

**Practical Manual**  
**ZOO101 (Biological Techniques)**  
**Virtual University of**  
**Pakistan**

# **ZOO101 Practical Manuals**

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## **ZOO101 Practical Manuals**

### **Introduction:**

Molecular biology techniques are applicable not only to modern medical practice but also to the identification of genetically modified organisms, forensics, and quality assessment of laboratory animals, pharmacogenomics, and other fields.

This procedure manual encompasses most widely used molecular biology techniques namely DNA extraction, end-point and Real-Time PCR.

### **Safety Considerations:**

- ❖ Use personal protective equipment such as disposable gloves, lab coats, disposable masks, etc.
- ❖ Handle all sharps with care and dispose of sharps in the sharp's disposal containers.
- ❖ Handle hazardous chemicals and samples carefully. Blood and other body fluid must be considered potentially hazardous. Biological waste should be disposed of in the designated trash bags that could be incinerated later on.
- ❖ Decontaminate the work benches regularly and especially before and after work. For decontamination, wipe the surfaces with 10% bleach followed by water.

### **Minimum Standards and Controls:**

- ❖ During DNA extraction, PCR and other experiments appropriate standards and controls should be used to assure the quality of the results.
- ❖ Positive control to check the efficiency of the reagents, procedure and equipment.
- ❖ Negative Control to check the contamination

### **Validation of Critical Reagents and Procedures:**

All technical procedures and critical reagents should be tested and validated before performing the actual case work or research work experiments.

### **Calibration of Instruments:**

All instruments should be calibrated according to required schedule and before performing the validation studies, case work and research experiments

### **Practical 01:**

#### **Preparation of Slides (Dry Mount)**

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Making microscopic slides is a great skill. The first step in making Microscopic slides is to choose the type of mount. The "mount" is simply the way in which a specimen is placed on the slide.

When we are making microscopic slides there are three types of mounts we can Prepare. These are wet mount, dry mount, and prepared mount. Each mount has its own Pros and cons.

### **Dry Mount Microscope Slides:**

#### **Instructions:**

Dry mounts microscope slides are the easiest to make.

#### **Material:**

- Glass slide
- Cover slips.

Dry mounts work best for samples like:

- Pollen
- Hair
- Feathers
- Or even dust particles caught in a microfilm filter.

### **Steps to Make Dry mount Slides:**

The basic steps to Make dry mount slides are very simple. Place the sample on the slide and cover. The cover will protect both your sample and lenses (Objective).



#### **Note:**

These mounts are temporary unless you seal the cover slip in some way. It is harder to see the more complex structures of some species. For that reason, you will have to learn how to make a prepared mount.

### **Practical 02:**

#### **Preparation of Slides (Wet Mount):**

#### **Instructions:**

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Wet mount microscopic slides have a lot of advantages. To see intricate structures the liquid refraction makes it much easier. The specimen is viewable in both the natural color and mobility patterns if the specimen is alive.

### **Procedure to Make Wet Mount Microscope Slides:**

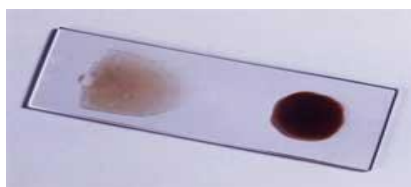
To make wet mount,

1. Place a few drops of your desired liquid on the slide.
2. Add the specimen to your liquid.
3. Then place a few more drops on top of it. This ensures that the specimen will be covered in the liquid.

Looking at an aquatic specimen like algae, use the water in which the specimen is exist in.

4. Next apply the cover slip. This is done very softly in order to avoid the formation of air bubbles. Slowly lower the cover down at an angle, and it will ultimately be held in place by surface tension. If the cover slip is instable on the bottom half of the slide, you have used too much liquid.

There are a variety of liquids that are suitable for making a wet mount, from tap water to glycerin. The important thing to consider is your type of sample. Some samples don't do well when faced with certain liquids. Be sure to choose your liquid sensibly to match your sample.



### **Note:**

Wet mounts have weaknesses as well. Finding a moving specimen can be a problem. The slides also incline to dry out under the light of the microscope. If your wet mounts are drying out before you are ready, apply an extra drop of liquid under the cover slip.

### **Practical 03:**

### **Observation of Wet Mounts of Human Cheek Cells Employing Bright and Dark Field Microscopy**

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### **Introduction:**

Observing human cheek cells under a light microscope is a simple way to quickly view a human cell structure. Many educational facilities use the procedure as an experiment for students to explore the principles of microscopy and the identification of cells. Observation uses a wet mount process that is straightforward to achieve by following an effective preparation method. You can replicate the observational experiment at home with any standard light microscope with magnification settings of X-40 and X-100.

### **Procedure:**

Swab the inside of your cheek with the non-sharp end of a toothpick. Place the toothpick at the bottom of the cheek and move the toothpick up horizontally to collect cheek cells. Be careful not to scrape the inside of the cheek too hard because the epithelial lining is delicate. Place the swabbed end of the toothpick onto the middle of a microscope slide. Add a single droplet of water squeezed from a plastic pipette onto the center of the slide. Rotate the toothpick in the water to release the human cheek cells. Add one drop of methylene blue onto the water and cell solution to stain the cheek cells for observation. Position a cover slip at a 45-degree angle just inside the left edge of the solution. Move your fingers down and to the right to place the cover slip over the cheek cell mixture.

Check for tiny air bubbles under the cover slip and lightly push the cover slip downwards to release any air bubbles you find. Place the edge of a paper towel on any solution outside of the cover slip to absorb the excess moisture. Mount the human cheek cell slide on the light microscope viewing platform.

Choose the X-40 magnification setting on the light microscope and look through the viewing lens. Turn the focusing dial to adjust the focus until you see a clear and crisp image. Observe the human cheek cells by looking for irregularly-edged circular structures with a dark center, or nucleus.

Change the magnification up to X-100 on the light microscope, and refocus the lens for image clarity if necessary. Observe the increased cell detail that the extra magnification provides.

Observe the different details inside the human epithelial cheek cell, noting the cell membrane and nucleic structures inside the cell cytoplasm.

### **Tip:**

Use iodine as an alternative to methylene blue.

### **Warning:**

Be careful when handling cover slips because they break easily.

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### Practical 04:

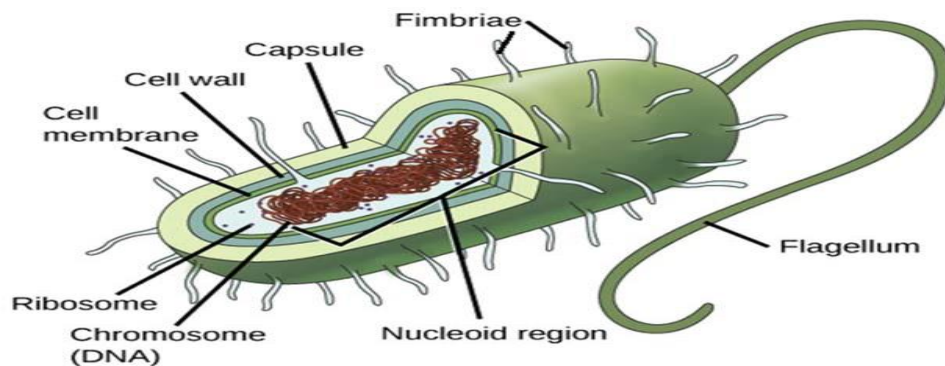
#### Measurement Cell Size: Prokaryotic (Bacterial Cell) Cell Size.

#### Components of prokaryotic cells:

There are some key ingredients that a cell needs in order to be a cell, regardless of whether it is prokaryotic or eukaryotic. All cells share four key components:

1. The **plasma membrane** is an outer covering that separates the cell's interior from its surrounding environment
2. **Cytoplasm** consists of the jelly-like cytosol inside the cell, plus the cellular structures suspended in it. In eukaryotes, cytoplasm specifically means the region outside the nucleus but inside the plasma membrane.
3. **DNA** is the genetic material of the cell
4. **Ribosomes** are molecular machines that synthesize proteins.

Despite these similarities, prokaryotes and eukaryotes differ in a number of important ways. A **prokaryote** is a simple, single-celled organism that lacks a nucleus and membrane-bound organelles. We'll talk more about the nucleus and organelles in the next article on eukaryotic cells, but the main thing to keep in mind for now is that prokaryotic cells are not divided up on the inside by membrane walls, but consist instead of a single open space. The majority of prokaryotic \text {DNA} DNA, N, A is found in a central region of the cell called the **nucleoid**, and it typically consists of a single large loop called a circular chromosome. The nucleoid and some other frequently seen features of prokaryotes are shown in the diagram below of a cut-away of a rod-shaped bacterium.



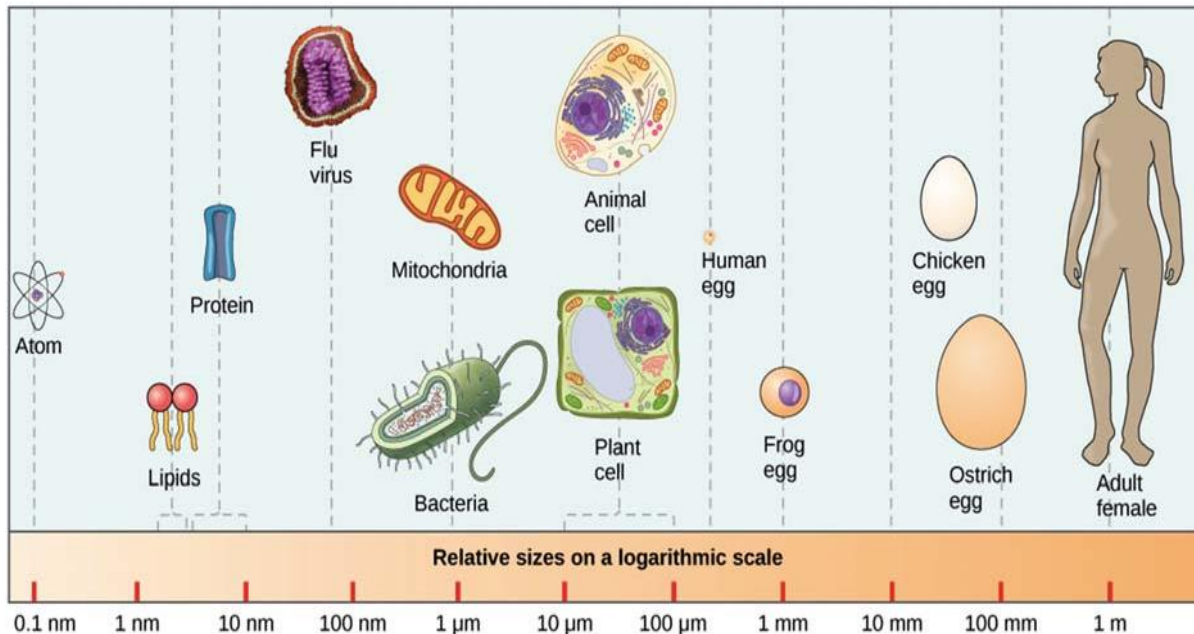
#### Cell Size:

Typical prokaryotic cells range from 0.1 to 5.0 micrometers ( $\mu\text{m}$ ) in diameter and are significantly smaller than eukaryotic cells, which usually have diameters ranging from 10 to 100  $\mu\text{m}$ . The figure below shows the sizes of prokaryotic, bacterial, and eukaryotic,

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plant and animal, cells as well as other molecules and organisms on a logarithmic scale. Each unit of increase in a logarithmic scale represents a 10-fold increase in the quantity being measured, so these are big size differences we're talking about!

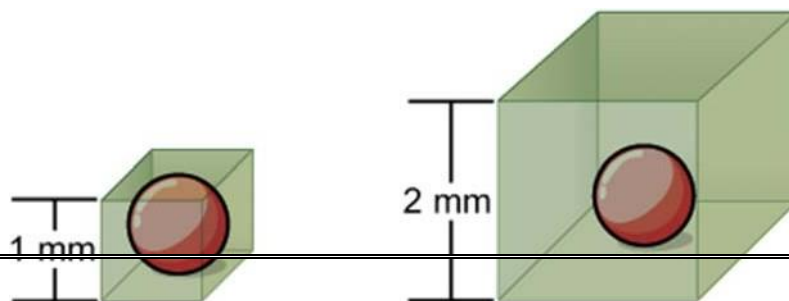
Graph showing the relative sizes of items from, in order, atoms to proteins to viruses to bacteria to animal cells to chicken eggs to humans.



With a few cool exceptions—check out the single-celled seaweed *Caulerpa*—cells must remain fairly small, regardless of whether they're prokaryotic or eukaryotic. Why should this be the case? The basic answer is that as cells become larger, it gets harder for them to exchange enough nutrients and wastes with their environment. To see how this works, let's look at a cell's **surface-area-to-volume ratio**.

Suppose, for the sake of keeping things simple, that we have a cell that's shaped like a cube. Some plant cells are, in fact, cube-shaped. If the length of one of the cube's sides is  $l$ , the surface area of the cube will be  $6l^2$ , and the volume of the cube will be  $l^3$ . This means that as  $l$  gets bigger, the surface area will increase quickly since it changes with the square of  $l$ . The volume, however, will increase even faster since it changes with the cube of  $l$ .

Thus, as a cell gets bigger, its surface-area-to-volume ratio drops. For example, the cube-shaped cell on the left has a volume of  $1 \text{ mm}^3$  and a surface area of  $6 \text{ mm}^2$  with a surface-area-to-volume ratio of six to one, whereas the cube-shaped cell on the right has a volume of  $8 \text{ mm}^3$  and a surface area of  $24 \text{ mm}^2$  with a surface area-to-volume ratio of three to one.



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Image of two cubes of different sizes. The cube on the left has 1 mm sides, while the cube on the right has 2 mm sides.

Surface-area-to-volume ratio is important because the plasma membrane is the cell's interface

with the environment. If the cell needs to take up nutrients, it must do so across the membrane, and if it needs to eliminate wastes, the membrane is again its only route.

Each patch of membrane can exchange only so much of a given substance in a given period of time—for instance, because it contains a limited number of channels.

If the cell grows too large,

its membrane will not have enough exchange capacity (surface area, square function) to support the rate of exchange required for its increased metabolic activity (volume, cube function).

The surface-area-to-

volume problem is just one of a related set of difficulties posed by large cell size. As cells get larger, it also takes longer to transport materials inside of them. These considerations place a general upper limit on cell size, with eukaryotic cells being able to exceed prokaryotic cells thanks to their structural and metabolic features.

Some cells also use geometric tricks to get around the surface-area-to-volume problem. For instance, some cells are long and thin or have many protrusions from their surface, features that increase surface area relative to volume<sup>3</sup>.

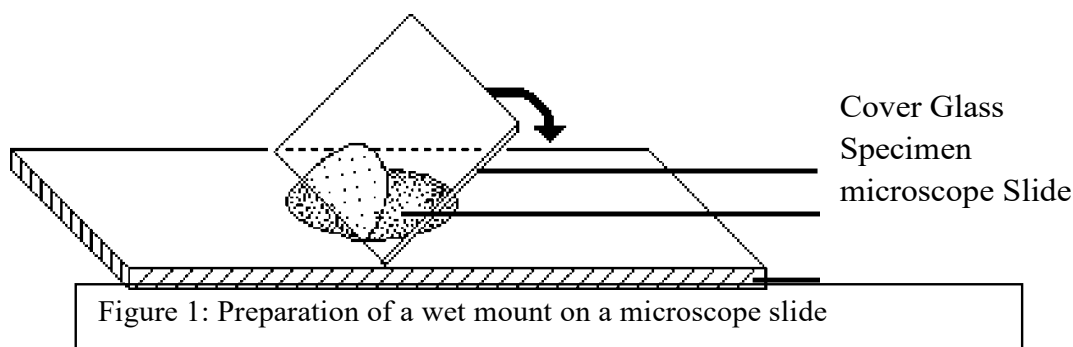
### **Practical 05:**

### **Measurement of Cell Size: Eukaryotic (Human Cheek Cell) Cell Size.**

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### Estimating the Sizes of Cells:

You will now observe various types of cells. You may use prepared slides, or you may make your own slides. To prepare a wet mount: Obtain a clean glass slide. Place the object you wish to observe on the slide. You may use forceps or a toothpick to manipulate small objects into the proper orientation. Use a pipette to add a drop of water (in some cases, you may add a drop of stain instead of water). Now put one edge of a cover glass into the edge of the drop of water and carefully lower the cover glass into the drop of water as shown in Figure 1. By carefully lowering the cover glass into the drop of water you should be able to prevent any air bubbles from being formed.



#### A. Instructions for the particular types of cells you will observe:

Human cheek cells -- the inside of your cheek is covered with epithelial cells. These cells are constantly worn away and replaced by new cells. Live cells are easily dislodged from the surface.

Have a slide and cover slip at hand. Gently scrape the inside of your cheek with a toothpick. Rub the scrapings onto the center of a microscope slide. These cells need to be stained with methylene blue to make them easily visible. Try to use about half a drop of methylene blue; just hold the squeeze-bottle over the slide and let a small drop dribble onto the cells. It is not necessary to add water to the slide. Place the cover glass as shown above.

**For each slide** that you observe follow steps 1 through 4: Using a pencil,

1. Draw a circle to represent the field of view. (Use a compass or a petridish.)
2. Carefully and accurately make a **scale drawing** of 2 or 3 representative cells.
3. Beside each drawing neatly record the following information:
  - a. total magnification
  - b. diameter of field of view
  - c. estimated number of cells that will stretch across the diameter of the field of view,
  - d. estimated length of an individual cell\*

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\*To figure the length of one cell, divide the number of cells that cross the diameter of the field of view into the diameter of the field of view.

Diameter of field of view = length of one cell Estimated number of cells that cross the diameter

For example, if the diameter of the field is 5 mm and you estimate that 50 cells laid end to end would cross the diameter, then  $5 \text{ mm} / 50 \text{ cells} = 0.1 \text{ mm/cell}$ . So, each cell would be 0.1 mm long.

A note about units of measurement: Items this small are often measured in microns (millionths of a meter) rather than millimeters (thousandths of a meter).

$1 \text{ micron} = 1/1000 \text{ mm}$  OR  $1 \text{ micron} = 0.001 \text{ mm}$

$1 \text{ mm} = 1000 \text{ microns}$

To convert from millimeters to microns, just multiply the measurement in millimeters by 1000.

For example, a cell that is 0.1 mm long is also 100 microns long. A cell that is 0.05 mm long is 50 microns long.

By using microns at this scale, one is often able to use whole numbers rather than decimals. When making your graph or solving the problem below, using microns as the unit of measurement will make your task easier.

IV. Construct a bar graph comparing the **lengths** of each cell type you have seen today.

### Practical 06:

### Recording of Microscopic Observations with the Help of Camera Lucid

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The Lucida camera, (Fig. 15.5), when attached with a Compound microscope, helps to draw microscope images of paper objects. It works on a simple optical principle that reflects light rays with a prism and a plane mirror.

A small image of the object is displayed by a prism on the plane screen and when from the image is displayed on the plane paper. The observer moves the pencil to the lines of the image and draws a precise and reliable picture of the object on paper.

There are three main parts of the camera lucida.

1. Ring attachment.
2. Prism.
3. mirror.

The attachment ring attaches the lucida of the camera and the body tunnel to the microscope. The prism sits above the eyeball when the tool is attached to a microscope.

The observer now sees a picture of an object under a microscope using a prism that displays the image horizontally on the plane screen. The plane's mirror, attached to the end of the arm, rotates, and is positioned at a  $45^\circ$  angle to the prism and plane paper.

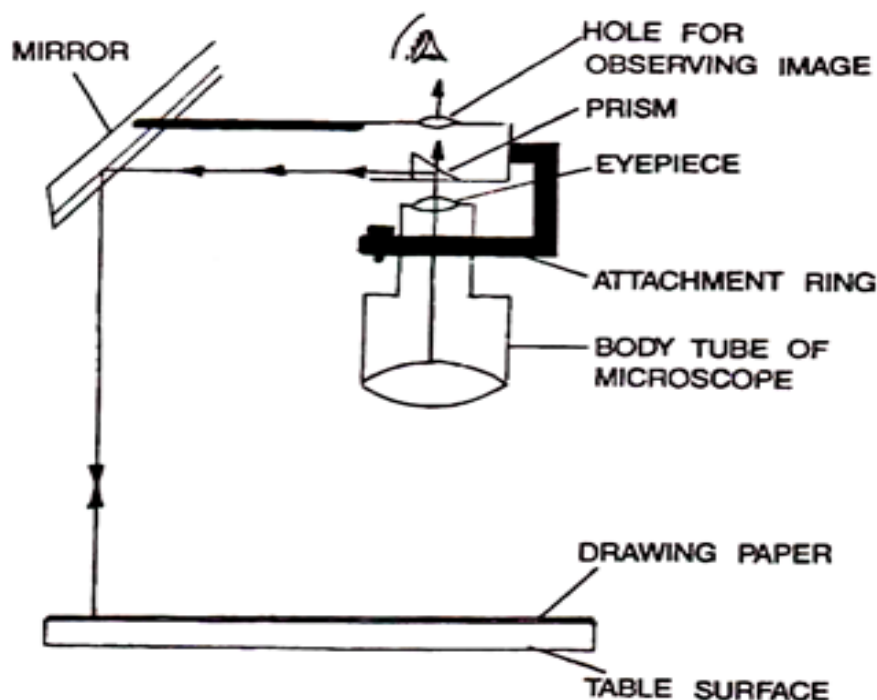


FIG. 15.5. Diagrammatic representation of a camera lucida.

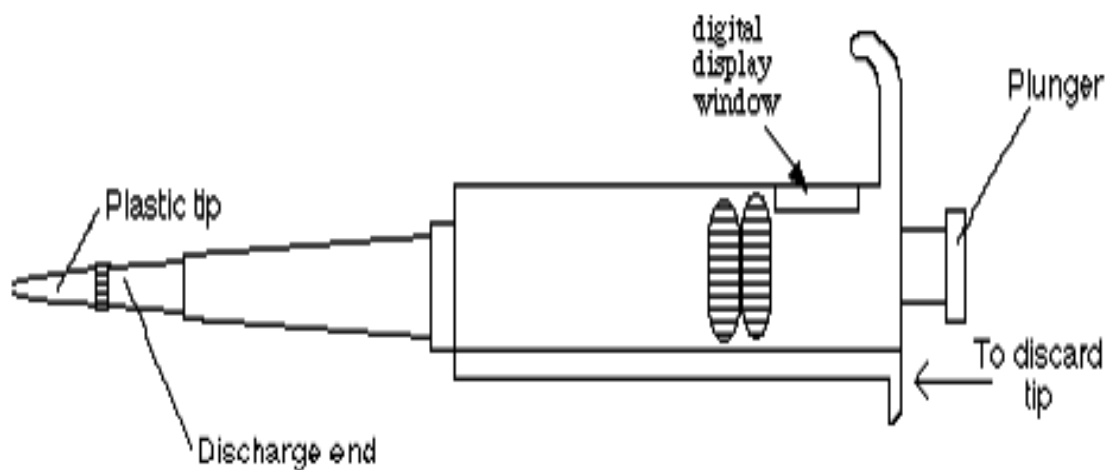
### Practical 07:

#### Liquid Handling: Proper Use of Pipettes.

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### Sample Delivery with Variable Automatic Micropipettes:

The micropipette is used to transfer small amounts ( $< 1\text{ ml}$ ) of liquids. The scales on micropipettes are in microliters ( $1000\mu\text{l} = 1\text{ ml}$ ). The brand of micropipettes we will be using is made by Rainin and called a "Pipetman". These are very expensive, delicate instruments costing \$250-300 apiece. We have four sizes identified by the number on the round button on the plunger. The value is the maximum volume in microliters that can be transferred with that size pipette. They are used in conjunction with disposable sterile plastic tips.



### The Pipetman

The following is an illustration of a micropipette:

### Protocol 08:

### Liquid Handling: Proper of Micropipettes.

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### Using a Micropipette:

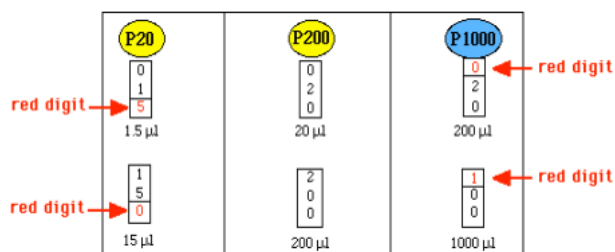


NEVER exceed the upper or lower limits of these pipettes. The limits are:

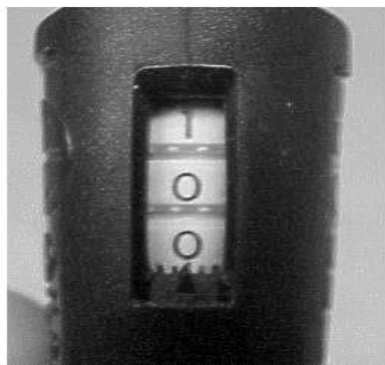
1. P10: 1.0 - 10.0  $\mu$ l
2. P20: 2.0 - 20.0  $\mu$ l
3. P200: 20 - 200  $\mu$ l
4. P1000: 200 - 1000  $\mu$ l

Look at the front face of the pipette and you will see a window with three digits inside. The diagram below shows the MAXIMUM value that can or should be dialed in on each size pipet. To exceed these values will put the pipet out of calibration. Beside each "window" below is the number it represents. Please take the time to learn how to read them so as to avoid damaging them by dialing values out of their range.

The examples below show how to read the volume on the micropipette:



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<http://abacus.bates.edu/~ganderso/biology/resources/pipet.html>

| P1000    |        | P200 or P100 |       | P20      |         |
|----------|--------|--------------|-------|----------|---------|
| <b>1</b> | 1000's | <b>2, 1</b>  | 100's | <b>2</b> | 10's    |
| <b>0</b> | 100's  | <b>0</b>     | 10's  | <b>0</b> | 1's     |
| <b>0</b> | 10's   | <b>0</b>     | 1's   | <b>0</b> | 1/10ths |

### Rule of Thumb:

- ❖ Always select the SMALLEST size pipette that will handle the volume you wish to move to achieve the greatest accuracy. Accuracy decreases as you use unnecessarily large pipettes for small volumes.
- ❖ Set the desired volume by turning the centrally located rings clockwise to increase volume or counterclockwise to decrease volume. P10: Maximum volume 10 $\mu$ l. Accurate between 1 $\mu$ l and 10 $\mu$ l. Numbers on the micropipette (typically black-black-red) are read as XX.X $\mu$ l. The change in color indicates the position of the decimal point. P20: Maximum volume 20 $\mu$ l. Accurate between 2 $\mu$ l and 20 $\mu$ l. Numbers on the micropipette (typically black-black-red) are read as XX.X $\mu$ l. The change in color indicates the position of the decimal point. P200: Maximum volume 200 $\mu$ l. Accurate between 20 $\mu$ l and 200 $\mu$ l. Numbers on the micropipette (one color) are read as XXX $\mu$ l. P1000: Maximum volume 1000 $\mu$ l (= 1 ml). Accurate between 200 $\mu$ l and 1000 $\mu$ l. Numbers on the micropipette (typically red-black-black) are read as X.XX ml. Note that this micropipette reads milliliters while the other two read microliters.
- ❖ Load a sterile tip. Use blue tips for P1000 pipettes and clear tips for P200 and all smaller sizes. Use filter tips when performing PCR or working with RNA. Close the tip box to maintain sterility. NOTE: Do not allow the pipette tip to touch any object (including your gloves, clothes, hair, skin, bench).

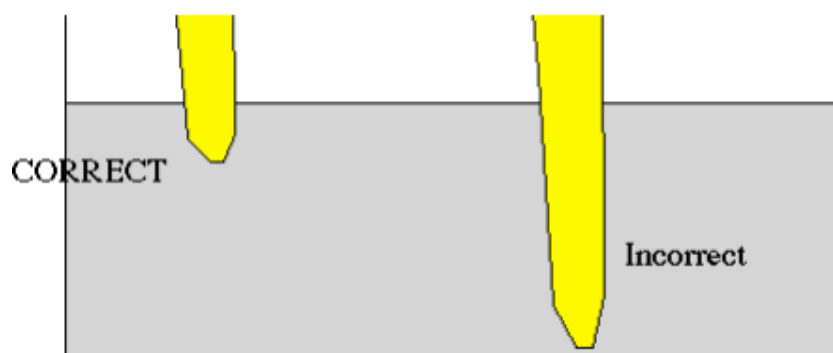
### Load the sample:

- ❖ The plunger will stop at two different positions when it is depressed. Push the plunger down

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slowly to the point of first resistance: this is the load volume. 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.

- ❖ While holding the plunger at the load volume set point, put the tip into the solution so that it is immersed just enough to cover the end (3-4mm), not as deep as possible.
- ❖ Slowly release the plunger to draw up the liquid making sure to keep the tip immersed. 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.
- ❖ Visually inspect the load to make sure it is correct - there should be no air space in the distal end tip



- ❖ Deliver the sample. 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 pipette. 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 pipette, only when expelling the last drop. To deliver the volume,
  - Place the tip into the receiving vessel.
  - Depress the plunger to the point of initial resistance
  - Wait 1 second
  - Continue to press the plunger all the way to the bottom - this expels all the liquid.
  - THEN, WITHOUT RELEASING THE PLUNGER, withdraw the tip.
- ❖ Discharge the tip. While holding the tip over an appropriate waste receptacle, press the discharge slider on the back of the grip.

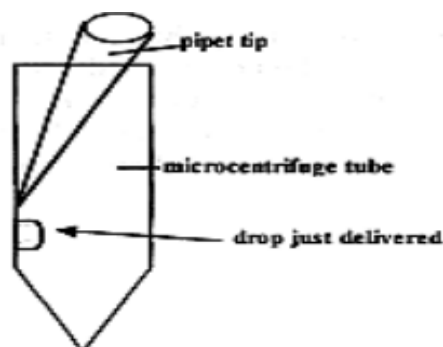
### Small Volumes Technique:

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With small volumes, especially the 1-

10  $\mu$ l range, you must keep track of the droplets your pipet.

Carefully expel the liquid droplet on the sidewall of the tube so that you can see it, drawing the tip away/out carefully BEFORE releasing the plunger. If adding to a larger volume, flush the tip with the solvent liquid after expelling the droplet to make sure you get all the delivery liquid. With small volumes you'll usually need to centrifuge and then



vortex the tube to get a good mixing of the reagents.

### **A Simple Check for Proper Calibration:**

Check the calibration of your micropipette by using the fact that 1 ml of deionized (or distilled) water has a mass of 1 g. Pipet a range of volumes spanning the pipette's useable range and weigh them on a top loading balance having at least 3 decimal place accuracy. Pipets having greater than 5% error should be recalibrated.

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### **Protocol 09:**

#### **Hematoxylin and Eosin Staining (Harris' Hematoxylin and Eosin).**

H&E stain, HE stains or hematoxylin and eosin stain, is a popular staining method in histology. It is the most widely used stain in medical diagnosis. For example, when a pathologist looks at a biopsy of a suspected cancer, the histological section is likely to be stained with H&E and termed H&E section, H+E section, or HE section.

The staining method involves application of hemalum, which is a complex formed from aluminum ions and oxidized hematoxylin. These colors nuclei of cells (and a few other objects, such as keratohyalin granules) blue. Materials colored blue by hemalum are often said to be basophilic, but this is an incorrect use of the word. The nuclear staining is followed by counterstaining with an aqueous or alcoholic solution of eosin Y, which colors eosinophilic other structures in various shades of red, pink and orange.

#### **Harris' Hematoxylin and Eosin (H&E) Staining Protocol**

##### **Solutions and Reagents**

###### **Acid Alcohol Solution (1%)**

- Hydrochloric acid, 1 ml
- 70% ethanol, 50 ml
- Mix well.

###### **Ammonia Water Solution (0.2%)**

- Ammonium hydroxide (concentrated), 2 ml
- Distilled water, 1000 ml
- Mix well.

###### **Lithium Carbonate Solution (Saturated):**

- Lithium carbonate 1.54 g
- Distilled water 100 ml
- Mix well

###### **Eosin-Phloxine B Solution**

Prepare the stock solutions first, and then create the working solution as needed.

###### **Eosin Stock Solution**

- Eosin Y, 1 g
- Distilled water, 100 ml
- Mix to dissolve.

###### **Phloxine Stock Solution**

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- Phloxine B, 1 g
- Distilled water, 100 ml
- Mix to dissolve.

### **Eosin-Phloxine B Working Solution**

- Eosin stock solution, 100 ml
- Phloxine stock solution, 10 ml
- Ethanol (95%), 780 ml
- Glacial acetic acid, 4 ml
- Mix well.

### **Hematoxylin Solution (Harris)**

Potassium or ammonium (alum), 100 g Distilled water, 1000 ml

1. Heat to dissolve. Add 50 ml of 10% alcoholic hematoxylin solution and heat to boil for 1 minute.
2. Remove from heat and slowly add 2.5 g of mercuric oxide (red).
3. Heat to the solution and until it becomes dark purple color.
4. Cool the solution in cold water bath and add 20 ml of glacial acetic acid (concentrated).
5. Filter.

### **Staining Procedure**

1. Deparaffinize sections, 2 changes of xylene, 10 minutes each.
2. Re-hydrate in 2 changes of absolute alcohol, 5 minutes each.
3. 95% alcohol for 2 minutes and 70% alcohol for 2 minutes.
4. Wash briefly in distilled water.
5. Stain in Harris hematoxylin solution for 8 minutes.
6. Wash in running tap water for 5 minutes.
7. Differentiate in 1% acid alcohol for 30 seconds.
8. Wash running tap water for 1 minute.
9. Bluing in 0.2% ammonia water or saturated lithium carbonate solution for 30 seconds to 1 minute.
10. Wash in running tap water for 5 minutes.
11. Rinse in 95% alcohol, 10 dips.
12. Counterstain in eosin-phloxine solution for 30 seconds to 1 minute.
13. Dehydrate through 95% alcohol, 2 changes of absolute alcohol, 5 minutes each.
14. Clear in 2 changes of xylene, 5 minutes each.
15. Mount with xylene based mounting medium.

### **Results:**

Nuclei should be blue, cytoplasm pink to red.

### **Practical 10:**

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### Gram's Staining.

This is the most important differential staining technique in bacteriology. It enables us to divide bacteria into two distinct groups, Gram-positive and Gram-negative, according to a particular staining procedure (the technique is given a capital letter, since it is named after its originator, H.C. Gram). The basis of the staining reaction is the different structure of the cell walls of Gram-positive and Gram-negative bacteria.

Heat fixation of air-dried bacteria causes some shrinkage, but cells retain their shape. Gram staining should be carried out using light smears of young, active cultures, since older cultures may give variable results. In particular, certain Gram-positive bacteria may stain Gram-negative if older cultures are used.

### Safety Notes Which we have to care about in Gram Staining:

1. The Gram-staining procedure involves toxic dyes and flammable solvents: avoid skin contact and extinguish any naked flames (e.g. Bunsen).
2. The catalase and oxidase reagents are irritants and could be harmful if swallowed. Avoid skin contact and ingestion.

### Assessing the Gram status of an unknown bacterium:

If a pure culture gives both Gram-positive and Gram-negative cells, identical in size and shape, it can be regarded as a Gram-positive organism that is demonstrating Gram-variability.

### Preparation of a heat-fixed, Gram-stained smear

#### Preparation of a heat-fixed smear.

The following procedure will provide you with a thin film of bacteria on a microscope slide, for staining.

1. Take a clean microscope slide and pass it through a Bunsen flame twice, to ensure it is free of grease. Allow to cool.
2. Using a sterile inoculating loop, place a single drop of water in the center of the slide and then mix in a small amount of sample from a single bacterial colony with the drop, until the suspension is slightly turbid. Smear the suspension over the central area of the slide, to form a thin film. For liquid cultures, use a single drop of culture fluid, spread in a similar manner.
3. Allow to air-dry at room temperature, or high above a Bunsen flame: air-drying must proceed gently, or the cells will shrink and become distorted.
4. Fix the air-dried film by passage through a Bunsen flame. Using slide holder or forceps, pass the slide, film side up, rapidly through the hottest part of the flame (just above the blue cone). The temperature of the slide should be just too hot for comfort on the back of your

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hand: note that you must not overheat the slide or you may burn yourself (you will also ruin the preparation).

5. Allow to cool: the smear is now ready for staining.

### **Gram-staining procedure:**

The version given here is a modification of the Hucker method, since acetone is used to decolorize the smear.

#### **Note:**

Note that some of the staining solutions used are flammable, especially the acetone decolorizing solvent, you must make sure that all Bunsens are turned off during staining. The procedure should be carried out with the slides suspended over a sink, using a staining rack.

1. Flood a heat-fixed smear with 2% w/v crystal violet in 20% v/v ethanol: water and leave for 1 min.
2. Pour off the crystal violet and rinse briefly with tap water. Flood with Gram's iodine (2 g KI and 1 g I<sub>2</sub> in 300 mL water) for 1 min.
3. Rinse briefly with tap water and leave the tap running gently.
4. Tilt the slide and decolorize with acetone for 2–3 s: acetone should be added dropwise to the slide until no color appears in the effluent. This step is critical, since acetone is a powerful decolorizing solvent and must not be left in contact with the slide for too long.
5. Immediately immerse the smear in a gentle stream of tap water, to remove the acetone.
6. Pour off the water and counterstain for 10–15 s using 2.5% w/v safranin in 95% v/v ethanol: water.
7. Pour off the counterstain, rinse briefly with tap water, then dry the smear by blotting gently with absorbent paper: all traces of water must be removed before the stained smear is examined microscopically.
8. Place a small drop of immersion oil on the stained smear: examine directly (without a coverslip) using an oil-immersion objective.

Gram-positive bacteria retain the crystal violet (primary stain) and appear purple while Gram-negative bacteria are decolorized by acetone and counterstained by the safranin, appearing pink or red when viewed microscopically. Other decolorizing solvents are sometimes used, including ethanol: water, ethyl ether: acetone and acetone: alcohol mixtures. The time of decolorization must be adjusted, depending upon the strength of the solvents used, e.g. 95% v/v ethanol: water is less powerful than acetone, requiring around 30s to decolorize a smear.

### **Staining bacterial spores:**

These are relatively impermeable to dyes, and require heat treatment and specialized stains.

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### **Performing the oxidase test:**

Never use a nichrome wire loop, as this will react with the oxidase reagent, giving a false positive result.

This Gram-variability is due to autolytic changes in the cell wall of Gram- positive bacteria. Developing spores are often visible as unstained areas within older vegetative cells of *Bacillus* and *Clostridium*. Other stains are required to demonstrate sub-cellular structures such as, capsules or flagella.

### **Practical 11: Handling of Centrifuge Machines.**

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The Centrifuge is a ubiquitous instrument in biomedical laboratories and a basic knowledge of the theory of centrifugation is more than useful. Centrifuge performance can be classified as low-speed, high-speed and ultra-speed. Usual applications include the separation of serum or plasma from red blood cells, the separation of precipitated solids from the liquid phase of a mixture, or the separation of liquids of varying density.

### **Principles of Centrifugation:**

Particles suspended in a fluid move, under the influence of gravity, towards the bottom of a vessel at a rate that depends, in general, on their size and density. Centrifugation is a technique designed to utilize centrifugal forces, which are greater than the force of gravity, to speed up the sedimentation rate of particles. This is achieved by spinning the vessel containing the fluid and particles about an axis of rotation so that the particles experience a force acting away from the axis. The force is measured in multiples of the Earth's gravitational force and is known as the relative centrifugal field (RCF) or, more commonly, the 'g' force.

### **Types of Centrifuges**

#### **Low-Speed Instruments:**

Low-speed centrifuges have maximum rotor speeds of less than 10,000 rpm, which do not require the rotors to be run in a vacuum, and there are instruments with a temperature control facility. Most instruments now include a sensor that will detect any imbalance when the rotor is running and cut off power to the drive mechanism if imbalance is present. Low-speed instruments are used to separate serum or plasma from red blood cells, and to harvest and purify chemical precipitates, intact cells, nuclei, large mitochondria and large plasma-membrane fragments.

#### **High-Speed Instruments:**

In general, high-speed centrifuges are capable of rotor speeds up to 21,000 rpm, although the new generation of super-speed instruments are capable of rotor speeds of 30,000 rpm, in which RCFs of 120,000 xg are possible. These instruments require refrigeration systems to overcome the heat generated by the friction of the spinning rotor, and the higher-speed machines must incorporate vacuum systems. High-speed centrifuges are used in the separation of a number of cell constituents and in the isolation and purification of viruses.

#### **Ultracentrifuges:**

Ultracentrifuges are capable of speeds in excess of 30,000 rpm and RCFs of over 600,000 xg.

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They can be used in the isolation and purification of membrane components such as the endoplasmic reticulum and Golgi membrane, endosomes, ribosomes, DNA and RNA. Once again, refrigeration and vacuum systems are necessary.

### Instrument Components

#### Rotor:

The design of most centrifuges allows the drive system to accept rotors of different sizes and capacities, although most instrument rotors are now capable of accepting a large range of tube sizes through the use of adaptors. Rotors have three basic designs: horizontal (pic. 1), in which the tubes are carried in buckets that can swing outwards to a horizontal position and can operate at speeds to about 3000 rpm; fixed angle (pic. 2), in which the sample tubes are held at a fixed angle to the vertical position and can attain much higher speeds (approximately 7000 rpm) because of the aerodynamic construction of the rotor; and vertical (pic. 3), in which the tubes are fixed in the vertical position. In general, the horizontal rotor offers advantages to the clinical laboratory because sedimentation of large particles (e.g. red blood cells) is efficient at low force and because a flat sediment is produced.

The load on the rotor should always be balanced before operating the centrifuge, particularly when using high-speed instruments in which the buckets and caps are often numbered so that they can be matched on opposite sides of the rotor.

The load must be balanced both by equal mass and by centers of gravity across the center of rotation. Thus, it is important not to run the centrifuge with buckets, carriers or shields missing from the unit, and not to exceed the maximum rated speed of the rotor in use.

Most modern rotors have microprocessor-controlled automatic rotor identification so that it is impossible to set the speed beyond the safety limit for that rotor.

Horizontal Rotor

Centrifuge fixed angle rotor

Centrifuge vertical rotor



#### Motor:

In general, centrifuge motors are high-torque, series-wound DC motors, the rotation of

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which increases as the voltage is increased. The rotor shaft is driven directly or through a gyro, although occasionally a pulley system is used. Electrical contact to the commutator is provided by graphite brushes, which gradually wear down as they press against the commutator turning at high speed, and thus should be replaced at specified intervals. Modern centrifuges have induction drive motors that have no brushes to change. The shaft of the motor turns through sleeve bearings located at the top and bottom of the motor. Most instruments contain sealed bearings that are permanently lubricated, while others require periodic application of oil or grease.

The speed of the centrifuge is controlled by a potentiometer that raises and lowers the voltage supplied to the motor. The calibrations on the speed control are often only relative voltage increments and should never be taken as accurate indicators of speed. Therefore, periodic recalibration is required.

### **Imbalance detector:**

Some instruments have an internal imbalance detector that monitors the rotor during operation, causing automatic shutdown if rotor loads are severely out of balance.

### **Tachometer:**

A tachometer indicates the speed in rpm. Most modern centrifuges use electronic tachometers, in which a magnet rotates around a coil to produce a current that can be measured.

### **Safety lid:**

Modern centrifuges must have a door-locking mechanism to prevent the lid from being opened while the instrument is running. If there is a power failure or the safety latch fails for some reason it may be necessary to trip the door-locking mechanism manually to retrieve the samples. Manufacturers' instructions should be checked for the exact procedure required.

### **Refrigerator:**

A centrifuge generates heat as it rotates and if samples are temperature labile then a refrigerated centrifuge should be used. Some centrifuges enable the rotor and chamber to be precooled before run.

### **Braking System:**

Braking devices are incorporated to provide rapid rotor deceleration. Modern instruments have an electrical braking system that functions by reversing the polarity of the electrical current to the motor. Other machines may have a mechanical brake.

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### **Centrifuge Tubes:**

It is advisable to use a conical-bottomed tube in a swing-out bucket rotor for the sedimentation of cells. This tube type will retain the pellet of cells more effectively as the supernatant is removed. All tubes for use with high-speed motors are round-bottomed. Pyrex glass tubes can withstand forces of around 2000 xg, while Corextubes can be used up to 12,000 xg. Polycarbonate or polyallomer are the most common plastic tubes in use but great care must be taken when using organic solvents. Manufacturers usually provide extensive information about solvent, salt and pH resistance, as well as sterilization procedure.

### **Centrifuge Use:**

There are a few important guidelines for operating a centrifuge, even a small one. Following them can prevent damage to the centrifuge and possible serious injury to you and others. The work surface must be level and firm. Do not use the centrifuge on an uneven or slanted work surface. Balance the tubes in the rotor! If you want to run a tube with 10 mL of liquid, put another tube with 10 mL of water in the opposing hole on the rotor. If the liquid has a higher or lower density than water, you must balance the tubes by mass, not volume.

Do not open the lid while the rotor is moving. Even though many centrifuges have a "safety shutoff" if the lid is opened, the only thing this does is stop powering the rotor. The rotor will still spin due to its own inertia for a while until friction slows and eventually stops it. If you see it wobbling or shaking, turn it off or pull the plug. A little vibration is normal, but excessive amounts can mean danger. FIRST, double check that you correctly balanced the tubes. If the answer is yes and the wobbling still happens, contact the manufacturer or dealer and get the unit serviced. Do NOT continue to run a centrifuge that wobbles visibly when the rotor is spinning.

Wear a face shield and / or safety goggles if you have to work anywhere near a centrifuge that's in use. Do not bump, jar, or move the centrifuge while the rotor is spinning. Make sure you don't have the cord dangling from a table edge where someone could catch their foot in it and pull down the centrifuge.

### **Preventive Maintenance:**

If the bearings on the upper and lower ends of the motor shaft are not of the sealed type then they should be lubricated as per the manufacturer's instructions.

Brushes should be removed regularly and checked for wear; they should be replaced if they are worn to more than one-half of their original length. When reinserting used brushes, replace them in the same orientation. New brushes should be broken in by slowly accelerating the unloaded unit to mid-speed and then allowing it to run for a period of time.

The rotor, buckets and shields or carriers should be examined for signs of mechanical stress (eg cracks, corrosion). Some manufacturers etch the expiry date on the rotor and this should be checked periodically.

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Regularly lubricate the contact areas between the centrifuge buckets and the pins.

Regularly check the condition of the O-ring on the tie-down nut on top of the rotor, and replace it if worn or damaged.

Always follow a manufacturer's specific instructions.

### **Practical 12:**

#### **Paper Chromatography of Amino Acids.**

##### **Materials Required:**

Whatman filter paper (12x22 cm), Butan-1-ol, Acetic acid, ninhydrin spray (2% solution of

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ninhydrin in ethanol), Capillary tube, beaker (500 ml), oven.

### Theory

Chromatography is an analytical method used for separation of different biomolecule on the basis of their chemical properties. In paper chromatography, a biomolecule (or mixture of biomolecules) is spotted on a piece of filter paper and is placed in an organic solvent. The hydrophobic organic solvent moves up the paper by capillary action. As the solvent reaches the biomolecule, the biomolecule starts to move up the paper. The degree of movement of biomolecule up the paper is associated to its relative affinity for paper (hydrophilic in nature) and the solvent (hydrophobic in nature).

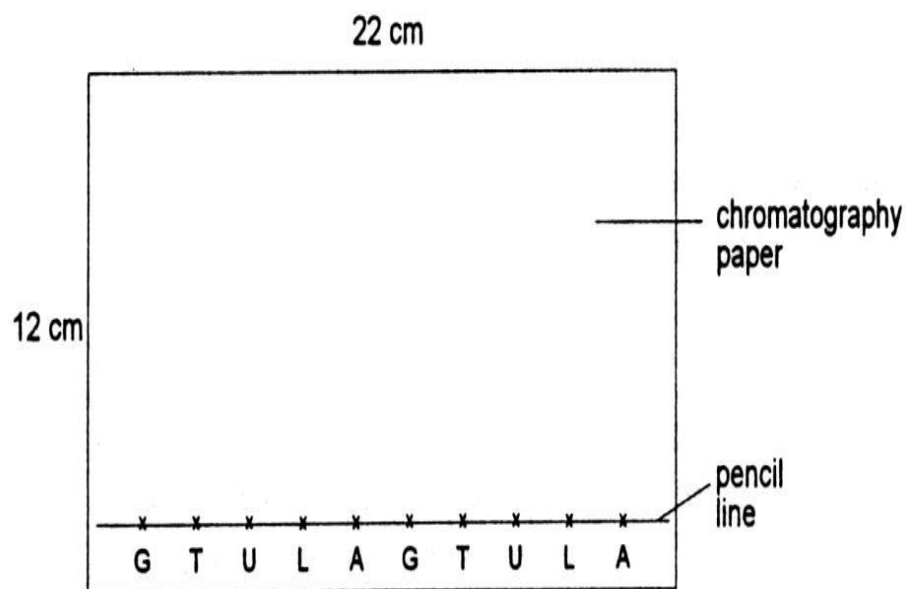
Paper chromatography is very valuable method for characterizing amino acids. Different amino acids migrate at different rates on the paper because of difference in their R groups. The rate of movement of a biomolecule during paper chromatography is known as its relative mobility (Rf). Rf is also known as retardation factor. Rf is simply “the distance the biomolecule moved through the filter paper divided by the distance the moved by solvent through the paper”.

$$R_f = \text{Distance travelled by sample} / \text{Distance travelled by solvent}$$

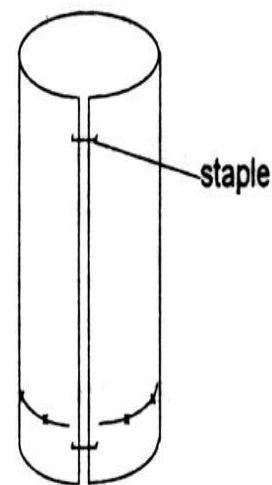
### Procedure:

- 1) Place enough chromatography solvent [Butan-1-ol: Acetic acid: Water in 60: 15: 25 ratios] in tank or beaker to achieve a depth of about ½ inch. Cover tank and allow solvent to saturate the atmosphere in the tank for at least 30 minutes.
- 2) Draw a line across the bottom of the chromatography sheet about 2.5 cm from the bottom edge of the chromatography paper with the aid of lead pencil.
- 3) Take a small volume of amino acid into the capillary and deposit it onto the paper by touching the capillary to the line drawn. The spot should not be larger than ¼ inch in diameter and the two spots should be separated by 2 cm. Allow the spots to dry.
- 4) After drying, roll the paper into a cylinder and staple so that the ends do not touch.

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**Figure 1**



**Figure 2**

### **Development of the Chromatogram:**

1. Place the cylinder (chromatography paper) in a chromatography chamber/beaker (under the hood). Cover the chamber and allow the solvent to migrate up the paper for 60-90 minutes or till the solvent line is about an inch from the top of the sheet.
2. Remove the chromatography paper from the tank and mark the solvent front line with pencil. Allow it to dry.
3. Spray the paper with Ninhydrin solution (used to detect the location of amino acids).
4. Dry the chromatography paper in a drying oven for about 100°C for 3-4 minutes to allow the color to develop. Amino acids give blue/purple color when they react with ninhydrin.
5. Measure the distance the solvent migrated and the distance each of the amino acids migrated. Calculate the relative mobility (R<sub>f</sub>)/retardation factor for each amino acid.

$R_f = \text{distance the amino acid migrated} / \text{distance the solvent migrated}$

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### Practical 13:

### Thin Layer Chromatography of Amino Acids.

#### Materials Required:

#### Reagents:

1. 2% solution of individual amino acids.
2. Solvent mixture of normal butanol, acetic acid and water in the ratio 12:3:5 by volume.
3. Ninhydrin reagent.

#### Requirements:

1. TLC plate.
2. TLC chamber.
3. Capillary tubes.
4. Reagent spray bottle.
5. Conical flasks.
6. Beakers.

#### Procedure:

1. Pour the solvent mixture into the TLC chamber and close the chamber.
2. The chamber should not be disturbed for about 30 minutes so that the atmosphere in the jar becomes saturated with the solvent.
3. Cut the plate to the correct size and using a pencil (never use an open) gently draw a straight line across the plate approximately 2 cm from the bottom.
4. Using a capillary tube, a minute drop of amino acid is spotted on the line.
5. Allow the spot to dry.
6. Spot the second amino acid on the plate [enough space should be provided between the spots].
7. Repeat the above step for spotting the unknown acid.
8. Place the plate in the TLC chamber as evenly as possible and lean it against the side (immerse the plate such that the line is above the solvent). Allow capillary action to draw the solvent up the plate until it is approximately 1 cm from the end.
9. Remove the plate and immediately draw a pencil line across the solvent top.
10. Under a hood dry the plate with the aid of a blow dryer.
11. Spray the dry plate with ninhydrin reagent.
12. Dry the plates in hot air oven at 105°C for 5 min. [Ninhydrin will react with the

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faded spots of amino acids and make them visible as purple colored spots.]

13. After some time, mark the center of the spots, then measure the distance of the center of the spots from the origin and calculate the R<sub>f</sub> values.

R<sub>f</sub> value can be calculated using the formula:

$$R_f = \frac{\text{distance moved by the substance from origin}}{\text{distance moved by solvent from origin}}$$

The R<sub>f</sub> values with butanol-acetic acid-water solvent are as follows: alanine 0.24, glutamic acid 0.25, glycine 0.2, leucine 0.58, valine 0.4, lysine 0.58, tyrosine 0.42.

### Differences Encountered in a Real Laboratory:

In an actual laboratory setting, there are certain important steps that are not necessarily applicable in a virtual lab.

1. Always wear lab coat and gloves when you are in the lab. When you enter the lab, switch on the exhaust fan and make sure that all the reagents required for the experiment are available. If it is not available, prepare the reagents using the components shown in the reagent preparation.
2. Care should be taken while handling reagents like Ninhydrin reagent. This reagent is a strong oxidizing agent and should not be inhaled or spilled on hands or other body parts. Accidental spill of this reagent will cause severe itching sensation. Wash the spilled area with cold water and inform the lab assistant immediately.
3. Hold the TLC plates by their side. Ensure that you do not touch the developing part of the TLC plate, because your fingerprints will also get developed causing the result to be unclear.
4. Make certain that the spots applied to the plate are above the surface of the eluting solvent.
5. Before applying the second spot make sure that the previously applied spot is dried.
6. Spot the components with proper space in between.
7. Ensure that the chamber is saturated with the solvent vapor before you place the TLC plate in it.
8. Given enough time for the solvent to advance up the plate.

The top of the solvent must not advance up to or beyond the edge of the plates.

### Practical 14:

#### Spectrophotometric Estimation of Glucose.

Glucose Color Reagent and the Glucose Standard are irritants. Hydrochloric acid is a corrosive. Use gloves and goggles.

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### Materials:

Spectrophotometer (340-600 nm), 0.1, 1.0, and 10 mL serological pipettes, 15 x 125 mixing tubes cuvettes, 0.1 N Hydrochloric acid, Glucose Kit (Sigma 115-A), 500 mg/dl Glucose standard (Sigma G3761), Grape Kool-Aid, test solution Blank 1, Blank 2 Grape Kool-Aid Glucose.

### Introduction:

Diabetes mellitus is a serious incurable disease that affects approximately 16 million U.S. citizens, although only 10 million have been diagnosed. It is a major cause of death in the United States, and its complications include kidney failure, blindness, and lower limb amputations. Diabetes mellitus is caused by the inability of body cells to uptake glucose. This inability can be caused either by low levels of insulin, a hormone necessary for the movement of glucose across cell membranes, or by a defect in the insulin-binding receptors on cell membranes. Glucose is a monosaccharide that is used by cells as an energy source, although when it is not available, most cells in the body use fatty acids as their fuel. After a meal, glucose is absorbed into the bloodstream, elevating the concentration of glucose found in the blood. Insulin is released as a result, and all cells switch to burning glucose. Diabetes is diagnosed by measuring the amount of glucose in blood after an 8-hour fast from food. In normal healthy individuals, the fasting glucose ranges between 70 – 110 mg/dl. If the fasting blood glucose level is high (greater than 126 mg/dl on two different occasions) and symptoms such as frequent urination or excessive thirst are present, a diagnosis of diabetes is made.

### Procedure:

#### Spectrophotometric Technique

1. Plug in the spectrophotometer and turn it on by turning the left dial clockwise. Allow the spectrophotometer to warm up at least 5 minutes before proceeding.
2. Set the wavelength dial to 600 nm. This is a wavelength of light that gets absorbed readily by the pigment in the Grape Kool-Aid.
3. With no vial in the spectrophotometer, the light path is closed. Therefore, no light is transmitted, or in other words there is an infinite absorption. With the on/off dial (left dial) set the needle to read infinity ( $\infty$ ) on the absorbance scale.
4. Now fill a cuvette (a small test tube that is 12 mm in diameter and 100 mm in length) with Blank 1. This solution should contain all the constituents except the substance to be measured. Since we are measuring Grape Kool-Aid, Blank 1 contains the other constituents, sugar and water, in the same proportion as are found in the Grape Kool-Aid.
5. Insert the cuvette containing Blank 1 into the sample chamber. As you do the light path will be

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opened. This blank solution does not contain any Grape Kool-Aid, and so the absorbance should be set to zero. Use the right knob to set the absorbance to zero.

6. Next fill a cuvette with Grape Kool-Aid test solution. Insert it into the spectrophotometer and record its absorbance in question number one of your assignment.
7. Next, fill a cuvette with Blank 2 solution and repeat steps 5 and 6. Is there a difference in the measurements of the same solution? How can you explain it?

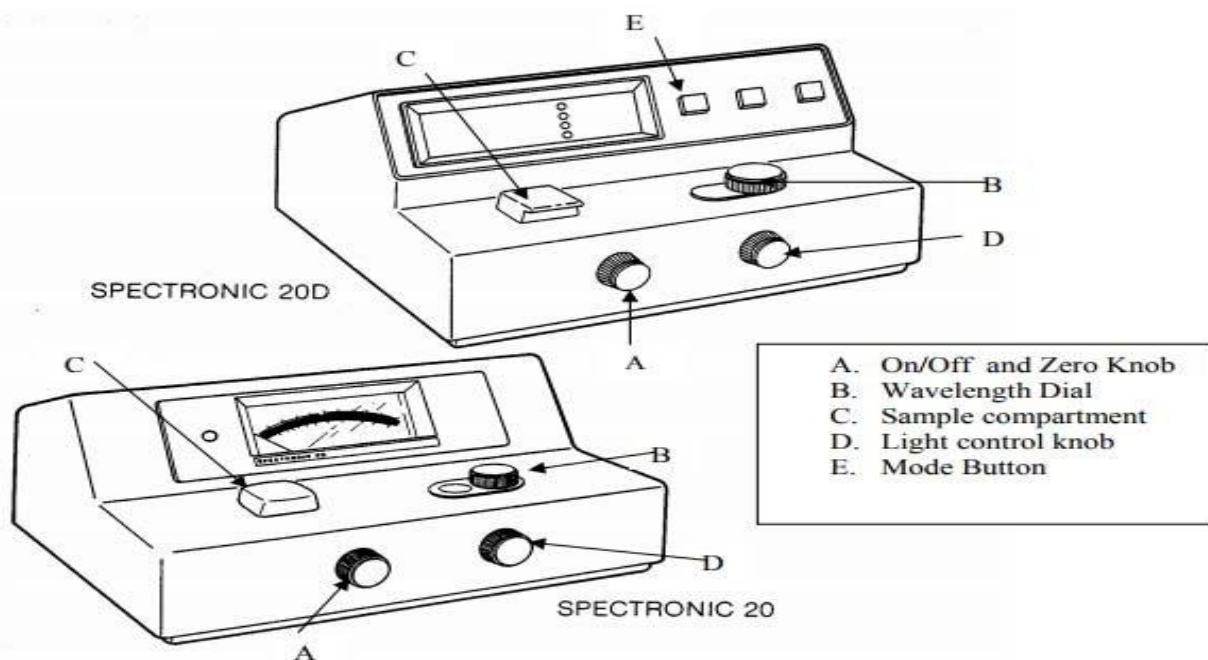


Fig. 2-2. Diagram of Spec 20 and Spec 20D Spectrophotometers

### Glucose Assay:

Now we will use the spectrophotometer to measure the concentration of glucose (sugar) in Kool-Aid. When you measured the absorbance of Kool-Aid, you made a direct measurement. The Kool-Aid was a colored product that the spectrophotometer could easily measure. In the glucose assay we will use, you will not measure the concentration of glucose directly. Instead, you will measure the absorbance of colored product created when glucose reacts with the Glucose Assay Reagent. The amount of colored substance is directly proportional to the amount of glucose present. You will create a standard curve that translates the absorbance of the colored substance into concentration of glucose and will use it to determine the amount of glucose in Kool-Aid.

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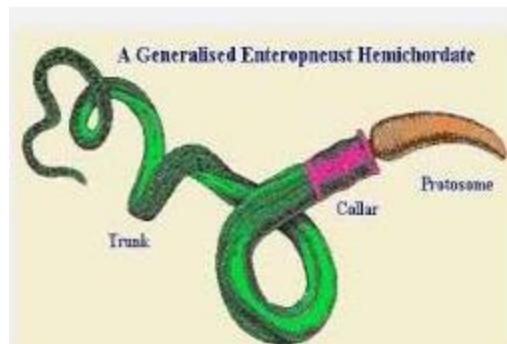
### Practical 15:

### Preservation of Representative Animals of Various Phyla(Vertebrates).

#### Lower Chordates

#### Hemichordata

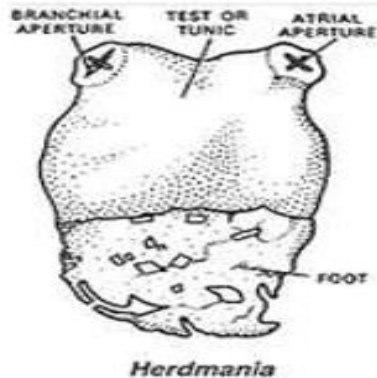
- For narcotization, place specimens in containers of sea water and permit them to expand completely.
- Then add, drop by drop over a period of an hour or more enough absolute alcohol to make a 10% solution.
- Complete relaxation usually takes from 4 to 6 hours. When insensible the specimen may be killed in 5% formalin.
- Specimens may be preserved either in 70% ethyl or isopropyl alcohol or 5% formalin.



#### Urochordata

- For narcotization expand specimens in a large volume of sea water and then
- Add a small quantity of chromacetic fixative to the surface of the water. This will diffuse into the water and kill specimens within 30 minutes to 24 hours.
- Formalin may be tried in place of chrom-acetic fixative.
- The water is then siphoned off without disturbing the specimens and large quantities of chromacetic fixative or 5% formalin is added for its preservation.

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### Fishes

#### Killing fishes

- Leave the fish in high doses of the anaesthetizing solution. anaesthetized fish relax and can be preserved in a more natural state. Fixation
- Specimen, is placed in a wide-mouthed glass, filled with the fixative solution.
- Fish should be inserted into jars head first to make them easier to remove from the jars in the laboratory.
- Care must be taken not to pack too many fish in one jar. The specimen should float freely in the jar to avoid curling or bending.
- Before immersing large specimens, fixative should be injected directly into the body cavity to facilitate penetration and preservation of the internal organs.
- Optimum fixation times for fish under six inches in length is one to two weeks. For fish over six inches in length preservation time ranges from two to four weeks

#### Washing

Following fixation, the specimens are thoroughly rinsed and put into 50 % isopropyl alcohol. The decanted formalin can be reused. The specimen can be rinsed by putting the jar under running water. Alternately, the jar is filled with water. After several hours, the water is decanted and fresh water is added. This is repeated for several days until no formalin odor can be detected. If during rinsing, the specimen shows signs of deterioration, it is transferred directly to 50% isopropyl alcohol for final preservation.

#### Preservation

- 10% formalin - slightly acidic and will de-calcify and soften bony structures. The addition of a buffering agent helps to slow down this process.
- Alcohols, such as ethanol and iso-propanol, are also commonly used to fix and preserve fish specimens, especially if skeletal structures such as otoliths are to be examined.
- Freeze in dry ice or liquid nitrogen. This is one of the best methods to preserve the colors and tissues of the specimen.

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### Amphibia

- **Fixative** – Formalin
- **Washing** – in alcohol to soak out the formalin before transfer. The alcohol should be changed after 24 hours
- **Preservation**- The following solutions are quite satisfactory for preservation; - 5% formalin; - 70% ethyl alcohol or - 40 to 50% isopropyl alcohol.



### Reptiles- Snakes and Lizards

#### Killing

- Drowned in warm water, but this is a slow process
  - Injecting them with 10% Nembutal solution/ ether/ chloroform Fixation - Fixation requires from 48 hours to 1 week.
  - **Small lizards** - Formalin injected in the body cavity or through a cut made on the left ventral side of the body.
  - **Larger lizards** - should also be injected in each leg segment and just underneath the skin at the base of the tail.
  - **Snakes** - injected with 10% formalin every inch along the length of the cavity, or else they are cut on the left ventral surface of the body. These cuts should measure 1 and 2 inches in length, should penetrate into the body cavity and should be about 1 inch apart.
- Preservation
- **Small and medium size reptiles** - 6% formalin; large specimens 10% formalin.

### Reptiles – Turtles and Crocodiles Turtles

- The specimens are killed by the same method as that of snakes and lizards.

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- For preserving small specimens, when the specimen is dead the head, tail, and limbs are pulled out of the shell so that they are exposed.
- Slits are made between the neck and forelimbs and between the tail and hind limbs. Formalin is injected into the head, neck and limbs.
- The specimen is placed directly in a jar with 10% formalin. After several days the solution may be replaced by 6 to 8% formalin solution.
- Big sized specimens may be kept as dried or wet dry specimens.

### Crocodilians

Small specimens should be positioned, fixed and preserved like lizards' Large specimens are skinned.

### Birds

- **Study skins:** The most traditional preparation is a study skin, in which almost all of the body inside the skin is removed and replaced with cotton so that the final result resembles a bird lying on its back with its wings folded. This stereotypic posture was developed to enable many skins to be kept together in cabinets to protect them from insect and light damage.
- **Complete skeleton:** If a complete skeleton is desired a flat skin may be prepared: all bones, muscle, digestive and other soft tissue is carefully removed and the feathers and skin are stretched flat and dried.
- **ROM:** A more recent preparation method pioneered by the Royal Ontario Museum removes all bones for a complete skeleton while also producing a round skin without bill or legs called a ROM.
- **Shmoo:** If one set of wing and leg bones remain with the skin the preparation is called a shmoo in North America.



### Mammals

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- Entire fluid-preserved animals (for studying anatomy and histology)
- Study skins with accompanying skulls / partial skeletons (some bones remain in the skin), for studying pelage color, hair quality and molting patterns.
- Mounted skins with accompanying partial or entire skeleton (some bones may remain in the skin, dependent on the method of preservation) or freeze-dried specimens,
- Entire skeletons, for studying anatomy, geographic variation or for age determination. At least one male and one female skeleton per species is recommended.

### **Field techniques**

- In field the specimen is preserved in 10 % buffered formalin (tissue - formalin solution ratio of at least 1:12) or 70-90% alcohol.
- For preservation in formalin the body cavity can be filled with formalin solution by injection until it is turgid and firm
- Some formalin may also be injected under the skin, into the body cavity, larger muscles and organs.
- Removal of the intestine prior to storage of the animal in alcohol is recommended.

### **Long term storage in fluid**

- After fixation in formalin, transfer into alcohol is the most desired alternative. For permanent liquid storage of specimens in alcohol, the following procedure is followed.
- After fixation in 10% buffered formalin solution the specimen is to be washed by keeping it in slowly flowing water for 24 hours for removal of formalin remnants.
- Next, the specimen is kept in distilled water for about 30 minutes. Exchanging the water twice produces the best results.
- With the formalin completely removed, the specimen can be transferred into 50 % alcohol for 30 minutes, then into 70% alcohol for some time.
- For long term storage in a collection, a final transfer into 80% alcohol is recommended.

### **Skins and mounts**

- The condition of the specimen will determine whether or not skin or mounted specimen can be prepared.
- If for instance decomposition of the skin has loosened the hair of a carcass so much that it can easily be pulled out or removed by rubbing ("slipping" fur), it will be very difficult or impossible to produce a study skin or mounted specimen.

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- The standard method of preservation for mammals is that of the round mount for small mammals and the tanned pelt for larger specimens.
- The taxidermy mount i.e., life like pose is not used in museum collections, since each specimen requires much more space for storage.
- Very small mammals such as shrews can be preserved in liquid and dried.

### **Preparation of skins in the field**

- After removing the skin from the animal, as much flesh as possible should be removed, but without damaging the skin with hair roots.
- Then the skin can be dried in the sun, or if necessary, high over a fire, either hung on a line or stretched between pegs.
- Salting the skin will speed the drying process and temporarily preserve the skin. Areas that still have flesh or fat should be salted thoroughly.
- Powdered borax can be put on the skin to further preserve it - alternatively cold ashes from a fire can be used.
- When the skin is nearly dry, it should be folded with the hairy sides together.
- Laboratory preparation of skins earlier dried in the field It consists of
- Relaxation of the dry skin by soaking it in lukewarm tap water, usually overnight.
- Brief washing of the relaxed skin with soap and water.
- Rinsing of the skin in a degreasing agent such as varsol or carbon-tetrachloride; if greasy, the skins are allowed to stand in it for half an hour or so.
- Drying of the skin in sawdust, using compressed air to assist the drying and to blow the sawdust out of the hair.

### **Preparing skeletons**

- The first step is to remove as much of the flesh from the bone as manually possible.
- The specimens can be further processed using a variety of techniques including: - enzyme solutions /maceration (with and without heat, detergents, ammonia, or bleaches)/burial in sand, soil, or manure/ dermestid beetle colonies
- After any of these treatments, further rinsing and some hand cleaning may be necessary. If cleaned by beetles, the specimens usually are fumigated, frozen, or rinsed in alcohol to kill the insects.
- Then the bones may be soaked in ammonia or chlorine bleach solutions to deodorize. The resulting specimens are then dried (with or without heat).
- Sometimes large long bones are drilled and the marrow is removed to reduce the potential for migration of fats and oils out of the bone.

## **ZOO101 Practical Manuals**

### **Practical 16:**

### **Preservation of Representative Animals of Various Phyla (Invertebrates).**

#### **Fixation**

- The fixation of biological specimens involves the coagulation of cell contents into insoluble substances with the purpose to prevent autolysis and the degradation of tissue. Fixation coagulates and stabilizes protein in specimens so that they do not distort or deteriorate during preservation, study and storage.
- A good fixation is generally achieved in a brief amount of time (hours to days) and as soon as the animal is collected.
- Formalin is generally the preferred fluid for fixation and is widely used. Invertebrates typically require only 4% formalin.
- In fish, amphibians, reptiles, birds, and mammals, the ratio of formalin to carcass must be at least 12 to 1 to assure a good fixation.

#### **Preservation**

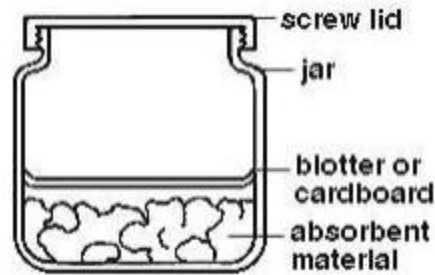
- Preservation is any process that serves to keep the dead body of an organism from decay, in part or in whole, presumably to be studied later.
- Both vertebrates and invertebrates can be preserved in fluid or as dry specimens.
- The archival preservation fluid that has been used the longest and is generally preferred is alcohol.
- The standard is 70-75% ethyl alcohol or ethanol.
- 40-50% isopropyl alcohol is used on some animal taxa. It tends to make them brittle over time. For this reason, one needs to buffer it with a few drops of glycerin and a pinch of calcium carbonate.
- "Sorting solution" (1.5 parts propylene phenoxetol, 5.0 parts propylene glycol, 10.0 parts full strength Formaldehyde, & 83.5 parts distilled water) has been used successfully for the long-term storage of some taxa.

#### **Invertebrate Killing methods**

##### **Killing Jars**

- Liquid killing agents are ethyl acetate, ether, chloroform, and ammonia water. Ethyl acetate is most widely used. All of these chemicals are extremely volatile and flammable and should never be used near fire.

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- Solid killing agents are the cyanides potassium cyanide, sodium cyanide, or calcium cyanide. They are dangerous, rapid acting poisons with no known antidote and hence are to be handled with extreme care.
- Absorbent material Plaster of Paris/Cotton

### **Freezing**

- Due to environmental and safety concerns, and the well-being of the specimen also, these methods have been done away with and are today being replaced by freezing.
- After the specimens have been collected, they can be transported home or to the lab in a plastic zip-lock bag or paper envelopes.
- The specimens are then carefully placed into a portion of the freezer where they will not be damaged. Leaving invertebrates in the freezer for prolonged periods of time however may damage the specimen.
- They are to be frozen only long enough to render them immobile.

### **Dry Method**

#### **Foraminifera and radiolaria**

- Amoebas and their relatives, including foraminifera and radiolaria, belong to the subphylum Sarcodina.



- Both groups produce skeletal casts or shells, casts of radiolaria are siliceous (silica), those of the forams are calcareous (calcite).

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**Micropaleontology slide**

### **Sponges**

- Wear hand gloves
- Put under clear, running water rinse and squeeze to drain out excess water
- For removing the smell, the sponge is then placed in a container of alcohol with a lid for 48 hours.
- Before drying large specimens, labels are attached by means of string, threaded through the body of the sponge.
- Dry it in the sun
- When thoroughly dried, sponges are kept in small cardboard boxes supported with tissue paper.
- Paradichlorobenzene or naphthalene flakes may be added to dry containers.
- Stony corals
- First, the animals using the coral as a home must be removed.
- This is achieved by soaking the coral in fresh water, as the animals are saltwater animals and cannot survive for more than three days in fresh water. Then, the coral are to be soaked in two parts bleach and one part water for 48 hours.
- For preserving the coral specimen, it should be soaked in alcohol and let dry, but most coral is fine after cleaning.

### **Coral**

A hard-stony substance secreted by certain marine coelenterates as an external skeleton, typically forming large reefs in warm seas, e.g. brain coral, star coral, staghorn coral, elkhorn coral, and pillar coral.

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### **Horny Coral**

- Specimens are dipped in neutral formalin for 15 minutes and then dried in a warm but shaded place.
- Prior to drying branches are so arranged that they will take up the least amount of space in a museum tray.
- After the specimens are thoroughly dried up, they are kept in light proof boxes with a few crystals of paradichlorobenzene.



### **Dry shells of Mollusca**

- The first step is to clear the shell of any dead tissue, which is a cause of bad odor.
- Most shells can be cleaned by coating them on the inside and outside or filling it completely with a dry solution of - three parts salt, one part baking soda and freezing them in zipper bags.
- An alternative to the process of cleaning dead tissue is to boil the shells.

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### **Shells of Mollusca**

- After removing the dead tissue, the shells are sorted out into sturdy and fragile shells.
- Sturdy shells are soaked in a container having four cups of water mixed with four cups of bleach and one tablespoon of dishwashing liquid. They are periodically scrubbed with a toothbrush to remove any seaweed, algae, barnacles and debris attached to them.
- Fragile and bivalve seashells are washed in mild dish detergent. After the seashell is clean, it is removed from the solution, rinsed well, and dried gently with paper towel. They are then air dried or sun dried.
- Some collectors like to use baby oil on a well-dried shell to bring out its luster.

### **Echinoderms (asteroids and ophiuroids)**

- Clean in a mild detergent solution that is mostly water and very little detergent and dry in the sun.
- Soak the starfish immediately in rubbing alcohol for 48 hours and forego cleaning it. It is then allowed to dry in the sun.
- Soak it in formalin, which is 1-part formaldehyde and 5 parts water, and then dry it properly
- While drying it is necessary to put weight on every arm of the starfish, or else they will curl up.



### **Insect Preservation**

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### 1. Relaxing methods

- Flat plastic container with an airtight lid makes an ideal relaxing dish.
- Base lined with moist cotton wool or synthetic sponge saturated with water and covered with a blotting paper.
- Phenol crystal/few drops of Dettol added to prevent fungal growth
- A sheet of blotting paper is also placed on the inside of the lid to avoid condensed water to fall over the specimens.
- The dry insects are kept in the relaxing dish and the dish sealed and left at least for a day.
- Alternatively, robust insects (e.g. Most beetles) can be quickly relaxed by dropping them into near boiling water. Small specimens will soften in a few seconds, whereas large ones require a minute or two. Large moths and butterflies can also be relaxed by injecting a 10% solution of ammonia or hot water into the thorax.



**Relaxing Chamber**

### 2. Insect Pins

#### **English:**

The English pins are 18 to 30 mm long and quite stout. These can be handled well with pinning forces and preferred for staging or double mounting.

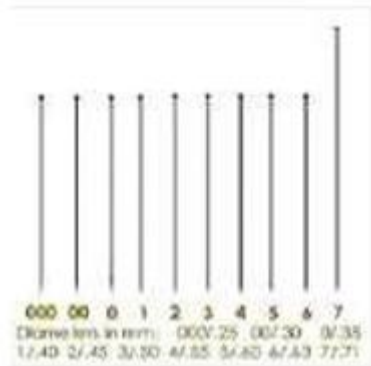
#### **Continental:**

The continental pins came in three sizes a. 35mm long (numbers 000,00,0 and 1 to 7) b. 38 mm long (numbers 8 to 10) and c. 50mm long (numbers 11 and 12). These are good for direct pinning.

#### **Mintutennadein:**

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The Minutennadein are very fine, short (numbers 10, 15, 20 or 22 mm) with or without heads and used for only double mounting of very small insects.



### **Mounting Insects**

#### **Pinning Blocks:**

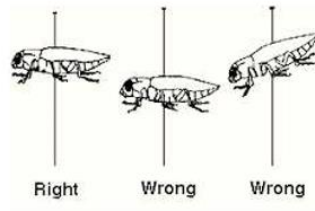
It is important that all the specimens and labels are placed at a uniform height on the pins. Setting/Spreading: Moths, butterflies, lacewings and dragonflies set with both pairs of wings spread, whereas Grasshoppers, cockroaches, mantids, stick insects and occasionally bees are set with only one pair of wings extended.



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### Mounting large insects

Insects longer than around 8 mm are mounted by direct pinning.



### Mounting small insects

**Card points:** Small bugs, wasps and flies

**Card Platforms:** Small insects, particularly certain beetles and parasitic wasps

**Minuten pins:** This method is also called double mounting or staging.

1. Gelatine capsules
2. Glueing to pins



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### Preservation of Invertebrates

#### Wet method

| <u>Phylum</u>                    | <u>Anaesthizing</u>                                                                           | <u>Fixative</u>                                                      | <u>Preservative</u>                                                                                              |
|----------------------------------|-----------------------------------------------------------------------------------------------|----------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------|
| <u>Porifera</u>                  | Not necessary                                                                                 | Two changes of 50% Alcohol                                           | 70% alcohol                                                                                                      |
| <u>Cnidaria &amp; Ctenophora</u> | Few crystals of menthol added to water in which animals have been allowed to extend           | 20% buffered formalin                                                | 10% formalin or 70% alcohol                                                                                      |
| <u>Platyhelminthes</u>           | Hot alcohol                                                                                   | 70-90% alcohol                                                       | 70-90% alcohol solution                                                                                          |
| <u>Nematoda</u>                  |                                                                                               | 3-5% hot formalin solution or hot 70-90% alcohol                     | 10% formalin or 70% alcohol                                                                                      |
| <u>Nematophora</u>               |                                                                                               | 3-5% formalin                                                        | 3-5% formalin                                                                                                    |
| <u>Bryozoa</u>                   | Slow addition of menthol crystals to water in which animals have been allowed to extend       | Rapid addition of commercial formalin or <u>Bouin's fluid</u>        | 5% formalin solution or 70-90% alcohol (colonies which produce calcified tubes should not be stored in formalin) |
| <u>Annelida</u>                  | 5-10% alcohol for 10-15 minutes                                                               | 4% formalin                                                          | 70-90% alcohol                                                                                                   |
| <u>Arthropoda</u>                |                                                                                               | <u>Freezer-killing jar</u> ; 70-90% alcohol                          | Non-insects-70-90% alcohol<br>Insects-drying and pinning                                                         |
| <u>Mollusca</u>                  | Menthol crystals to water                                                                     | Marine-5% neutral formalin<br>Freshwater and terrestrial-70% alcohol | Marine-5% neutral formalin<br>Freshwater and terrestrial-70% alcohol                                             |
| <u>Echinodermata</u>             | Menthol or magnesium sulfate crystals in water ( <u>holothuroidea</u> and brittle stars only) | 10-12% buffered formalin                                             | 70-90% alcohol; some can be dried                                                                                |

#### Parasites

- Arthropods preserved in 70% alcohol in glass vials with screw caps lined with anti-evaporation inserts made of plastic. - Mites, ticks, lice, fleas, louse flies, and bugs can be dropped straight into alcohol. - Nest fly larvae should be killed by immersion

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in boiling water prior to storage in alcohol or, better yet, immersed for 24 hours in KAAD solution (1-part kerosene, 10 parts 95% alcohol, 2 parts acetic acid, 14 parts dioxane).

- If specimens are to be stored in alcohol for a long time (years), 5-10% glycerin should be added to the alcohol to prevent hardening of specimens
- To prevent small vials from drying out in long-term storage, they can be placed in an inverted position between layers of cotton in a larger jar of alcohol, so long as the caps are not loosened in the process. This ensures that the fluid in the vials will be the last to evaporate should they go unchecked.
- If specimens are to be mounted on slides, they should first be washed several times with 70% alcohol to remove the glycerol.