

DETECTION OF β-GALACTOSIDASE ACTIVITY IN TISSUES/CELLS BY CYTOCHEMICAL STAINING

β-galactosidase normally uses galactose as substrate. However, a chromogenic compound, X-gal, (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) also acts as a good substrate. The yellowish colored X-gal is converted by β-galactosidase into its indolyl derivative which in turn is oxidized to the blue dye 5,5’-dibromo-4,4’-dichloro-indigo. Potassium ferricyanide is used as a catalyst for oxidation of the indolyl product. Potassium ferrocyanide is also added in equal amount to prevent overoxidation to a colorless compound. Without the added oxidizing agents, initial products of β-galactosidase reaction, the indole monomers, appear to diffuse out of the cell before they could be oxidized and dimerized by molecular oxygen.

MATERIALS REQUIRED

Late 3rd instar larvae of stock; incubator set at 37°C; dissection instruments and Stereobinocular microscope; Microscope slides and coverglasses (22 or 24mm²), DPX

SOLUTIONS

*Drosophila* Ringers’ or Poels’ salt solution (pH 6.8) ✓

- NaCl 86mg
- KCl 313mg
- CaCl₂.2H₂O 116mg
- NaH₂PO₄.2H₂O 88mg
- K₂CO₃ 18mg
- MgSO₄.7H₂O 513mg
- Dist. H₂O 100ml

Adjust pH to 7.0 with 1M NaOH and filter. ✓

1M Phosphate Buffer (pH 8.0) ✓

- 1M Na₂HPO₄ 93.2ml
- 1M NaH₂PO₄ 6.8ml

Wash Buffer (50mM Phosphate Buffer pH 8.0) ✓

- 1M Phosphate Buffer 5ml
- Dist. Water 95ml

2.5% Glutaraldehyde in 50mM Phosphate Buffer ✓

- Glutaraldehyde 250µl
- 50mM Phosphate Buffer (pH 8.0) 9.75ml

**X-Gal stain** ✓

- 5% X-Gal (in Dimethylformamide) 60µl (0.3%)
- 100mM K₃[Fe(CN)₆] 20µl (2mM)
- 100mM K₄[Fe(CN)₆] 20µl (2mM)
- 1M Phosphate buffer (pH 8.0) 50µl (50mM)
- Dist. Water 850µl

- 50% Glycerol 5ml RT
- Glycerol 5ml RT
- Dist. Water 5ml RT

- C. dehyd - 07h 50m
- G7526-10m²
- Genade 1 8.7. aqueous 36
- For electron microscopy
PROCEDURE
X-Gal Staining
1. Dissect the larvae in Ringer's (or Poels' Salt) soln and remove unwanted tissues like cuticle, fat body etc. The desired tissues are transferred to a fresh cavity slide.
2. Remove excess Ringer's soln and fix tissues in 2.5% Glutaraldehyde for 10min at room temperature.
3. Remove fixative and wash 2x with wash buffer.
4. Remove the wash buffer and add 50μl of X-Gal staining solution. Cover the cavity slide and keep overnight in a moist chamber in dark at 37°C.
5. Remove the X-Gal stain, wash the tissues with wash buffer and briefly refix with aceto-methanol.
6. Transfer tissues to a fresh slide in a drop of 50% glycerol; spread the tissues as desired and carefully mount with a clean coverglass. Seal the edges with DPX mountant.

OBSERVATIONS
The tissues stain bluish-black while the control tissues show very little β-galactosidase activity.

DISCUSSION
Some staining may be seen in control tissues due to normal developmental enzyme activity or due to the larvae already being mildly stressed due to some reasons.

PRECAUTIONS
The larvae etc that are used in these experiments must be grown under stress-free conditions (growth temperature lower than 25°C and grown in uncrowded conditions with rich food).

REFERENCES
5% Glycerol dehydrate in 50mM phosphate buffer

0mM phosphate buffer

Dilute 1M phosphate buffer to 20 times.

2.5% Glycerol dehydrate.

Original is 8% C.D.

So if we dilute to 2.2 times = 2.2mL 50mM buffer + 0.8mL C.D.

And total...
IN VITRO CULTURE OF THE EARLY CHICK EMBRYO

INTRODUCTION

The chick embryo has been an important and favourite model for the study of vertebrate development. Chick embryos can be made available throughout the year. Various techniques are available for growing the isolated chick blastoderm in culture up to 4th day of incubation. These techniques allow very close observations and fine operations on the earliest stages of development. Later stages are usually studied in ovo through windows cut in the shell.

After obtaining the freshly fertilized eggs from the hatchery, further development can be delayed by keeping the eggs cool at 10°C to 15°C for a few days, if necessary and then can be incubated for certain time period to get desired developmental stage. Optimum temperature for storage is 7°C to 15°C. Outside this range the incidence of subsequent embryonic abnormalities is increased and hatchability is reduced. Storing the eggs under optimum temperature conditions for 1-2 weeks does little harm, but if kept longer, hatchability falls off rapidly. Violent and prolonged shaking of eggs reduces hatchability and increases the incidence of all types of malformations. The very important factor during incubation is the correct temperature. The optimum temperature at 60% relative humidity is 37°C ± 0.5°C.

The water available to the embryo during the entire period of incubation is that stored initially in the egg and this is continually being reduced by evaporation. Excessive dry air in the incubator reduces the hatchability. To maintain humidity, a tray filled with water is kept in the incubator.

Advantages of using chick embryos:
1. Easy to culture
2. Available throughout the year.
3. Treatment with exogenous factors can be administered easily.
4. Observations up to 72 hrs in culture conditions are possible.
5. The avian system is much closer to mammalian system than other vertebrate systems.

Disadvantages of using chick embryos
1. Only a small number of embryos can be studied, treated or cultured at one time.
2. Early mixing of cells. Tracing of cells becomes difficult as there are no cell specific markers.

MATERIALS REQUIRED

Biological:
Freshly laid white leghorn chicken eggs (hatching)

Glassware (Dry sterilization):
✓ Cake dish - 1. small cake dish (23 cm x 14 cm) for up to 20 eggs
  2. big cake dish (21 cm x 21 cm) for more than 20 eggs
✓ Petri plates - 1. small petri plate (9 cm diameter) for growing cultures.
  2. big petri plate (20.5 cm diameter) for cleaning embryos
✓ Watch glasses - 7.5 cm diameter (one per culture).
✓ Beakers - 250 ml (2 numbers).
  150 ml (2 numbers)
✓ Measuring cylinder - 100 ml (1 number)
Glass rings - 1. small (18 mm) for culturing embryos up to stage 4.
   2. big (22 mm) for culturing embryos of higher developmental stages.
* Conical flasks - 100 ml (2 numbers) for 1% glucose solution
   500 ml (2 numbers) for solutions A and B1.
   150 ml (1 number) for solution B2.
* Pasteur pipettes - bent pipettes (2 numbers)
   blunt pipette (1 number)

Chemicals:
For Pannet Compton saline -
Sodium chloride, potassium chloride, calcium chloride (dihydrate), magnesium chloride,
sodium dihydrogen phosphate - 2 hydrate, di-sodium hydrogen phosphate.
For fixation of embryos -
1. For histology  ✔Saturated picric acid (75 ml)
   ✔Formaldehyde solution (25 ml)
   ✔Glacial acetic acid (5 ml)
2. For whole mounts  ✔Ethanol (3 parts)
   ✔Acetic acid (1 part)

Other requirements:
1. Incubator set at 37°C
2. Rubber bulbs
3. Bowl for discarding yolk, albumin and shells
4. Absorbent cotton
5. Dissection instruments - 1 pair of curved scissors, 2 pairs fine forceps, 2 pairs blunt forceps
6. Aluminium foil
7. Microscope slides

For grafting experiments - 2 fine tungsten needles, spirit lamp, match box.
For morphogenetic movement experiments - resin (anion exchanger) beads soaked in vital
dyes like neutral red, crocein scarlet, tungsten needles.

Solution preparations:
A. Pannet Compton saline:

Solution A:
- ✔NaCl 24.22 gms
- ✔KCl 3.10 gms
- ✔CaCl₂.2H₂O 3.08 gms
- ✔MgCl₂.6H₂O 2.54 gms
Dissolve in 200 ml distilled water and autoclave.

Solution B1:
\( \text{Na}_2\text{HPO}_4 \quad 1.875 \text{ gms dissolve in 360 ml distilled water and autoclave.} \\

\text{Solution B2:} \\
\text{NaH}_2\text{PO}_4\cdot2\text{H}_2\text{O} \quad 0.16 \text{ gms dissolve in 80 ml distilled water and autoclave.} \\

\text{Solution B:} \quad 88 \text{ ml of solution B1 + 8 ml of solution B2} \\

\text{1% Glucose solution:} \\
13.5 \text{ gms of glucose dissolve in 1350 ml distilled water and autoclave.} \\

To the above 1350 ml glucose solution add the following slowly with constant stirring: \\
60 \text{ ml of solution A} \\
90 \text{ ml of solution B} \\

\text{PROCEDURE} \\
\text{Fixation of Chick embryos:} \\
1. Take 0.9% saline in a petri plate. \\
2. Lift the embryo along with the ring using forceps and immerse it immediately in the 0.9% saline. \\
3. Using a Pasteur pipette, pass slow jets of saline inside the ring so as to separate the blastoderm from vitelline membrane. \\
4. After complete separation of the embryo from the vitelline membrane, take the embryo on a slide immersed in saline. \\
5. Pour a few drops of saline on the top of the embryo to stretch the embryo properly on the slide. \\
6. After good spreading of the embryo, remove the remaining saline on the slide carefully using a Pasteur pipette. \\
7. Pour acetic acid: alcohol fixative drop by drop on the embryo so as to avoid wrinkles or folds. \\
8. After about 8 min, transfer embryo in a vial containing acetic acid: alcohol fixative for overnight fixation. \\
9. Next day, remove the fixative and store the embryos in 70% ethanol.
IMMUNOSTAINING

INTRODUCTION

Immunostaining is based on the detection of antigens by antibodies. Antigens are in general large cellular molecules, such as proteins, polysaccharides and nucleic acids. In immunostaining the antibody that interacts with the tissue antigen is known as primary antibody. The first antigen-antibody complex can be detected by suitable markers.

Since the antigen-primary antibody complexes are small, their detection becomes often difficult. Therefore, secondary labeled antibodies specific to the primary antibodies are used to detect the primary complex. If the primary antibody is an IgG made in rabbit, then the secondary antibody used will be an anti-rabbit IgG made in goat or sheep.

The molecules used to label or tag the secondary antibody are either an enzyme or a fluorescence molecule. A chemical is said to be fluorescent if it absorbs light at one wave length (the excitation wave length) and emits light at a specific and longer wave length within the visible spectrum. Three very useful fluorescent dyes are rhodamine, Cy3, which emit red light, and fluorescein, which emits green light. These dyes have a low nonspecific affinity for biological molecules and they can be chemically coupled to purified antibodies specific to almost any desired macromolecules: a fluorescent dye-antibody complex, when added to a permeabilized cell or tissue, will bind to the chosen antigens, which then light up when illuminated by the exciting wave length.

MATERIALS REQUIRED

Poels' salt solution- [86mg NaCl, 313mg KCl, 116mg CaCl₂·2H₂O, 88mg NaH₂PO₄, 18mg KHCO₃, 513mg MgSO₄·7H₂O, 100ml distilled water, pH 6.8]

10X PBS - [175.0 mM NaCl, 84.1 mM Na₃HPO₄, 18.6 mM NaH₂PO₄, pH 7.4]

Prepare 1X PBS by diluting 1:10 with dH₂O.

PBST - [1X PBS, 0.1% Triton X-100, 0.1% BSA]

PBS-PFA - [1X PBS, 4% paraformaldehyde]

Dissolve at 60°C and cool down to room temperature.

Blocking solution - 1X PBS, 0.1% Triton X-100, 0.1% Tween 20, 0.1% BSA, 10.0% fetal calf serum, 0.1% Deoxycholate and 0.02% thiomersal.

Primary Antibody

Secondary Antibody

DAPI

PROCEDURE

1. Dissect tissues in Poels' salt solution (pH-7.0) or 1X PBS (pH-7.4).
2. Transfer tissues to cavity slide in IX PBS.
3. Fix in freshly prepared PBS-PFA for 20 mins at room temperature.
22. Safranin, Aceto Orcein, Aceto Carmine and Trypan blue stain
23. Entellan mountant, Immersion oil

**PROCEDURE**

The DNA of interest is amplified by PCR, eluted and sequenced.

For cDNA labeled probe, RNA is extracted from tissue of interest and cDNA is prepared.

**Labeling of probe DNA with digoxigenin dUTP by random priming method**

1-3μg of DNA can be labeled per standard reaction

1. Take the required amount of DNA (linear) in an eppendorf tube and denature by heating in boiling water for 10min. Quickly chill the tube on ice.
2. Add the following in sequence to the same eppendorf tube

   a. Hexanucleotide mix 2μl
   b. dNTP labeled mix 2μl
   c. Distilled water to make 19μl
   d. Klenow enzyme (3-5 units) 1μl

   Final volume 20μl

3. Incubate at 37°C for 1h
4. Stop reaction by adding 0.8ml of 0.5M EDTA (Final concentration 20mM)
5. Precipitate labeled DNA by adding the following:
   2.0μl of salmon sperm DNA (10mg/ml)
   2.5μl of 4M Lithium Chloride
   75μl of pre chilled (-20°C) ethanol
   Leave at -70°C for 2hrs

6. Centrifuge at 12,000rpm for 30min at 4°C
7. Decant supernatant and wash the pellet with 70% ethanol
8. Dry at room temperature or lyophilize
9. Dissolve in required amount of TE. The labeled probe can be stored at -20°C for at least 2 years.

**Checking the efficiency of DIG-labeling**

1. Take a small piece of nylon membrane, wet it with 2xSSC and dot blot (under vacuum) 10pg, 1pg and 0.1pg of the prepared probe. Let it dry at room temperature (~30min) following which cross-link the probe DNA with the membrane either by 3-4min exposure to UV on a transilluminator or by heating the filter at 70°C for 2h.
2. Wash the filter briefly in Buffer I
3. Incubate in Buffer II for 30min at room temp (to block the membrane surface for non-specific binding of the antibody used in next step)
4. Briefly rinse in Buffer I
5. Incubate in Anti-DIG Antibody-Enzyme conjugate (1µl in 4ml of Buffer I) for 30min at room temp
6. Wash twice at 15min interval in Buffer I
7. Briefly rinse in Buffer III
8. Put the blot in a small polythene bag and working in dim light add color developing solution (4.5µl of NBT and 3.5µl of BCIP in 1ml of Buffer III) and seal the bag
9. Incubate the blot within the sealed bag in dark (e.g., by wrapping with aluminium foil) till desired level of colored signal is visible. When adequate signal is obtained, remove the blot from the bag and put in Buffer IV to stop reaction (under optimal conditions of probe labeling, 0.1pg of probe gives a detectable signal within 30min). The blot can be stored in Buffer IV or in dry condition

**Processing of prepared slides prior to hybridization**

1. Dip the prepared slides (with the desired cytological preparation) for about 5sec in a freshly prepared 0.1% poly L-lysine.
2. Arrange the slides in a moist chamber containing filter papers soaked in 2x SSC. Place 100µl of RNase (100µg/ml in 2X SSC) over the preparation on each slide and cover with 22mm² coverglasses (no air bubbles should be trapped) to remove RNA from preparations. Incubate the slides at room temperature for 2 hr
3. Remove the coverglasses gently by dipping slides into a beaker containing 2x SSC. Coverglasses will fall in solution
4. Wash slides in 2X SSC (3 times 5min each), in 70% ethanol (2 times 10min each) and in 95% ethanol for 5min. Air dry. Slides can be stored at this stage, if required
5. Place slides in 0.07N NaOH for exactly 5min to denature chromosomal DNA
6. Wash slides in 3 changes of 70% ethanol (10min each) and 2 changes of 95% ethanol (5min each). Air dry. Slides are now ready for hybridization

**HYBRIDIZATION MIXTURE**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formamide</td>
<td>500µl</td>
</tr>
<tr>
<td>20X SSC</td>
<td>250µl</td>
</tr>
<tr>
<td>DIG labeled probe (10-20ng/slide)</td>
<td>as required</td>
</tr>
<tr>
<td>H₂O to make TOTAL volume to</td>
<td>1000µl</td>
</tr>
</tbody>
</table>

The total volume of hybridization mix that is prepared depends upon the number of slides being processed.

**Hybridization**

1. Denature labeled probe DNA by placing the tube in boiling waterbath for 10min. Add the desired amount of denatured probe to the hybridization mix.
2. Add 20µl of hybridization mixture containing 10-20ng of labeled probe. Place a coverglass over the hybridization mixture and seal the edges with DPX. *No air bubbles should be trapped*
3. Incubate slides at 37°C in a closed moist chamber. Allow hybridization to proceed for 12-14hrs (overnight)

**Washing**
1. Peel off DPX sealing with the help of forceps. Remove coverglasses by dipping slides in 2X SSC
2. Wash slides in 1x SSC, (3 times 15min each) at 60°C

Color detection

1. Rinse slides for 1min in buffer I (100mM Tris pH7.5, 150mM NaCl)
2. Place them in buffer II (0.5% W/V Blocking reagent in Buffer I) and leave for 30min
3. Wash again in buffer I for 1min
4. Incubate in anti-Digoxigenin antibody alkaline phosphatase conjugate (diluted 1: 5000 in buffer I) for 30min
5. Wash in buffer I (2 times 20min each)
6. Rinse in buffer III (Tris pH 9.5 100mM, NaCl 100mM, MgCl2 50mM)
7. Prepare fresh color reaction reagent. Put 20-30μl of color reaction reagent on the slide, cover with a coverglass, seal with DPX and leave the slide in a dark chamber at room temperature for 1-12h, depending upon the time required for optimal signal development.
8. Stop reaction in Buffer IV (10mM Tris pH 8.0, 1mM EDTA) after observing slides under microscope
9. Air dry and counter-stain with Safranin by dipping the slides in the staining solution for 5 to 10 sec followed by 2-3 washes in clean distilled water. Air dry.
(Alternatively, stain the slides with 2% aceto-orcein / aceto carmine and Trypan blue for 5-6min. Following the staining, quickly rinse slides in two changes of 70% alcohol and air dry).
10. Mount dried slides with Entellan.
Alternatively, mount the slides temporarily using the stop buffer or the immersion oil; after examination the coverglasses are removed and slides cleaned by rinsing in distilled water if mounted with the stop buffer or with xylene if immersion oil was used. Mounting with alcohol-based mountants (e.g., DPX) causes fading of color.
OBSERVATIONS
Hybridization of the probe results in appearance of purplish-blue color deposit at the site of hybridization. The specific chromosome region that shows the hybridization signal can be identified by referring to standard polytene chromosome maps. For tissues, a specific purplish-blue color deposit at the site of hybridization

PRECAUTIONS
Washing after the hybridization and after antibody binding must be adequate so that all of the excess probe and the antibody are removed. Incomplete washing at any step would generate undesirable background.

Staining of chromosomes must be controlled so that the hybridization signal is not masked.

Care must be taken to avoid trapping of air bubbles while mounting coverglasses at the various steps since any trapped bubble would not permit the reaction in the local region and thereby prevent the hybridization signal.
IMMUNOSTAINING

INTRODUCTION

Immunostaining is based on the detection of antigens by antibodies. Antigens are in general large cellular molecules, such as proteins, polysaccharides and nucleic acids. In immunostaining the antibody that interacts with the tissue antigen is known as primary antibody. The first antigen-antibody complex can be detected by suitable markers.

Since the antigen-primary antibody complexes are small, their detection becomes often difficult. Therefore, secondary labeled antibodies specific to the primary antibodies are used to detect the primary complex. If the primary antibody is an IgG made in rabbit, then the secondary antibody used will be an anti-rabbit IgG made in goat or sheep.

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MATERIALS REQUIRED

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10X PBS - [175.0 mM NaCl, 84.1 mM Na2HPO4, 18.6 mM NaH2PO4, pH 7.4]
Prepare 1X PBS by diluting 1:10 with dH2O.

PBST - [1X PBS, 0.1% Triton X-100, 0.1% BSA]

PBS-PFA - [1X PBS, 4% paraformaldehyde]
Dissolve at 60°C and cool down to room temperature.

Blocking solution - 1X PBS, 0.1% Triton X-100, 0.1% Tween 20, 0.1% BSA, 10.0% fetal calf serum, 0.1% Deoxycholate and 0.02% thiomersal.

Primary Antibody

Secondary Antibody

DAPI

PROCEDURE

1. Dissect tissues in Poels’ salt solution (pH-7.0) or 1X PBS (pH-7.4).
2. Transfer tissues to cavity slide in IX PBS.
3. Fix in freshly prepared PBS-PFA for 20 mins at room temperature.
4. Wash the tissues in PBST for 10 min (x3)
5. Incubate the tissues in blocking solution at room temperature for 2 hrs.
6. Add LoxL1 primary antibody at a dilution of 1:10 in blocking solution and incubate at 4°C over night.
7. Take off the supernatant. This can be saved for second use.
8. Rinse once with PBST and then wash 2 X 10 min each in PBST.
9. Add AlexaFluor-488 conjugated secondary antibody (or a desired secondary antibody) diluted in blocking solution and incubate for 2 hrs at room temperature with gentle shaking.
10. Take off the supernatant. Wash as in step 8 and counter stain in DAPI for 10 min.
11. Rinse with PBS and mount in mounting medium seal and store at -20°C till further observation.
12. Observe under a fluorescence microscope at the desired excitation wavelength of light.

OBSERVATIONS
Green fluorescence will be observed at those specific sites where the antigen-primary antibody complex is present when viewed at 494nm wave length of light if AlexaFluor-488 conjugated antibody is used. Blue (DAPI) stained nuclei can be observed if excitation wave length 359 nm is used.
GENETIC
ENGINEERING
Genetic Engineering Lab  
Exercise I  
Instructor: Manjusha Dixit

Goal: Designing primers for a given gene

Requirement: Computer, internet connection

Method:
1. Be familiar with the guidelines to design the primers
2. Choose a gene of interest
3. Get the sequence from NCBI/ UCSC genome browser
4. Design primer
5. Check specificity using NCBI Basic Local Alignment Search Tool (BLAST) program

Practice 1 (Day 1)

1. Use primer blast to design primers for any gene of your interest
   (i) for gDNA flanking single nucleotide polymorphism (SNP) site
   (ii) for real time PCR using cDNA
2. Check specificity with BLAST
3. Report in lab record

Practice 2 (Day 2)

1. Use primer blast to design primers for amplification of given whole gene from an expression vector (complete gene + vector sequence and location of gene have been sent to you by email)
2. Check specificity with BLAST
3. Report in lab record
Genetic Engineering Lab
Exercise II
Instructor: Manjusha Dixit

Goal: Setting up the PCR Reaction and optimization of PCR conditions

Requirement: 0.2 ml, 0.5, and 1.5 ml tubes, pipette tips (all types, DNase RNase free), Buffer, dNTPs, primers, Taq polymerase, TAE/TBE buffer, EtBr, DNA template, DNA ladder, loading Dye, Gel Electrophoresis apparatus, PCR machine, pipettes (0.1 μl to 1 ml), refrigerated micro-centrifuge

Procedure:

1. For a Single 25 μl PCR Reaction (see details below):

   10x PCR buffer 
   dNTP mixture (10 mM each dNTP)  
   5' primer (10 μM) 
   3' primer (10 μM) 
   Taq DNA polymerase (5 U/μl stock)  
   DNA template  
   Sterile Water  
   25 mM MgCl2 (optional)

2. Use various annealing temperatures and/or MgCl2 conc. to optimize the PCR condition
3. Report final result (reaction conditions and Reaction component conc.) in record

Setting up the reaction:

1. Thaw 10x buffer, dNTPs, and primers. Keep on ice.
2. INCLUDE A BLANK REACTION TUBE
3. Prepare Master Mix
4. Use 0.2 ml thin walled centrifuge tubes for reaction. Label top of tubes with sample #
5. Pipet mix reagent (except template) into each tube.
6. Pipet DNA samples into appropriate tubes, using a new tip for each sample.
7. Mix tube well. Spin for 5 seconds in microfuge to remove liquid from sides of tube.
8. Place tubes in PCR machine, program machine, and begin PCR reaction.
9. Analyze the PCR reaction products by agarose gel electrophoresis of a 5 μl aliquot from the total reaction. The products should be readily visible by UV transillumination of the ethidium bromide-stained gel.
Expected PCR product size: .................bp

10. Store reaction products at -20 °C until needed.
EXPERIMENT: Digestion of DNA insert and vector with restriction endonucleases

Requirements: PCR amplified insert, *EcoRI*, *NotI*, BSA 100X, NEB *EcoRI* Buffer, nuclease free water, microfuge tubes, water bath, pipettes, tips, gel extraction kit, vector (pcDNA 3.1) etc.

Procedure: 1. Combine the following in the given qty in microfuge tube in given order, on ice:

Reaction mix-I (Total volume 50 μl)

- 5 μl 10x NEB *EcoRI* Buffer
- ..........μl *EcoRI* (1U/μg of DNA)
- ..........μl *NotI* Enzyme (1U/μg of DNA)
- ..........μl DNA (PCR product)
- 0.5 μl BSA (100X)
- ..........μl NFW (upto 50 μl)

Reaction Mix II- Digest 1 μg of vector in similar way

2. Put the reaction mix at 37°C for 3-4 hours.

3. Inactivation of enzymes- 65°C for 20 minutes.
   - It can be stored at -20°C (optional)

4. Purification by gel extraction (Use Qiagen gel extraction kit)
   - It can be stored at -20°C (optional)
EXPERIMENT : Ligating vector and insert DNA

Requirements: Agarose, purified insert DNA, purified vector DNA, Restriction endonucleases, heating block/water bath, electrophoresis unit, Ligase Mixture [Tris-Cl 1μl (1M, pH-7.6), MgCl₂ 2μl (100mM), DTT 1μl (200mM), ATP 1μl (10mM), Nuclease free water 4.5μl, Ligase enzyme 1 unit] etc.

Procedure:

1. Combine 50 ng of vector with a 3-fold molar excess of insert. Adjust volume to 10 μl with ddH₂O.
2. Add 10 μl of 2X Quick Ligation Buffer and mix.
3. Add 1μl of Quick T4 DNA Ligase and mix thoroughly.
4. Centrifuge briefly and incubate at room temperature (25°C) for 5 minutes.
5. Chill on ice, then transform or store at -20°C.

Note- Do not heat inactivate. Heat inactivation dramatically reduces transformation efficiency.
Goal: Transformation of Competent E. coli, selection of E. coli colonies with insert

Materials Needed
Microbiological supplies (i.e. plates, spreaders), Cloning reaction mixture (From previous step), E. coli, chemically competent, S.O.C. Medium, Positive control, 42°C water bath, selective LB plates containing 100 µg/ml ampicillin for each transformation, 37°C shaking and non-shaking incubators, agarose. EtBr, TAE/TBE, electrophoresis unit, Restriction enzymes

Preparing for Transformation
• Equilibrate a water bath to 42°C.
• Warm the vial of S.O.C. Medium to room temperature
• Warm selective LB plates at 37°C for 30 minutes
• Thaw one vial of E.coli cells on ice for each transformation

Chemical Transformation Protocol
1. Add 2 µl of the Cloning reaction into a vial of Chemically Competent E. coli with a sterile pipette tip and mix gently. Do not mix by pipetting up and down.
2. Incubate cells/plasmid mix on ice for 5-30 minutes.
Note: Longer incubations on ice seem to have a minimal effect on transformation efficiency.
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250 µl of room temperature S.O.C. Medium.
6. Cap the tube tightly and shake the tube horizontally at 200 rpm in a 37°C shaking incubator for 1 hour.
7. Spread 50-100 µl from each transformation on a pre warmed selective LB plate.
8. Incubate overnight at 37°C. Plate two different volumes to ensure that at least one plate contains well-spaced colonies. Incubate plates overnight at 37°C.
9. Pick single colony (at least five different colonies from each plate) and inoculate 5 ml of LB broth containing 200 g/l ampicillin or 1mg/5ml. Optional: Use a 15ml conical tube with a loosened cap and a piece of tape to hold it in place. Shake at 250 RPM 37°C overnight.
10. Use miniprep kit (Qiagen) for isolation of DNA.
11. Quantitate DNA from each colony using nanodrop (Thermo)
12. Digest the DNA products using protocol in lab exercise III
13. Run on 1.5% agarose gel, check for the presence of insert, record picture
14. Note colonies with presence of insert.
15. Keep DNA from each colony in -20°C until further use.
Goal: Transfection of HeLa cells.

Requirement: DMEM serum free, DMEM (10% FBS), Haemocytometer, 12 well plate, 18 mm cover slip, Trypsin-EDTA, 1X PBS, Lipofectamine 2000, OPTI-MEM, Plasmids for transfection, Cell culture plates, serological pipettes.

Procedure

Day 1:

1. Discard the media from 80% confluent HeLa cells containing T 75 flask. Wash the cells with 1X PBS (10 ml). Discard the PBS.
2. Add 3-5 ml of Trypsin-EDTA and incubate at 37°C for 2-5 mins once the cells are detached add 2-5 ml of media to neutralize the Trypsin.
3. Spin the suspension at 1000 rpm for 5 minutes.
4. Count the cells using Haemocytometer after suspending the cells in 1 ml DMEM (10% FBS).
5. For 12 well plates seed 8 x 10^5 cells/well in 2 ml complete DMEM media with cover slips (18mm) in each well.
6. Incubate the plates at 37°C and 5% CO₂ for 12-18 hours.

Day 2

1. For each transfection dilute 2 μg of plasmid DNA in 100 ul of OPTI-MEM (Solution A) and 4 ul of Lipofectamine 2000 in 100 ul of OPTIMEM (Solution B).
2. Incubate both the solution at room temperature for 15 minutes.
3. Mix both the solution at 1:1 ratio and incubate at room temperature for 30 minutes.
4. Replace the media of 12 well plates with serum free DMEM 0.8 ml after washing with 1X PBS.
5. Add the 200 ul transfection mix to the cells.
6. Incubate the cells at 37°C and 5% CO₂ for 5 hours.
7. After 5 hours add 1 ml of DMEM (20% FBS) and incubate at 37°C and 5% CO₂ for 18-24 hours.
8. Replace the media after 24 hours and go for the downstream processing after 48 hrs of transfection.
Genetic Engineering Lab
Exercise - 11
Instructor: Manjusha Dixit

Goal: Immunofluorescent staining of Transfected HeLa cells.

Requirement:
- 4% paraformaldehyde
- 1 X PBS
- 0.1% Triton X 100
- 0.1% PBS-T buffer
- 5% BSA
- Primary antibody
- Alexa flour labeled secondary antibody
- 1 uM DAPI
- Fluoromount G
- Glass slides
- Cover slips
- Fluorescent microscope

Procedure:

1. Remove the media from the 12 well plate and wash the cells with 1 X PBS
2. Fixation of cells: Add 500 ul of paraformaldehyde in the well plates with 500 ul of 1 X PBS. Incubate the cells at 37°C for 15 minutes
3. Wash the cover slips twice with 1 X PBS after removing paraformaldehyde
4. Membrane permeabilization step: Rinse in 0.1% Triton X-100 in PBS for 20 min at room temperature (RT). Followed by wash with 0.1% PBS-T
5. Blocking step: Incubate in freshly prepared blocking solution (5% BSA in PBS) for 30 min at RT.
6. Primary antibody (Ab) incubation step: Incubate overnight in primary antibody. Primary antibody should be prepared in the blocking solution (step 3). Dilution of primary Ab should be determined by using series of dilutions. Duration of incubation: overnight at 4°C or 6 h at room temperature. Followed by wash in 1 X PBS, 3 times for 5 mins each.
7. Secondary antibody incubation step: Incubate the cover slips in fluorescence conjugated secondary Antibody prepared in blocking solution (step 3) for 4-6 h at RT. Followed by wash in 1 X PBS, 3 times for 5 minutes each.
8. DAPI Staining: Incubate the cover slips in 1-5 uM DAPI for 20 minutes to stain the nucleus. Wash the cover slips 3 times with 1 X PBS 5 minutes each.
9. Mounting step: Mount with Fluoromount G and place cover-slip over it.
10. Observation step: Observe under fluorescence microscope and store the slides at 4°C.