**Experiment 1** for the CSS451 (2010)

**Sterile techniques and plant micropropagation**

**Part 1: Make 200 ml liquid LB Medium (Luria-Bertani Medium)**

1. Add about 150 ml of distilled water to the 200 (or 500 ml) plastic beaker.
2. Add the contents for 200 ml LB (see the table). Put on the stirrer to dissolve the components completely.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto-Tryptone</td>
<td>10 (2g)</td>
<td>gram (g)</td>
</tr>
<tr>
<td>Bacto-yeast extract</td>
<td>5 (1g)</td>
<td>gram (g)</td>
</tr>
<tr>
<td>NaCl</td>
<td>10 (2g)</td>
<td>gram (g)</td>
</tr>
<tr>
<td>ddH2O to</td>
<td>1 (200 ml)</td>
<td>litre (L)</td>
</tr>
<tr>
<td>Total volume</td>
<td>1 (200 ml)</td>
<td>litre (L)</td>
</tr>
<tr>
<td>pH</td>
<td>7.0</td>
<td></td>
</tr>
<tr>
<td>Bacto-agar</td>
<td>15 g per liter (for solidified medium)</td>
<td>Gram (g)</td>
</tr>
<tr>
<td>autoclave to sterilize</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3. Add distilled water to the 200 ml. Adjust the pH to 7.0.
4. Pour 20 ml to each of the 6 vials. Put lids on the vials. The remaining LB is transferred to a 150 ml glass flask and covered with 2 layers of aluminum foil.
5. Put a piece of autoclave tape on three lids and the aluminum foil before autoclaving for 20 minutes at 121°C 15 lb in-2. The other three vials with LB will not be autoclaved.
6. For each group, observe the autoclaved LB (3 vials) and unautoclaved LB (3 vials) after one week LB in the tissue room at 25°C.
Part 2: Sterile air in the laminar flow cabinet vs open air

1. Switch on the Laminar flow cabinets and let it run for over 15 min.
2. Each group will be provided with 4 LB plates.
3. Remove the lids of two LB plates in the cabinet and let the plates open for 5 min. Similarly, leave the other two plates open in the open air for 5 min.
4. Close all the dishes and seal them with parafilm.
5. Label the plates and put them in the tissue culture room for one week.
6. Observe the plates and take the results.

Part 3: Plant micropropagation

Materials: Transgenic and wild type tobacco plants. Magenta boxes each with 50 ml MS plus 100 mg/L kanamycin

1. Switch on the Laminar flow cabinets and let it run for over 15 min.
2. Burn the forceps and scalpels for 30 s. Cool down the tools.
3. Take the plant out from a Magenta box and put it in a sterile perti dish (DO NOT LET YOU PLANT TOUCH ANY UNSTERILE SPOT).
4. Cut the plants into segments following the diagram using the sterile scalpel.
5. Insert individual segment into the medium in each Magenta box.
6. Each group does two boxes for wild type plants and two for transgenic plants.
7. Culture the plants in the tissue culture room.
8. Observe the results after two weeks.
Part 4: Learn how to use a Pipetman

(This should be review for most of you.)

Parts:

1. Volume adjustment dial
2. Tip ejector button
3. Plunger button
4. Stainless steel micrometer
5. Digital volume indicator
6. Stainless steel ejector arm (removable)
7. Plastic shaft
8. Disposable yellow or blue tip

Pointers:

1. 

   Never rotate the volume adjustment knob past the upper or lower range of the pipetman.

   The ranges are:

   P-20 - up to 20 ul - up to .02 ml
   P-200 - 20 to 200 ul - up to .2 ml
   P-1000 - 200 to 1000 ul - up to 1.0 ml

2. Never use a pipetman without a tip in place - this can ruin the inner workings.
3. Never lay the pipetman down on its side when it contains liquid. This liquid could run into the pipetman.
4. Never let the plunger snap back after withdrawing or ejecting fluid as this could damage the piston.
5. Never immerse the barrel of the pipetman into fluid. Always use a disposable tip.

   (This should be review for most of you.) This part of the stroke is the calibrated volume that you see on the digital micrometer.

Directions:

1. Check the top of the pipetmen plunger button to make sure that you have the pipetman that you need. We have three sizes. Refer to the ranges listed above.

2. Rotate the volume adjustment knob until the digital indicator reaches the desired volume.

3. Firmly, place a disposable tip on the shaft of the pipetman.

4. Press down the plunger to the First Stop. (You will be able to push past this point, but there is enough resistance to stop the movement if you try to be aware of it.)
5. Hold the pipetman vertically and immerse the disposable tip into the sample. Use a small beaker of water. It is only necessary to place the tip in to a depth of several millimeters.

6. Allow the plunger button to return slowly to its original position. Do not allow the button to snap up.

7. Wait a couple of seconds to ensure that the full volume of the sample is drawn into the tip.

8. Withdraw the tip from the sample.

9. To dispense the sample: place the tip against the side wall of the receiving tube and push the plunger down to the first stop. Wait 2-3 seconds, then depress the plunger to the second stop in order to expel any residual sample in the tip.

10. While the plunger is still pushed down, remove the pipetman from the tube and allow the plunger to slowly return to its original position.

11. Discard the disposable tip by pushing the ejector button. (Be careful where you point.)

**Sample Pipetman settings:**

![Pipetman settings](image)

- P-20 = 17.4 µl
- P-200 = 57 ul
- P-1000 = 970 ul

**How to Use a Micropipettor**

The micropipettor is used to transfer small amounts (< 1 ml) of liquids. The scales on micropipettors are in microliters (1000µl = 1 ml). The brand of micropipettors we will be using is made by Rainin and called a "Pipetman". They come in three sizes which are capable of pipetting three ranges of volumes: **P20** = 0.5-20 µl, **P200** = 20-200 µl, and **P1000** = 200-1000 µl. They are used in conjunction with disposable (often sterile) plastic tips; the smaller two micropipettors (P20 and P200) require the yellow tips and the P1000 pipettor uses the larger blue tips. The following is an illustration of a micropipettor:
Directions for use of the micropipettor:

1. **Never exceed the upper or lower limits of these pipettors.** They are very expensive and delicate instruments which we cannot afford to damage. The limits are:
   - P20: 0.5 to 20.0 µl
   - P200: 20 to 200 µl
   - P1000: 200 to 1000 µl

2. Set the desired volume by turning the centrally located rings clockwise to increase volume or counterclockwise to decrease volume. Some examples are provided below:

   ![Image of micropipettor with rings and red digits]

   - **P20**:
     - 0: 0 µl
     - 1: 5 µl
     - 2: 20 µl
     - 3: 15 µl
   - **P200**:
     - 0: 0 µl
     - 1: 200 µl
     - 2: 20 µl
     - 3: 200 µl
   - **P1000**:
     - 0: 0 µl
     - 1: 1000 µl
     - 2: 200 µl
     - 3: 100 µl

3. Place a tip on the discharge end of the pipettor. NOTE: If sterile conditions are necessary do not allow the pipet tip to touch any object (including your hands).

4. The plunger will stop at two different positions when it is depressed. The first of these stopping points is the point of initial resistance and is the level of depression that will result in the desired volume of solution being transferred. Because this first stopping point is dependent on the volume that is being transferred, the distance you have to push the plunger to reach the point of initial resistance will change depending on the volume being pipetted. The second stopping point can be found when the plunger is depressed beyond the initial resistance until it is in contact with the body of the pipettor. At this point the plunger cannot be further depressed. This second stopping point is used for the complete discharging of solutions from the plastic tip. You should not reach this second stop when drawing liquid into the pipettor, only when expelling the last drop. Before continuing, practice depressing the plunger to each of these stopping points until you can
easily distinguish between these points.

5. Depress the plunger until you feel the initial resistance and insert tip into the solution, just barely below the surface of the liquid and not as deep as possible.

6. Carefully and \textit{slowly} release plunger. NOTE: If the solution you are pipetting is viscous, allow the pipet tip to fill to final volume before removing it from solution to avoid the presence of bubbles in the plastic tip which will result in an inaccurate volume.

7. Discharge the solution into the appropriate container by depressing plunger. This time, depress the plunger to the point of initial resistance, wait one second, and then continue pressing the plunger as far as it will go in order to discharge the entire volume of solution.

8. Remove tip by pressing down on the tip discarder.

\textbf{REMEMBER TO CHANGE TIPS BETWEEN SOLUTIONS TO AVOID MIXING OR CONTAMINATING THE SOLUTIONS USED!!}