Zoo505

CELL & MOLECULAR BIOLOGY

LABORATORY MANUAL

Contents:

Practical 1
Study of Prokaryotic cell

Principle

Study of prokaryotic and eukaryotic cells, as well as the similarities and dissimilarities among them.

Materials:

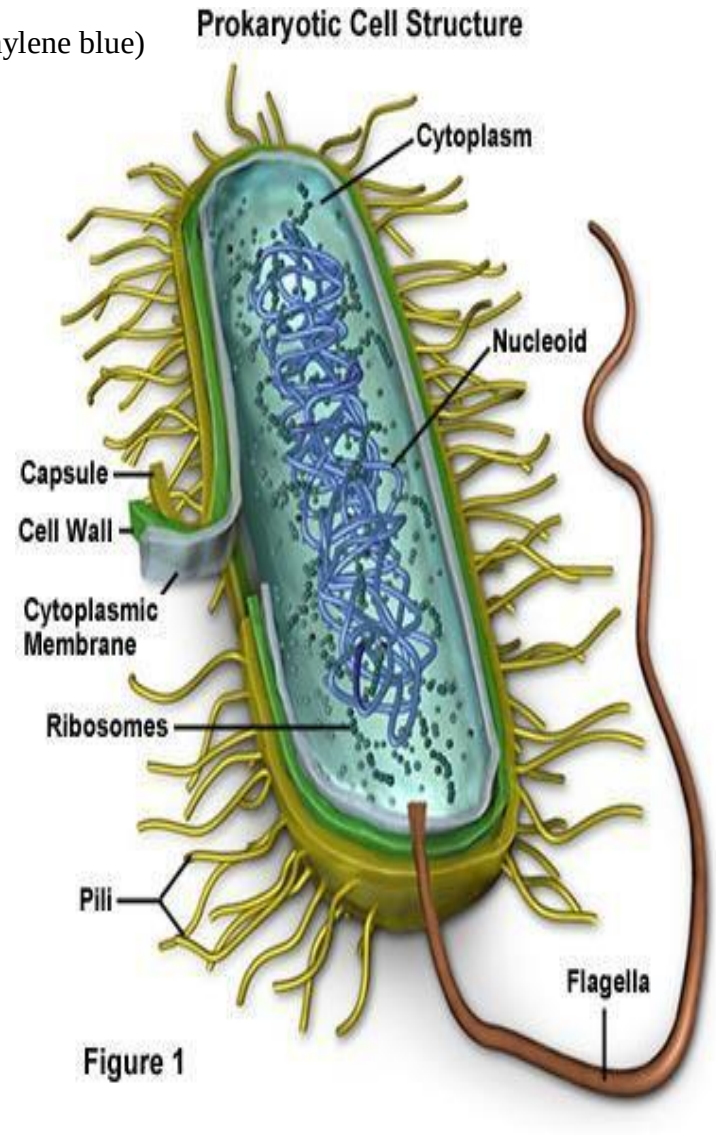
- Microscope
- Specimen
- Simple staining dyes (crystal violet/ methylene blue)
- Slides
- Water beaker

Procedure:

- Clean and adjust the microscope carefully.
- Prepare the specimen by simple staining technique.
- Fix the smear slide under microscope.
- Notice and write observations of respective cells examined by microscope.

Study of Prokaryotic cell under microscope

Organisms having no well-defined nucleus are called prokaryotes. Examples are Monera or Bacteria and Archaea. Typical structure of a prokaryotic cell and its components observed under microscope are given in *figure 1*.



A typical structure of bacterial cell

Cell Wall

Provide shape and protection to cell.

Pilli

Organ for the exchange of genetic material from one bacterium to another.

Capsule

Sticky projections that cover cell wall and provide protection from phagocytosis, chemicals and dehydration.

Flagellum

A “whip-like;” structure that helps in movement.

Plasma Membrane

A thin, flexible asymmetrical “sac” that holds the cytoplasm and serves as a passageway for anything that enters or leaves the cell such as nutrients and gases.

Cytoplasm

Contains organelles for different functions inside the cell.

Nucleoid or Nuclear Body

Area of the cytoplasm where the DNA strand is located

Plasmids

Extra chromosomal piece of DNA. Plasmids are often the site of genes that code for resistance to antibiotics.

Ribosomes

The function of prokaryotic ribosomes widely depends on the bacteria.

Practical 2
Eukaryotic, Plant and Animal cells

Study of Eukaryotic cell under microscope

Organisms having well-defined nucleus and membrane bound organelles are called Eukaryotes. Examples are Plants and Animals. Typical structures of Eukaryotic cells including plant and animal cell and its components are given below in *figure 2.*

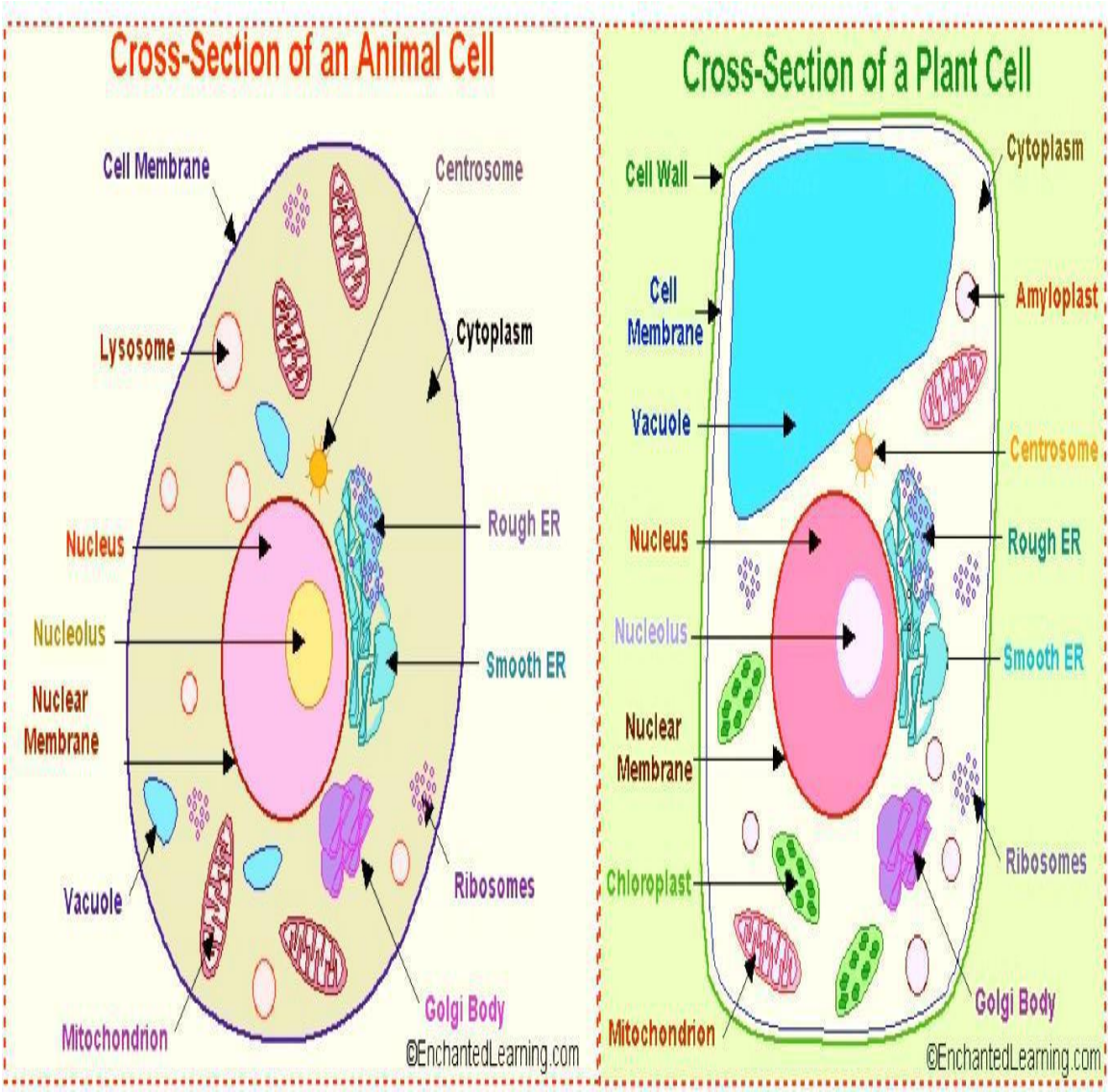


Figure 2 representing a typical animal and plant cell

Similarities and dissimilarities in plant and animal cell

Organelles	Plant Cell	Animal Cell
Cell Wall	Cell wall made of cellulose is present in almost all cells.	Cell wall is absent.
Plastids	Plastids like leucoplasts, chloroplast and chromoplasts are present.	No plastids found.
Chloroplasts	Plants cells have chloroplasts to prepare their own food.	Chloroplasts completely absent.
Vacuoles	Cell sap containing vacuoles are present.	Vacuoles are usually absent or one or more small vacuoles are seen.
Lysosomes	Lysosomes not evident.	Lysosomes occur in cytoplasm.
Nucleus	Due to the presence of the vacuole at the center of the cell, nucleus may be located at the edge of the cell.	Nucleus is usually located centrally.
Golgi bodies	Plant cells have many simpler units of Golgi complex, called dictyosomes.	Animal cells have a single highly elaborate Golgi complex.
Endoplasmic reticulum	Present	Present
Ribosomes	Present	Present
Mitochondria	Present	Present
Centrioles	Present only in lower plant forms.	Present

Practical 3
Observation of wet mounts of human cheek cells employing bright and dark field microscopy

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:

1. Swab the inside of your cheek with the non-sharp end of a toothpick.
2. 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.
3. Place the swabbed end of the toothpick onto the middle of a microscope slide.
4. Add a single droplet of water squeezed from a plastic pipette onto the center of the slide.
5. Rotate the toothpick in the water to release the human cheek cells.
6. Add one drop of methylene blue onto the water and cell solution to stain the cheek cells for observation.
7. Position a cover slip at a 45 degree angle just inside the left edge of the solution.
8. Move your fingers down and to the right to place the cover slip over the cheek cell mixture.
9. Check for tiny air bubbles under the cover slip and lightly push the cover slip downwards to release any air bubbles you find.
10. Place the edge of a paper towel on any solution outside of the cover slip to absorb the excess moisture.
11. Mount the human cheek cell slide on the light microscope viewing platform.
12. 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.

Tip: Use iodine as an alternative to methylene blue.

Warning: Be careful when handling cover slips because they break easily.

Practical 4

Study of cellular reproduction

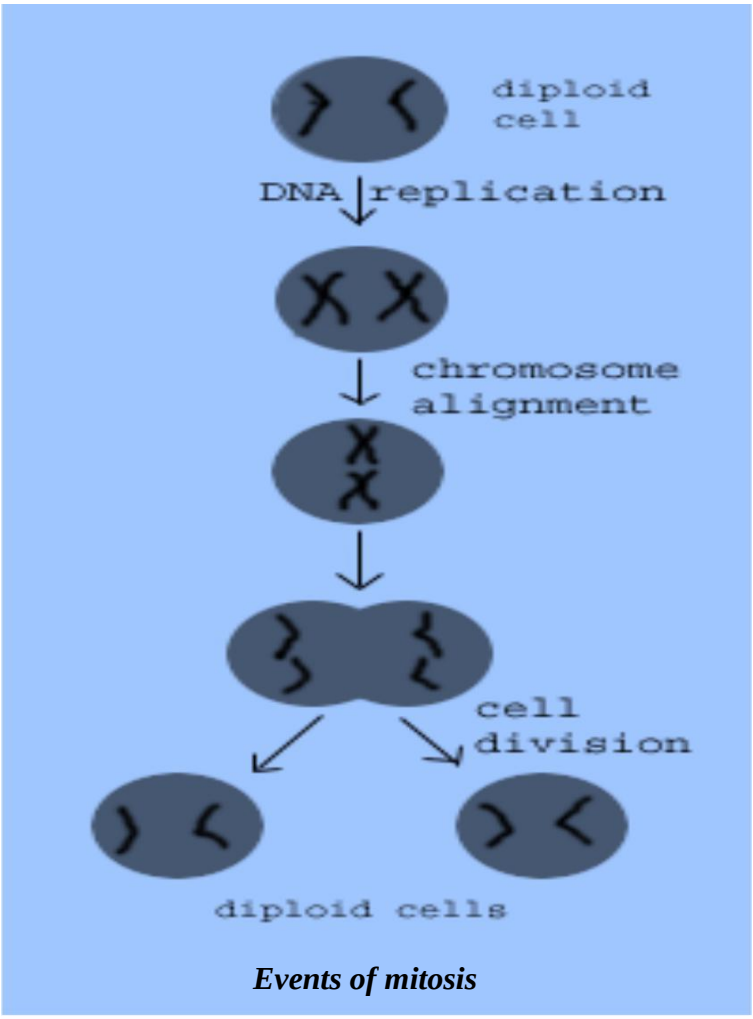
Introduction:

Cellular reproduction is the process by which organisms are reproduce asexually by fission or spore formation and sexually by formation of gametes through cell division. Cell division is also a source of tissue growth and repair in multicellular organisms. Mitosis is responsible for reproducing somatic cells and meiosis is responsible for reproducing germ cells.

Mitosis

In single-cell organisms, mitosis is the only form of cellular reproduction. One round of mitosis yields two genetically identical cells. In bacteria, this process results in an entirely new, independent organism. This is classified as asexual reproduction because it does not require sex for the creation of new organisms. In multi-cellular organisms, mitosis only occurs in somatic cells, which comprise all cells in an organism excluding germ cells.

Cells that undergo mitosis duplicate their chromosomes, resulting in cells with two times their normal haploid or diploid numbers (4*N*chromosomes). Newly-synthesized chromosomes remain closely associated with their like-chromosome. These two identical chromosomes are called sister chromatids. Once duplicated, sister chromatids separate such that one copy of each chromosome lines up on opposite ends of the cell. The cell then pinches in the center until it breaks into two different cells. A nucleus then forms around the chromosomes in each cell to yield two cells with the same original number of chromosomes as the preexisting cell.



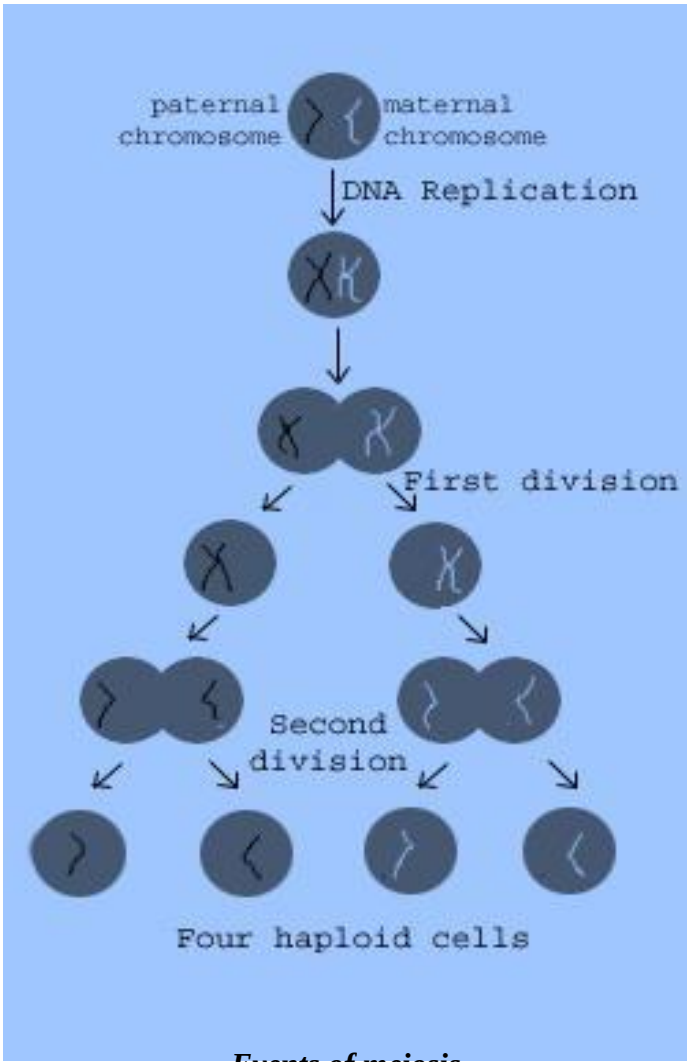
Events of mitosis

Meiosis

There are two major differences between mitosis and meiosis. First, meiosis involves not one, but two cell divisions. Second, meiosis leads to the production of germ cells, which are cells that give rise to gametes. Germ cells are different from somatic cells in a critical way. Whereas somatic cells are diploid, meaning they have two copies of each chromosome, germ cells are haploid. The haploid nature of germ cells is vital to the process of sexual reproduction.

There are two different sex cells or gametes: sperm and eggs. Males produce sperm and females produce eggs. Because they are produced from germ cells, gametes are likewise haploid. In order to create a new individual via sexual reproduction, a sperm cell needs to activate an egg by joining it in a fertilization process. When these two haploid cells unite, a diploid cell results. This specialized cell can then develop into a new individual. The sexual reproductive process just described ensures that the resulting offspring will have an equal maternal and paternal genetic contribution.

As we mentioned earlier, higher-order cells contain homologous pairs of chromosomes--one from the father and the other from the mother. In meiosis, as in mitosis, the maternal and paternal homologues are replicated during DNA replication yielding two pairs of sister chromatids. After the first cell division, each of the resulting cells contains a pair of sister chromatids—-one maternal pair and the other paternal. Unlike mitosis, meiosis does not end after one division; it continues with a second cell division. In this division, the sister chromatids are separated yielding four total haploid cells.



Events of meiosis

Practical 5
Study of Mitosis: Smear/Squash preparation of Onion roots

Introduction:

The genetic information of plants, animals and other eukaryotic organisms resides in several (or many) individual DNA molecules, or chromosomes. For example, each human cell possesses 46 chromosomes, while each cell of an onion possesses 8 chromosomes. All cells must replicate their DNA when dividing. During DNA replication, the two strands of the DNA double helix separate, and for each original strand a new complementary strand is produced, yielding two identical DNA molecules. DNA replication yields an identical pair of DNA molecules (called sister chromatids) attached at a region called the centromere.

DNA replication in eukaryotes is followed by the process called mitosis which assures that each daughter cell receives one copy of each of the replicated chromosomes. During the process of mitosis, the chromosomes pass through several stages known as prophase, metaphase, anaphase and telophase. The actual division of the cytoplasm is called cytokinesis and occurs during telophase. During each of the preceding stages, particular events occur that contribute to the orderly distribution of the replicated chromosomes prior to Cytokinesis.

The stages of Mitosis

Prophase:

During prophase, the chromosomes supercoil and the fibers of the spindle apparatus begin to form between Centrosome located at the pole of the cells. The nuclear membrane also disintegrates at this time, freeing the chromosomes into the surrounding cytoplasm.

Prometaphase:

During Prometaphase, some of the fibers attach to the centromere of each pair of sister chromatids and they begin to move toward the center of the cell.

Metaphase:

At metaphase the chromosomes have come to rest along the center plane of the cell.

Anaphase:

During anaphase, the centromeres split and the sister chromatids begin to migrate toward the opposite poles of the cell.

Telophase:

During telophase, the chromosomes at either end of the cell cluster begin to cluster together, which facilitates the formation of a new nuclear membrane. This also is when cytokinesis occurs, leading to two separate cells. One way to identify that telophase has begun is by looking for the formation of the cell plate, the new cell wall forming between the two cells.

Objective:

Better understand the process and stages of mitosis.

Prepare your own specimens of onion root in which you can visualize all of the stages of mitosis.

Viewing mitosis in onion root tips

Why use onion roots for viewing mitosis?

- The roots are easy to grow in large numbers.

- The cells at the tip of the roots are actively dividing, and thus many cells will be in stages of mitosis.
- The tips can be prepared in a way that allows them to be flattened on microscopes slide ("squashed") so that the chromosomes of individual cells can be observed.
- The chromosomes can be stained to make them more easily observable.

Regions of Onion Root tips

There are three cellular regions near the tip of an onion root.

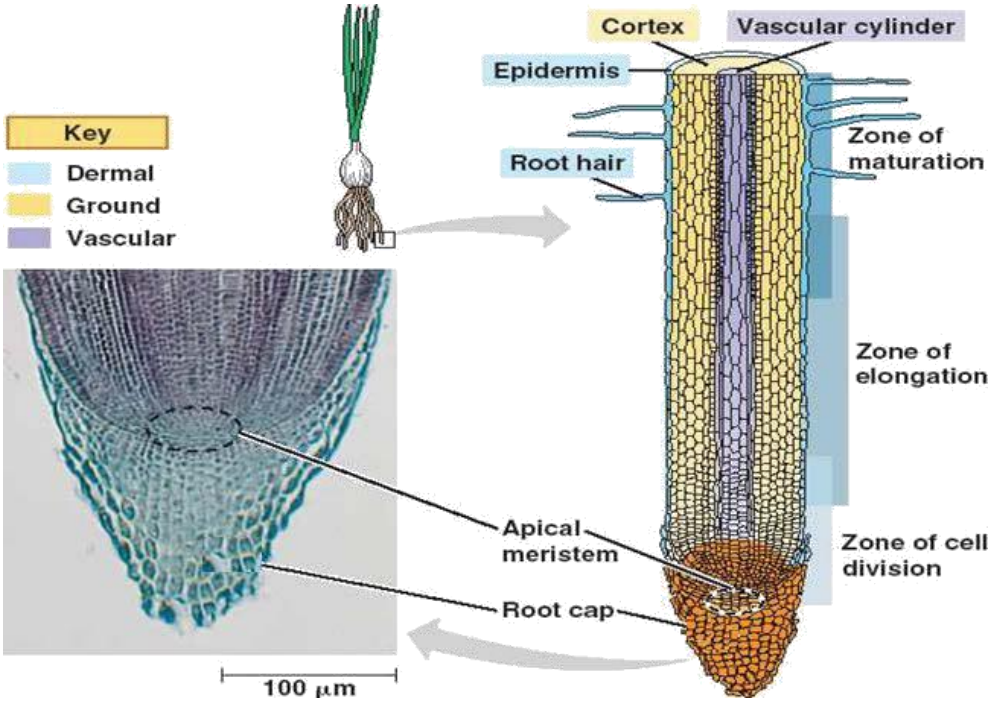
- ❖ The root cap contains cells that cover and protect the underlying growth region as the root pushed through the soil.

- ❖ The region of cell division (or meristem) is where cells are actively dividing but not increasing significantly in size.

- ❖ In the region of cell elongation, cell are increasing in size, but not dividing.

Viewing Chromosomes

Chromosomes generally are not visible as distinct entities in non-dividing cells, since the DNA is uncoiled, but the process of mitosis is facilitated by supercoiling of the chromosomes into a highly compacted form. Supercoiled chromosomes can be visualized in cells, particularly if they are treated with a DNA-specific stain, such as the Feulgen stain.



Structure of Onion root tip

Materials:

- Onion plant with root
- Feulgen stain
- 1 N HCl
- Scissors
- Forceps
- Razor blade
- Pasture pipette
- 1.5 ml microfuge tubes
- Dissection probe with wooden back
- Microscopic slides and cover slips
- Water bath
- Light Microscope

Procedure for preparing root tip squashes

While actively growing onions are present in the lab for you to observe, you will be provided with roots that have been previously harvested and treated with a fixative to stabilize the cells. You will work in groups of two for this lab exercise. The first step will be to 'soften' the roots so that they later can be spread on a microscope slide.

- Using scissors, cut 2 roots tips about 1 cm long, and transfer them into a plastic micro-tube. (One of the rots will be an extra one.)

- Fill the tube about 2/3 full with 1N HCl from a dropper bottle**. *** Caution: Work with the HCl carefully, it is a strong acid. *****

- Place the tube in a 60OC water bath, and allow the roots to incubate for 12 minutes. 4. After the 12 minute incubation period, remove the tube from the water bath.

Rinse the roots in H2O:

- Using forceps carefully transfer the root tips to a small petri plate.

- Using a plastic 'squeeze' pipet, carefully remove the HCl from the micro-tube and transfer it to the "discard flask".

- Rinse the root tips 3 times with water from the dropper bottle, disposing of the rinses in the discard flask.

Staining the chromosomes:

- After removing the water from the third rinse, cover the root with the Feulgen stain**. *****

  Caution: Although the Feulgen stain does not appear colored, it will strongly stain skin and clothing. ***
- Incubate the roots in the stain for 12 minutes. During this time the very tip of the root will begin to turn red as the DNA stains the numerous small actively dividing cells at the tip.

Remove the stain and again rinse the roots:

- Using a plastic 'squeeze' pipet, carefully remove the Feulgen stain and discard it in the discard flask. Again, rinse the root tips 3 times with water.

Preparing the root tip squash:

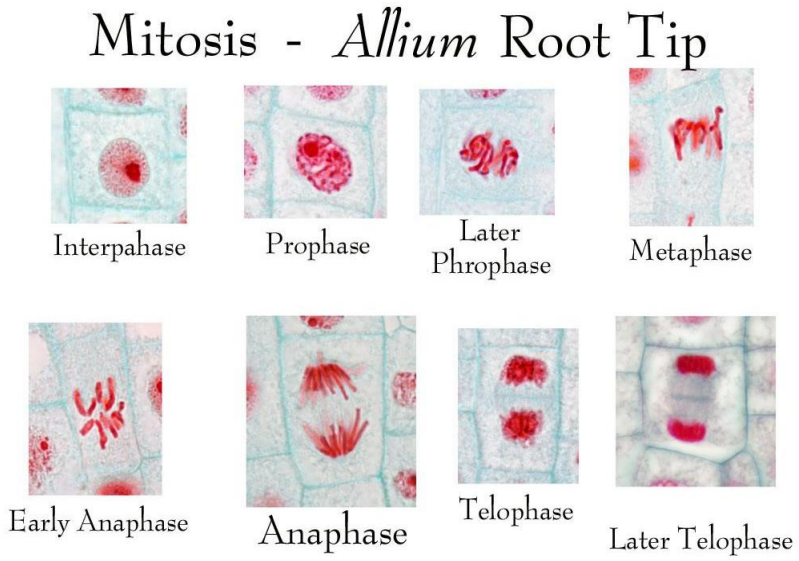
- Transfer a root to the center of a clean microscope slide and add a drop of water.

- Using a razor blade cut off most of the unstained part of the root, and discards it.

- Cover the root tip with a cover slip, and then carefully push down on the cover slide with the wooden end of a dissecting probe. Push hard, but do not twist or push the cover slide sideways. The root tip should spread out to a diameter about 0.5 – 1 cm.

Observations of onion root tip squash:

Scan the microscope under the 10x objective. Look for the region that has large nuclei relative to the size of the cell; among these cells will be found cells displaying stages of mitosis. Examples are shown in the figure to the right. Switch to the 40X objective to make closer observations. Since prophase and Prometaphase are difficult to distinguish, classify all these cells as prophase. Record your observations in the table provided.



Mitosis in Onion Root Tip

Results Onion root tip squash

1. **Find and draw a cell showing each stage of mitosis.**

Prophase	Metaphase	Anaphase	Telophase

2. What is a distinguishing visible feature of each stage of mitosis?

Prophase:

Metaphase:

Anaphase:

Telophase:

Practical 6

Staining of Bacteria (Gram Staining)

Staining

A technique that is used to define and examine different types of microbes.

Types of staining techniques

1. **Simple stain techniques.**

Simple staining is performed with basic dyes such as crystal violet or methylene blue.

2. **Differential stain techniques.**

Differential stain technique distinguishes two kinds of organisms. There are two types of this technique; one is Gram stain technique while other is acid fast technique.

Gram stain technique

A most commonly used technique for study of microbes like bacteria.

Principle

This technique separates bacteria into two groups, Gram-positive bacteria and Gram-negative bacteria.

Materials:

- Clean glass slides
- Inoculating loop
- Bunsen burner
- Bibulous paper
- Microscope
- Lens paper and lens cleaner
- Immersion oil
- Distilled water
- 18 to 24 hour cultures of organisms

Reagents:

Primary Stain	- Crystal Violet
Mordant	- Grams Iodine
Decolourizer	- Ethyl Alcohol
Secondary Stain	- Safranin

Procedure:

Smear preparation

A small sample of microorganisms is placed on a slide and permitted to air dry. The smear is heat fixed by quickly passing it over a flame. Heat fixing kills the organisms, makes them adhere to the slide, and permits them to accept.

Primary stain

Apply crystal violet as primary stain

Mordant

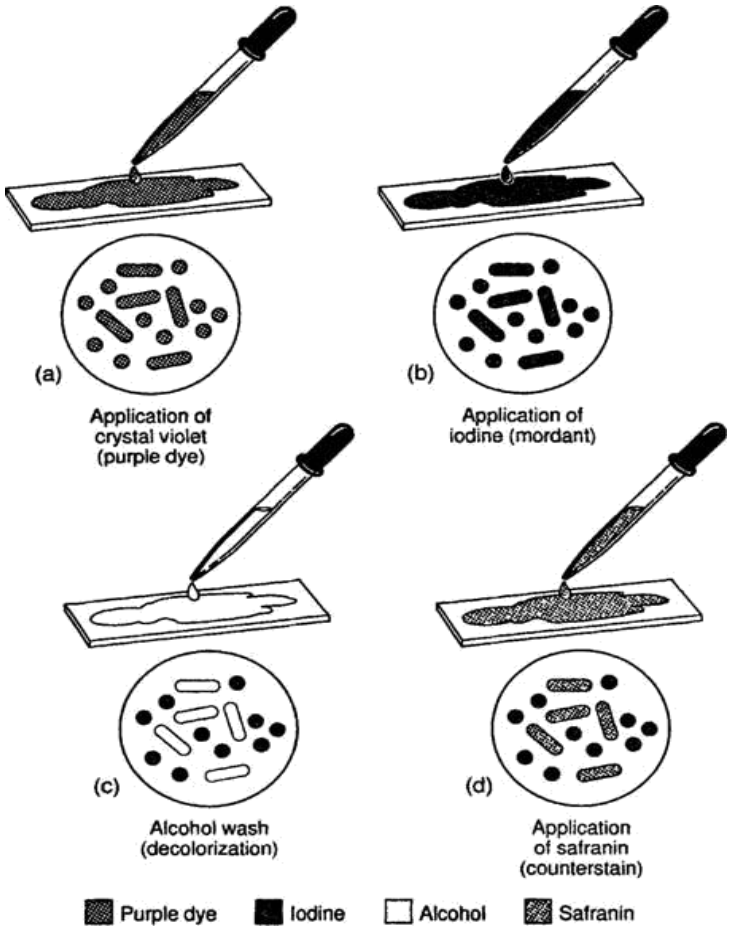
Use iodine as a mordant

Decolourizer

Wash with ethyl alcohol that acts as a decolourizing agent. Gram-positive bacteria retain the crystal-violet iodine stain; however, the Gram-negative bacteria lose the stain.

Secondary stain

The Gram-negative bacteria subsequently stain with the Safranin dye, the counterstain. These bacteria appear red under the oil-immersion lens, while Gram-positive bacteria appear blue or purple, reflecting the crystal violet retained during the washing step.



***The Gram staining procedure used for differentiating bacteria into two groups*.**

Practical 7
DNA Extraction from Whole Blood

Principle

The extraction of DNA involves three main steps that are cell lysis, protein separation, and DNA purification. Cell lysis is usually performed by incubation of cell in buffer containing detergent and protease. Cellular proteins are salted out or phase separated using organic solvents. Finally DNA is isolated and purified either by alcohol precipitation or adsorption with silica and elution.

Reagents required

3. TE buffer (10mM Tris, 2mM EDTA, pH 8.0)
4. TEN buffer (10 mM Tris, 2mM EDTA, 400mM NaCl)
5. 10% SDS
6. Proteinase-K solution 20mg/ml
7. 6M NaCl
8. Phenol-Choloroform-Isoamylalcohol (PCI) (25:24:1)
9. Absolute Ethanol or Isopropanol
10. 75% Ethanol
11. Low TE buffer (10mM Tris, 0.2mM EDTA)

Consumables required

- Filter barrier tips 200 µl
- Filter barrier tips 1000 µl
- Wide bore tips 1000 µl
- Falcon tubes 15 ml
- Microcentrifuge tubes 1.5 ml

Equipment required

- Centrifuge for 15 ml falcon tubes
- Microcentrifuge for 1.5 ml tubes
- Adjustable micropipettes 1 ml and 200 µl

Procedure

1. Add 1 ml chilled TE buffer to 200 µl blood. Mix by inverting the tube several times.
2. Spin at 4000 rpm for 15 min at room temperature.
3. Discard the supernatant and add 900 µl chilled TE buffer. Re-suspend the pellet by vigorous shaking by hand.
4. Spin at 4000 rpm for 15 min at room temperature.
5. Discard the supernatant and add 800 µl TE buffer. Re-suspend the pellet by vigorous shaking by hand.
6. Spin at 4000 rpm for 15 min at room temperature.
7. Discard the supernatant and add 200 µl TEN/A1 buffer, 20 µl SDS (10% solution) and 10 µl Proteinase-K solution. Re-suspend the pellet by shaking and vortex mixing.

8. Incubate the mixture at 56°C overnight.
9. Next day, place the tubes on ice and add 50 µl 6M NaCl. Shake the tube vigorously and place on ice again for 15 min.
10. Spin at 4000 rpm for 15 min to pellet down the salts and proteins.
11. Transfer the supernatant in a fresh properly labeled 1.5-ml centrifuge tube.
12. Add equal volume of chilled isopropanol and invert the tubes gently till DNA is visible.
13. Spin at 8000 rpm for 1 min at room temperature. Discard supernatant.
14. Add 200 ml absolute ethanol and vortex for 15 sec.
15. Spin at 8000 rpm for 1 min at room temperature.
16. Add 200 ml 75% ethanol and vortex for 15 sec.
17. Spin at 8000 rpm for 1 min at room temperature.
18. Discard the supernatant and add 100 µl low TE buffer or sterile distilled water to dissolve the DNA pellet. Incubate at 72°C for 30 min.
19. Store DNA at -20°C.

Alternate steps for protein precipitation

First 8 steps are same as above.

9. Add equal volume of Phenol-Choloroform-Isoamylalcohol (PCI) solution. Mix the contents by inverting gently. Leave at room temperature for 5 min.
10. Centrifuge at 13000 rpm for 10 min to form three layers.
11. Carefully take upper aqueous layer containing DNA with 1ml pipette and transfer to a fresh properly labeled 1.5 ml centrifuge tube.

Follow step 12 onwards as given in the inorganic protocol.

NOTE: For more purification, organic and inorganic protein precipitation can be combined i.e., Precipitation by 6M NaCl followed by the phenol-chloroform-isoamyl alcohol purification.

Practical 8
DNA quantification by spectrophotometry using NanoDrop

Principle:

Nucleic Acids (nucleotides, RNA, ssDNA, and dsDNA) all absorb light at 260 nm wavelength; therefore spectrometry at 260nm is useful to quantify DNA or RNA in solutions according to Beer-Lamberts law.
Readings should be taken at wavelengths of 260 nm and 280 nm. The reading at 260 nm allows calculation of the concentrate on of nucleic acid in the sample.

1 O.D. at 260 nm for double-stranded DNA = 50 ng/µl of dsDNA
1 O.D. at 260 nm for single-stranded DNA = 20-33 ng/ul of ssDNA
1 O.D. at 260 nm for RNA molecules = 40 ng/µl of RNA

The reading at 280 nm gives the amount of protein in the sample.
Pure preparations of DNA and RNA have OD260/OD280 values of 1.8 to 2.0, respectively.
If there is contamination with protein or phenol, this ratio will be significantly less than the values given above, and accurate quantitation of the amount of nucleic acid will not be possible.
So typically, dilute sample 1 µl in 100 µl so the dilution factor is 100. Put whole 100 µl in spectrophotometer cuvette. The DNA concentration read will then be:

OD260 X 50 ng/ul x dilution factor

For example, if have OD260 = 1.6. Then the concentration is:

1.6 x 50 ng/ul x 100 = 8000 ng/ul or 8 ug/ul.

Equipment Required

- NanoDrop 2000
- Vortex Mixer
- Pipettes covering 1-1000µL range
- Cuvets and sample tubes

Procedure

Nucleic acid samples can be easily checked for concentration and quality using the NanoDrop 2000/2000c spectrophotometer. To measure nucleic acid samples select the Nucleic Acid application from the home screen.

Nucleic Acid Calculations

For nucleic acid quantification, the Beer-Lambert equation is modified to use a factor with units of ng-cm/microliter.

The modified equation used for nucleic acid calculations is the following:

c = (A * ε)/b
c = the nucleic acid concentration in ng/microliter
A = the absorbance in AU
2. = the wavelength-dependent extinction coefficient in ng-cm/microliter **b**= the pathlength in cm

The generally accepted extinction coefficients for nucleic acids are:

- Double-stranded DNA: 50 ng-cm/µL
- Single-stranded DNA: 33 ng-cm/µL
- RNA: 40 ng-cm/µL

When the pedestal mode is selected, the NanoDrop 2000/2000c spectrophotometer uses short path lengths between 1.0 mm to 0.05 mm to enable measurement of concentrated samples without dilution.
Note: Absorbance data shown in reports is archived as displayed on the software screen. The Nucleic Acid application absorbance values are normalized to a 1.0 cm (10.0 mm) path for all pedestal and cuvette measurements.

Measurement Concentration Ranges

The NanoDrop 2000/2000c will accurately measure purified dsDNA samples <15,000 ng/µL without dilution. The software automatically utilizes the optimal path length to measure the absorbance of each sample. Refer to ―Measurement Ranges‖ for additional information.
The small sample volume option is available when samples have 10 mm equivalent absorbance values of 3.0 or higher (>150 ng/µL dsDNA.)

Unique Screen Features

The right pane displays features specific to the Nucleic Acid application. Task bars in the left pane not described below are described in ―Software Overview.‖
The spectral display shows data for the current sample normalized to a 10 mm path for all measurements including measurements made with any cuvette path length. The following features are to the right of the spectral display:

- **Sample ID** - field into which a sample ID is entered. The appropriate sample ID should be entered prior to each measurement.

- **Type** - a drop down list from which the user may select the (color-keyed) type of nucleic acid being measured. Options include DNA-50 for dsDNA, RNA-40 for RNA, and ssDNA-33 for single-stranded DNA.
Additional options include Oligo DNA and Oligo RNA which utilize the appropriate extinction coefficient based upon user-defined base sequences. The Custom option allows the user to enter an extinction coefficient between 15 and 150.

8 **Conc** - concentration based on absorbance at 260 nm and the default or user defined extinction coefficient. Concentration units may be selected from the adjacent drop-down box. Refer to ―Nucleic Acid Calculations‖ for more details.

9 **A260** - displays absorbance at 260 nm normalized to a 10 mm pathlength.

10 **A280** - displays absorbance at 280 nm normalized to a 10 mm pathlength.

11 **260/280** - ratio of absorbance at 260 nm and 280 nm. The ratio of absorbance at 260 and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as ―pure‖ for DNA; a ratio of ~2.0 is generally accepted as ―pure‖ for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. See ―260/280 Ratio‖ in ―Diagnostics and Troubleshooting‖ for more details on factors that can affect this ratio.

12 **260/230** - ratio of absorbance at 260 nm and 230 nm. This is a secondary measure of nucleic acid purity. The 260/230 values for a ―pure‖ nucleic acid are often higher than the respective 260/280 values and are commonly in the range of 1.8-2.2. If the ratio is appreciably lower, this may indicate the presence of co-purified contaminants.

13 **Baseline correction** - if selected, the default wavelength for the bichromatic normalization is 340 nm. The user can manually enter a different wavelength for the bichromatic normalization of the absorbance data. In either case, the baseline is automatically set to the absorbance value of the sample at the selected wavelength. All wavelength data will be referenced off this value.
Note: If a baseline correction is not selected, the spectra may be offset from the baseline and the calculated concentration will change accordingly.

Making Nucleic Acid Measurements

1. Select the **Nucleic Acid** application from the main menu. If the wavelength verification window appears, ensure the arm is down and click **OK**.

2. Select the type of sample to be measured from the Type drop-down list. The default setting is DNA-50.

3. Choose the concentration units from the drop-down list adjacent to the color coded concentration box. The default units are ng/µL.

4. A default wavelength of 340 nm is automatically used for a bichromatic normalization. Select an alternative reference wavelength or choose not to have the spectrum normalized by de-selecting the **baseline correction** box.
- Select the file drop-down option **Use current settings as default** as a convenient way to limit set-up time for each new workbook.

5. Select **Add to report** to automatically include all measurements in the current report. The default setting is for all samples to be added to reports. The **Add to report** checkbox must be selected prior to a measurement to save the sample data to a workbook.

6. Select **Overlay spectra** to display multiple spectra at a time.

7. Establish a blank using the appropriate buffer. The blank solution generally is the buffer that the molecule of interest is suspended or dissolved in. This solution should be the same pH and of a similar ionic strength as the sample solution.
Pedestal Option: Pipette 1-2 μL of the appropriate blanking solution onto the bottom pedestal, lower the arm and click the **Blank** button.

Cuvette Option (Model 2000c only): Insert the cuvette noting the direction of the light path indicated by the etched arrow. The optical beam (2 mm) is directed 8.5 mm above the bottom of the cuvette. Refer to the cuvette manufacturer for volume recommendations.

Note: The arm must be down for all measurements, including those made with cuvettes. It is recommended that cuvettes be removed from the instrument prior to making a pedestal measurement to ensure that the pedestal arm can move to the proper starting position.
8. Enter a Sample ID in the appropriate field, load the first sample as described for the blank above and click **Measure**.

Note: A fresh aliquot of sample should be used for each measurement.

After the measurement:

- Simply wipe the upper and lower pedestals using a dry laboratory wipe and the instrument is ready to measure the next sample.
- When using the cuvette option, remove the cuvette, rinse thoroughly and dry between samples.

Practical 9
Polymerase Chain Reaction (PCR) amplification

Principle:

Polymerase Chain Reaction is an in-vitro method for exponential amplification of a target portion of template DNA, which involves incorporation of nucleotides by DNA polymerase during thermal cycling

Reagents Required:

- PCR master mix including Taq polymerase, dNTPs, $MgCl_2$ and buffer.
  or
- Taq DNA Polymerase, dNTPs, $MgCl_2$ and PCR buffer separately.
- PCR primers (Forward and Reverse)
- PCR grade water
- Negative and Positive Controls

Equipment Required:

- Thermal Cycler with analysis software
- Vortex Mixture
- Microcentrifuge
- Pipettes
- PCR safety cabinet

Consumables:

5. PCR tubes/strips/plates according to equipment compatibility and requirement
6. Filtered pipette tips
7. 1.5 ml centrifuge tubes

Procedure:

- Label the PCR tubes for samples and controls. In case of quantification experiments, tube will also be labeled for standards.
- Thaw the PCR reagents and prepare PCR reaction mix. A generalized recipe of PCR is given in the following table. The amount of ingredients may vary according to the desired protocol and manufacturer's instructions. Calculate the volume of total reaction mix required for the whole batch including samples, controls and standards.

Table 4.1: Preparation of PCR Reaction Mix

Reagent	Stock Conc. (M1)	Final Conc. In Reaction Mix (M2)	Volume per Reaction (V1)
PCR buffer	10x	1x	5 µl
Forward Primer (dilution)	10 µM	0.5 µM	2.5 µl
Reverse Primer (10 µM dilution)	10 µM	0.5 µM	2.5 µl
dNTP mix	10 mM each	0.2 mM each	1 µl
$MgCl_2$	25 mM	2 mM	4 µl
Taq DNA Polymerase	5 U/ µl	1.25 U	0.25 µl
Sterile distilled H_2O	To make up volume		31.75 µl
Total			47 µl

Or

Table 4.2 PCR using prepared 2x Master Mix

Reagent	Volume per Reaction
2x PCR Master Mix	25 µl
Forward Primer (10 µM dilution)	2.5 µl
Reverse Primer (10 µM dilution)	2.5 µl
Sterile distilled H_2O	17 µl
Total	47 µl

Mix the reagent by gentle vortex followed by short spin.

- Aliquot the reaction mix in the individual PCR reaction tubes/well.
  Add the template i.e; sample/ control in the appropriate labeled tube. The volume of template varies according to the protocol in use. In the above example, 3 µl templates will be added to each tube so that 50 µl total reaction volume (V2) is achieved. The DNA concentration of the template should be known so that the optimum input quantity of the

template DNA can be used for PCR reaction. Optimal amounts of template DNA in the 50 µl reaction volume are 0.01-1 ng for both plasmid and phage DNA, and 0.1-1 µg for genomic DNA. Higher amounts of template increases the risk of generation of non-specific PCR products. Lower amounts of template reduces the accuracy of the amplification.

4. Open the PCR machine's software and edit run parameters e.g. run ID, user ID, sample IDs, sample volume and cycling conditions according to desired protocol. A generalized 3 step cycling protocol for PCR is given below
5.

Table 4.3 PCR cycling conditions

Step	Temperature	Duration	No. of cycles
Initial Denaturation	95^{o}C	3 min (in case of Hot Startitmay be prolonged upto 10 min)	1
Denaturation	94^{o}C	10 sec	35
Annealing	50-60^{o}C	20 sec	
Extension	72 ^{o}C	30 sec	
Final Extension	72 ^{o}C	5 min	1
Final hold	25 ^{o}C	Hold	1

- Place the sample tubes in the thermal cycler, close the lid and Run the program.
- After the completion of the PCR, remove the tubes from the thermal cycler and proceed for agarose gel electrophoresis or other downstream application. Otherwise store the PCR products at -20 ^{o}C.

Practical 10
Agarose Gel Electrophoresis

Principle

Nucleic acid molecules are size separated by the aid of an electric field where negatively charged molecules migrate toward anode (positive) pole. The migration flow is determined solely by the molecular weight where small weight molecules migrate faster than larger ones (Sambrook & Russel 2001). In addition to size separation, nucleic acid fractionation using agarose gel electrophoresis can be an initial step for further purification of a band of interest. Extension of the technique includes excising the desired ―band‖ from a stained gel viewed with a UV transilluminator (Sharp et al.,1973)

Equipment Required

- An electrophoresis chamber and power supply
- Gel casting trays, which are available in a variety of sizes and composed of UV-transparent plastic. The open ends of the trays are closed with tape while the gel is being cast, then removed prior to electrophoresis.
- Sample combs, around which molten agarose is poured to form sample wells in the gel.
- Transilluminator (an ultraviolet lightbox), which is used to visualize ethidium bromide-stained DNA in gels. NOTE: always wear protective eyewear when observing DNA on a transilluminator to prevent damage to the eyes from UV light.
- Pipettes ---- covering 1 to 100 ul range

Reagent Required

- Electrophoresis buffer, usually Tris-acetate-EDTA (TAE) or Tris-borate-EDTA (TBE).
- DNA sizing standard/ladder
- Loading buffer, which contains something dense (e.g. glycerol) to allow the sample to "fall" into the sample wells, and one or two tracking dyes, which migrate in the gel and allow visual monitoring or how far the electrophoresis has proceeded.
- Ethidium bromide, a fluorescent dye used for staining nucleic acids. *NOTE: Ethidium bromide is a known mutagen and should be handled as a hazardous chemical – wear gloves while handling.*

Procedure:

To pour a gel, agarose powder is mixed with electrophoresis buffer to the desired concentration, then heated in a microwave oven until completely melted. Most commonly, ethidium bromide is added to the gel (final concentration 0.5 ug/ml) at this point to facilitate visualization of DNA after electrophoresis. After cooling the solution to about 60C, it is poured into a casting tray containing a sample comb and allowed to solidify at room temperature.
After the gel has solidified, the comb is removed, using care not to rip the bottom of the wells. The gel, still in its plastic tray, is inserted horizontally into the electrophoresis chamber

and just covered with buffer. Samples containing DNA mixed with loading buffer are then pipetted into the sample wells, the lid and power leads are placed on the apparatus, and a current is applied. You can confirm that current is flowing by observing bubbles coming off the electrodes. DNA will migrate towards the positive electrode, which is usually colored red.

The distance DNA has migrated in the gel can be judged by visually monitoring migration of the tracking dyes. Bromophenol blue and xylene cyanol dyes migrate through agarose gels at roughly the same rate as double-stranded DNA fragments of 300 and 4000 bp, respectively. When adequate migration has occurred, DNA fragments are visualized by staining with ethidium bromide. This fluorescent dye intercalates between bases of DNA and RNA. It is often incorporated into the gel so that staining occurs during electrophoresis, but the gel can also be stained after electrophoresis by soaking in a dilute solution of ethidium bromide. To visualize DNA or RNA, the gel is placed on an ultraviolet trans-illuminator. Be aware that DNA will diffuse within the gel over time, and examination or photography should take place shortly after cessation of electrophoresis.

Migration of DNA Fragments in Agarose

Fragments of linear DNA migrate through agarose gels with a mobility that is inversely proportional to the log10 of their molecular weight. In other words, if you plot the distance from the well that DNA fragments have migrated against the log10 of either
their molecular weights or number of base pairs, a roughly straight line will appear.
Circular forms of DNA migrate in agarose distinctly differently from linear DNAs of the same mass. Typically, uncut plasmids will appear to migrate more rapidly than the same plasmid when linearized. Additionally, most preparations of uncut plasmid contain at least two topologically-different forms of DNA, corresponding to supercoiled forms and nicked circles. The image to the right shows an ethidium-stained gel with uncut plasmid in the left lane and the same plasmid linearized at a single site in the right lane.

Several additional factors have important effects on the mobility of DNA fragments in agarose gels, and can be used to your advantage in optimizing separation of DNA fragments. Chief among these factors are:
Agarose Concentration: By using gels with different concentrations of agarose, one can resolve different sizes of DNA fragments. Higher concentrations of agarose facilitate separation of small DNAs, while low agarose concentrations allow resolution of larger DNAs.

Voltage: As the voltage applied to a gel is increased, larger fragments migrate proportionally faster than small fragments. For that reason, the best resolution of fragments larger than about 2 kb is attained by applying no more than 5 volts per cm to the gel (the cm value is the distance between the two electrodes, not the length of the gel).
Electrophoresis Buffer: Several different buffers have been recommended for electrophoresis of DNA. The most commonly used for duplex DNA are TAE (Tris-acetate- EDTA) and TBE (Tris-borate-EDTA). DNA fragments will migrate at somewhat different rates in these two buffers due to differences in ionic strength. Buffers not only establish a pH, but provide ions to support conductivity. If you mistakenly use water instead of buffer, there will be essentially no migration of DNA in the gel! Conversely, if you use concentrated buffer (e.g. a 10X stock solution), enough heat may be generated in the gel to melt it.
Effects of Ethidium Bromide: Ethidium bromide is a fluorescent dye that intercalates between bases of nucleic acids and allows very convenient detection of DNA fragments in gels, as shown by all the images on this page. As described above, it can be incorporated into agarose

gels, or added to samples of DNA before loading to enable visualization of the fragments within the gel. As might be expected, binding of ethidium bromide to DNA alters its mass and rigidity, and therefore its mobility.