Practical Manual

Zoology

Under

Star college scheme

Of

Department of Biotechnology
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By

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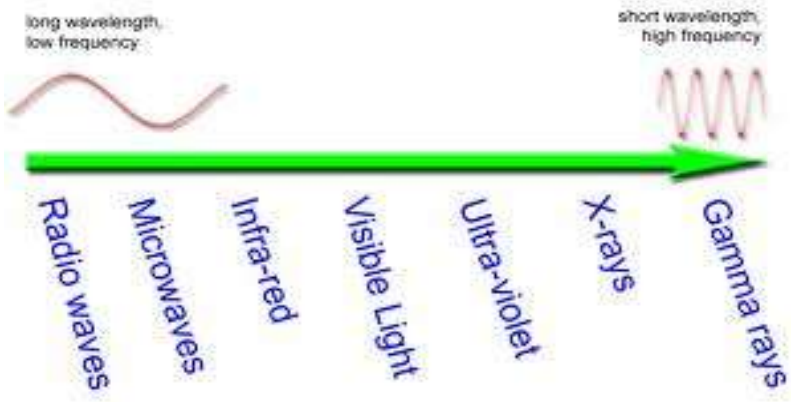
Contents

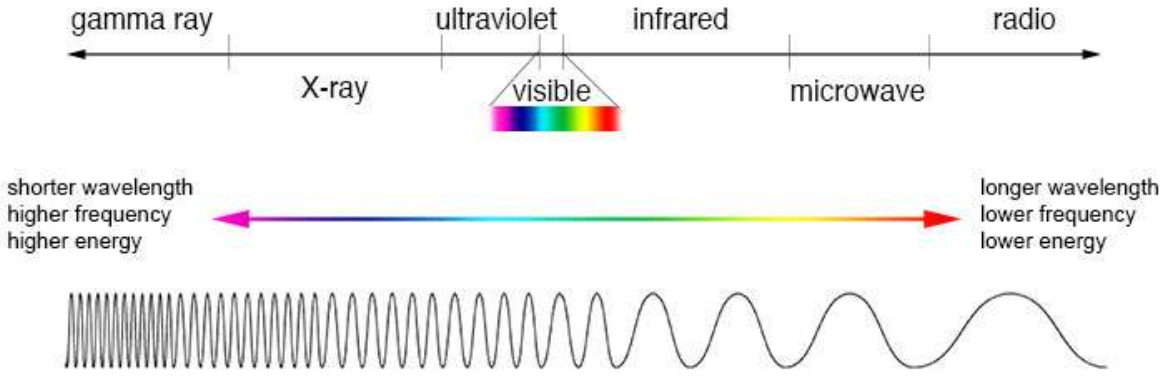
COLORIMETER

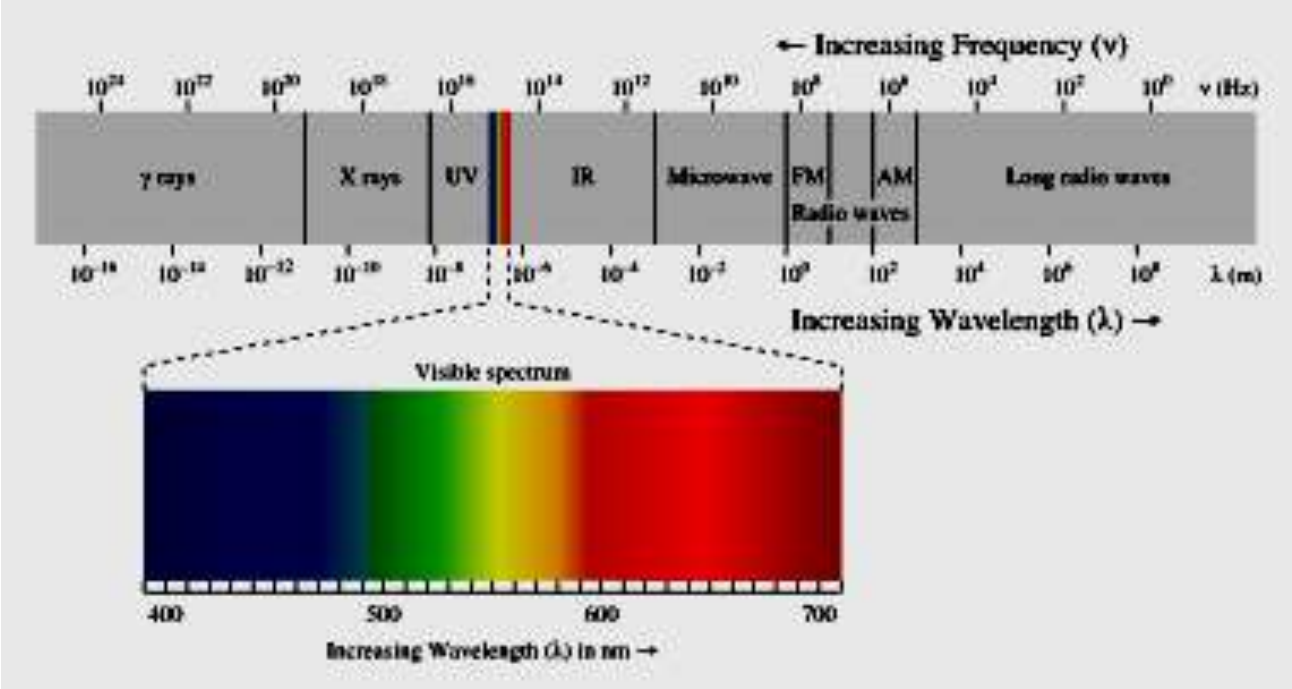
About light:

Visible light (commonly referred to simply as **light**) is electromagnetic radiation that is visible to the human eye, and is responsible for the sense of sight. Visible light has a wavelength in the range of about 380 nanometres (nm), or 380×10^{-9} m, to about 740 nanometres – between the invisible infrared, with longer wavelengths and the invisible ultraviolet, with shorter wavelengths.

Electromagnetic spectrum







V - Violet
I - Indigo
B - Blue
G - Green
Y - Yellow
O - Orange
R - Red

Aim :- Working of Colorimeter

A **colorimeter** is a device used in colorimetry.

The method of estimation of conc of coloured solute in a solution by comparing its colour intensity with that of a standard solution containing a known conc of the same solute is known as colorimeter.

Principle:-

Visible light of same intensity and an identical wavelength is passed through both solutions. When white light is passed through a colored solution, some wavelengths are absorbed more than others.

It obeys Lambert's and Beer's law, which states that the concentration of a solute is proportional to the absorbance.

♣ Beer's Law:

According to Beer's law when monochromatic light passes through the colored solution, the amount of light transmitted decreases exponentially with increase in concentration of the colored substance.

$$\mathbf{I_t = I_o e^{-KC}}$$

♣ Lambert's Law

According to Lambert's law the amount of light transmitted decreases exponentially with increase in thickness of the colored solution.

$$\mathbf{I_t = I_o e^{-KT}}$$

Therefore, together Beer-Lambert's law is:

$$\mathbf{I_t / I_o = e^{-KCT}}$$

where,

I_t = intensity of transmitted light
I_o = intensity of incident light
e = base of neutral logarithm
K = a constant
C = concentration
T = thickness of the solution

(fig. Digital Colorimeter)

The essential parts of a colorimeter are :

This apparatus comprises of the following parts :-

1) Light source usually tungsten.
2) Filter (the device that selects the desired wavelength)
3) Sample chamber or cuvette chamber (the transmitted light passes through compartment wherein the solution containing the colored solution are kept in cuvette, made of glass or disposable plastic)
4) Detector (this is a photosensitive element that converts light into electrical signals) (The spectrophotometer also works on a similar principle except the fact that it has UV light)

Procedure to operate:-

1) Switch on the instrument at least 10 minutes before use to allow it to stabilize.
   2)Select the most appropriate filter for the analysis and insert it in the light path.
3) Place the reagent blank solution (everything present in test cuvette except the component to be determined) in the clean and dry cuvette, then adjust the optical density (OD) to zero or transmittance to 100. Put the OD knob on.
4) Now transfer the blank solution to its test tube.
5) Place the known sample in the cuvette and read the absorbance of the solution. Do the same for all known concentrations and record the OD.
6) Finally take the test solution in another cuvette and read the optical density.
7) After use turn off the instrument, rins and dry the cuvette then place them in their desired boxes.

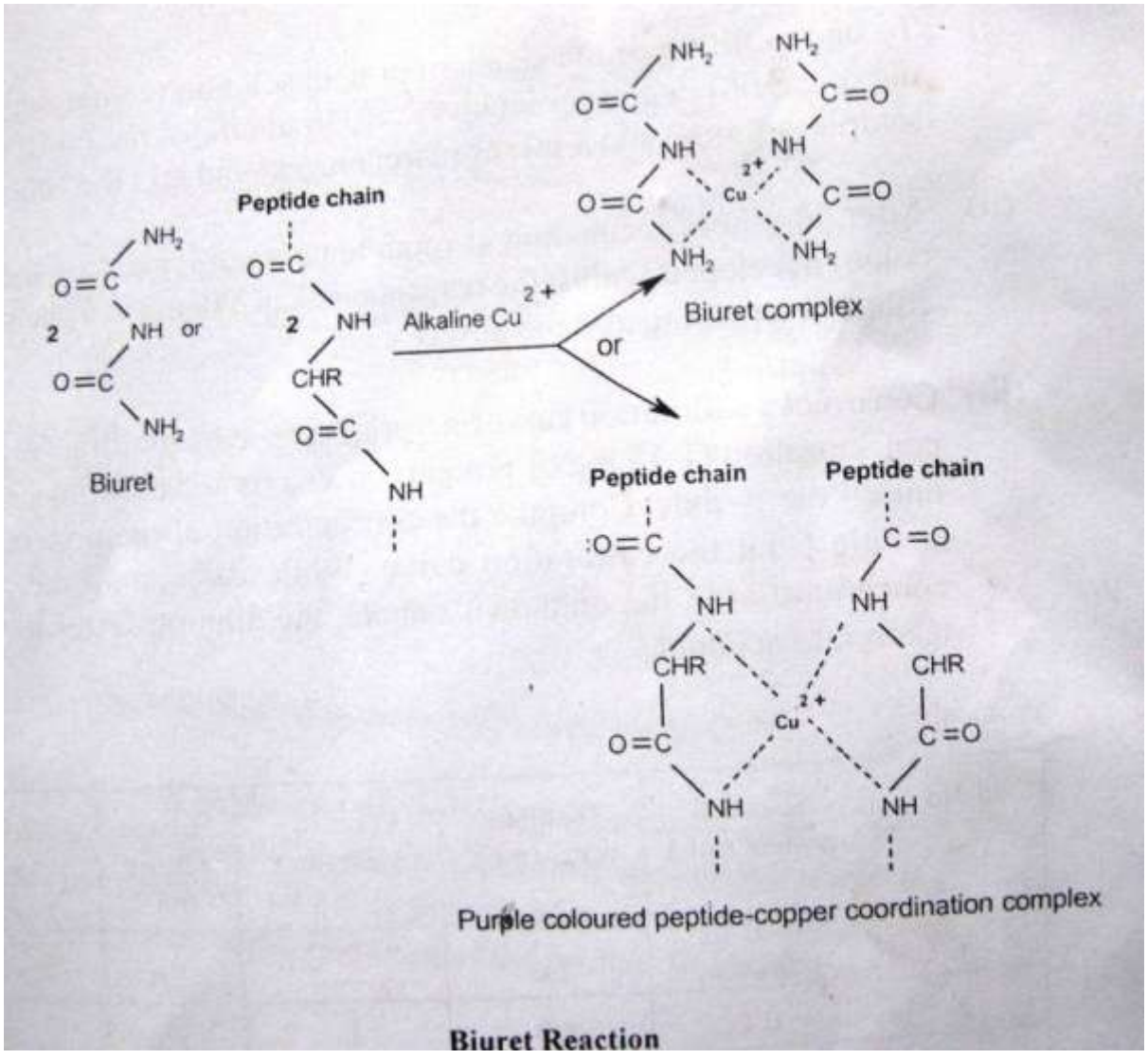
Observations:-

Results:-

Protein analysis

Determination of protein concentration by biuret method.

Principle:- The peptide bonds of the protein react with cupric copper (Cu2+) under alkaline condition to yield a purple / violet coloured complex, which shows an absorption maximum at 540 nm.



Biuret Reaction

Protein reference standard: BSA is used as a reference standard protein (10 mg/mL). Weigh accurately 1 g of BSA and transfer it to a clean and dry 100 mL volumetric flask. Suspend the protein in 50 mL of distilled water. Dissolve the

protein by adding few drops of 10 N sodium hydroxide solution and make up the volume to 100 mL.

$$\text{normality, } N = \left(\frac{\text{gram equivalents}}{\text{liters solution}}\right)$$

Reagents:- Biuret reagent: Dissolve 1.5 g of cupric sulphate in 250 mL distilled water. Separately, weigh and dissolve 6 g of sodium potassium tartrate in 250 mL of distilled water. Mix the cupric sulphate and tartrate solutions in a 1 L beaker. To this solution add 300 mL of 10% (w/v) sodium hydroxide solution, with constant stirring. Make up the volume to 1L with distilled water using a volumetric flask. Store the biuret reagent in a plastic container.

Procedure

(i) Take 12 test tubes. Mark one test tube as B (Blank),ten test tubes as S1,S2,S3,S4,S5,S6,S7,S8,S9,S10 respectively (Standard 1 -10) , one test tube as T (Test) .

(ii) Make additions as follows:--

Sr. No.	Standard protein (mL)	Distilled water (ml)	Conc. Of Casein (mg)	Biuret reagent (ml)	Absorbance at 540 nm
1	Blank	1.0	--	5.0	
2	0.1	0.9	1	5.0	
3	0.2	0.8	2	5.0	
4	0.3	0.7	3	5.0	
5	0.4	0.6	4	5.0	
6	0.5	0.5	5	5.0	
7	0.6	0.4	6	5.0	
8	0.7	0.3	7	5.0	
9	0.8	0.2	8	5.0	
10	0.9	0.1	9	5.0	
11	1.0	--	10	5.0	
12	Test 1.0 ml	--	(to be	5.0	

mix the contents.

(ii) After 30 min of incubation at room temperature, measure the violet colour developed against the reagent blank at 540 nm in a photometer/colorimeter and record the absorbance.

(iii) Construct a calibration curve on a graph paper, by plotting the protein concentration (1-10 mg of protein) on x- axis and absorbance at 540 nm on the y- axis. Compute the concentration of the protein in the sample from the calibration curve.

Observation table:

Sr. No.	Standard protein (mL)	Distilled water (ml)	Conc. Of Casein (mg)	Biuret reagent (ml)	Absorbance at 540 nm
1	Blank	1.0	--	5.0	
2	0.1	0.9	1	5.0	
3	0.2	0.8	2	5.0	
4	0.3	0.7	3	5.0	
5	0.4	0.6	4	5.0	
6	0.5	0.5	5	5.0	
7	0.6	0.4	6	5.0	
8	0.7	0.3	7	5.0	
9	0.8	0.2	8	5.0	
10	0.9	0.1	9	5.0	
11	1.0	--	10	5.0	
12	Test 1.0 ml	--	(to be determined)	5.0	

Result : in given sample protein concentration is found as _____mg/ml

Workout : Estimate the protein content in egg-white (lien's egg) and report the value of protein as g%.

Estimation of Glucose

Glucose analysis was done by O-Toluidine method. For glucose analysis from sample, O-Toluidine reagent had been used.

Procedure :--

In each test tube, 5 ml of O-Toluidine reagent had been taken. In the test tube marked as blank, 0.1 ml of distilled water was added. In the test tube marked as standard, 0.1 ml of glucose working standard (1 ml. of stock standard in 9 ml benzoic acid saturated.) was added which was prepared from glucose stock standard (glucose 1 gm., benzoic acid 250 mg. in 100 ml of distilled water) and in test tubes mark as test, 0.1 ml of test sample were added from each sample tube.

All the test tubes then kept in boiling water bath exactly for 8 minutes. After 8 min. all the test tubes were moved from water bath and kept in cold water in a beaker. Optical density were measured in 630 nm in UV spectrophotometer with blank set as zero. Calculations were done for percentage of glucose per 100 ml.

Formula used to calculate the percentage of glucose in tissues:

Optical density of test/optical density of standard × 100

Estimation of Cholesterol

Cholesterol analysis was done by Liebermann Burchard method. For analysis of Cholesterol from tissues, glacial acetic acid aldehyde free was used.

In test tube marked as Blank, 6ml of glacial acetic acid had been added then 0.1 ml of distilled water was added followed by 4 ml color reagent (10% $FeCl_3$ 0.5ml was taken in 50 ml measuring cylinder and volume was make up with H_2SO_4 to the mark).

In test tube marked as standard, 5 ml of glacial acetic acid aldehyde free was taken. 1 ml of cholesterol working standard (10 ml of cholesterol stock standard in 50ml glacial acetic acid extra pure) was added in test tube which was prepared from cholesterol stock standard(1mg cholesterol in 1 ml distilled water) followed by 4 ml of color reagent.

In test tubes marked with test, 5 ml of acetic acid aldehyde free was taken. 0.1 ml of test sample were added from each sample tube followed by 4 ml of color reagent in each test tube. All the test tubes were allowed to cool at room temperature. Absorbance was measured in 630 nm in UV Spectrophotometer against the reagent Blank. Blank was set as zero. Calculations were done for percentage of cholesterol present per 100 ml.

Formula used to calculate the percentage of cholesterol in tissues:

Optical density of test/optical density of standard × 0.2×100/0.1

ANIMAL CELL CULTURE TECHNIQUE:

Introduction

Cell culture has become one of the major tools used in life sciences today. This guide is designed to serve as a basic introduction to animal cell culture. It is appropriate for laboratory workers who are using it for the first time, as well as for those who interact with cell culture researchers and who want better understanding of the key concepts and terminology in this interesting and rapidly growing field.

What is cell and tissue culture?

Tissue culture is the general term removal of cells, tissues or organs from an animal or plant and their subsequent placement into an artificial environment conducive to growth. This environment usually consists of a suitable glass slide of plastic culture vessel containing a liquid or semi-solid medium that supplies the nutrients essential for survival and growth. The culture of whole organs or intact organ fragments with the intent of studying their continued function or development is called organ culture. When the cells are removed from the organ fragments prior to, or during cultivation, thus disrupting their normal relationship with neighboring cells, it is called Cell Culture.

Primary Culture:

When cells are surgically removed from an organism and placed into a suitable culture environment, they will attach, divide and grow. This is called Primary Culture.

Secondary Culture (a subculture derived from a primary culture)

When the cells in the primary culture vessel have grown and filled up all of the available culture substrate, they must be sub cultured to give them room for continued growth. This is usually done by removing them as gently as possible from the substrate. The cell suspension can then be subdivided and placed into new culture vessels, is called as Secondary Culture. An alternative to establishing cultures by primary culture is to buy established cell culture (secondary cell culture) from organizations such as ATCC, NCCS, Pune.

1. Determination of cell viability and cell count:

Trypan blue staining is a simple way to evaluate cell membrane integrity and thus cell viability. The isolated and washed lymphocytes are checked for viability and cell count using a hemocytometer. The purpose is to determine the cell density of viable cells in the culture. The viable and non-viable cell density can be determined with the use of dye (Trypan Blue) exclusion assay. The viable or living cells do not dye while non-viable or dead cells do so because of disrupted cell membrane. Non-viable or dead cells do not have the metabolic capability to expel the intruding dye.

Material required:

Reagents:

1. Trypan blue dye solution (0.5%)
2. Phosphate buffered saline
3. RPMI-1640 medium

Apparatus:

1. Eppendorf tubes
2. Micropipette and tips

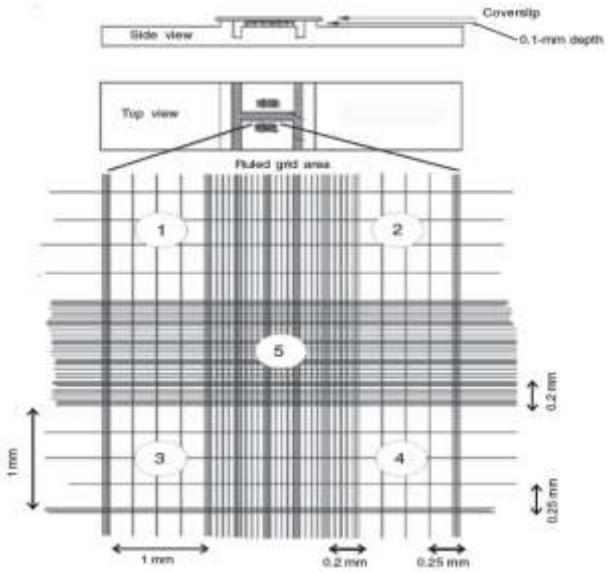
Equipments:

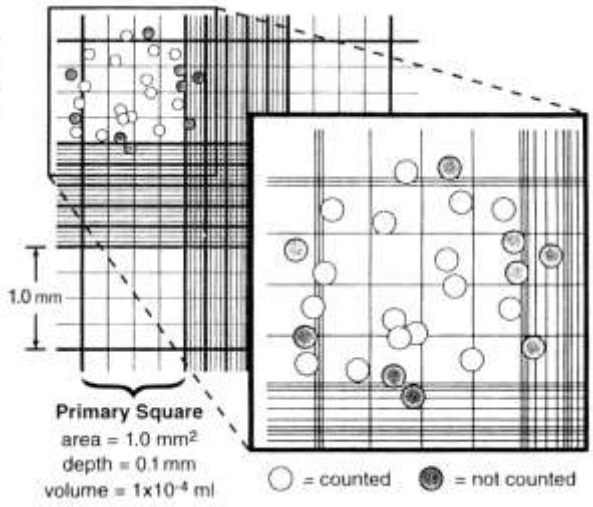
1. Light microscope
2. Hemocytometer with coverslip

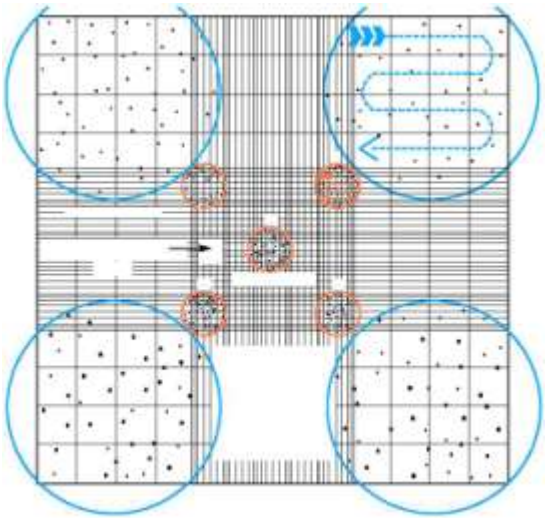
Method:

1. Take 50µl of washed cell suspension in microcentrifuge tube.
2. Add 400µl PBS and 10 µl of Trypan Blue solution to the cell suspension and mix thoroughly.
3. Trypan blue- cell suspension is transferred to a chamber of hemocytometer with cover slip using a micropipette. It was done carefully by touching the edge of the cover slip with pipette tip and allowing the chamber to fill by capillary action. Care was taken to neither overfill nor under fill the chamber.

4. Count all the viable cells (colorless opaque) in the four WBC counting chamber of hemocytometer under light microscope. Occasionally non-viable cells with blue stained nucleus were also observed.







Calculation:

Cell count/ml= Average cell count in four chambers x 10 (dilution factor) x 10^4(Constant)

If average cell count is 50 then,

Cell count/ml = 50 x10 x 10^4= 5 x 10^6cells/ml.

Viability staining:

1. Prepare a cell suspension of at least 10^6cells/ml by trypsinizing (adherent cells) or centrifugation (suspension cells) and resuspension.
2. Add 0.5 ml of 0.4% (w/v) trypan blue and 0.3 ml of PBS or HBSS to 0.1ml of cell suspension mix thoroughly, and let stand for 1-2 min.
3. At least 10^6 cells/mlare required for accurate counting.

Note: Blue –stained cells are non-viable and unstained cells are viable.

Viability % = No. of viable cells/Total no. Of cells X 100

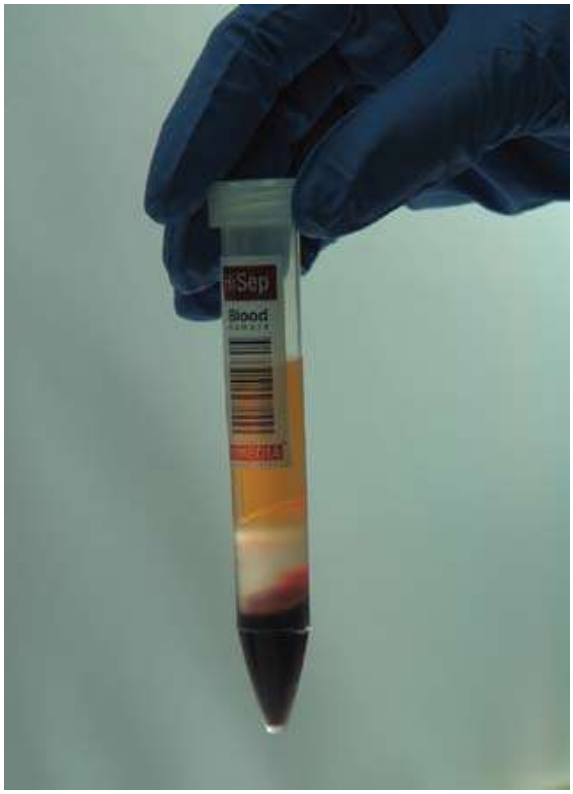
Primary Culture: Culturing of Peripheral blood lymphocytes

Isolation of Lymphocytes:

Peripheral blood lymphocytes from heparinised blood were isolated by gradient centrifugation using HIMEDIA HISep LSM 1077.

Density Gradient method:

It is simple and rapid method for separation of lymphocytes from whole blood. In this method and anticoagulant-treated blood is layered on Hisep solution and centrifuged, resulting in layers of different cell types due to differential migration. The bottom layers of different cell types due to differential migration. The bottom layer contains erythrocytes, the layer above contains Hisep, then lymphocytes and platelets, and the top layer contains plasma. The lymphocytes sit between the plasma and Hisep solution due to their lower density compared to the pelleted erythrocytes.



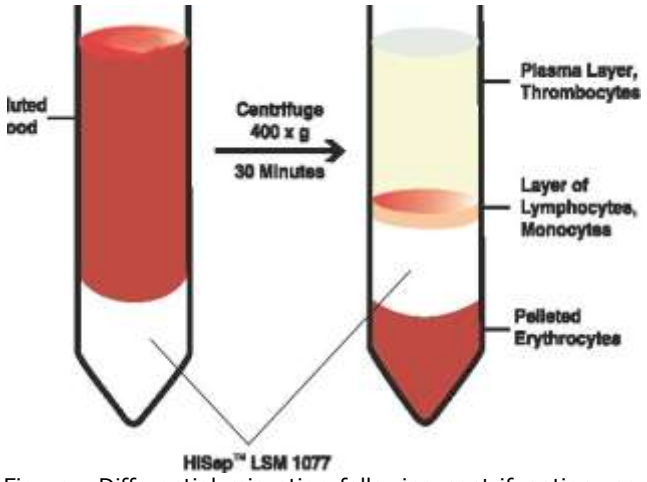


Figure . Differential migration following centrifugation resu in the formation of several cell layers. Mononuclear c (lymphocytes and monocytes) and platelets are contained the banded plasma-LSM interphase due to their density, a the pellet that is formed contains mostly erythrocytes a granulocytes, which have migrated through the gradient the bottom of the tube. Lymphocytes are recovered aspirating the plasma layer and then removing the ce Excess platelets, HiSep™ LSM 1077, and plasma can then removed by cell washing with isotonic phosphate buffe saline.

Materials required:

Reagents:

1. HISep LSM 1077
2. Phosphate buffered saline
3. RPMI-1640 medium

Glassware/ Apparatus:

1. 15ml centrifuge tubes
2. Eppendorf tubes
3. Micropipette and tips
4. Centrifuge tube stand

Equipments:

1. Refrigerated/ cold centrifuge (REMI)
2. Light microscope
3. Biosafety cabinet

Method:

1. Take 2ml heparinised blood from a consenting volunteer and dilute in 4ml of RPMI-1640.
2. Layer blood carefully over 2-3ml HISep LSM 1077in a centrifuge tube (care has to be taken not to disturb the Hisep below. So, add sample slowly by the walls).
3. Centrifuge at 1000 rpm for 20-25 minutes.
4. Aspirate upper plasma layer.
5. Then take out the lymphocytes from the buffy coat and put it in another centrifuge tube (care should be taken not to take RBCs).
6. Wash the lymphocytes twice with RPMI medium.
7. Suspend the lymphocyte pellet in 1ml of RPMI medium and check the cells for viability and do the cell count by hemocytometer.

3. Passaging/ Sub culturing

After seeding a cell in a culture dish it grows and multiplies in presence of the supplied culture medium, they cover the tissue culture dish, and there is decrease in the nutrient, accumulation of toxic metabolites.

Passaging is a process of diluting cell number in order to keep cells actively growing otherwise; the cells will become unhealthy and stop growing.

Passaging of adherent cells:

Since adherent cells grow in a single layer (called monolayer) attached to the tissue culture dish, it is required to detach it from the cell culture dish for Passaging. Trypsinization is a process in which adherent cells were detached from the cultured dish with the help of protease enzyme treatment which digest the extracellular matrix, and bring the cells in the form of the suspension culture. After getting the cell in the form of suspension culture it was passaged similar to suspension culture.

Trypsinisation:

1. Observe the culture under microscope for contamination if any and condition of the cells. (**Passage only healthy cells**).
2. Aspirate the medium and discard.
3. Wash cells with medium without serum twice.The volume of the medium should be approximately the same as the volume of medium used for culturing the cells.Repeat the step if necessary.
4. Add enough TVPG or trypsin solution to cover the monolayer, and rock the flask/dish 4-5 times to coat the monolayer.
5. Give another wash of TVPG or trypsin.
6. Tighten the flask cap and observe under inverted microscope until cells detach. If cells have not dislodged, loosen the flask cap and return the flask/dish to the incubator for a few minutes.
7. Once dislodged, resuspend the cells in growth medium containing serum. Use medium containing serum. Use medium containing the same percentage of serum as used for growing the cells.
8. Gently pipette the cells up and down to disrupt cell clumps, count, and replate at desired density in fresh flask/dishes.

IMPORTANT:

Do not leave cells in trypsin or TPVG solution for extended periods of time. Do not force the cells to detach before they are ready to do so, or clumping may occur. Overly confluent cultures, senescent cells, and some cell lines may be difficult to

trypsinize. Increase the length of trypsin exposure should help to dislodge resistant cells. Some cell lines will resist this treatment and will produce cell clumps. Such cell clumps can be broken up by repeatedly pipetting the cells up and down in a syringe with a needle attached. This should be performed as gently as possible to avoid damaging the cells. If pipette too vigorously, the cells will become damaged. Ensure that pipetting does not create foam. The serum inactivates the trypsin activity.

Size of flask	Volume of TPVG or trypsin	Volume of Medium
T25	2ml	5ml
T75	5ml	15ml
T 150-180	10ml	30ml

TPVG = 0.25% trypsin, 0.02% EDTA, 0.1% glucose in phosphate buffered saline.

Sub culturing suspension cell lines

1. Observe the culture under microscope for contamination if any and condition of the cells. Passage only healthy cells.
2. Gently pipette the cells and transfer to centrifuge tube and spin at 200Xg for 10min. Aspirate the spent medium and discard.
3. Gently pipette the cells up and down to disrupt cell clumps and count the cells. Collect the pellet and resuspend the cells in fresh complete medium at appropriate density and seed them in new flasks.
4. Alternatively fresh medium can be added to obtain necessary cell density and cells are seeded in new flask.

IMPORTANT:

Cell clumps can be broken up by repeatedly pipetting the cell up and down in using wide bore pipette. This should be performed as gently as possible to avoid damaging the cells. If pipetted too vigorously, the cells will become dogged. Ensure that pipetting does not create foam.

Adhered cells

1ml (0.25% Trypsin + 0.02% EDTA in phosphate buffer)

Cell detachment

Suspension culture

Passaging

4. Preparation of primary chick Embryo Fibroblast cultures

1. For primary chick embryo cell cultures select embryos from 9 to 11 day-old fertilized eggs derived from specific-pathogen-free flocks. Mark the air space.
2. Swab to disinfect the egg with 70% ethanol or tincture iodine.
3. Place the egg on rack with the air sac and uppermost.
4. With gentle but firm strokes with sterile scalpel or sterile blunt thumb forceps break open the shell over the air sac end.
5. Remove the egg shell membrane with sterile forceps.
6. Lift out the embryo by holding the neck with forceps and place it in a sterile disposable Petridish with sterile balance salt solution.
7. Wash the embryo with excess of BSS at least three times.
8. Remove the heads, legs and wing tips of the embryos with sterile scissors.
9. Wash the embryos in BSS.
10. Open the body cavity of each embryo with the sterile forceps and remove the liver and the bulk of the other viscera.
11. Gently squeeze the remainder of the embryo with the forceps to remove as much blood as possible.
12. Pick the washed embryos out of the wash solution with the forceps. Drain them momentarily, and place them in a sterile, dry Petridish. Mince the embryos thoroughly by cutting with sharp, sterile scissors.
13. Place the minced tissue in a 250ml sterile trypsinizing flask. Add 30 ml of 0.20% trypsin solution (prewarmed to approximately 350C), and trypsinize for 5 min at room temperature.

14. Carefully decant the supernatant suspension and discard. To the remaining fragments in the trypsinizing flask, add 70 ml of 0.20% trypsin and trypsinize for another 40 min.
15. Allow big fragments to settle and transfer to the centrifuge bottle to stop the action of the trypsin on the cells.
16. Centrifuge at approximately 250 x g for 10 minutes andcarefully remove the supernatant fluids.
17. Resuspend the cells in growth medium to a concentration of approximately 800,000 cells per ml.
18. Seed 1 ml per tube or 5ml per flask and incubate at 37^0C.

5. Chromosome Preparation

1. Seed the cells in flask and culture for 48 hours.
2. Treat cells with colchicines (0.5 ug per ml) and incubate at 37^0C for 2 hours. Observe the cells, they will be rounded off and may be floating, if not, continue to incubate and check every half hour.
3. Shake the bottle to dislodge the colchicines arrested metaphase cells and collect them along with the medium.
4. Trypsinize the remaining cells in bottle and spin at 1000 rpm for 5minutes.
5. Discard the supernatant and resuspend the cell pellet in 5 ml of 0.5%KCL and keep at 37^0C for20 minutes to well the cells.
6. Centrifuge at 1000 rpm for 10 min. Aspirate and discard all but 0.3 ml of supernatant. Mix by hand to break up the cell pellet. It is important to get the cell pellet completely resuspended, but the cells are fragile and easily broken.
7. Slowly add 2ml of fixative (chilled Methanol + Acetic acid: 3+1 volume) letting it run down the side of the tube so that it layers on top of the cell suspension. Cap the tube. Mix rapidly by hand so that all the cell suspension is fixed evenly; if it is mixed too slowly, clumps may form. Incubate at 40^0 C for 15-20 minutes.

MOLECULAR TECHNIQUES:

I. Isolation of Deoxyribose Nucleic Acid (DNA)

Introduction

Extraction and purification of nucleic acids is the first step in most molecular biology studies and in all recombinant DNA techniques. Here the objective of nucleic acid extraction methods is to obtain purified nucleic acids from various sources with the aim of conducting a GM specific analysis using the Polymerase Chain Reaction (PCR). Quality and purity of nucleic acids are some of the most critical factors for PCR analysis. In order to obtain highly purified nucleic acids free from inhibiting contaminants, suitable extraction methods should be applied. The possible contaminants that could inhibit the performance of the PCR analysis are listed in Table 1. In order to avoid the arising of a false negative result due to the presence of PCR inhibitors in the sample, it is highly recommended to perform a control experiment to test PCR inhibition. For this purpose, a plant-specific (eukaryote or chloroplast) or species-specific PCR analysis is commonly used.

Table 1:**Some inhibitors of the PCR process Inhibitor Inhibiting concentration**

INHIBITOR	INHIBITING CONCENTRATION
SDS	> 0.005%
Phenol	> 0.2%
Ethanol	> 1%
Isopropanol	> 1%
Sodium acetate	> 5 mM
Sodium chloride	> 25 mM
EDTA	> 0.5 mM
Hemoglobin	> 1 mg/ml
Heparin	> 0.15 i.u./ml
Urea	> 20 mM

Reaction mixture	> 15%

As a wide variety of methods exist for extraction and purification of nucleic acids, the choice of the most suitable technique is generally based on the following criteria:

• Target nucleic acid

• Source organism

• Starting material (tissue, leaf, seed, processed material, etc.)

• Desired results (yield, purity, purification time required, etc.)

• Downstream application (PCR, cloning, labelling, blotting, RT-PCR, cDNA synthesis, etc.)

Extraction methods

The principles of some of the most common methodologies used today for the extraction and purification of nucleic acids are described in the following sections.

The extraction of nucleic acids from biological material requires cell lysis, inactivation of cellular nucleases and separation of the desired nucleic acid from cellular debris. Often, the ideal lysis procedure is a compromise of techniques and must be rigorous enough to disrupt the complex starting material (e.g. tissue), yet gentle enough to preserve the target nucleic acid.

Common lysis procedures include:

• Mechanical disruption (e.g. grinding, hypotonic lysis)

• Chemical treatment (e.g. detergent lysis, chaotropic agents, thiol reduction)

• Enzymatic digestion (e.g. proteinase K)

Cell membrane disruption and inactivation of intracellular nucleases may be combined. For instance, a single solution may contain detergents to solubilise cell membranes and strong chaotropic salts to inactivate intracellular enzymes. After cell lysis and nuclease inactivation, cellular debris may easily be removed by filtration or precipitation.

Purification methods

Methods for purifying nucleic acids from cell extracts are usually combinations of two or more of the following techniques:

- Extraction/precipitation
- Chromatography
- Centrifugation
- Affinity separation

A brief description of these techniques will be given in the following paragraphs.

Extraction/Precipitation

Solvent extraction is often used to eliminate contaminants from nucleic acids. For example, a combination of phenol and chloroform is frequently used to remove proteins. Precipitation with isopropanol or ethanol is generally used to concentrate nucleic acids. If the amount of target nucleic acid is low, an inert carrier (such as glycogen) can be added to the mixture to increase precipitation efficiency. Other precipitation methods of nucleic acids include selective precipitation using high concentrations of salt ("salting out") or precipitation of proteins using changes in pH.

Chromatography

Chromatography methods may utilise different separation techniques such as gel filtration, ion exchange, selective adsorption, or affinity binding. Gel filtration exploits the molecular sieving properties of porous gel particles. A matrix with defined pore size allows smaller molecules to enter the pores by diffusion, whereas bigger molecules are excluded from the pores and eluted at the void volume. Thus, molecules are eluted in order of decreasing molecular size. Ion exchange

chromatography is another technique that utilises an electrostatic interaction between a target molecule and a functional group on the column matrix. Nucleic acids (highly negatively charged, linear polyanions) can be eluted from ion exchange columns with simple salt buffers. In adsorption chromatography, nucleic acids adsorb selectively onto silica or glass in the presence of certain salts (e. g. chaotropic salts), while other biological molecules do not. A low salt buffer or water can then elute the nucleic acids, producing a sample that may be used directly in downstream applications.

Centrifugation

Selective centrifugation is a powerful purification method. For example ultracentrifugation in self-forming CsCl gradients at high g-forces has long been used for plasmid purification. Frequently, centrifugation is combined with another method. An example of this is spin column chromatography that combines gel filtration and centrifugation to purify DNA or RNA from smaller contaminants (salts, nucleotides, etc.), for buffer exchange, or for size selection. Some procedures combine selective adsorption on a chromatographic matrix (see above paragraph “Chromatography”) with centrifugal elution to selectively purify one type of nucleic acid.

Affinity separation

In recent years, more and more purification methods have combined affinity immobilisation of nucleic acids with magnetic separation. For instance, poly(A) + mRNA may be bound to streptavidin-coated magnetic particles by biotin-labelledoligo(dT) and the particle complex removed from the solution (and unbound contaminants) with a magnet. This solid phase technique simplifies

nucleic acid purification since it can replace several steps of centrifugation, organic extraction and phase separation with a single, rapid magnetic separation step.

DNA extraction using Kit columns

A costly however, an effective method of extracting high quality amplifiable genomic DNA from whole blood, urine, dried blood spot, buffy coat and tissue biopsy samples. Refrigerated samples and reagents from the kit are brought to room temperature before starting the procedure. Check the following equipments and reagents are ready:

• Water bath at 56°C.

• Buffer AE or dd.H2O for elution.

• Buffer AWl, Buffer AW2, and QIAGEN Protease.

• If a precipitate has formed in Buffer AL, dissolve by incubating at 70oC

• All centrifugation steps should be carried out at room temperature

Extraction Protocol

Pipette **20µl** of **Protease** into the bottom of a 1.5ml microcentrifuge tube.

⇩

Add **200 µl** of the sample to the microcentrifuge tube.

⇩

Add **200µl buffer AL (lysis buffer)** to the sample. Mix by pulse-vortexing for 15sec.

⇩

Incubate at **56°C** for **10 min**.

(DNA yield reaches a maximum after lysis for 10 min at 56°C, but longer incubation times will not have negative effect on DNA extraction)

⇩

Briefly centrifuge the 1.5ml microfuge tube to remove drops from the inside of the lid.

⇩

Add **200µl of ethanol (96-100%)** to the sample and mix again by pulse-vortexing. After mixing, briefly centrifuge the 1.5ml microfuge tube to remove drops from the inside of the lid

⇩

Carefully transfer the mixture from the previous step to QIAamp spin column (in a 2ml collection tube) without wetting the rim, close the cap, and centrifuge at 6000xg (8000rpm) for 1 min. Place the QIAamp spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.

⇩

Carefully open the QIAamp spin column and add **500µl Buffer AW1** without wetting the rim. Close the cap and centrifuge at 6000xg (8000rpm) for 1min. Place the QIAamp spin column in a clean 2ml collection tube (provided), and discard the collection tube containing the filtrate.

⇩

Carefully open the QIAamp spin column and add **500µl Buffer AW2** without wetting the rim. Close the cap and centrifuge at full speed (20,000xg; 14000rpm) for 3 min. Continue with the next step that is optional to eliminate any chance of possible buffer AW2 carryover or directly go to step after that and continue.

⇩

(Optional): Place the QIAamp spin column in a new 2 ml collection tube (not provided) and discard the collection tube containing the filtrate. Centrifuge at full speed for 1min.

⇩

Place the QIAamp spin column in a clean 1.5ml microfuge tube (not provided), and discard the collection tube containing the filtrate.

⇩

Carefully open the QIAamp spin column and add **200µl Buffer AE or distilled water**. Incubate at room temperature for 5min, and then centrifuge at 6000xg (8000rpm) for 1min

Quantification of DNA:

1. The extracted DNA is now referred asstock DNA and isstored at -20^{0}C.
2. After isolation of DNA, quantization is done by diluting 10 µl of stock DNA in 990 µl of MQW in fresh eppendorf tube and was vortex for ½ minute.
3. Using a UV - spectrophotometer the estimate of the purity of DNA sample was obtained by calculating the ratio between the readings at 260 nm and 280 nm . A ratio of $OD_{260}/_{280}$ of 1.8 indicates that the preparation is pure.

Table showing Example for OD ratio.

Sample	A/260	A/280	A260/280	A260 X 5 = x	1/x	MQW
10 BL	0.111	0.065	1.7	0.55	1.8	98.2

By diluting 1.8 µl of stock genomic DNA in 98.2 µl of MQW make the quantity 10ng/ µl. (A= Absorbance). Using this method, the all genomic DNAs were diluted and working stock was prepared for PCR amplification.

II. Agarose Gel Electrophoresis

Introduction

Electrophoresis through agarose or polyacrylamide gels lies near the heart of molecular biology and is used to separate, identify, and purify DNA fragments. The technique is simple, rapid to perform, and capable of resolving fragments of DNA that cannot be separated adequately by other procedures, such as density gradient centrifugation.

Gel electrophoresis is a method that separates macromolecules on the basis of size, electric charge and other physical properties. The term electrophoresis describes the migration of charged particles under the influence of an electric field. “Electro” refers to electricity and “Phoresis”, from the Greek word phoros, meaning, "to carry across." Thus, gel electrophoresis refers to a technique in which molecules are forced across a span of gel, motivated by an electrical current.

The driving force for electrophoresis is the voltage applied to electrodes at either end of the gel. The properties of a molecule determine how rapidly an electric field can move it through a gelatinous medium.

Many important biological macromolecules (e.g. amino acids, peptides, proteins, nucleotides and nucleic acids) possess ionisable groups and, at any given pH, exist in solution as electrically charged species either as cations (+) or anions (-). Depending on the nature of the net charge, the charged particles will migrate either to the cathode or to the anode. For example, when an electric field is applied across a gel at neutral pH, the negatively charged phosphate groups of the DNA cause it to migrate toward the anode.

Electrophoresis through agarose is a standard method used to separate, identify and purify DNA fragments. The technique is simple, rapid to perform, and capable of

resolving fragments of DNA that cannot be separated adequately by other procedures. Furthermore, the location of DNA within the gel can be determined by staining with a low concentration of ethidium bromide, a fluorescent intercalating dye.

The following sections will outline the physical principles, components (gel matrix, buffer, loading buffer and marker) and procedures for the preparation of agarose gel electrophoresis (Sambrook et al., 1989).

Physical principles of agarose gel electrophoresis

Gel electrophoresis is a technique used for the separation of nucleic acids and proteins. Separation of macromolecules depends upon two variables: charge and mass. When a biological sample, such as DNA, is mixed in a buffer solution and applied to a gel, these two variables act together. The electrical current from one electrode repels the molecules while the other electrode simultaneously attracts the molecules.

The frictional force of the gel material acts as a "molecular sieve", separating the molecules by size. During electrophoresis, macromolecules are forced to move through the pores and their rate of migration through the electric field depends on the following:

- the strength of the field
- the size and shape of the molecules
- the relative hydrophobicity of the samples
- the ionic strength & temperature of the buffer in which the molecules are moving.

To completely understand the separation of charged particles in gel electrophoresis, it is important to look at the simple equations relating to

electrophoresis. When a voltage is applied across the electrodes, a potential gradient, E, is generated and can be expressed by the equation:

E = V/d (1) where V, measured in volts, is the applied voltage and d the distance in cm between the electrodes.

When the potential gradient, E, is applied, a force, F, on a charged molecule is generated and is expressed by the equation:

F = Eq (2) where q is the charge in coulombs bearing on the molecule. It is this force, measured in Newtons that drives a charged molecule towards an electrode. There is also a frictional resistance that slows down the movement of charged molecules. This frictional force is a function of:

- the hydrodynamic size of the molecule
- the shape of the molecule
- the pore size of the medium in which electrophoresis is taking place
- the viscosity of the buffer

The velocity v of a charged molecule in an electric field is a function of the potential gradient, charge and frictional force of the molecule and can be expressed by the equation:

v = Eq / f (3) where f is the frictional coefficient.

The electrophoretic mobility, M, of an ion can then be defined by the ion's velocity divided by the potential gradient:

$$M = v / E \qquad (4)$$

In addition, from equation (3) one can see that electrophoretic mobility M can be equivalently expressed as the charge of the molecule, q, divided by the frictional coefficient, f:

$$M = q / f \qquad (5)$$

When a potential difference is applied, molecules with different overall charges will begin to separate due to their different electrophoretic mobilities. The

electrophoretic mobility is a significant and characteristic parameter of a charged molecule or particle and depends on the pK value of the charged group and the size of the molecule or particle. Even molecules with similar charges will begin to separate if they have different molecular sizes, since they will experience different frictional forces.

Linear double stranded DNA migrates through gel matrices at rates that are inversely proportional to the log10 of the number of base pairs. Larger molecules migrate more slowly because of the greater frictional drag and because of the less efficient movement through the pores of the gel.

The current in the solution between the electrodes is conducted mainly by the buffer ions with a small proportion being conducted by the sample ions. The relationship between current I, voltage V, and resistance R is expressed as in Ohm's law:

$$R = V / I \qquad (6)$$

This equation demonstrates that for a given resistance R, it is possible to accelerate an electrophoretic separation by increasing the applied voltage V, which would result in a corresponding increase in the current flow I. The distance migrated will be proportional to both current and time. However, the increase in voltage, V, and the corresponding increase in current, I, would cause one of the major problems for most forms of electrophoresis, namely the generation of heat. This can be illustrated by the followingequation in which the power, W, (measured in Watts) generated during the electrophoresis is equal to the product of the resistance times the square of the current:

$$W = I2R \qquad (7)$$

Since most of the power produced in the electrophoretic process is dissipated as heat the following detrimental effects can result:

- an increased rate of diffusion of sample and buffer ions leading to broadening of the separated samples
- the formation of convection currents, which leads to mixing of separated samples;
- thermal instability of samples that are rather sensitive to heat (e.g. denaturation of DNA)
- a decrease of buffer viscosity hence a reduction in the resistance of the medium

Components of agarose gel electrophoresis

Agarose

Agarose, a natural colloid extracted from seaweed, is a linear polysaccharide (average molecular mass ~12,000 Da) made up of the basic repeated unit agarobiose, which comprises alternating units of galactose and 3, 6-anhydrogalactose.

Agarose is very fragile and easily destroyed by handling. Agarose gels have large "pore" sizes and are used primarily to separate large molecules with a molecular mass greater than 200 kDa.

Agarose gels process quickly, but with limited resolution since the bands formed in the agarose gels tend to be fuzzy/diffuse and spread apart. This is a result of pore size and cannot be controlled. Agarose gels are obtained by suspending dry powdered agarose in an aqueous buffer, then boiling the mixture until the agarose melts into a clear solution. The solution is then poured onto a gel-tray and allowed to cool to room temperature to form a rigid gel. Upon hardening, the agarose forms a matrix whose density is determined by its concentration.

Electrophoresis buffer

The electrophoretic mobility of DNA is affected by the composition and ionic strength of the electrophoresis buffer. In the absence of ions, electrical conductance is minimal and DNA migrates slowly, if at all. In a buffer of high ionic strength electrical conductance is very efficient and a significant amount of heat is generated. In the worst circumstance, the gel melts and the DNA denatures.

Several buffers are available for electrophoresis of native double-stranded DNA. These contain EDTA (pH 8.0) and Tris-acetate (TAE), Tris-borate (TBE), or Trisphosphate (TPE) at a concentration of approximately 50 mM (pH 7.5 - 7.8). Electrophoresis buffers are usually prepared as concentrated solutions and stored at room temperature. TBE was originally used at a working strength of 1x for agarose gel electrophoresis. However, a working solution of 0.5x provides more than enough buffering power and almost all agarose gel electrophoresis is now carried out using this buffer concentration.

Agarose concentration

A DNA fragment of a given size migrates at different rates through gels depending on the concentration of agarose. For a specific concentration of agarose and/or buffer, it is possible to separate DNA segments containing between 20 and 50,000 bp. In horizontal gels, agarose is usually used at concentrations between 0.7% and 3% (see Table 1).

Table 1. Recommended agarose gel concentration for resolving linear DNA molecules

% agarose	DNA size range (bp)
0.75	10.000 - 15.000
1.0	500 - 10.000
1.25	300 - 5000

1.5	200 - 4000
2.0	100 - 2500
2.5	50 - 1000

DNA Ladders and Markers

DNA ladders and markers are available for sizing double-stranded, single-stranded, or supercoiled DNA. A variety of these DNA ladders and markers are also available in the ready-to-load format.

1 Kb plus DNA Ladders

The 1 Kb plus DNA Ladder is a powerful tool for estimating the molecular weight of linear double-stranded DNA fragments. The 1 Kb Plus DNA Ladder has 12 evenly spaced bands ranging from 1 kb to 12 kb, a quick orientation band at 1,650 bp that forms a distinct doublet with the 2 kb band, and seven bands of round-number sizes below 1 kb. This wide size range is perfect for downstream cloning applications as well as PCR and other molecular biology applications.

1 Kb plus Compatibility

The 1 Kb Plus DNA Ladder is compatible with all agarose gels for DNA fragment analysis. The ladder is provided in a purified format without premixed loading buffer.

Easy-to-remember band sizes

Broad size range (100 bp–12 kb)
Room temperature–stable for easy storage

The 1 Kb Plus DNA Ladder can be visualized with DNA stains such as SYBR® Safe stain, ethidium bromide, or similar DNA stains. In addition, the 1 Kb Plus DNA Ladder can be radiolabeled using T4 polynucleotide kinase, T4 DNA polymerase, DNA polymerase I, or Klenow fragment, and any radiolabeled dNTP.

100 bp DNA Ladders

The 100 bp DNA Ladder is suitable for sizing double-stranded DNA from 100 to 1,500 bp. Prepared from a plasmid containing repeats of a 100 bp DNA sequence, the ladder consists of 15 blunt-ended fragments ranging in length from 100 to 1,500 bp, in 100 bp increments, and an additional fragment at 2,072 bp. This ladder is ideal for determining the size of PCR products.

DNA Stains

There are several different stains that can be used to visualize and photograph DNA after separation by gel electrophoresis. The following list explains some of the choices of stains, and the differences between them.

Molecular Probes® fluorescent nucleic acid gel stains—SYBR® Gold, SYBR® Green I, SYBR® Green II, and SYBR® Safe dyes—are highly sensitive reagents for staining DNA in electrophoresis gels. These gel stains provide greater sensitivity, with lower background fluorescence, than the conventional ethidium bromide gel stain.

Ethidium Bromide (EtBr) Dye for DNA and RNA Detection

Ethidium bromide is the most commonly used dye for DNA and RNA detection in gels. Ethidium bromide is a DNA intercalator, inserting itself between the base pairs in the double helix. Ethidium bromide has UV absorbance maxima at 300 and

360 nm, and an emission maximum at 590 nm. The detection limit of DNA bound to ethidium bromide is 0.5 to 5.0 ng/band.

Structure, chemistry, fluorescence

As with most fluorescent compounds, ethidium bromide is aromatic. Its core heterocyclic moiety is generically known as a phenanthridine, an isomer of which is the fluorescent dye acridine. The reason for ethidium bromide's intense fluorescence after binding with DNA is probably not due to rigid stabilization of the phenyl moiety, because the phenyl ring has been shown to project outside the intercalated bases. In fact, the phenyl group is found to be almost perpendicular to the plane of the ring system, as it rotates about its single bond to find a position where it will impinge upon the ring system minimally. Instead, the hydrophobic environment found between the base pairs is believed to be responsible. By moving into this hydrophobic environment and away from the solvent, the ethidiumcation is forced to shed any water molecules that were associated with it. As water is a highly efficient fluorescent quencher, the removal of these water molecules allows the ethidium to fluoresce.

Applications

Ethidium bromide is commonly used to detect nucleic acids in molecular biology laboratories. In the case of DNA this is usually double-stranded DNA from PCRs, restriction digests, etc. Single-stranded RNA can also be detected, since it usually folds back onto itself and thus provides local base pairing for the dye to intercalate. Detection typically involves a gel containing nucleic acids placed on or under a UV lamp.

Since ultraviolet light is harmful to eyes and skin, gels stained with ethidium bromide are usually viewed indirectly using an enclosed camera, with the fluorescent images recorded as photographs. Where direct viewing is needed, the viewer's eyes and exposed skin should be protected. In the laboratory the intercalating properties have long been used to minimize chromosomal condensation when a culture is exposed to mitotic arresting agents during harvest. The resulting slide preparations permit a higher degree of resolution, and thus more confidence in determining structural integrity of chromosomes upon microscopic analysis. Ethidium bromide has also been used extensively to reduce mitochondrial DNA copy number in proliferating cells.

Loading buffer

The DNA samples to be loaded onto the agarose gel are first mixed with a loading buffer usually comprising water, sucrose, and a dye (e.g. xylene cyanole, bromophenol blue, bromocresol green, etc.). The maximum amount of DNA that can be loaded depends on the number of fragments. The minimum amount of DNA that can be detected by photography of ethidium bromide stained gels is about 2 ng in a 0.5-cm wide band.

If there is more than 500 ng of DNA in a band of this width, the slot will be overloaded, resulting in smearing. The loading buffer serves three purposes:

- Increases the density of sample ensuring that the DNA drops evenly into the well
- Adds colour to the sample, thereby simplifying the loading process

- Imparts a dye to the sample that, in an electric field, moves toward the anode at a predictable rate

Experimental

Caution: Ethidium bromide is a powerful mutagen/carcinogen and is moderately toxic. Gloves should always be worn when handling solutions and gels containing ethidium bromide.

Equipment

- Horizontal electrophoresis unit with power supply
- Microwave oven or heating stirrer
- Micropipettes
- 1.5 ml reaction tubes
- Balance capable of 0.1 g measurements
- Spatulas
- Rack for reaction tubes
- Glassware
- Transilluminator (UV radiation, 312 nm)
- Instruments for documentation (e.g. Polaroid camera or a video recorder)

Reagents

- Agarose, suitable for DNA electrophoresis
- Tris[hydroxymethyl] aminomethane (Tris)
- Boric acid
- Na2EDTA
- Ethidium bromide
- Sucrose
- Xylene cyanole FF
- DNA markers: Lambda DNA *Eco*RI/*Hind*III digested *(or other similar suitable marker)*
- 100 bp DNA ladder

10x TBE buffer (1 litre)	
Tris [hydroxymethyl] aminomethane (Tris)	54.0 g
Boric acid	27.5 g
Na2EDTA	7.44 g

- Mix reagent to deionised water to obtain a 1 litre solution at pH 8.3
- Store at room temperature

6x loading buffer (10 ml)	
Xylene cyanole FF	0.025 g
Sucrose	4 g

- Add sucrose and Xylene cyanole FF to deionised water to obtain 10 ml of solution.
- Mix the solution, autoclave and store at 4°C.

Loading of Agarose Gel

Prepare samples and marker for genomic DNA as follows:

	sample	**marker**
	water 3 µl	water 6 µl
	loading buffer 2 µl	loading buffer 2 µl
	sample 5 µl	λ DNA EcoRI / HindIII 2 µl
	Total 10 µl	Total 10 µl
Prepare samples and marker for PCR products as follows:		

sample		**marker**	
loading buffer	2 µl	100 bp DNA ladder	1 5 µl
sample	8 µl		
	10 µl		

Load 10 µl of each sample into consecutive wells and the appropriate DNA marker into the first and last lane

⇩

Close the lid of the gel tank and attach the electrical leads so that the DNA will migrate toward the anode and apply a voltage of 5 - 10 V/cm

⇩

Run the gel until the xylene cyanole has migrated the appropriate distance through the gel (~ 40 - 60 minutes)

⇩

Turn off the current; remove the leads and the lid from the gel tank. Place the gel on a UV lightbox and photograph the gel

Discard the gel into the provided ethidium bromide solid waste bin

III. Polymerase Chain Reaction (PCR) – A Noble WinningTechnology

Introduction:

Polymerase Chain Reaction was developed in 1984 by the American biochemist, Kary Mullis. Kary Mullis received the Nobel Prize and the Japan Prize in 1993 for the development of this technology. However the basic principle of replicating a piece of DNA using two primers had already been described by Gobind Khorana in 1971. Progress was limited by primer synthesis and polymerase purification issues.

PCR since then has become the most common and often indispensable technique used in medical and biological research labs for a variety of applications. It is an extremely powerful technique which rapidly became one of the most widely used techniques in molecular biology because it is quick, inexpensive and simple.

The technique amplifies specific DNA fragments from minute quantities of source DNA material. As PCR is quick in generating unlimited copies of any fragment of DNA, it is a scientific development that actually deserves timeworn superlatives like "revolutionary" and "breakthrough". Furthermore, many important contributions to the development and application of PCR technology have been made; however the present literature attempts to review and make you understand the basics of PCR.

Principle:

The basic PCR principle is simple. As the name implies, it is a chain reaction: One DNA moleculeis used to produce two copies, then four, then eight and so forth. This continuous doubling is accomplished by specific proteins known as polymerases, enzymes that are able to string together individual DNA building blocks to form long molecular strands.

To do their job polymerases require a supply of DNA building blocks, i.e. the nucleotides consisting of the four bases adenine (A), thymine (T), cytosine (C) and guanine (G). They also need a small fragment of DNA, known as the primer, to which they attach the building blocks as well as a longer DNA molecule to serve as a template for constructing the new strand. If these three ingredients are supplied, the enzymes will construct exact copies of the templates. PCR is a method used to

acquire many copies of any particular strand of nucleic acids. It's a means of selectively amplifying a particular segment of DNA.

The segment may represent a small part of a large and complex mixture of DNAs *e.g.* a specific exon of a human gene. Therefore it can be thought of as a molecular photocopier (or molecular Xerox machine).

PCR can amplify a usable amount of DNA (visible by gel electrophoresis) in ~2 hours. The PCR product thus obtained can be digested with restriction enzymes, sequenced or cloned. PCR can amplify a single DNA molecule, *e.g.* from a single sperm. The polymerase chain reaction relies on the ability of DNA copying enzymes to remain stable at high temperatures.

PCR has transformed the way that almost all studies requiring the manipulation of DNA fragments may be performed as a result of its simplicity and usefulness. Mullis's original PCR process was very inefficient since it required a great deal of time, vast amounts of DNA-Polymerase, and continual attention throughout the PCR process. Due to developments in the technology, it has shown to have wide utility for researchers interested in;

- Forensic analysis – paternity.
- Genetics – human, animals, plants and microbes.
- monitoring of therapeutic treatments
- Diagnostic applications, and for myriad of never ending research and clinical applications.

Process:

Typically, PCR consists of a series of 20-40 repeated temperature changes, called cycles, with each cycle commonly consisting of 2-3 discrete temperature steps, usually three (fig important). The cycling is often preceded by a single temperature step (called *hold*) at a high temperature (>90°C), and followed by one hold at the end for final product extension or brief storage. The temperatures used and the length of time they are applied in each cycle depend on a variety of parameters. These include the enzyme used for DNA synthesis, the concentration of divalent ions and dNTPs in the reaction, and the melting temperature (Tm) of the primers.

Initialization step:

This step consists of heating the reaction to a temperature of 94–96 °C (or 98 °C if

extremely thermostable polymerases are used), which is held for 1–9 minutes. It is only required for DNA polymerases that require heat activation by hot-start PCR.

Denaturation step:

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

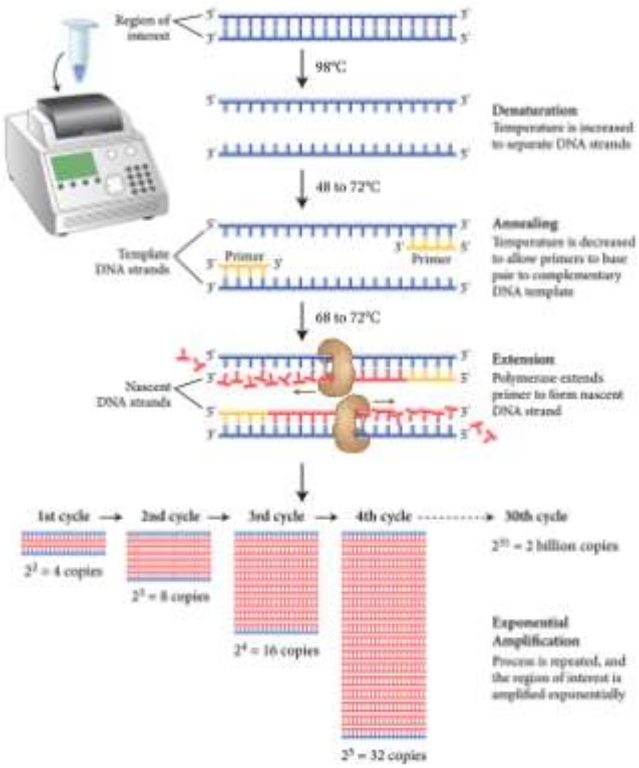
Annealing step:

The reaction temperature is lowered to 50–65 °C for 20–40 seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3-5 degrees Celsius below the Tm of the primers used. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA formation.

Extension/elongation step:

The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75–80 °C, and commonly a temperature of 72 °C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5′ to 3′ direction, condensing the 5′-phosphate group of the dNTPs with the 3′-hydroxyl group at the end of the nascent (extending) DNA strand.

The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. As a rule-of-thumb, at its optimum temperature, the DNA polymerase will polymerize a thousand bases per minute. Under optimum conditions, i.e., if there are no limitations due to limiting substrates or reagents, at each extension step, the amount of DNA target is doubled, leading to exponential (geometric) amplification of the specific DNA fragment.



Final elongation:

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

Final hold:

This step at 4–15 °C for an indefinitetime may be employed for short-termstorage of the reaction.

Necessities of PCR Reaction:

A basic PCR set up requires several components and reagents. These components include:

- DNA template that contains the DNA region (target) to be amplified.
- Two primersthat are complementary to the 3′(three prime) ends of each of the sense andanti-sense strand of the DNA target.
- Taq polymerase or another DNA polymerasewith a temperature optimum at around 70 °C.
- Deoxynucleoside triphosphates (dNTPs; nucleotides containingtriphosphate

groups), thebuilding-blocks from which the DNA polymerase synthesizes a new DNA strand.

- Buffer solution,providing a suitable chemical environment for optimum activity andstability of the DNA polymerase.
- Divalent cations,HYPERLINK "http://en.wikipedia.org/wiki/Magnesium" magnesium or manganeseions; generally $Mg^{2+}$is used, but Mn^{2+}can beutilized for PCR-mediated DNA mutagenesis, as higher Mn^{2+}concentration increases the error rate during DNA synthesis
- Monovalent cationpotassiumions.

The PCR is commonly carried out in a reaction volume of 10–200 µl in small reaction tubes (0.2–0.5 ml volumes) in a thermal cycler. The thermal cycler heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction.

Many modern thermal cyclers make use of the **Peltier effect**, which permits both heating and cooling of the block holding the PCR tubes simply by reversing the electric current. Thin-walled reaction tubes permit favourable thermal conductivity to allow for rapid thermal equilibration. Most thermal cyclers have heated lids to prevent condensation at the top of the reaction tube. Older thermal cyclers lacking a heated lid require a layer of oil on top of the reaction mixture or a ball of wax inside the tube.

Variations on the basic PCR technique

Touchdown PCR (Step-down PCR):

A variant of PCR, aimed at reducing nonspecific background by gradually lowering the annealing temperature as PCR cycling progresses. The annealing temperature at the initial cycles is usually a few degrees (3-5°C) above the Tm of the primers used, while at the later cycles, it is a few degrees (3-5°C) below the primer Tm.

The higher temperatures give greater specificity for primer binding, and the lower temperatures permit more efficient amplification from the specific

products formed during the initial cycles.

Allele-specific PCR:

A diagnostic or cloning technique based on single-nucleotide variations (SNV not to be confused with SNP) (single-base differences in a patient). It requires prior knowledge of a DNA sequence, including differences between alleles, and uses primers whose 3′ ends encompass the SNV (base pair buffer around SNV usually incorporated).

PCR amplification under stringent conditions is much less efficient in the presence of a mismatch between template and primer, so successful amplification with an SNP-specific primer signals presence of the specific SNP in a sequence.

Nested PCR:

It increases the specificity of DNA amplification, by reducing background due to non-specific amplification of DNA. Two sets of primers are used in two successive PCRs. In the first reaction, one pair of primers is used to generate DNA products, which besides the intended target, may still consist of non-specifically amplified DNA fragments.

The product(s) are then used in a second PCR with a set of primers whose binding sites are completely or partially different from and located 3′ of each of the primers used in the first reaction. Nested PCR is often more successful in specifically amplifying long DNA fragments than conventional PCR, but it requires more detailed knowledge of the target sequences.

Hot start PCR:

A technique that reduces non-specific amplification during the initial set up stages of the PCR. It may be performed manually by heating the reaction components to the denaturation temperature (e.g., 95°C) before adding the polymerase. Specialized enzyme systems have been developed that inhibit the polymerase's activity at ambient temperature, either by the binding of an antibody or by the presence of covalently bound inhibitors that dissociate only after a high-temperature activation step.

Hot-start/cold-finish PCR is achieved with new hybrid polymerases that are

inactive at ambient temperature and are instantly activated at elongation temperature.

Multiplex-PCR:

It consists of multiple primer sets within a single PCR mixture to produce amplicons of varying sizes that are specific to different DNA sequences. By targeting multiple genes at once, additional information may be gained from a single test-run that otherwise would require several times the reagents and more time to perform.

Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction, and amplicon sizes. That is, their base pair length should be different enough to form distinct bands when visualized by gel electrophoresis.

1. Polymerase Chain Reaction

Sr no	**Constituents**	**Stock concentration**	**Volume added (µl)**	**Final concentration**
1	MilliQ	-	13.8	-
2	Buffer	10X	2.5	1X
3	dNTP	10 mM	1.0	Variable
4	Forward primer	10pmol/µl	1.0	10pmol/µl
5	Reverse primer	10pmol/µl	1.0	10pmol/µl
6	Taq polymerase	5U/µl	0.2	1U/µl
7	DNA template	20ng	5.0	100ng
		Total Volume		**25 µl**

The above tabulation is the flow-sheet for preparation of the master mix for PCR reaction. The addition of the components must be in the order maintained in the table.

2. Programming Thermal Cycler

Process	Temperature	Time	Cycle/Hold	Repeat
Initial Denaturation	95°C	5min	Hold	Only once
Denaturation	95°C	30 sec	Cycle	35 times
Annealing	56°C	30 sec	Cycle	
Extension	72°C	30 sec	Cycle	
Final Extension	72°C	5min	Hold	Only Once

The steps followed while preparing the program on thermal cycler will be different from machine to machine. A basic flow of events is presented in the table and in the flowchart.

Set up the Initial Denaturation Temperature.

⇩

Set up the time for denaturation process.

⇩

Set up the hold for the Initial Denaturation Process.

⇩

Set up the number of cycle s of the process.

⇩

Set up the time for denaturation process within the cycle.

⇩

Set up the time duration for annealing process within the cycle.

⇩

Set up the time duration for extension process within the cycle.

⇩

Set up the time duration and Hold duration for Final Extension process.

IV. Restriction Fragment Length Polymorphism (RFLP) –Biological Scissors

Restriction enzymes also known as restriction endonucleases (REases) are the basic tools of molecular biology. They are enzymes that cleave double-stranded deoxyribonucleic acids (DNAs) in a sequence-specific manner and are ubiquitously present among prokaryotic organisms. They form part of the restriction-modification systems, which usually consist of an endonuclease and a methyltransferase. Restriction endonucleases have been the workhorse of molecular biology for the past 30 years.

They catalyse the breakage of phosphodiester bonds on DNA backbones at specific sites and, together with their companion methyltransferases, are part of bacterial defence systems against the invasion of bacteriophages. Their working principle as well as their applications since their discovery has greatly impacted and enhanced genetic engineering which this review article hopes to elaborate.

Basics of Restriction Enzymes

Restriction endonucleases degrade foreign DNA upon its introduction into a cell. These enzymes recognize particular base sequences, called ***recognition sequences*****or*** ***recognitionsites***,in their target DNA and cleave that DNA at defined positions. Once inside a bacterialhost, the restriction enzymes selectively cut up foreign DNA in a process called restriction. To cut the DNA, a restriction enzyme makes two incisions, once through each sugar-phosphate backbone (i.e. each strand) of the DNA double helix. The restriction endonucleases catalyse the breakage of the phosphodiester bonds on DNA backbones at specific sites and, together with their companion methyl transferases, are part of bacterial defence systems against the invasion of bacteriophages.

The most well studied class of restriction enzyme comprises of the so adenosine triphosphate (ATP) hydrolysis-dependent, translocation of double-stranded DNA towards the stationary enzyme bound at the recognition sequence. Following restriction, the enzymes are thought to remain associated with the DNA at the target site, hydrolyzing copious amounts of ATP.

Typically ~1520 hydrogen bonds are formed between a dimeric restriction enzyme and the bases of the recognition sequence, in addition to numerous van der Waals contacts to the bases and hydrogen bonds to the backbone, which may also be water mediated. The recognition process triggers large conformational changes of the enzyme and the DNA, which lead to the activation of the catalytic centres. In many restriction enzymes the catalytic centres, one in each subunit, in which the two carboxylates are responsible for Mg2+ binding, the essential cofactor for the great majority of enzymes. Cleavage in the two strands usually occurs in a concerted fashion and leads to inversion of configuration at the phosphorus. The products of the reaction are DNA fragments with a 3'-OH and a 5'-phosphate.

Classification of Restriction Enzymes:

RestrictionModification (R-M) enzymes are classified into three main groups designated types **I, II & III**; based on their subunit structure, cofactor requirements, sequence recognition andcleavage position.

Type II systems are the most well known, having found widespread use in molecular cloning.They comprise separate methylases and homodimeric restriction endonucleases which recognize short, usually palindromic sequences of 4-8 basepairs (bp) in the presence of Mg2+ and cleave the DNA within or in close proximity to the recognition sequence. Cleavage is catalytic and typically occurs either within, or immediately adjacent to the palindromic sequence.

Type III RM enzymes are tetramericholoenzymes that possess sequence-specificmethylation, restriction and DNA dependent nucleoside triphosphatase activities. The sequence recognized is 56bp in length and cleavage typically occur 2527bp away from, and to one side of the recognition sequence.

Type I are the most complex of the group,the first R-M systems discovered. Type I restrictionendonucleases (REs) consist of three different subunits: methylase (M), restriction (R) and specificity (S) encoded by the *hsdM*, -*R* and -*S* genes, where hsd refers to Host Specificity for DNA. Together, they form an intriguing, multifunctional complex which can either restrict or modify DNA. Here, the mode of action of the complex is dictated by the methylation state of the recognition sequence.

A fully methylated site results in no action being taken and in enzyme dissociation; hemi-methylated target sequences direct the enzyme into a protective methylation mode producing fully methylated DNA, while unmethylated DNA shifts the enzyme into a destructive (and protective) restriction mode. It is in this protective mode that type I enzymes restrict foreign DNA and thereby maintain the integrity of the host genome.

Process of RFLP:

Restriction Fragment Length Polymorphism (RFLP) is a difference in homologous DNAsequences that can be detected by the presence of fragments of different lengths after digestion of the DNA (genomic DNA or PCR product or plasmid) samples in question with specific restriction endonucleases.Most RFLP markers are **co-dominant** (both alleles in heterozygous sample will be detected) and highly **locus-specific**.

The RFLP probes are frequently used in genome mapping and in variation analysis (genotyping, forensics, paternity tests, hereditary disease diagnostics, etc.).

PCR-RFLP

Isolation of sufficient DNA for RFLP analysis is time consuming and labor intensive. However, PCR can be used to amplify very small amounts of DNA, usually in 2-3 hours, to the levels required for RFLP analysis. Therefore, more samples can be analyzed in a shorter time. An alternative name for the technique is Cleaved Amplified Polymorphic Sequence (CAPS) assay.

Variation Analysis

Genetic variation or polymorphism: is the regular and simultaneous occurrence, in a singleinterbreeding population of two or more discontinuous genotypes. The concept includes differences in genotypes ranging in size from a single nucleotide site (Single Nucleotide Polymorphism) to large nucleotide sequences visible at a chromosomal level.

Genotype: is the genetic constitution of the individual, comprising the ALLELES present ateach GENETIC LOCUS.

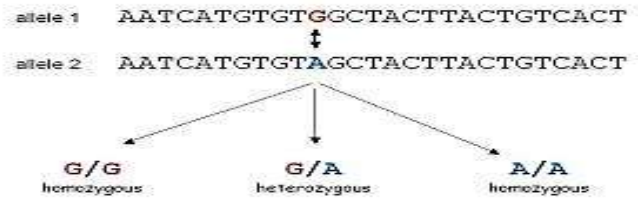
Genotyping: is the method used to determine individuals' specific ALLELES or SNPS (singlenucleotide polymorphisms).

Basic classes of DNA variation

- Insertion-Deletion Length Polymorphism (INDEL)
- Single Nucleotide Polymorphism (SNP)
- Simple Sequence Repeat (SSR) Length Polymorphism (Mini- and Micro- Sattellites)

Single nucleotide polymorphism

A single nucleotide variation in a genetic sequence, that occurs at appreciable frequency in the population.



Importance of genetic variation analysis

- early diagnosis, prevention and treatment of human diseases
- systematics and taxonomy
- population, quantitative, and evolutionary genetics
- plant and animal breeding
- identifying individuals and populations (paternity and forensic analysis)

SNP genotyping technologies

Primer Extension

- Common Primer Extension (CPE) reaction, a method for SNP genotyping that involves annealing a primer to 3' end of DNA fragment adjacent to a SNP site followed by its extension using dideoxynucleotide terminators (ddNTPs) to preven further incorporation of nucleotides. The identity of the extended base is determined either by fluorescence or mass to reveal SNP genotype.

- Allele-Specific Primer Extension (ASPE) uses extension of allele-specific primers with PCR amplified template, and the extended products are analyzed for fluorescence to determine SNP genotype.

Hybridization

- Gene Chip® array (Affymetrix, CA)
- TaqMan® genotyping assay (Applied Biosytems) combines hybridization and 5' nuclease activity of polymerase coupled with fluorescence detection.

Ligation

- Molecular Invertion Probes (MIP)

Enzymatic cleavage

- Restriction Fragment Length Polymorphism (RFLP)
- Invader® assay (Third Wave,TM Technologies, WI)

Selection of Restriction Enzymes:

Step 1

Identify restriction enzyme sites on your vector by looking at a restriction map. The restriction map will tell you which enzymes will cut your vector, and where.

Step 2

Choose a restriction enzyme that also has a site present on your gene insert, by looking at the sequence of the insert. Ensure the restriction site is at a position on your insert that is outside the gene of interest, so you do not lose any part of the gene.

Step 3

Ensure that there are no duplicates of the restriction site anywhere in your gene insert or vector. This will cause multiple cuts in your DNA and give you misleading data.

Step 4

Try to choose restriction enzymes that cut with sticky ends, rather than blunt ends. Sticky ends occur when the enzyme cuts double stranded DNA in a staggered manner, leaving a single stranded overhang that facilitates attachment with an insert cut in the opposite manner. Blunt ends occur when the double-stranded DNA is cut in a smooth manner, and these are more difficult to attach.

Step 5

Choose a different restriction enzyme for both ends of your insert to ensure it is inserted into the vector in the proper orientation and to ensure the vector does not re-attach to itself.

Step 6

Try to choose two restriction enzymes which function well in the same buffer system and temperature. If this is not possible, run each digestion separately.

Note: A similar chemistry is applied for SNP genotyping with certain variations. The variations dependon the nature and type of change which is supposed to be recognized by the restriction enzyme of choice

Protocol:

Serial no	Constituents	Stock concentration	Volume (µl) 1X reaction	Final concentration
1	MilliQ	-	21.5	-
2	Buffer	10X	3	1X
3	RE (Hha I)	10U/µl	0.5	1U/µ
4	PCR product	-	5	Depending on Amplification
	Total volume		**30**	

Prepare 0.2ml PCR tubes properly labelled for the number of samples whose RFLP is to be performed.

In a 0.5ml tube prepare the above mentioned master mix except the PCR product. The master mixis for a single tube.

Calculate the volume of the master mix according to the number of tubes considered for RFLP.

Dispense the 1X volume of the master mix in each of the tube with PCR product selected for RFLP.

Mix the tube by gently spinning in a table spinner.

Incubate the tubes in water at the activation temperature suggested in the Restriction enzyme manual provided by the company.

On completing the incubation for activation temperature, incubate the tubes at the denaturation temperature suggested by the manual for Restriction Enzyme provided by the company.

Load the entire sample in the tubes on 1% agarose gel to observe the digestion.

V. Primer Designing – Concepts to Expertise

General Concepts for PCR Primer Design

PCR is a technology born of the modern molecular biology era. The enzyme used for PCR, Taq DNA polymerase, supplied with the 10x buffer, is purchased as a cloned product, and the nucleoside triphosphates are ultrapure, buffered, and available at a convenient concentration. Yet, with all of these commercially available starting materials, PCR still fails, particularly for the novice. Assuming that all of the reagents have been added in the proper concentrations, two critical PCR components are left to the researcher.

The first is the nucleic acid template, which should be of sufficient quality and contain no inhibitors of Taq DNA polymerase (although when it comes to template purity, PCR is more permissive than many other molecular biology techniques).

The second is the selection of the oligonucleotide primers. This process is often critical for the overall success of a PCR experiment, for without a functional primer set, there will be no PCR product. Although the selection of a single primer set may be trivial, the construction of primer sets for applications such as multiplex or nested PCR becomes more challenging.

The manual selection of optimal PCR oligonucleotide primer sets can be quite tedious and thus lends itself very naturally to computer analysis. The primary factors that affect the function of the oligonucleotides their melting temperatures as well as possible homology among primers are well defined and straightforward tasks that are easily encoded in computer software.

Once the computer has provided a small number of candidate primer sets, the task of selection can be (and still is) performed manually. In this approach, the researcher is taking advantage of the raw speed of computer calculations, trying all possible permutations of a primer's placement, length, and relation to the other primers that meet conditions specified by the user. From the thousands of combinations tested by the computer, a software program can present just those that are suitable for the needs of the experiment.

Thus, the overall "quality" (as defined by the user in program parameters) of the

primers selected is almost guaranteed to be better than the handful chosen and hand-tested by the research without computer assistance.

As with any tool, understanding its function will make the end product more useful. A wide range of programs have been written to perform primer selection, varying significantly in selection criteria, comprehensiveness, interactive design, and user-friendliness. There are also commercially available specialty primer design software programs that offer enhanced user interfaces, additional features, and updated selection criteria, as well as primer design options that have been added to larger, more general software packages.

Although most people would agree that application of analytic computer software to a well-defined problem is a smart thing to do, not all researchers are convinced that PCR primerselection is a nontrivial task or that the selection rules that make a primer amplify efficiently are even well defined. Even though many of the rules discussed have been fine-tuned by collective empirical wisdom, most are based on firm theoretical ground, if not common sense.

The purpose of this chapter is to explain basic rules of oligonucleotide primer design. With this understanding of primer selection criteria, the information deduced by primer design software can be rationally interpreted and manipulated to fit your experimental needs.

PARAMETERS USED IN BASIC PCR PRIMER DESIGN

Primer design is aimed at obtaining a balance between two goals: specificity and efficiency of amplification. Specificity is defined as the frequency with which a mispriming event occurs. Primers with mediocre to poor specificity tend to produce PCR products with extra unrelated and undesirable amplicons as visualized on an ethidium bromide-stained agarose gel. Efficiency is defined as how close a primer pair is able to amplify a product to the theoretical optimum of a twofold increase of product for each PCR cycle.

Given a target DNA sequence, analysis software attempts to strike a balance between these two goals by using preselected default values for each of the primer design variables. These variables, listed below, have predictable effects on the specificity and efficiency of amplification. Depending on the experimental

requirements, these "primer search parameters" can be adjusted to override the default values that are meant to be effective for only general PCR applications.
For example, in medical diagnostic PCR applications, search parameters and reaction conditions would be adjusted to increase specificity at the cost of some efficiency, because avoiding false-positive results is a higher priority in this case than producing large quantities of amplified product. By carefully considering the following parameters when using primer design software, more effective selection of primers will be achieved.

Primer Length

The specificity is generally controlled by the length of the primer and the annealing temperature of the PCR reaction. Oligonucleotides between 18 and 24 bases tend to be very sequence specific if the annealing temperature of the PCR reaction is set within a few degrees of the primer Tm (defined as the dissociation temperature of the primer/template duplex). These types of oligonucleotides work very well for standard PCR of defined targets that do not have any sequence variation.
The longer the primer, the smaller the fraction of primed templates there will be in the annealing step of the amplification. In exponential amplification, even a small inefficiency at each annealing step will propagate to produce a significant decrease in amplified product. In summary, to optimize PCR, the utilization of primers of a minimal length that ensures melting temperatures of 54°C or higher will provide the best chance for maintenance of specificity and efficiency.
Short oligonucleotides of 15 bases or less are useful only for a limited amount of PCR protocols such as the use of arbitrary or random short primers in mapping simple genomesand in the subtraction library protocol described by certain research references. Depending on the genome size of the organism, there is a minimum primer length. In general, it is best to build in a margin of specificity for safety. For each additional nucleotide, a primer becomes four times more specific; thus, the minimum primer length used in most applications is 18 nucleotides. Clearly, if purified cDNA is being used, or genomic DNA is not present, the length could be reduced because the risk of nonspecific primer/template interactions will be greatly reduced.

Yet, it is generally a good idea to design primers such that the synthesized oligonucleotides can be used in a variety of experimental conditions (18- to 24-mers), and the small marginal cost of oligonucleotides with four to five additional

bases makes it worth the expense.

The upper limit on primer length is somewhat less critical and has more to do with reaction efficiency. For entropic reasons, the shorter the primer, the more quickly it will anneal to target DNA and form a stable double-stranded template to which DNA polymerase can bind. In general, oligonucleotide primers 28--35 bases long are necessary when amplifying sequences where a degree of heterogeneity is expected. This has proved to be generally useful in two types of applications:

1. In amplifying sequences encoding closely related molecules, such as isoforms of a protein or family of proteins within a species, as well as in the cloning of the homologous gene from a different species, and

2. In amplifying the sequences of viruses such as HIV-1, where the possibility of having a set of primers with perfect complementary to all the templates (in this example, all HIV-1 isolates) is not expected.

In both cases, one first uses primer design software to compare all available related sequences and, in such a manner, determines the DNA region with the least amount of sequence variability. These regions serve as starting places for selecting the primers. In some instances, the researcher already knows the function of the encoded protein and the domains essential to performing that function. In these cases, comparing available sequences in the regions critical for the functional activity of the related proteins within the family will aid in defining the sequences for designing new primers.

Examples include the PCR cloning of an enzyme or receptor with a similar structure and function from a related species using the available structural data. With the amino acid sequence information and the help of codon usage tables for different species, both primers, and at least one of them, could be designed around the "conserved sequence." When selecting primers to amplify DNA from a different species, sequences at the 5'- or 3'-untranslated regions of the mRNA should be avoided because they may not necessarily have high degree of homology.

The placement of the 3' end of the primer is critical for a successful PCR reaction. If a conserved amino acid can be defined, the first 2 bases of the codon, or 3 bases

in the case of an amino acid encoded by a single codon (methionine and tryptophan), can serve as the 3' end. Perfect base-pairing between the 3' end of the primer and the template is optimal forobtaining good results; minimal mismatch should exist within the last 5 to 6 nucleotides at the 3' end of the primer.

Attempts to compensate for the mismatches between the 3' end of the primer and the template by lowering the annealing temperature of the reactions do not improve the results, and failure of the reaction is almost guaranteed. With this concept in mind, one should evaluate all possible strategies in the design of primers when the nucleotide sequence of the template to be amplified is not known with certainty. Cases like the one described above are routinely encountered when the researcher wishes to amplify a cDNA using information from a partial protein sequence.

Several approaches that include the use of degenerate oligonucleotide primers covering all possible combinations for the bases at the 3' end of the primer in the pool, as well as the use of inosine to replace the base corresponding to the third or variable position of certain amino acid condons, have been successful for cDNA cloning and for detection of sequences with possible variations. Much of this type of PCR study is empirical, and different primers may have to be synthesized to obtain the desired match.

Longer primers could also arise when extra sequence information, such as a T7 RNA polymerase-binding site, restriction sites, or GC clamp, is added to primers. In general, the addition of unrelated sequences at the 5' end of the primer does not alter the annealing of the sequence-specific portion of the primer. In some cases, when a significant number of bases that do not match the template sequence are added to the primer, four to five cycles of amplification can be performed at a lower annealing temperature followed by the rest of the cycles at the annealing temperature, calculated with the assumption that the sequence at the 5' end of the primer is already incorporated into the template.

Additional bases at the 5' end of the primers are frequently added when the researcher needs to clone the PCR product. In these cases, the restriction enzyme sites of choice will be the ones that do not cut within the DNA at sites other than the primer.

To ensure sub cloning of the whole amplified fragment of unknown sequence as a

single piece, addition of sites for enzymes that recognize 6 bases or the addition of partially overlapping recognition sites for different enzymes is recommended. An important consideration when adding restriction sites to a primer is the fact that most enzymes require two or three nonspecific extra bases 5' to their recognition sequence to cut efficiently, adding to the length of the non template- specific portion of the primer.

Another drawback of long primer sequences is in the calculation of an accurate melting temperature necessary to establish the annealing temperature at which the PCR reaction is to be performed.

For primers shorter than 20 bases, an estimate of Tm can be calculated as

$$\mathbf{Tm = 4\ (G + C) + 2\ (A + T)}$$

Whereas for longer primers the Tm requires the nearest- neighbour calculation, which takes into account thermodynamic parameters and is employed by most of the available computer programs for the design of PCR primers.

The Terminal Nucleotide in the PCR Primer

Kwok and colleagues have shown that the 3'-terminal position in the primer is essential for controlling mispriming. For certain applications as described above, this chance of mispriming is useful. The other issue concerning the 3' ends of the PCR primers is the prevention of homologies within a primer pair. Care has to be taken that the primers are not complementary to each other, particularly at their 3' ends. Complementarity between primers leads to the undesirable primer-dimer phenomena in which the PCR product obtained is the result of the amplification of the primers themselves.

This sets up a competitive PCR situation between the primer-dimer product and the native template and is detrimental to the success of the amplification. In cases when multiple primer pairs are added in the same reaction (multiplex PCR), it is very important to check for possible complementarity of all the primers added in the reaction. Generally, the computer programs do not allow primer pairs with 3'-end homologies and, in conjunction with the hot start technique, the chances of formation of primer-dimer products are greatly reduced.

Reasonable GC Content and Tm

PCR primers should maintain a reasonable GC content. Oligonucleotides 20 bases long with a 50% G + C content generally have Tm values in the range of 56-62~ this provides a sufficient thermal window for efficient annealing. Within a primer pair, the GC content and T m should be well matched. Poorly matched primer pairs can be less efficient and specific because loss of specificity arises with a lower T m and the primer with the higher T m has a greater chance of mispriming under these conditions. If too high a temperature is used, the primer of the pair with the lower T m may not function at all. This matching of GC content and T m is critical when selecting a new pair of primers from a list of already synthesized oligonucleotides within a sequence of interest for a new application. For this reason we advocate the adoption of standardized criteria for primer selection within a laboratory. By planning ahead, it is easier to mix and match selected primers, as they will all have similar physical characteristics.

PCR Product Length and Placement within the Target Sequence

All of the computer programs provide a place for selecting a range for the length of the PCR product. In general, the length of the PCR product has an impact on the efficiency of amplification. The length of a PCR product for a specific application is dependent in part on the template material. Clinical specimens prepared from fixed tissue samples tend to yield DNA that does not support the amplification of large products. From pure plasmid or high-molecular-weight DNA, it is relatively straightforward to obtain products >3 kb.

For the purpose of detecting a DNA sequence, generally PCR products of 150-1000 bp are produced. The specifics of the size of the desired products often depend on the application. If the purpose is to develop a clinical assay to detect a specific DNA fragment, a small DNA amplification product of 120-300 bp may be optimal. The product should be specific and efficient to produce while containing enough information for use in a capture probe hybridization assay. Products in this size range can be produced using the two-step amplification cycling method, thereby shortening the length of the amplification procedure.

Other PCR approaches have different optimal product lengths. For example, for the purpose of monitoring gene expression by quantitative RNA PCR, the product

must be large enough that a competitive template can be constructed and both can be easily resolved on a gel. These products generally are in the 250- to 750-bp range. Here, the issue is maximizing the efficiency of both the reverse transcriptase step and the PCR.

In terms of placement of the PCR primers within a cDNA sequence, two specific points should be kept in mind. The first is to try to keep the primers and product within the coding region of the mRNA. This is the unique sequence that is responsible for the production of the protein, unlike the 3'-noncoding region that will share homologies with many different mRNAs. The second point is to try to place the primers on different exons. In so doing, the RNA-specific PCR product will be different in size from one arising from contaminating DNA.

If the purpose of the PCR is to clone a specific region of a gene or cDNA, then the size of the PCR product is preselected by the application. Here, the computer program can provide information about selected primer sets that flank the desired area. In some instances, when the complete sequence is required for further experiments and the PCR product to be obtained is above the ideal length, or the template is not of the best quality, overlapping PCR fragments can be amplified by designing primers flanking unique restriction sites in the template sequence. The production of a fragment containing the entire sequence will then be obtained by cutting and pasting the amplified pieces.

When approaching this kind of application it is important beforehand to think about the ideal method for cloning the PCR products and how the clone will be used in the future. For example, if one wishes to utilize restriction endonuclease sites at the end of primers as described above, it is important to be sure that these enzymes do not cut within the amplified region. Software programs can provide this information.

A Simple Rule for Non-computer-based Selection

Occasionally, the PCR primers must be selected from very much defined regions at the 3' and 5' ends of a specific sequence. A simple method of primer design is to choose regions that are deficient in a single nucleotide. By selecting primers in this way, the chance of extensive primer-primer homology is reduced. Here, again, care must be taken to have a balanced primer pair in terms of length and base

composition so that the T m of the primers are well matched.

Utilizing primer design software

It is important to stress that the primer selection parameters described here are general and are not necessarily implemented in the same manner among the different primer selection software. Thus, two programs using slightly different selection algorithms will rarely, if ever, select the exact same primers, even if the basic parameters are equivalently set. These discrepancies are attributable to differences in the calculation methods and the order in which the selection criteria are applied. For example, calculating the temperature of primer/template annealing can be performed in one of several ways.

The original formula of Suggs and co-workers,

Tm=2°C X (A+T) + 4°C X (G+C),

is popular for its simplicity and roughly accurate prediction of oligonucleotide Tm. More recently, Rhylick et.al. Implemented Tm prediction based on nearest-neighbour thermodynamic parameters, which appear to be slightly more accurate. Other programs base primer annealing temperatures on formulas originally developed for DNA fragments > 100 nucleotides long. Thus, specifying a desired primer annealing temperature to be 60~ will produce different primers from the exact same target sequence.

Further work by Rychlik and co-workers produced an empirically derived equation for the optimal annealing temperature of a primer pair that depends on nearest neighbour calculations.

Wu et al. have also empirically derived an equation, based on primer length and GC content, to determine optimal oligonucleotide annealing temperature. These examples illustrate how something as basic as primer Tm calculation can vary among the programs.

Second, different programs attack the task of primer selection very differently, applying selection criteria to reduce the number of possible primers that the program must consider while not eliminating potentially good candidates. For example, the program by Lowe and colleagues only considers primers that have a

3'-end CC, GG, CG, or GC dinucleotide, which may increase priming efficiency while allowing the user to specify a range of primer lengths.

In contrast, the program by Rychlik and Rhoads does not impose this requirement, but instead checks primers of a single length specified by the user. Both of these approaches eliminate potentially good primers but will, in most cases, produce an adequate number of primers that meet all of the conditions considered to be important by the investigators. The researcher using the computer software should keep in mind that the broader the selection parameters are made, the more cases the computer must consider, significantly affecting the timerequired for primer searches. This is one reason that search parameters should be kept as narrow and specific as possible when clearly dictated by experimental design. More restrictive search parameters usually result in faster searches and produce primers of greater quality.

In programs that attempt more difficult selection tasks, such as choosing primers that are highly conserved across many species, or section of degenerate primers from protein sequences, the basic criteria for primer section often must be relaxed before the software finds any suitable primer pairs. Using one of the available software programs in conjunction with the information presented here should result in the selection of a good primer set; the next task is the preparation of a good nucleic acid template.

Database Information – Necessary for Bioinformatics Data work

Databases are the collection of data which is systematically organised. Biological databases are the data sets which contain data obtained from the biological experiments. The biological data is organised in specific databases depending on the type of data. The advances on the modern experimental techniques have generated large amount of data. This poses an urgent requirement to organise the data in biological databases.

A biological database is a large, organized body of persistent data, usually associated with computerized software designed to update, query, and retrieve components of the data stored within the system. A simple database might be a single file containing many records, each of which includes the same set of information. For example, a record associated with a nucleotide sequence database typically contains information such as contact name; the input sequence with a description of the type of molecule; the scientific name of the source organism from which it was isolated; and, often, literature citations associated with the sequence.

Sources of biological data: The major sources of biological include scientific experiments, published literature, high throughput experiment technology and computational analyses. The advanced scientific areas like genomics, proteomics, metabolomics, microarrays, gene expression and phylogenetics have contributed for the generation of vast amount of data.
The biological data falls into two major categories: raw data and the processed data. Raw data (source data or atomic data) is data that has not been processed for use. A distinction is sometimes made between data and information to the effect that information is the end product of data processing. Raw data that has undergone processing is referred to as cooked data or processed data. The raw data has the potential to become "information," which requires selective extraction, organization, analysis and formatting for presentations.

What are databases?

- A collection of information, usually stored in an electronic format that can be searched by a computer.

- Convenient method of vast amount of information
- Allows for proper storing, searching & retrieving of data.
- Before analysing them we need to assemble them into central, shareable resources
- Generally separated into application areas. Example: human resource database, sales database, accounting database etc

Why databases?

- Means to handle and share large volumes of biological / any data
- Support large-scale analysis efforts
- Make data access easy and updated.
- Link knowledge obtained from various fields of biology and medicine.

Properties of databases:

- The experimental and theoretical data that is organized so that its contents can be easily accessed, Managed, updated and retrieved.
- The activity of preparing a database can be divided in to: Collection of data in a form which can be easily accessed and making it available to a multi-user system
- Most of the databases have a web-interface to search for data
- Common mode to search is by Keywords
- User can choose to view the data or save to your computer
- Cross-references help to navigate from one database to another easily

Data entry and quality control:

- Scientists (teams) deposit data directly
- Appointed curators add and update data
- Are erroneous data removed or marked?
- Type and degree of error checking
- Consistency, redundancy, conflicts, updates

Major category of data available in the databases:

- Nucleotide sequences
- Protein sequences
- Proteins sequence patterns or motifs
- Macromolecular 3D structure
- Gene expression data
- Metabolic pathways

Type of databases	Information
Bibliographic databases	Literature
Taxonomic databases	Classification
Nucleic acid databases	DNA information
Genomic database	Gene level information
Protein databases	Protein information
Protein families, domains and functional sites	Classification of proteins and identifying domains
Enzymatic / metabolic pathways	Metabolic pathways

Classification of databases:

Biological databases are classified in different ways. They are classified based on the specific type of data they contain or nature of data. The classification of the databases based on the type of data is summarised as follows.

The databases are also classified depending on the nature of the data and whether they have undergone any processing or not. Based on this criterion the databases are classified as primary, secondary and composite databases.

- Primary databases: Experimental results directly into database
- Secondary databases: results of analysis of primary databases

- Aggregate of many databases / composite databases: o Links to other data items
- Combination of data o Consolidation of data
- Primary databases Contain sequence data such as nucleic acid or protein
- Example of primary databases include:
- Protein Databases
- SWISS-PROT, TREMBL, PIR
- Nucleic Acid Databases
- EMBL, Genbank, DDBJ
- Secondary databases: Known as pattern databases. They contain results from the analysis of the sequences in the primary databases
- Example of secondary databases include: PROSITE, Pfam, BLOCKS and PRINTS
- Composite databases: Combine different sources of primary databases. Make querying and searching efficient and without the need to go to each of the primary databases.
- Example of composite databases include: NRDB – Non-Redundant Database and OWL
- Nucleic acid databases: The important nucleic acid databases include GenBank, DDBJ and EMBl.

Introduction to MS Word

A Table Of Contents Manually

You can type table of contents entries and use tabs to get the dotted lines, or dot leaders, between each entry and its page number. For a faster way to create a table of contents, see Create a table of contents automatically.

1. Type the first entry.
2. Press TAB and then type the page number for the first entry.
3. Select the tab stop character.

Note If you can't see the tab stop character, click the **Home** tab, and then click **Show/Hide** in the **Paragraph** group.

4. On the **Page Layout** tab, click the **Paragraph Dialog Box** launcher.
5. Click **Tabs**.
6. Under **Tab stop position**, type where you want the page number to be.

Note To view the ruler so that you can type the right-margin location, click the **View Ruler** button at the top of the vertical scrollbar.

7. Under **Alignment**, click **Right**.
8. Under **Leader**, click the option that you want, and then click **OK**.
9. Press ENTER, and then type your next entry.
10. Press TAB, and then type the page number for your second entry.
11. Repeat until your table of contents is complete.

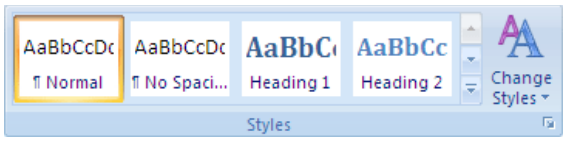
Important If you make changes to headings or pages in your document, you need to update the table of contents manually.

Create a table of contents automatically

The easiest way to create a table of contents is to use the built-in heading styles. You can also create a table of contents that is based on the custom styles that you have applied. Or you can assign the table of contents levels to individual text entries.

Mark entries by using built-in heading styles

1. Select the heading to which you want to apply a heading style.
2. On the **Home** tab, in the **Styles** group, click the style that you want.



For example, if you selected text that you want to style as a main heading, click the style called **Heading 1** in the Quick Style gallery.

- If you don't see the style that you want, click the arrow to expand the Quick Style gallery.
- If the style that you want does not appear in the Quick Style gallery, press CTRL+SHIFT+S to open the **Apply Styles** task pane. Under **Style Name**, click the style that you want.

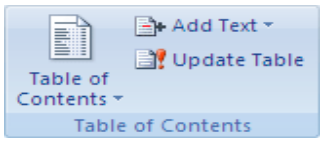
Create a table of contents

After you mark the entries for your table of contents, you are ready to build it.

Create a table of contents from built-in heading styles

Use this procedure if you created a document by using heading styles.

1. Click where you want to insert the table of contents, usually at the beginning of a document.
2. On the **References** tab, in the **Table of Contents** group, click **Table of Contents**, and then click the table of contents style that you want.



Note If you want to specify more options — for example, how many heading levels to show — click **Insert Table of Contents** to open the **Table of Contents** dialog box. To find out more about the different options, see Format the table of contents.

Create a table of contents from custom styles that you applied

Use this procedure if you already applied custom styles to your headings. You can choose the style settings that you want Word to use when it builds the table of contents.

1. Click where you want to insert the table of contents.
2. On the **References** tab, in the **Table of Contents** group, click **Table of Contents**, and then click **Insert Table of Contents**.
3. Click **Options**.
4. Under **Available styles**, find the style that you applied to the headings in your document.
5. Under **TOC level**, next to the style name, type a number from 1 to 9 to indicate the level that you want the heading style to represent.

Note If you want to use only custom styles, delete the TOC level numbers for the built-in styles, such as Heading 1.

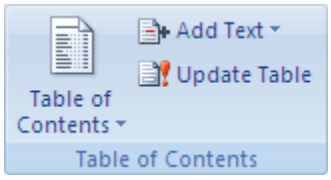
6. Repeat step 4 and step 5 for each heading style that you want to include in the table of contents.
7. Click **OK**.
8. Choose a table of contents to fit the document type:
    - **Printed document** If you are creating a document that readers will read on a printed page, create a table of contents in which each entry lists both the heading and the page number where the heading appears. Readers can turn to the page that they want.
    - **Online document** For a document that readers will read online in Word, you can format the entries in the table of contents as hyperlinks, so that readers can go to a heading by clicking its entry in the table of contents.
9. To use one of the available designs, click a design in the **Formats** box.
10. Select any other table of contents options that you want.

Format the table of contents

If you already have a table of contents in your document, you can change the options. To do this, you need to insert a new table of contents by using the **Table of Contents** dialog box.

1. Select the existing table of contents.

2. On the **References** tab, in the **Table of Contents** group, click **Table of Contents**, and then click **Insert Table of Contents**.



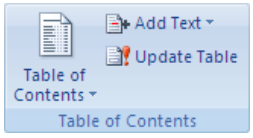
3. In the **Table of Contents** dialog box, do any of the following:
    - To change how many heading levels are displayed in the table of contents, enter the number that you want in the box next to **Show levels**, under **General**.
    - To change the overall look of your table of contents, click a different format in the **Formats** list. You can see what your choice looks like in the **Print Preview** and **Web Preview** areas.

- To change the way heading levels are displayed in the table of contents, click **Modify**. In the **Style** dialog box, click the level that you want to change, and then click **Modify**. In the **Modify Style** dialog box, you can change the font, the size, and the amount of indentation.

Update the table of contents

If you added or removed headings or other table of contents entries in your document, you can quickly update the table of contents.

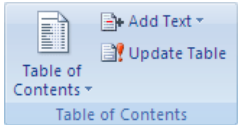
1. On the **References** tab, in the **Table of Contents** group, click **Update Table**.



2. Click **Update page numbers only** or **Update entire table**.

Delete a table of contents

1. On the **References** tab, in the **Table of Contents** group, click **Table of Contents**.



2. Click **Remove Table of Contents**.

Create a customized hyperlink to a document, file, or Web page

1. Select the text or picture that you want to display as the hyperlink.
2. On the **Insert** tab, in the **Links** group, click **Hyperlink**.

You can also right-click the text or picture and then click **Hyperlink** on the shortcut menu.

3. Do one of the following:
    - To link to an existing file or Web page, click **Existing File or Web Page** under **Link to**, and then type the address that you want to link to in the **Address** box. If you don't know the address for a file, click the arrow in the **Look in** list, and then navigate to the file that you want.
    - To link to a file that you haven't created yet, click **Create New Document** under **Link to**, type the name of the new file in the **Name of new document** box, and then, under **When to edit**, click **Edit the new document later** or **Edit the new document now**.

Note To customize the ScreenTip that appears when you rest the pointer over the hyperlink, click **ScreenTip** and then type the text that you want. If you don't specify a tip, Word uses the path or address of the file as the tip.

Create a hyperlink to a blank e-mail message

1. Select the text or picture that you want to display as the hyperlink.
2. On the **Insert** tab, in the **Links** group, click **Hyperlink**.

You can also right-click the text or picture and then click **Hyperlink** on the shortcut menu.

3. Under **Link to**, click **E-mail Address**.
4. Either type the e-mail address that you want in the **E-mail address** box, or select an e-mail address in the **Recently used e-mail addresses** list.
5. In the **Subject** box, type the subject of the e-mail message.

Notes

- Some Web browsers and e-mail programs might not recognize the subject line.
- To customize the ScreenTip that appears when you rest the pointer over the hyperlink, click **ScreenTip** and then type the text that you want. If you don't

specify a tip, Word uses "mailto" followed by the e-mail address and the subject line as the tip.

Tip You can also create a hyperlink to a blank e-mail message by typing the address in the document. For example, type **someone@example.com**, and Word creates the hyperlink for you, unless you turned off automatic formatting of hyperlinks.

Create a hyperlink to a specific location

To link to a location in the same document or in a different document, you must mark the hyperlink location or destination and then add the link to it.

Mark the hyperlink location

You can mark the hyperlink location by inserting a bookmark. If you're linking to a location in the same document, you can also use a heading style to mark a location.

Insert a bookmark

In the document that you want to link to, do the following:

1. Select the text or item to which you want to assign a bookmark, or click where you want to insert a bookmark.
2. On the **Insert** tab, in the **Links** group, click **Bookmark.**

3. Under **Bookmark name**, type a name.

Bookmark names must begin with a letter and can contain numbers. You can't include spaces in a bookmark name. However, you can use the underscore character to separate words — for example, **First_heading**.

4. Click **Add**.
5.

Apply a heading style

When you're linking to a location in the same document, you can apply one of the built-in heading styles in Word to the text at the location that you want to go to. In the current document, do the following:

1. Select the text to which you want to apply a heading style.
2. On the **Home** tab, in the **Styles** group, click the style that you want.

For example, if you selected text that you want to style as a main heading, click the style called **Heading 1** in the Quick Styles gallery.

Add a link to a specific location in the current document

1. Select the text or object that you want to display as the hyperlink.
2. Right-click and then click **Hyperlink** on the shortcut menu.
3. Under **Link to**, click **Place in This Document**.
4. In the list, select the bookmark or heading or that you want to link to.

Note To customize the ScreenTip that appears when you rest the pointer over the hyperlink, click **ScreenTip**, and then type the text that you want. If you don't specify a tip, Word uses "Current document" as the tip for links to headings. For links to bookmarks, Word uses the bookmark name.

Add a link to a specific location in another document

1. Select the text or object that you want to display as the hyperlink.
2. Right-click and then click **Hyperlink** on the shortcut menu.
3. Under **Link to**, click **Existing File or Web Page**.
4. Click the file that you want to link to, and then click **Bookmark**.
5. In the list, select the bookmark that you want to link to.

Note To customize the ScreenTip that appears when you rest the pointer over the hyperlink, click **ScreenTip** and then type the text that you want. If you don't specify a tip, Word uses "Current document" as the tip for links to headings. For links to bookmarks, Word uses the bookmark name.

Quickly create a hyperlink to another file

You can create a hyperlink quickly without having to use the **Insert Hyperlink** dialog box by dragging selected text or pictures from a Word document.

Important The text that you copy must come from a file that has already been saved.

Note You cannot drag drawing objects, such as Shapes, to create hyperlinks. To create a hyperlink for a drawing object, select the object, right-click, and then click **Hyperlink** on the shortcut menu.

Create a hyperlink by dragging content from another Word document

1. Save the file that you want to link to. This is the destination document.
2. Open the document where you want to add a hyperlink.
3. Open the destination document and select the text, graphic, or other item you want to go to.

For example, you might want to select the first few words of a section of a document that you want to link to.

4. Right-click the selected item, drag it to the task bar and rest over the icon of the document to which you want to add a hyperlink.
5. Release the right mouse button where you want the hyperlink to appear on the page, and then click **Create Hyperlink Here**.

Note The text, graphic, or other item that you selected is the link to the destination document.

Turn on or off automatic hyperlinks

You can turn automatic links on or off. If you're using automatic links and Word is displaying only part of the link path, you can change the settings to show the full path of hyperlink.

Turn on automatic hyperlinks

1. Click the **File** tab.
2. Click **Options**.
3. Click **Proofing**.
4. Click **AutoCorrect Options**, and then click the **AutoFormat As You Type** tab.
5. Select the **Internet and network paths with hyperlinks** check box.

Show the full path of hyperlinks

1. Click the **File** tab.

2. Click **Options**.
3. Click **Advanced**.
4. Under **General**, click **Web Options**, and then click the **File** tab.
5. Clear the **Update links on save** check box.

Turn off automatic hyperlinks

1. Click the **File** tab.
2. Click **Options**.
3. Click **Proofing**.
4. Click **AutoCorrect Options**, and then click the **AutoFormat As You Type** tab.
5. Clear the **Internet and network paths with hyperlinks** check box.

Remove a hyperlink

Do one of the following:

- Press CTRL+Z. You must press CTRL+Z immediately after you type the address, or URL, to delete a hyperlink from a typed URL.

Note If you press CTRL+Z a second time, the typed URL is deleted.

- Right-click the hyperlink, click Edit Hyperlink on the shortcut menu, and then click Remove Link.

Click to follow a link

By default, Word requires you to press CTRL and click to go to the destination of a hyperlink. This prevents you from suddenly going to a destination when you're trying to edit a document.

To change this setting so that you can click a link without having to press CTRL, do the following:

1. Click the **File** tab.
2. Click **Options**.
3. Click **Advanced**.
4. Under **Editing Options**, clear the **Use CTRL + Click to follow hyperlink** check box.

Introduction to MS Excel

Evolution of the Spreadsheet :

The first electronic spreadsheet, known as VisiCalc, was developed by Dan Bricklin and Bob Frankston. While an MBA student at Harvard, Dan used this spreadsheet concept to crunch numbers in a case study around Pepsi-Cola's marketing campaigns. This software soon became one of two "killer apps", (along with word processing) that influenced personal computer growth. Today the electronic spreadsheet is used in numerous fields for asking "what if" questions and providing immediate answers by using formulas to perform calculations. To read more about the development of electronic spreadsheets,

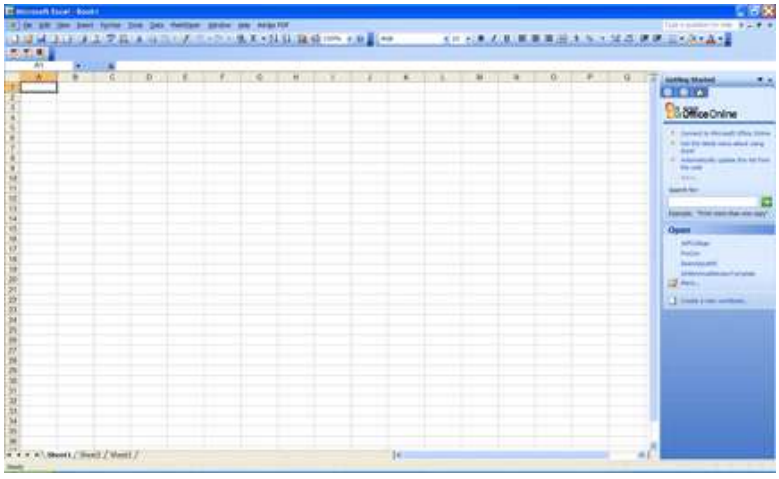
Workbook:

As spreadsheets became larger, they became more difficult to manage. The concept of a workbook was developed to manage this collection of spreadsheets. In this lesson, you have opened a sheet within a workbook in Excel. The default number of sheets included in a workbook is three but you can add as many sheets as needed depending on the memory available on your computer.

Lesson 1: Electronic Spreadsheet

Excel is an electronic spreadsheet. A spreadsheet program can store, manipulate, and create graphical representations of data. It can be integrated into the curriculum in lessons that have information that can be quantified.

Excel spreadsheet example - An empty spreadsheet with a task pane opened. Remember that a task pane has common tasks readily displayed.



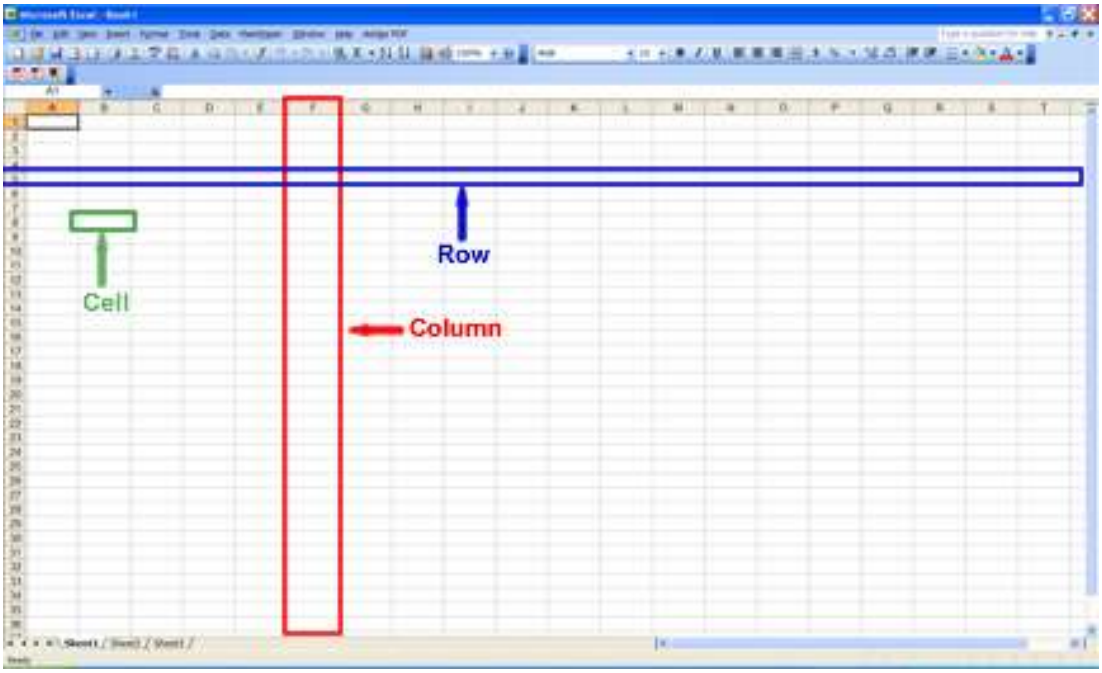
Activity 1: Opening Excel

In this activity you will be opening the spreadsheet program Microsoft Excel and entering text into an Excel document.

1. Turn on your computer.
2. ***Click on*** the **Start** button 

then click on **All Programs**> **Microsoft Office**> **Microsoft Excel** 

. *(For Macs go to the Finder Menu and click on Go then click on Applications. In the Application window click on the link to Microsoft Office. In the Microsoft Office window click Excel.)*



Elements of importance:

- Columns
- Rows
- Cells

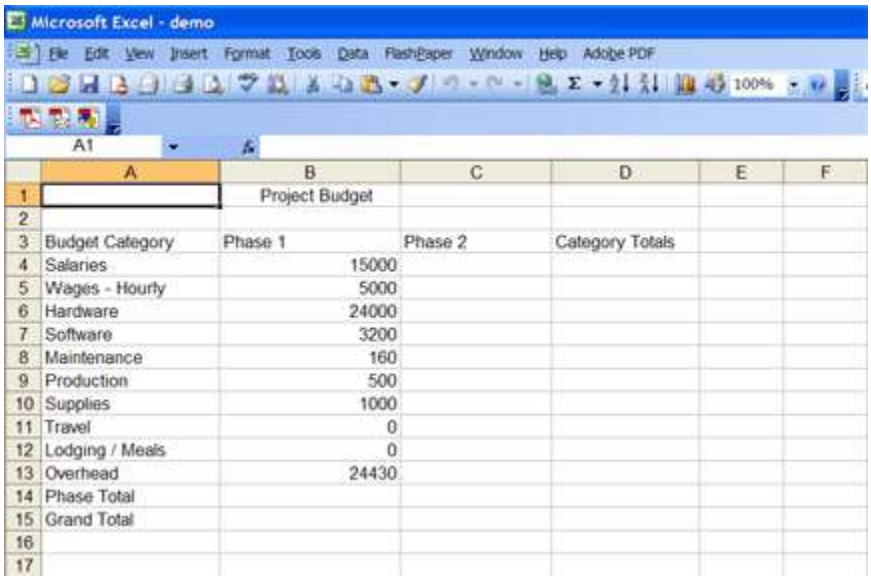
Activity 2: Downloading, Saving and Opening an Existing File

This activity will use a file entitled ***demoXP.xls*** that has already been created and saved on the Edutech site. To download and save this file:

1. Right click here
2. Select Save Target As... (In FireFox you will select Save Link As.) A *Save As* dialogue box will open allowing you to change the file name and the location where the file is saved. You should include your initials in the file name and choose a folder location where your course files are stored on the hard drive. In previous tutorials you should have created a course work folder and a CEP 810 folder. Save this file in your CEP 810 folder.
3. **Click on Save** and the demoXP.xls spreadsheet will be saved to your folder.

To Open the File:

1. In the main Excel menu bar **click once** on the **File** menu, scroll down and **click on Open**. (**File**> **Open**)
2. Navigate through your folder directory and click on the demoXP file. (You should have added your initials to the file name.)



	A	B	C	D	E	F
1		Project Budget				
2						
3	Budget Category	Phase 1	Phase 2	Category Totals		
4	Salaries	15000				
5	Wages - Hourly	5000				
6	Hardware	24000				
7	Software	3200				
8	Maintenance	160				
9	Production	500				
10	Supplies	1000				
11	Travel	0				
12	Lodging / Meals	0				
13	Overhead	24430				
14	Phase Total					
15	Grand Total					
16						
17						

Lesson 2: Spreadsheet Window - Elements Common with Microsoft Word

In this next lesson you will learn about the main elements of the Excel window. The elements shown below are **similar** to elements found in Microsoft Word.

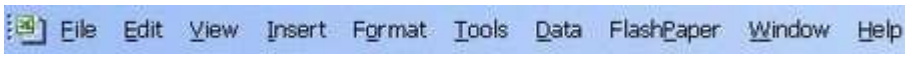
Title Bar

The **Title Bar** lists the name of the software program you have open and then lists the name of the specific document you are viewing. In this case, Microsoft Excel is open with

the document name of Book1

showing. Microsoft and other software use a default naming system of Book1, Book2 etc., to automatically name files until you change the name to a descriptive word meaningful to you. Because you have opened the demoXP file, your title bar should show the name of that document.

Menu Bar



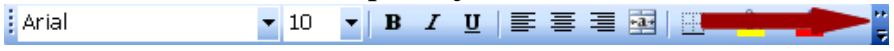
The terms (words) in the **Menu Bar** each represent a different submenu. To view the menu- mouse over the word in the menu bar and a pull down menu appears. The options in the pull down menu represent functions that are relevant to the term in the menu bar. If you see a downward pointing double arrow then you are viewing only the most frequently used options. Click on the double arrow to show all available options.

Standard Toolbar

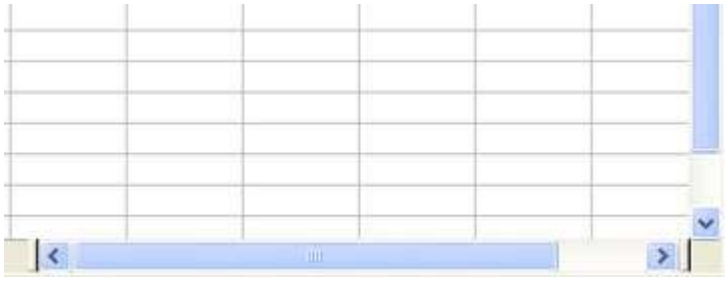
The **Standard Toolbar** has icons for frequently used items. This bar will change to reflect recently used icons as you use the software. You can move any toolbar to other locations in the spreadsheet by clicking and holding on the four vertical dots on the left side of the toolbar. The separator line, highlighted below, provides additional handles for moving the toolbar within the page.

Formatting Toolbar

The **Formatting Toolbar** allows you to quickly make formatting changes to text and cells. Specifically, these buttons will allow you to make changes to the appearance of your document. The icons in this bar will vary as you use the program. If the icon you want to use does not appear in this menu, click on the toolbar options icon at the far right of this bar to select the option you would want to add.

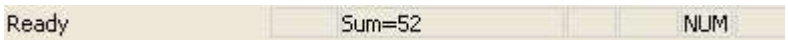


Scroll bar



The **Scroll Bar** allows the viewer to view different parts of the spreadsheet that may not be viewable because of the screen size. You can move the vertical scroll bar up or down and the horizontal scroll bar left or right.

Status Bar



The left side of the **Status Bar** shows the possible states you have for each cell; Ready, Enter, or Edit. The other cells show the result of formulas used in selected cells.

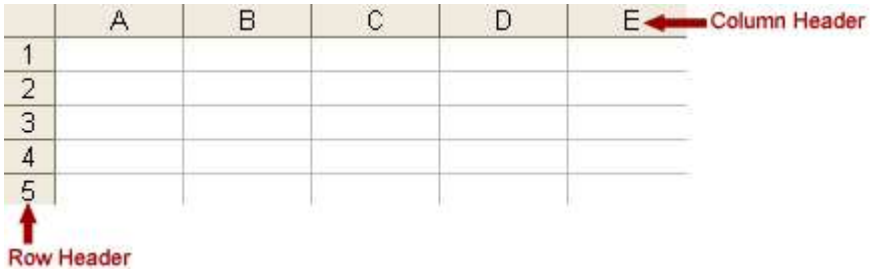
Lesson 3: Spreadsheet Window - Unique Elements

These elements are unique to Excel and other electronic spreadsheets.

Formula Bar

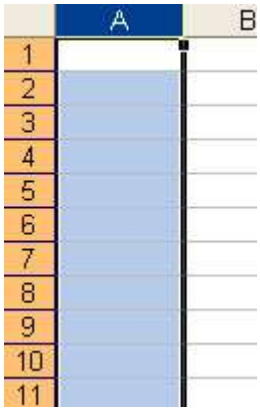
The **Formula Bar** shows the selected cell on the left. The ***fx*** box on the right provides an area for entering data or formulas into the cell.

Column & Row Header



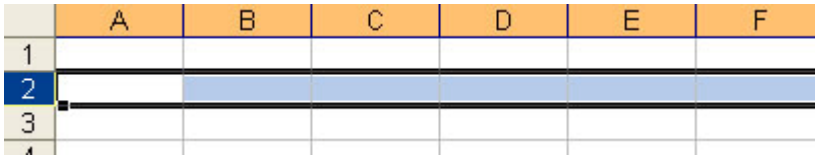
In the **Column & Row Header**, each ***column is labeled with a letter*** and each ***row is associated with a number***. The columns, which go from the top of the page to the bottom, just like a column on a building, begin with A and go through the alphabet repeatedly with a letter sequence of AA, AB, ...IV until 256 columns have been identified. The rows are numbered from 1 - 65,536.

Column



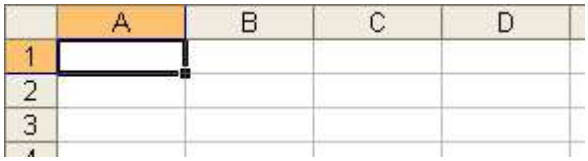
Each **Column** can be selected by clicking on the corresponding letter on the spreadsheet. Numerous columns can be selected by clicking and dragging the cursor over the letters.

Row



In turn, each **Row** can be selected by clicking on the corresponding number on the spreadsheet. Numerous rows can be selected by clicking and dragging the cursor over the numbers. A hint: When selecting many rows, start from the row furthest from the beginning and drag the mouse upward to control the "runaway mouse".

Cell



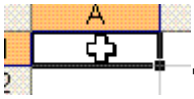
A **Cell** is the union of a column and row. The wide black line around the cell means that the cell has been selected using a single left click. A square box (AutoFill handle) appears on the lower right edge. We will talk more about the AutoFill in another lesson.

Tabs

The **Sheet Tabs** are located at the bottom of the spreadsheet and serve as navigation tools for the workbook. A sheet is a single spreadsheet. A workbook (excel file) may have multiple sheets. Three sheets are provided when you open the workbook but more can be

added by selecting Insert > Worksheet from the standard toolbar. The current worksheet will have the white background and extra sheets will have the gray tab. To change from one worksheet to another, just click on the worksheet tab you want to view. Sheet names can be easily changed by double clicking on the text "Sheet1" and typing the new name when the original letters are highlighted with a black background.
The arrows serve as navigation tools also. The vertical line with the left pointing arrow will take you to the leftmost sheet in the workbook. Correspondingly, the right pointing arrow with the horizontal line brings up the rightmost worksheet. The individual arrows move to the previous or next worksheet.

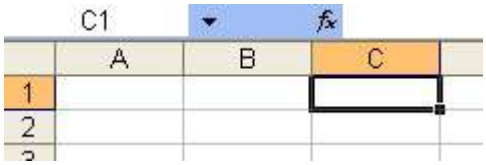
Cursor



The wide white **Plus Symbol** serves as a selecting cursor. With the cursor in this state, you can select a cell using a left click. The cell then becomes highlighted with the wide black rectangle

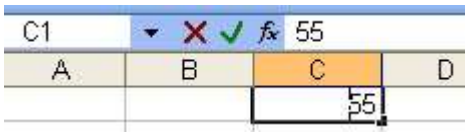
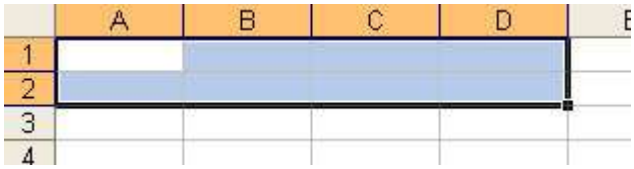
The blinking **I Beam or Insertion Point** is visible when the cell is in the Ready state and you have either double clicked into the cell or have put data into the cell. Even though the I Beam is common in other programs, the difference is that an extra action (double clicking or typing) is necessary for this to appear.

Nomenclature



The cell is named by listing the **column letter** first followed by the **row number**. In this example; C1 refers to the cell in the third column of the first row. Remember that the formula bar displays the name of the highlighted cell. If more than one cell is selected, the initial cell will be displayed followed by a colon and then the name of the

last cell (e.g., A1:D1).

Entry Bar	 There are two ways to activate the enter or edit mode of the cell. You can double click in the cell or single click in the **Formula Box** located in the formula bar. When you are in this enter or edit mode, additional icons appear next to the entry bar. The ✕ is used to delete content in the cell, the ✓ is used to accept the entry you have made and the *fx* is used to insert a formula. Note that the black line surrounding the cell becomes thinner when the cell has been activated.
Selected Cell(s)	 The wide black line surrounding the cell or selection of cells indicates the **Selected Cells.** Any changes made in the program will occur in those cells. Cells need not be adjacent to one another to be selected. This will be covered in later lessons.

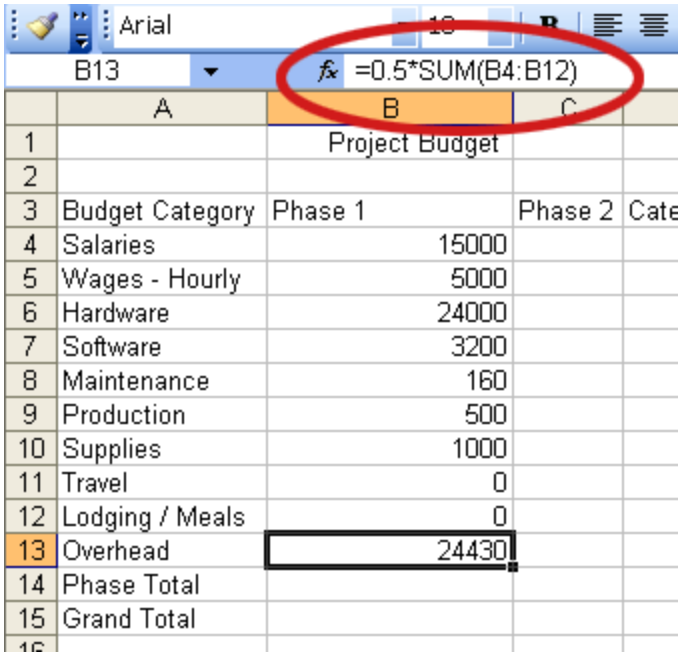
Lesson 4: Spreadsheet Cells

For this next lesson you will be working with the ***demoXP*** file. Please make sure you have the file open. You will be exploring some of the elements in an existing spreadsheet. Therefore for many of the steps in these activities there will be an accompanying explanation.

Remember - there are two levels when clicking in the cell - single click and double click

Activity 1: Values and Formulas

1. **Single click** on ***cell B13*** (You should see the data next to *fx* displayed like the image below.)



Res

ult: Cell B13 shows the value of 24430 but the formula bar displays the underlying formula of one-half of the sum of cells B4 through B12.

Adding Values

When keying in numbers, the keypad at the right hand side of the desktop keyboard is helpful. Make sure the Num Lock key has been selected.

After entering data in the cell, you can press Enter, TAB or any arrow key to move to adjacent cells. This will confirm the cell's data entry.

Edit mode allows you to edit a cell's content. There are two ways to accomplish this task.

- Double click on the cell and enter your data.
- Click on the cell and then click on the formula bar to make changes. The enter, cancel and function arguments will appear allowing you to accept, cancel your entry or add a formula from the formula bar

To begin adding values:

1. **Click on** cell ***C4***
2. **Key in 15000. Press** the **Enter** key or down arrow key to move to cell ***C5*** Key in the remaining values in the rest of this column... (Remember to press the enter key or down arrow after each value has been entered.)

   5000
   0
   0
   160
   1500
   1000
   2500
   2500

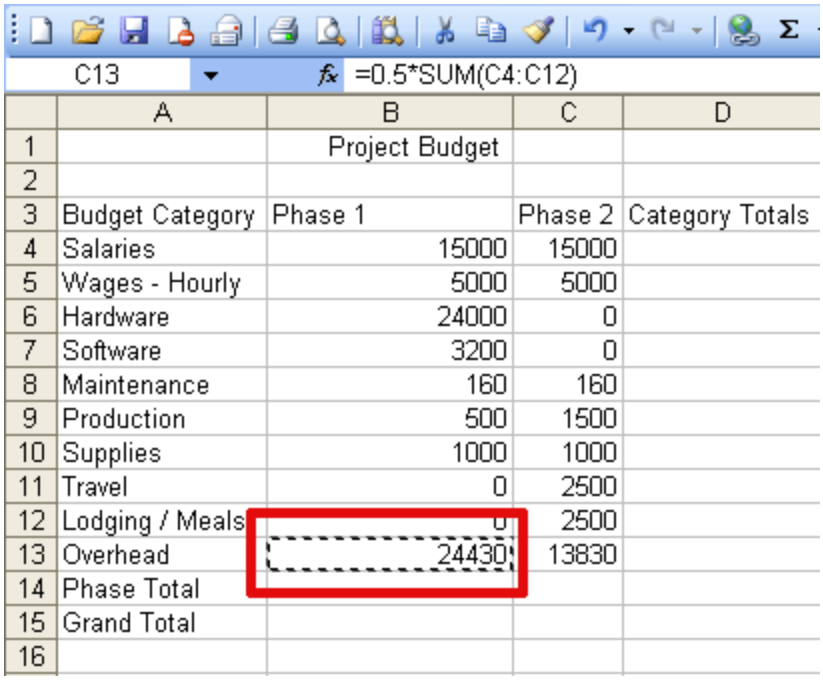
3. **Click on File**, then **Save**. Remember to periodically save your file.

Remember - if you single click on a cell to change data, you may overwrite current data by mistake - always double click on the cell or use the formula bar.

Activity 2: Copying and Pasting Formulas

In this activity you will learn how to copy a formula from one cell and paste it into another.

1. **Click on** cell ***B13***
2. In the ***Edit*** menu, select **Copy** (You should see a dotted line (marquee) around cell B13.)
3. **Click on** cell ***C13***
4. In the ***Edit*** menu, select **Paste**
5. **Press Enter or Esc** to stop the copying of subsequent cells (You will no longer see the dotted line around cell B13.) The number 13830 should appear in cell C13.



C13 =0.5*SUM(C4:C12)

	A	B	C	D
1		Project Budget		
2				
3	Budget Category	Phase 1	Phase 2	Category Totals
4	Salaries	15000	15000	
5	Wages - Hourly	5000	5000	
6	Hardware	24000	0	
7	Software	3200	0	
8	Maintenance	160	160	
9	Production	500	1500	
10	Supplies	1000	1000	
11	Travel	0	2500	
12	Lodging / Meals	0	2500	
13	Overhead	24430	13830	
14	Phase Total			
15	Grand Total			
16				

Notice the marquee around cell B13 reminding you that the content of this cell has been copied.

The formula box for the row and column labels is not case sensitive so typing b4 will result in B4 in the cell.

Activity 3: Entering Formulas

In this activity you will enter a formula that will add the columns B4 and C4

1. **Double click** on cell ***D4*** and type **=sum(b4:c4)**
2. **Press Enter** to confirm the entry

Note: Using the sum function is more flexible than simply adding the two columns together since the sum function will allow you to easily add additional columns if you were to include a Phase 3 to the formula. Example: [=(B13+C13+D13)] vs. [=SUM(B13:D13)] When using the sum formula, the colon (:) is used to select the adjacent cell and the comma (,) is used to select non-adjacent cells. This

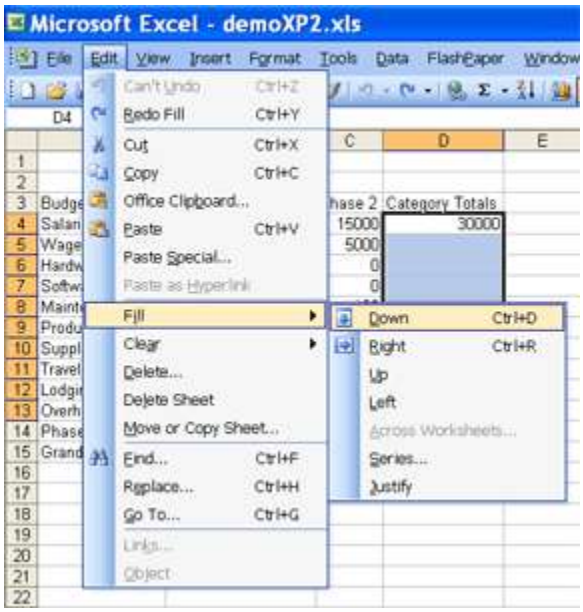
formula [=SUM(B4:C4,B6:C6)] would give the sum of the adjacent cells, B4 & C4 added to the sum of adjacent cells B6 & C6.

Result: The value displayed in the cell D4 should be 30000 with the cursor in a ready position in cell D5.

Activity 4: Copying Formulas into Multiple Locations

In this activity you will learn how to copy and paste a formula into multiple cell locations. You will copy and paste the formula from D4 into cells D5 through D13.

1. **Select** cells ***D4 through D13*** by either clicking on D4 and dragging down to D13 or clicking on D4 and holding down the Shift key and then clicking on D13.
2. In the ***Edit*** menu, select **Fill**>**Down**

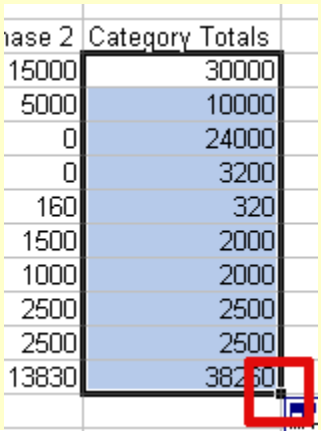


Result: The formula and values should be displayed in cells D5 through D13. Cell D13 should display the number 38260.

Additional Information: Fill Down can be used with adjacent cells and does not require the use of the clipboard which uses copy and paste. The Fill Down action provides a Relative Reference to the cells.

This means that the formula will remain the same but the cell letters and numbers will reflect the currently selected rows and columns. If you want to learn more about Relative vs. Absolute Reference in cells, check the help menu.

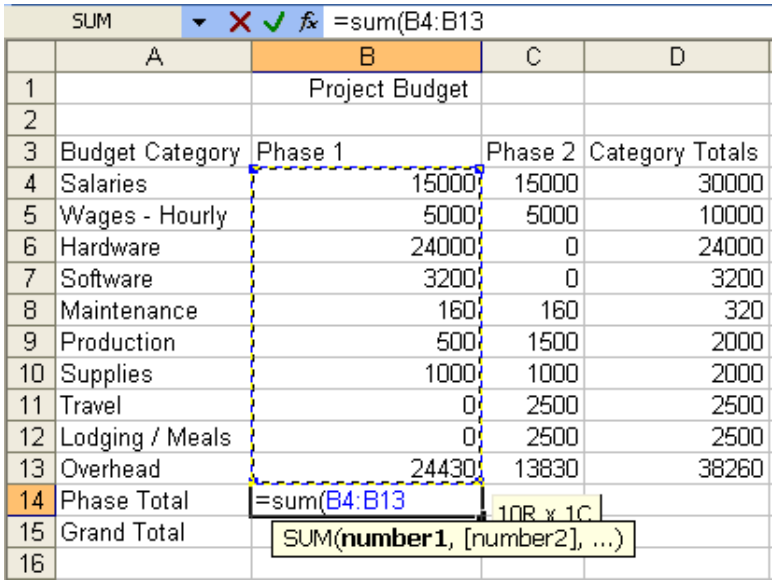
Alternative Method: An alternate way to complete the Fill Down is to select the cell to be copied, then click and hold the small black box or handle on the edge of the cell, then drag until you have selected all of the cells you want filled.



Activity 5: Entering Additional Formulas

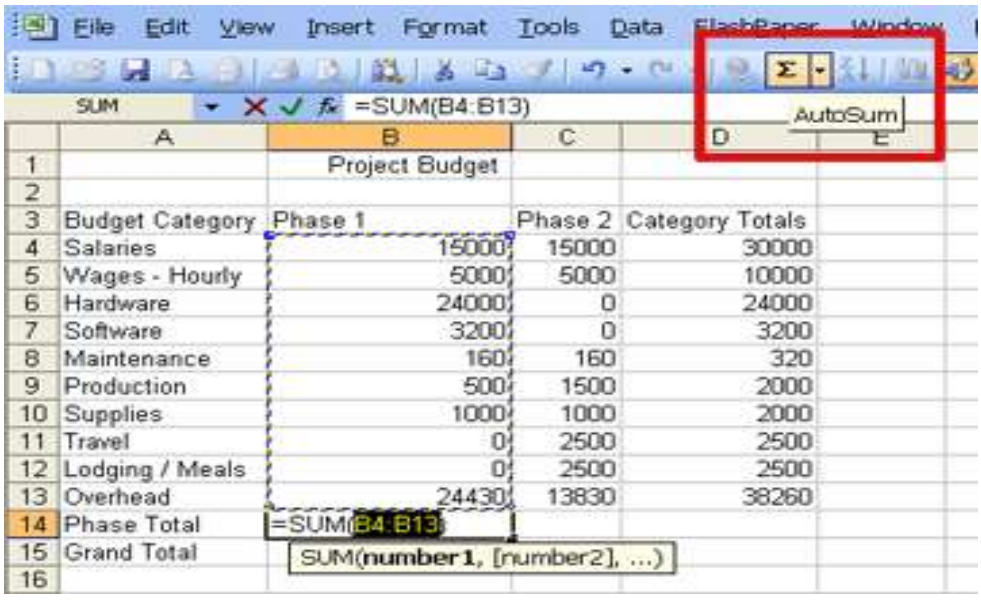
In this activity you will learn how to add additional formulas to the spreadsheet. You will enter a sum formula to total Phase 1, Phase 2, and the Grand Total.

1. **Click on *B14*** and enter *=sum(*
2. **Drag from *B4 through B13*** and **key in** a right parenthesis and **press Enter**



Result: The value of 73290 should be displayed in cell B14.

An alternate way to include the Sum formula is to click on cell B14 and then click on the Sigma symbol for summation and select and drag through B4 through B13. If you have double clicked on the cell, you will need to double click on the Sigma symbol to activate the formula.



Now you are ready to add the totals for Phase 2 and the Grand Total.

1. **Copy and paste** the formula from ***B14 into C14.*** Remember to click Enter or Esc to stop copying.
2. In cell ***C14***, **enter the formula to *sum column D***

The spreadsheet calculations are complete.

D16

	A	B	C	D
1		Project Budget		
2				
3	Budget Category	Phase 1	Phase 2	Category Totals
4	Salaries	15000	15000	30000
5	Wages - Hourly	5000	5000	10000
6	Hardware	24000	0	24000
7	Software	3200	0	3200
8	Maintenance	160	160	320
9	Production	500	1500	2000
10	Supplies	1000	1000	2000
11	Travel	0	2500	2500
12	Lodging / Meals	0	2500	2500
13	Overhead	24430	13830	38260
14	Phase Total	73290	41490	
15	Grand Total			114780
16				
17				

Introduction to MS PowerPoint

Basic tasks for creating a PowerPoint presentation

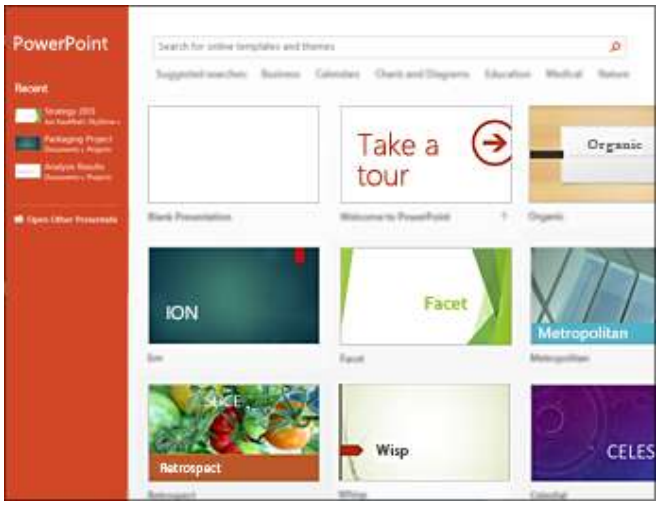
PowerPoint presentations work like slide shows. To convey a message or a story, you break it down into slides. Think of each slide as a blank canvas for the pictures, words, and shapes that will help you build your story.

- For a training course overview to help you create your first PowerPoint 2013 presentation, see Create your first PowerPoint 2013 presentation.
- For more information about the new features in PowerPoint 2013, see What's new in PowerPoint 2013.
- For information about what's new in Microsoft Office, see What's new in Office 2013

Choose a theme

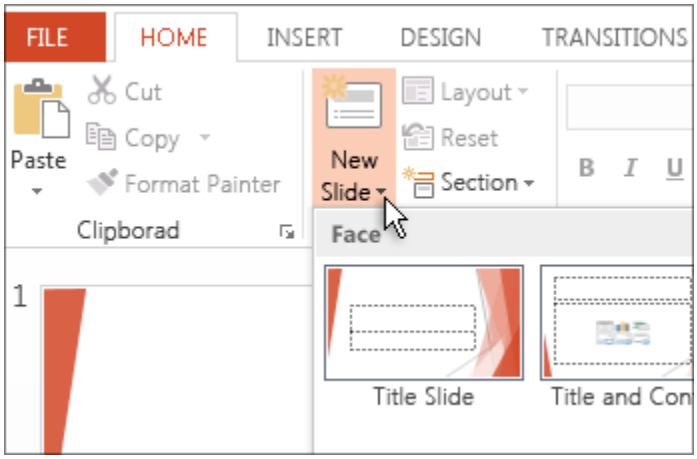
When you open PowerPoint, you'll see some built-in themes. A theme is a slide design that contains matching colors, fonts, and special effects like shadows, reflections, and more.

1. Choose a theme.
2. Click **Create**, or pick a color variation and then click **Create**.



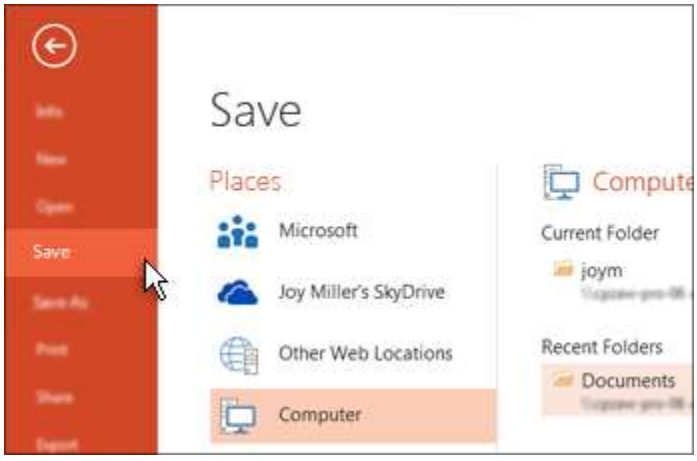
Insert a new slide

On the **Home** tab, click **New Slide**, and pick a slide layout.



Save your presentation

1. On the **File** tab, click **Save**.
2. Pick or browse to a folder.
3. In the **File name** box, type a name for your presentation, and then click **Save**.



Tip Save your work as you go. Hit **Ctrl+S** often.

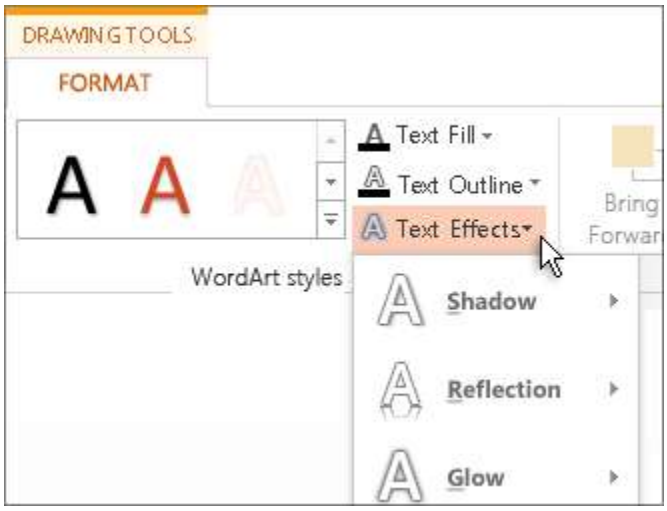
Add text

Click inside a text placeholder, and begin typing.

Format your text

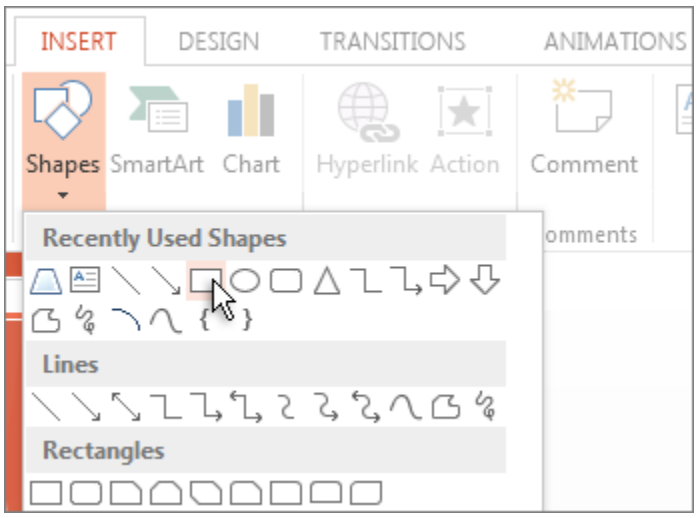
1. Select the text.
2. Under **Drawing Tools**, click **Format**.
3. Do one of the following:

- To change the color of your text, click **Text Fill**, and then choose a color.
- To change the outline color of your text, click **Text Outline**, and then choose a color.
- To apply a shadow, reflection, glow, bevel, 3-D rotation, a transform, click **Text Effects**, and then choose the effect you want.



Add shapes

1. On the **Insert** tab, click **Shapes**.
2. Pick the shape that you want, click anywhere on the slide, and then drag to draw the shape.



Tip To create a perfect square or circle (or constrain the dimensions of other shapes), press and hold Shift while you drag.

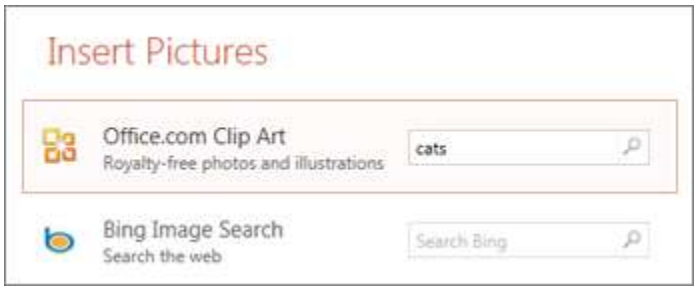
Add pictures

On the **Insert** tab, do one of the following:

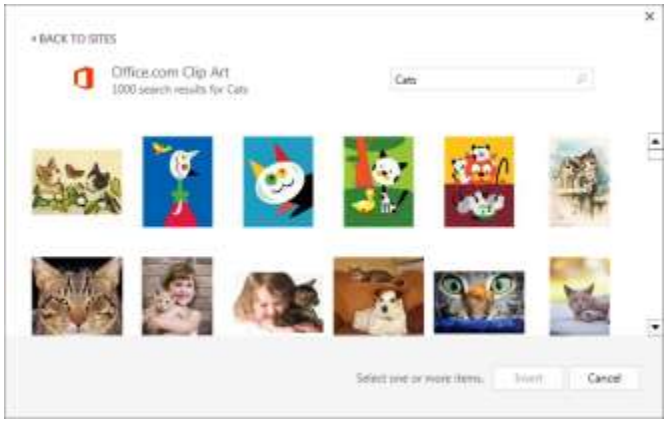
- To insert a picture that is saved on your local drive or an internal server, click **Pictures on my PC**, browse for the picture, and then click **Insert**.

- To insert a picture from Bing or the Office.com Clip Art gallery, click **Online Pictures**, and use the search box to find a picture.

For example, type **'Cats'** in the **Office.com Clip Art** search box.



Here's a sample of the cat pictures you'll see:



- Choose a picture, and then click **Insert**.

Add speaker notes

Slides are best when you don't cram in too much information. You can put helpful facts and notes in the speaker notes, and refer to them as you present.

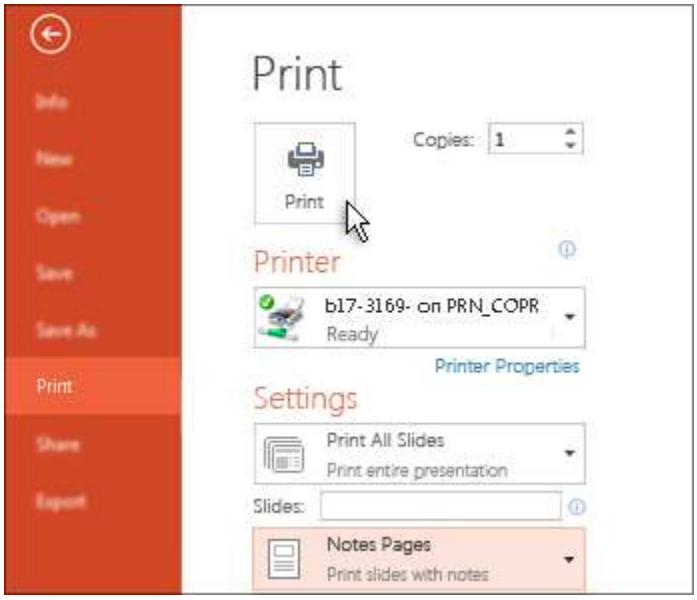
1. To open the notes pane, at the bottom of the window, click **Notes**.

2. Click inside the **Notes** pane below the slide, and begin typing your notes.

Read more: Add speaker notes to each slide in a presentation

Print your speaker notes

1. On the **File** tab, click **Print**.
2. Under **Print**er, choose the printer that you want to print to.
3. Under **Settings**, next to **Full Page Slides**, click the down arrow and under **Print Layout**, click **Notes Pages**.
4. Click **Print**.

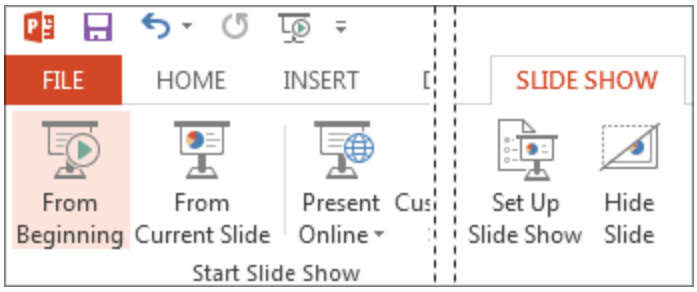


Read more: Print slides with or without speaker notes.

Give your presentation

On the **Slide Show** tab, do one of the following:

- To start the presentation at the first slide, in the **Start Slide Show** group, click **From Beginning**.



- If you're not at the first slide and want to start from where you are, click **From Current Slide**.
- If you need to present to people who are not where you are, click **Present Online** to set up a presentation on the web, and then choose one of the following options:
  - Present online using the Office Presentation Service
  - Start an online presentation in PowerPoint using Lync

INTRODUCTION TO INTERNET AND ITS APPLICATIONS

The Browser

As you surf the Web, you will come across sites that state, "This site is best viewed with…" and then name a particular browser. Many will even provide a link to a site where you can download the specified browser.

Sites make these recommendations because some browsers use special protocols, allowing site creators to offer extra features beyond the standard capabilities of hypertext markup language (HTML). Chief among these browsers are Netscape Navigator and Microsoft Internet Explorer.

Your Internet service provider will most likely give you a choice of browsers (if you have a SLIP/PPP account, you may use any browser you wish), so try out a couple, and use the browser that best suits your needs.

Connection Options

Until recently, the two primary methods of accessing the Internet were through a **network connection**, allowing users of local area networks (LANs) to go online through their school or workplace systems, and **dial-up connections** through a modem and phone line. However, new connection options allow for greater speeds and flexibility, while keeping costs to a minimum.

The following are some of the newer connection options that you might want to investigate:

- **Cable Internet**—These systems allow your computer to connect to the Internet through the same cable that carries your TV signal. Monthly service charges are usually not much more than standard modem connection costs, but you have to rent or purchase a "cable modem." Additionally, your computer will need an Ethernet card (a special circuit board that allows for network connections). Not all cable service operators offer this service; call your local operator for more information.
- **Satellite connections**—DirecPC (http://www.direcpc.com/) allows you to download Internet files via a satellite connection. This is an

efficient method for receiving large Web graphics and other items, but you still need a modem connection for other features. You must purchase the connection hardware as well as subscribe to the service.

- **Integrated Services Digital Network (ISDN)**—An ISDN line is a type of digital phone line that can transmit data many times faster than a conventional modem and phone line. To learn more about ISDN, go to http://www.isdn.ocn.com/index.shtml
- **Wireless connections**—Pagers, cellular phones and personal digital assistants (PDAs) now allow varying levels of Internet access, from notification of E-mail to limited Web connections. Many of these services remain in the experimental stage.
- **WebTV**—Introduced in late 1996, WebTV (http://www.webtv.net/) provides Web and E-mail access through ordinary television sets. The connection is made through a custom high-speed modem. You must purchase a special set-top unit for your TV, plus subscribe to the connection service. Recently, similar systems by other manufacturers have appeared on the market to compete with WebTV.

Getting Online

Once you have established your Internet account, you are now ready to "surf" the World Wide Web from your computer. To do so, perform the following steps (specific instructions will vary depending on your access provider and software):

1. Start up your computer, and make sure that your modem is on and connected to a telephone line.
2. Open your access software.
3. Initiate the connection. Many access software packages will display the status of the connection process, which generally takes between 15 and 30 seconds.
4. Once you have successfully connected, access and launch your Web browser.

If you have successfully accessed the Web, you will see in your browser window the *home page*, or the first page that your browser is set to access.

Often the home page is a site belonging to the manufacturer of the Web browser you're using. On most browsers, you can change the home page to a site that you'd like to access each time you begin a Web session.

Depending on the speed of your modem and the size or complexity of the page you're accessing, the time necessary to completely load a page can range from a few seconds to several minutes.

Finding Your Way Around

Now that you've gotten on the Web, you're probably wondering how to get to all those great resources that you've been hearing about. Fortunately, there are several strategies for moving about the Web:

- Type the URL address of a site into the entry field of your browser.

  Try it! Type the *complete* URL for the CenterSpan Web site below, then click on the "Go!" button:

This is the most basic method of accessing a Web site. However, you have to know exactly where you want to go, and then type in the address *precisely* in order to get there. The newer browsers can compensate for some typing (for instance, allowing you to omit the "http://" at the beginning of a URL), but some URLs are too complicated for a browser to second-guess.

If you simply want to explore and get to know the Web, any one of the following strategies are preferable:

- Click on **hyperlinks**  to move among resources; your home page probably has some links to interesting sites.
- Access a **jump site**, which is a page consisting mainly of categorized hotlinks to other sites. Again, your home page may function as a jump site.
- Access a **Web index**, which lets you access sites by typing in keywords.

Jump sites contain collections of special-interest sites that the author has included for certain reasons. Web indexes, however, usually contain much

more eclectic assemblies of Web resources. If you're on the Web and are not sure where to go, head to the nearest Web index.

Using a Web Index

To access and use a Web index, perform the following steps:

1. While logged on to the Web, type **http://www.yahoo.com/** in your browser's URL entry field.
2. Press ENTER or RETURN on your keyboard.

This will take you to Yahoo!, one of the most popular Web indexes. Underneath the Yahoo! logo, you will see a blank search entry form, as well as hotlinks of site categories.

3. Click on one of the category topics and follow the categories until you reach a site that matches your interests.

...OR...

4. Click your mouse in the entry form. This will place a blinking cursor in the form.
5. Type one or more words pertaining to information you'd like to locate on the Web.
6. Click on the SEARCH button to the right of the entry form, or press RETURN or ENTER on your keypad.

   After a few seconds, Yahoo! will return with a list of hotlinks that match your search criteria. The more specific your criteria, the fewer and more specific hotlinks you will see.
7. Scroll down the page and select the hotlink to a page that you'd like to explore [**NOTE:** Extensive search results will take up multiple pages].

If you would like to try searching with a Web index other than Yahoo!, select one of the index hotlinks listed at the bottom of each page of search results. When you select any one of these indexes, the keywords you entered for Yahoo! will remain in force, though the search results will be different simply because of the way that each Web index processes information.

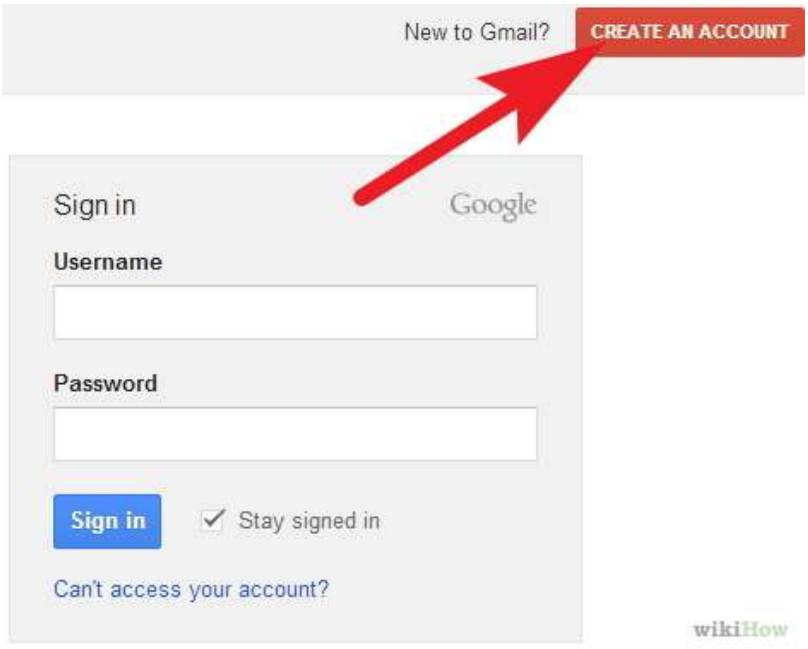
Where Are the Web Indexes?

In your searches, you will probably want to use various Web indexes, as they vary in both their content and the way in which they process search queries.

The following are the addresses for a few of the more popular Web indexes:

- **Yahoo!** (http://www.yahoo.com/)
- **Lycos** (http://www.lycos.com/)
- **WebCrawler** (http://www.webcrawler.com/)
- **InfoSeek** (http://www.infoseek.com/)
- **Alta Vista** (http://www.altavista.digital.com/)
- **Excite** (http://www.excite.com/)
- **HotBot** (http://www.hotbot.com/)
- **DejaNews** [for searching USENET newsgroup posts] (http://www.dejanews.com/)

Method 1 of 3: Choosing an Email Platform

1. 

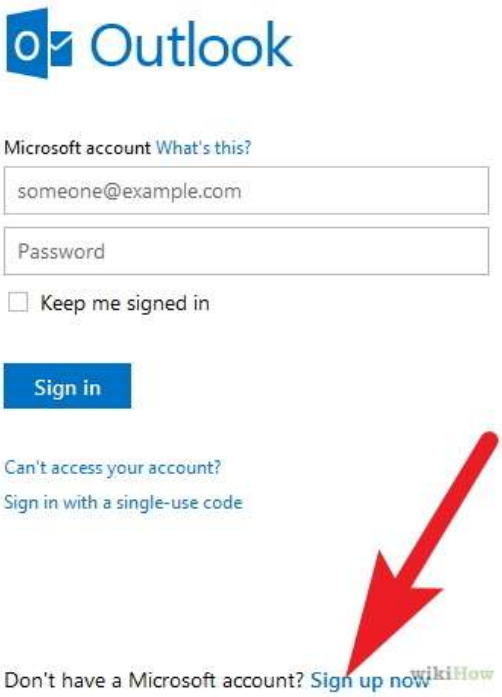
1

Set up a Gmail account to easily access all Google platforms. Gmail is an extremely straightforward interface to operate and offers 15 GB of free storage to share between your Google Drive, Gmail, and Google+ accounts. This means you almost never have to delete emails and can simply archive them in case you ever need to recover old messages.

- The best part about having a Google account is that it lets you access all of your Google platforms from any device, including Google Drive, which organizes text documents, photos, videos, and more, and allows you to share them with your contacts.
- Gmail allows you to organize your incoming mail into as many folders as you want for maximum organization.
- Gmail is very effective at blocking junkmail and also does not display banner ads that distract you as you construct emails.
- Gmail allows you to send 25MB of attachments in each email.
- Several features including RSS feeds, automatically saving drafts, Google search for within your mail, and linking other email accounts to your Gmail account are available with Gmail.
- Gmail also allows you to communicate with contacts in real time using instant messaging in your inbox. You can also video chat with up to nine people at once and call phone numbers from the Gmail interface.

Create a Yahoo! Mail account for fast email sending without complicated features. Yahoo! Mail updated to a new version in 2011 that runs twice as fast as the old version.

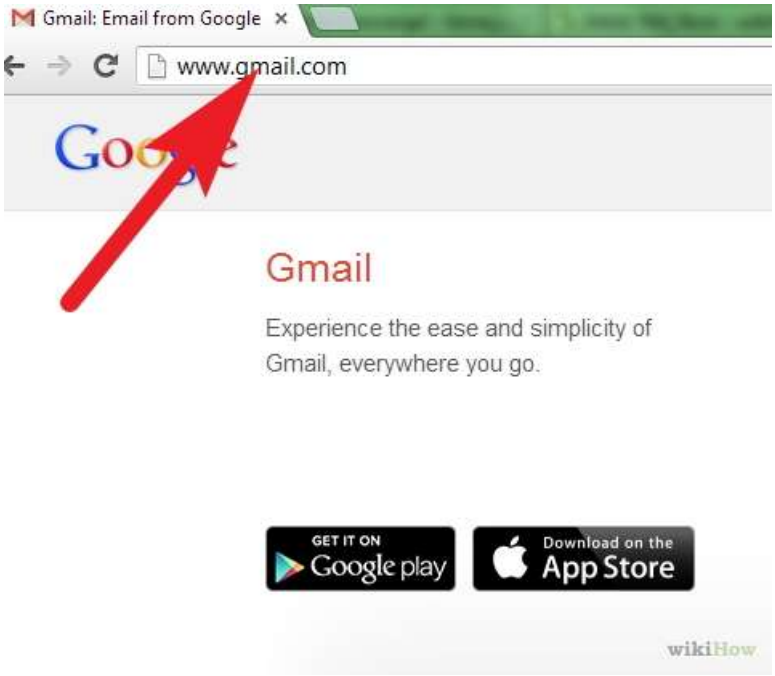
- Yahoo Mail works with Flickr and Picasa to transform links to online photos into embedded thumbnails and slideshows.[1]
- Yahoo! Mail offers unlimited data storage, which makes it great for sending photos and videos with large bandwidth.
- Unlike Gmail, however, Yahoo! allows banner ads and incorporates advertisements on your email interface, which can be distracting and annoying when trying to operate your email.
- A "Trending Now" link appears in the upper right-hand corner of the Yahoo! interface, which links to trending news topics but is unrelated to your email.



Make a Microsoft Outlook account to easily track shipping information and watch videos without leaving your email interface. Microsoft Outlook is the umbrella provider of Hotmail. If you are signing up for a new account, your address will be youremailaddress@outlook.com. Existing Hotmail users can retain the @hotmail.com address or make a new Outlook address.

- Outlook has a limited amount of storage space, though it does have the same basic features that the other email providers offer.
- Like Yahoo! Mail, Outlook automatically turns links to photos on Flickr or SmugMug into slideshows. It does not work with Picasa, however.
- Outlook lets you watch videos linked in your email to Hulu or YouTube without leaving your email interface. You can also track USPS packages without navigating to a separate website.[2]
- Outlook is also good for inter-office emailing because it includes an address book, calendar, task list, and virtual sticky notes. You can also easily create an appointment or a note and delegate work notifications to coworkers.

EditMethod 2 of 3: Setting Up an Account



Visit the website of the email provider of your choice. If you choose to use Gmail, simply visit www.gmail.com. The website for Yahoo! Mail is mail.yahoo.com, and Outlook is simply www.outlook.com.

Find the link on the homepage of your chosen provider that says "Sign Up" or "Create New Account." Click on the link and follow the prompts on the following pages. You will be asked for basic personal information and to create an email address.

- Identify your audience when coming up with an email address. If you are going to be using your email mostly for professional purposes, keep it simple and use your full name or a variation of your name.
- Don't use complicated addresses with several numbers. Doing so makes it harder for people to remember your address.
- Create a password that you can remember but is not obvious or too simple. Combine upper and lower case words, as well as numbers, and make a password that someone who knows you well would not be able to guess. Avoid using obvious number combinations such as birthdays, as these are relatively easy to figure out.

2.

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Fill out the required fields and set up any other features that the provider prompts you to set up. If you do not want to configure a Google+ account, for instance, there will always be a button somewhere on the page that allows you to skip that step or revisit the process at a later time.

Once you've created an account, you're ready to start sending and receiving email to your friends, family, and colleagues!

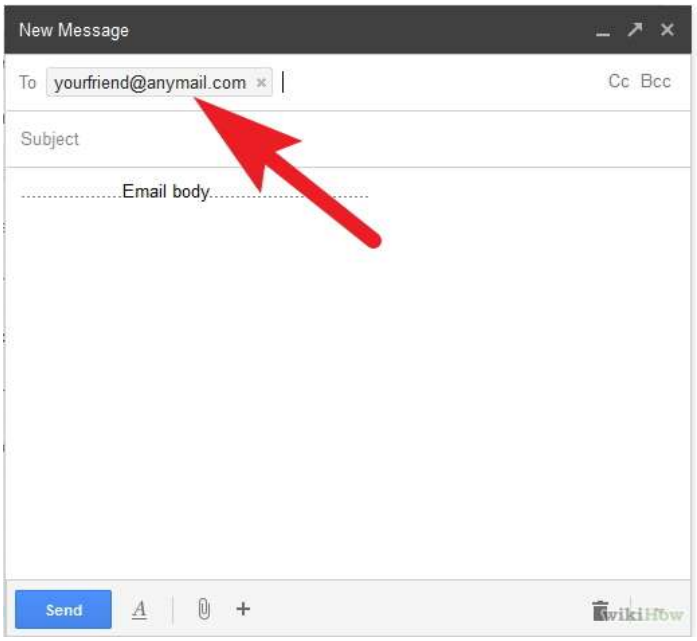
EditMethod 3 of 3: Sending an Email

Type in the URL of your email provider and log in with your username and password. If you forget either piece of login information, there will be a link underneath the login box that asks "Forgot Your Username/Password?" or something similar. Click the link and follow the steps to recover your lost information. You will most likely have to answer security questions to be able to reset your password.

Become familiar with your email interface. Spend some time clicking around your email interface and familiarizing yourself with the different features and functions available to you.

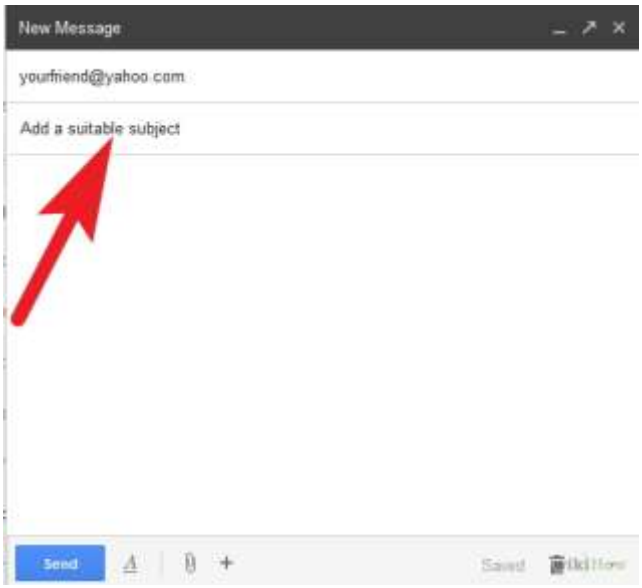
Find the button that allows you to compose a new message and click on it. It will usually be somewhere in the upper left-hand corner of your inbox and read "Compose," "Compose New Message," or simply "New Message." A new screen will pop up with a blank template for composing your message.

- In Gmail, you compose a new message by clicking on the red "Compose" button under the drop-down "Gmail" link in the upper left-hand corner of your inbox.



Enter your recipient's email address in the address bar that reads "To." This will likely be the first bar on the new message template.

- You can add multiple email addresses by separating each with a comma in the "To" address line.
- You can also Cc and Bcc as many email addresses as you want by adding them in the appropriate bars or clicking the "Cc" and "Bcc" tabs in Gmail to create new address bars. Cc stands for carbon copy and will send an exact copy of the email you are sending to your main address to the addresses that you Cc. When you use the Cc function, all of the recipients can see the addresses of the other people you sent the message to. When you Bcc (blind carbon copy), only the address in the "To" field can see all of the email addresses that the message was sent to.



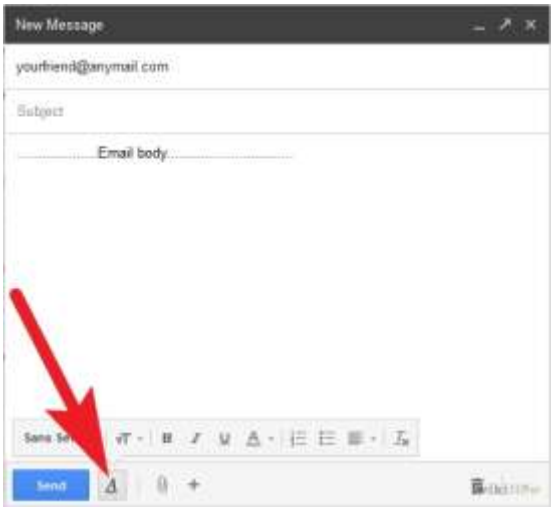
Enter a subject in the subject bar. Keep your subject line short and make sure that it appropriately summarizes the contents of the email you are sending.

- Subject lines that use all upper case letters, misuse numbers, or include too many symbols may look like spam emails to your recipients and therefore they may not want to open them.
- You do not have to put a subject in order to send an email. Leave the subject line blank if you prefer not to add one.

Click your cursor in the "Body" field of the message. This box may not be labeled as anything but will always be the largest block of white space on the new message template.

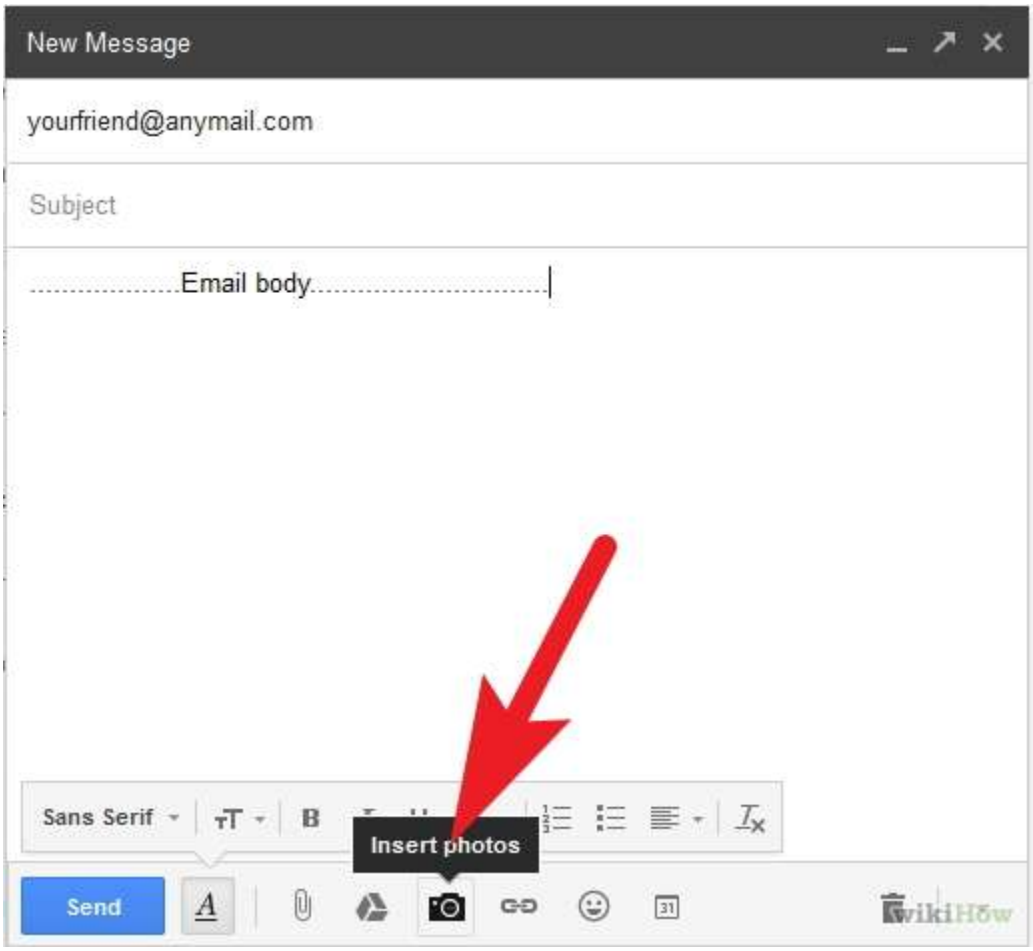
Type in the message you would like to send to your recipient(s). Start off with an introductory address such as "Dear Mrs. Smith" and press "return" twice to create a line of space between the two pieces of text. When you are finished with the body of the email, press "return" twice again and sign your email with a parting sign-off such as "Sincerely, [Your Name]" and be sure to hit return between the sign-off and your name.

- You can also add your contact information below your signature in order to make it easy for recipients to contact you in ways other than email. A common signature includes your job title, company, work or home address, phone number, and email.



Review your formatting options. Many email providers simply have a toolbar located between the subject line and the body of the email that allows you to perform simple formatting functions from choosing a font style, color, and size to creating a bulleted list.

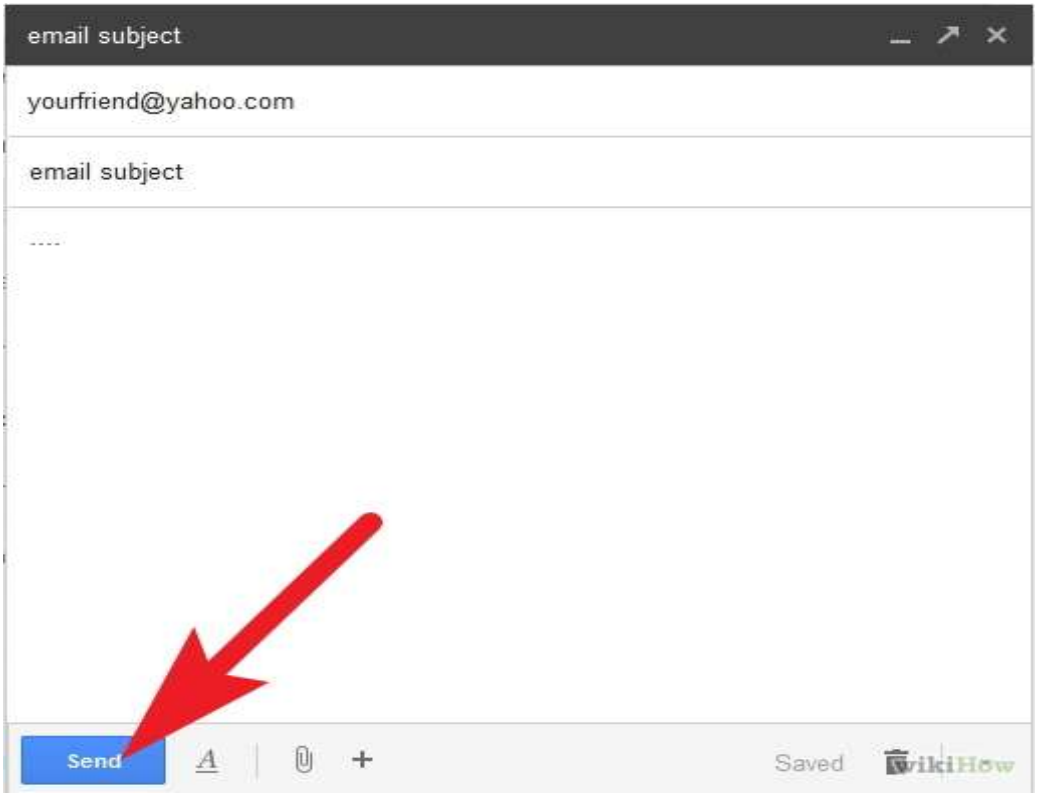
- Gmail's latest template simply has a capitalized, italicized, underlined "A" to the right of the "Send" button on the bottom left of the new message template. Click on the "A" to expand the toolbar and view formatting options.



Attach a photo, video, document, or other file. Most email interfaces either use a paperclip symbol in order to indicate where to attach your files, or have a button that says "Attach Files." It is usually located at the bottom of the body of the email or somewhere along the toolbar between the subject line and the body of the email. Locate the button and click on it to attach

files. A pop-up window will appear that allows you to access all of the files on your computer. Choose the files that you want to upload and click "Attach," "Open," or whatever affirmative option the drop-down menu gives you.

- Most email providers only allow a certain amount of data to be transferred per email. Gmail currently allows 25 MB per email. If all of the files that you want to send cannot fit in a single email, you may have to send multiple messages.



Click the "Send" button once you have finished crafting your email and adding any desired attachments or formatting options. The "Send" button is almost always located on the bottom of the message template on either the left or right side. Make sure your email sends before navigating your browser to another page.

Tips

- Connect your email account to your mobile device in order to receive and send emails remotely.
- Save drafts of your email as you type if the email is important. Gmail automatically saves drafts of your message for you, but other email providers may not.
- Create two separate email addresses if you want to use one for your professional contacts and one for friends and family. You can also create a separate email address for signing up for mailing lists so that your personal inbox is not spammed with advertisements and newsletters.
- If you need to send an email in a hurry, try incorporating Internet shorthand language into your email such as "BTW" to say "by the way" or "TTYL" to say "talk to you later." Make sure the person receiving the email also uses Internet slang and will understand what you are trying to say.
- Try to respond quickly to emails that you receive. Because most people are constantly connected to the Internet in some form, email users typically expect a response within 24-48 hours of sending an email if a response is required.