Lab 1

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Molecular biology

Preparation of laboratory solutions

1.1. Ratio and proportion

Ratio is the relationship between two quantities using division.

Example: a laboratory solution contains 58.8 gm of NaCl per litter express this ratio as a fraction.

The relationship can be expressed as

$$\frac{58.8\ gm}{1\ L}$$

Proportion is the statement that two ratio are equal.

Example: if 58.8 gm of NaCl is required to prepare 1 litter of it, how much grams you will need to prepare 2 litter of NaCl.

$$\frac{58.8\ gm}{1\ L} = \frac{X}{2\ L}$$

$$58.8\ gm * 2L = 1L * X$$

$$X = 117.6\ gm$$

- Homework
  1. If there are about 100 paramecia in a 20 ml water sample, then about how many paramecia would be found in 10 ml of this water?
  2. 10 ml of buffer are needed to fill a particular size test tube. How many ml are required to fill 37 of these test tubes?
  3. There are about $3* 10^9$ DNA base pairs in the human genome. Human chromosome 21 which contains 2% of the human genome. About how many b.p comprise chromosome 21?

1.2. Proportion Method of unit conversion.

1 Kg	=	1000 gm
1 gm	=	1000 mg
1 mg	=	1000 µg
1 L	=	1000 ml
1 ml	=	1000 µl
1 M	=	1000 mM
1 mM	=	1000 µM

How many µg in 150 gm?
How many µl in 5 L?

2. Concentration and Dilution

2.1. Concentration

Concentration is the amount of particular substance in a stated volume or sometimes mass of a solution or mixture.

2.1.1. Types of concentration expression and the calculation associated with each type.

1. (wt/ vol) fraction

$$\frac{2 \text{ gm NaCl}}{1 \text{ L water}}$$

Means that 2 gm of NaCl is dissolved in enough water so that the total volume of the solution is 1 liter.

- How could you make 300 ml of a solution that has a concentration of 10 gm of NaCl in 100 ml total volume

2. Percent (%)

- Prepare 2 % NaoH in 500 ml (% could be wt/vol or vol/vol)

❖ **Homework**

A solution has 5 µg/L of enzyme Q. how much enzyme Q is present in

1. 50 ml of solution 2. 100 µl of solution.
2. Molarity

   Molarity is a concentration expression that is equal to the number of moles of a solute that are dissolved per liter of solution.

$$M = \frac{wt}{m.wt} * \frac{v}{1000}$$

   ❖ How much solute is required to prepare 1 L of 1 mM solution of $CuSo_4$ (mwt = 159.61)

3. Part

   Parts solution tell you how many parts of each component to mix together. The parts may have any units but must be the same for all components of the mixture.

   Example: A solution that is 3:2:1 of ethylene: chloroform: isoamyle alcohol is

   3 parts of ethylene
   2 parts of chloroform
   1 parts of alcohol

   ❖ Prepare 50 ml of a solution that is 3:2:1 ethylene: chloroform: isoamyle alcohol.

2.2. Dilution

Dilution is when one substance (often but not always water) is added to another to reduce the concentration of the first substance.

$$M1V1=M2V2$$

$$C1V1=C2V2$$

❖ Homework

1. Prepare 50ml of 0.6 M NaOH from 1 M NaOh stock solution.
2. A recipe says to mix

10 X buffer A	1µl
Solution B	2µl
Water	7µl

What is the concentration of buffer A in the final solution.

Molarity Calculation Relating to DNA

It is necessary to know the molecular weight of a substance of interest in order to perform molarity calculations. The formula weight of a specific chemical compound is always the same so it is usually straightforward to find the formula weight of chemicals by looking at the label on their containers. **DNA is different** because its sequence and length vary depending on the source. A DNA molecules may be single stranded (SS) or double stranded (DS) and it may consist of anywhere from a few nucleotides to billions of base pairs. Therefore there is no single MW for all DNA molecules.

1. Molecular weight of DNA molecule with known sequence and length.

	Molecular weight of Nucleotides incorporated into Nucleic Acids
A in DNA	313.22 D
C in DNA	289.18 D
T in DNA	304.21 D
G in DNA	329.22 D
A+T	313.22+ 304.21= 617.43
G+C	289.18+ 329.22= 618.4
A in RNA	329.22 D
C in RNA	305.18 D
U in RNA	306.20 D
G in RNA	345.22 D

$MW = (N_c * 289.18) + (N_a * 313.22) + (N_t * 304.21) + (N_g * 329.22) - 61.96$

2. Molecular weight of DNA molecule with unknown sequence with known length. A single nucleotide, on the average, has a molecular weight of 330 D and a base pair on the average has a weight of 660 D.

❖ What is the MW of a DS DNA molecules that is 100 bp long?

Lab 2 Molecular Biology

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DNA extraction

DNA constitutes a small percent of the cell material and is usually localized in a defined part of the cell. In procaryotic cells DNA is highly condensed and localized in a structure called the nucleoid, which is not separated from the rest of the cell sap by a membrane. In eucaryotic cells the bulk of DNA is localized in the nucleus, which is separated from the rest of the cell sap by a complicated membrane structure. Usually approximately 90 percent of the DNA is localized in the nucleus (chromosomes); the rest can be separated into other organelles such as mitochondria or chloroplasts. In viruses and bacteriophages, DNA is encapsulated by the protein coat and constitutes between 30 and 50 percent of the total mass of the virus.

DNA extraction steps

1. **Breaking the cells open to release the DNA (cell lysis or cell disruption)**. This is commonly achieved by grinding or sonicating the sample. and put into a solution containing salt. The positively charged sodium ions in the salt help protect the negatively charged phosphate groups that run along the backbone of the DNA. A detergent is then added. The detergent breaks down the lipids in the cell membrane and nuclei. DNA is released as these membranes are disrupted
2. **Separating DNA from proteins and other cellular debris.** Often a protease is added to degrade DNA-associated proteins and other cellular proteins. Alternatively, some of the cellular debris can be removed by filtering the sample.
3. **Precipitating the DNA with an alcohol** — usually ice-cold ethanol or isopropanol. Since DNA is insoluble in these alcohols, it will aggregate together, giving a pellet upon centrifugation. This step also removes alcohol-soluble salt.
4. **Cleaning the DNA.** This can be achieved by adding ethanol 70%.
5. **DNA storage.** DNA usually re-suspend and stored with slightly alkaline Buffer.

Cell disruption

Extraction of DNA from plant and animal cells is much harder than from bacterial cells. Before proceeding DNA extraction, plant and animal cells must often be ground into tiny fragments either by mechanical (blender) or chemical (enzymes) methods. In case of Bacteria, the cell wall can be disrupted easily by **lysozyme**, an enzyme the degrades the peptidoglycan layer of the cell wall. A

successive treatment with **detergent** dissolves the lipid of the cell membrane. **Chelating agents**, such as EDTA, are also used especially with gram positive bacteria, to remove the mental ions that bind components of the outer membrane together.

DNA purification

Two general types of procedure are used for purification of DNA, **centrifugation** and **chemical** extraction. The principle of centrifugation is as follows. The sample is spun at high speed and the centrifugal force causes the larger or heavier components to sediment to the bottom of the tube.

1. Removal of Protein (deproteinization)

- **Deproteinization using Phenol: chloroform: isoamyl alcohol.**

DNA is then re-dissolved in an appropriate buffer solution. However, it still has a lot of protein and RNA mixed in with it. These are generally removed by chemical means. The organic solvents commonly used are phenol, chloroform and isoamyl alcohol. The method that uses phenol as the deproteinizing agent is called **Kirby method**. Use of chloroform isoamyl alcohol mixtures is named the **Marmur method**. Phenol, also known as carbolic acid, is very corrosive and extremely dangerous because it dissolves and denatures the proteins that make up 60 to 70 percent of all living matter. Consequently, phenol may be used to dissolve and remove all of the proteins from a sample of DNA.

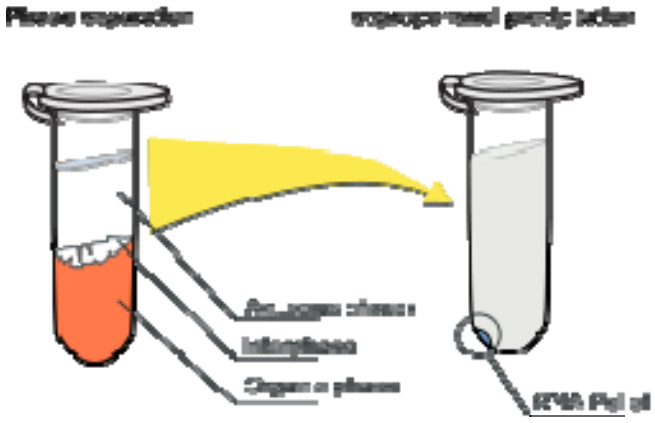


FIGURE 1 Phenol *Extraction Removes Proteins from Nucleic Acids*
Proteins can be removed from a solution of DNA or RNA by adding an equal volume of phenol. The phenol dissolves the proteins without disrupting the DNA or RNA. Since phenol is very dense, it forms a separate layer at the bottom of the tube. When the two solutions are shaken, the proteins dissolve into the phenol. The two layers separate again after a brief spin in the centrifuge. The top phase, which now contains just DNA and RNA, can be isolated.

Protein molecules generally contain many hydrophobic residues, which are concentrated in the center of the molecule. When an aqueous protein solution is mixed with an equal volume of phenol, some phenol molecules are dissolved in the aqueous phase. Yet the phenol molecules are extremely hydrophobic. Consequently, they tend to be more soluble in the hydrophobic cores of the protein than in water. As a result, phenol molecules diffuse into the core of the protein causing the protein

to swell and eventually to unfold or denature. The denatured protein, with its hydrophobic groups exposed and surrounded by micelles of phenol, is far more soluble in the phenol phase than in the aqueous phase. As a result, proteins are partitioned into the phenol phase leaving the nucleic acids in the aqueous phase.

- **Deproteinization using enzymes**

Proteins can be removed from DNA preparations using a protease that can digest all proteins. Two such enzymes are in use, Proteinase K and Pronase. Proteinase K and pronase are usually used in DNA purification procedures at final concentrations of 0.1–0.8 mg/ml. The difference between these two enzymes lies in their activities towards self; pronase is a self-digesting enzyme, whereas proteinase K is not. The fact that proteinase K is not a self-digesting enzyme makes it a more convenient enzyme to use than pronase, because it is unnecessary to continually add it during the prolonged course of the reaction. This treatment is used when a large amount of protein is present, i.e. right after cell lysis. The remaining proteins can be removed with a single extraction using organic solvent.

2. **Removal of Unwanted RNA**

Special enzymes remove contaminating RNA from a DNA sample. The enzyme **ribonuclease** degrades RNA into short oligonucleotides but leaves the giant DNA macromolecule unchanged. A mixture of DNA and RNA is first incubated with the ribonuclease at the optimal temperature for enzyme activity.

DNA precipitation

Next, an equal volume of 95% alcohol is added. The alcohol precipitates large macromolecules, including long chains of DNA, out of solution. However, the small RNA fragments remain dissolved. Next the DNA is precipitated at the bottom of the tube by centrifugation and the supernatant solution containing the RNA fragments is discarded (Fig.2).

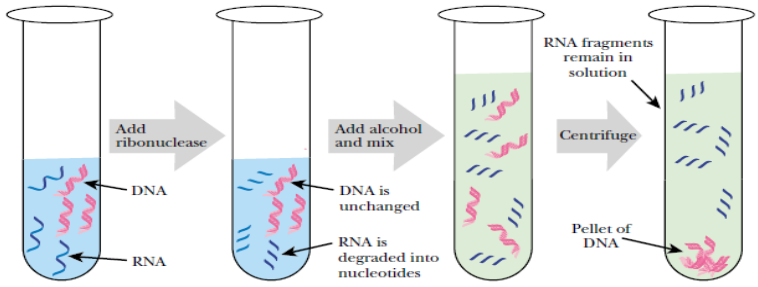


FIGURE2 Removal of RNA by Ribonuclease and DNA precipitation by alcohol. A mixture of RNA and DNA is incubated with ribonuclease, which digests all the RNA into small fragments and leaves the DNA unaltered. An equal volume of alcohol is added, and the larger pieces of DNA are precipitated.

Materials use in DNA extraction:

1- **Phenol/chloroform isoamyl alcohol :**use to remove proteins from nucleic acid samples and can be carried out in a manner that is very close to quantitative. Nucleic acids remain in the aqueous phase and proteins separate into the organic phase or lie at the phase interface.or DNA extraction with phenol/chloroform isoamyl alcohol pH 8 aqueous top phase contains the majority of DNA, interphase mostly proteins, and lower organic phase most of the RNA and lipids.

2- **Ethanol 95% or Isopropanol:** DNA is not soluble in alcohol. When alcohol is added to the mixture, all the components of the mixture, except for DNA, stay in solution while the DNA precipitates out into the alcohol layer.

3- **EDTA (Ethylene diamine tetra acetic acid) :** chelating agent to sequester divalent cations such as Mg^{2+} and Ca^{2+}. This stops Dnase enzymes from degrading the DNA..

4- **SDS (Sodium dodecyl sulfate):** is anionic detergent use to soluble the cell membrane to release cell contents.

5- **Pronase or Protenase k:** an enzymes use to breaks down or denatures protein

6- **Triton X-100** ($C_{14}H_{22}O(C_2H_4O)_n$) : is a nonionic surfactant , used in DNA extraction as part of the lysis buffer (usually in a 5% solution in alkaline lysis buffer).

Molecular biology lab.

Lab4. DNA extraction from Bacteria

Materials Required:

- Nutrient Broth
- *E. coli*
- Reagents
- TE buffer (pH 8.0)
- 10% SDS
- chloroform
- 5M Sodium Acetate (pH 5.2)
- 95% ethanol
- 70% ethanol
- Autoclaved Distilled Water
- Eppendorf tubes 2 ml
- Micropipette
- Microtips
- Microfuge

Preparation of Reagents:

1. **TE BUFFER (pH 8.0):** 10 mm Tris HCl (pH 8.0), 1 mm EDTA (pH 8.0)
2. **10% SDS:** Dissolve 10 g of SDS in 100 ml autoclaved distilled water.
3. **CHLOROFORM**
4. **5M SODIUM ACETATE:** Dissolve 41 g of sodium acetate in 100 ml distilled water and adjust pH with dilute acetic acid (pH 5.2).
5. **95% ETHANOL**
6. **70% ETHANOL**

PROCEDURE:

- 2 ml overnight culture is taken and the cells are harvested by centrifugation for 10 minutes
- 875 µl of TE buffer is added to the cell pellet and the cells are resuspended in the buffer by gentle mixing.
- 100 µl of 10% SDS is added to the cells.
- The above mixture is mixed well and incubated at 37° C for an hour in an incubator.
- 1 ml of chloroform is added to the contents, mixed well by inverting and incubated at room temperature for 5 minutes.
- The contents are centrifuged at 10,000 rpm for 10 minutes at 4° C.
- The highly viscous jelly like supernatant is collected using cut tips and is transferred to a fresh tube.
- The process is repeated once again with phenol-chloroform mixture and the supernatant is collected in a fresh tube.
- 100 µl of 5M sodium acetate is added to the contents and is mixed gently.
- 2 ml of 95% ethanol is added and mixed gently by inversion till white strands of DNA precipitates out.

- The contents are centrifuged at 5,000 rpm for 10 minutes.
- The supernatant is removed and 1ml 70% ethanol is added.
- The above contents are centrifuged at 5,000 rpm for 10 minutes.
- After air drying for 5 minutes 50 µl of TE buffer or distilled water is added.
- The concentration of DNA is determined using a spectrophotometer at 260/280 nm.
- The remaining samples are stored for further experiments.

Plant Genomic DNA Extraction using CTAB

Introduction

The search for a more efficient means of extracting DNA of both higher quality and yield has lead to the development of a variety of protocols, however the fundamentals of DNA extraction remains the same. DNA must be purified from cellular material in a manner that prevents degradation. Because of this, even crude extraction procedures can still be adopted to prepare a sufficient amount of DNA to allow for multiple end uses. DNA extraction from plant tissue can vary depending on the material used. Essentially any mechanical means of breaking down the cell wall and membranes to allow access to nuclear material, without its degradation is required. For this, usually an initial grinding stage with liquid nitrogen is employed to break down cell wall material and allow access to DNA while harmful cellular enzymes and chemicals remain inactivated by means of low temperature during the grinding of plant tissues. Once the tissue has been sufficiently ground, it can then be resuspended in a suitable buffer, such as CTAB (Cetyl Trimethylammonium Bromide). In order to purify DNA, insoluble particulates are removed through centrifugation while soluble proteins and other material are separated through mixing with chloroform and centrifugation. DNA must then be precipitated from the aqueous phase and washed thoroughly to remove contaminating salts. The purified DNA is then resuspended and stored in TE buffer or sterile distilled water. This method has been shown to give intact genomic DNA from plant tissue.

Plants are considered to be a “difficult” for DNA isolation due to its hard leaves, fibres and the presence of highly viscous polysaccharides which may interfere with the DNA purity. The presence of high amounts of contaminants, mainly phenolic compounds, polysaccharides, and secondary metabolites impedes the DNA isolation procedure and inhibits analytical studies on the isolated DNA. Phenolic compounds are powerful oxidizing agents and bind covalently to the extracted DNA, making it useless for most of molecular manipulations, so, high concentration of PVP (polyvinylpyrrolidone) mixed in the extraction buffer binds to phenolic compounds and helps in their removal. Polysaccharides are also problematic as they make the DNA unruly during pipetting and hinder the activity of polymerases, ligases, and restriction endonucleases. Co-precipitation of polysaccharide was avoided by adding higher concentrations of selective precipitants of nucleic acid, CTAB and NaCl. Long-tail surfactants (such as CTAB) produce a conformational change in the DNA from “random coil” to “compact globule”, making DNA precipitation more effective as well as its helps in a better cell lysis. The CTAB (which is cationic detergent) would form the Nucleic acid/CTAB complex which does not dissolve in solvents; later the nucleic acid could be separated from other cell components by isopropanol or ethanol precipitation. The excessive quantities of cellular proteins were managed by extended treatment with chloroform-isoamyl alcohol. In addition to the removal of proteins, this treatment also helped to remove different coloring substances such as chlorophy, pigments, and dyes The presence of EDTA in the extraction buffer acts to withdraw the Mg+2 ions required for nucleases activity. The presence of high NaCl concentration with isopropanol alcohol would act to precipitate the DNA.

Procedures:

- Approximately 0.7-2 g of fresh leaf tissue from date palm trees were cut into pieces and placed into a cold mortar.
- Liquid nitrogen was added to the mortar to freeze the tissue. The temperature of liquid nitrogen is -196°C or (-321° F), gloves were worn during grinding to protect the hands.
- The leaf tissue was crushed by grinding the pestle against the mortar in a circular motion. Grinding continued for a minute or two until the tissue becomes fine powder.
- The powder was transferred into a 100ml flask using a paint brush to get all of the tissue into the flask.
- A volume of 12 milliliters of extraction buffer were added to flasks covered and mixed by gently shaking in a water bath shaker at 68°C for 60 minutes. Flask contents were mixed several times while they were incubated. Flasks were cooled down for several minutes at room temperature.
- A volume of 10mL of chloroform/isoamyl alcohol solution was added to each flask and shaken for 5-10 minutes at room temperature (in the fume hood).
- The samples were then transferred to 50mL tubes and centrifuged at (4000 rpm) for 15 minutes.
- The upper phase was transferred into new 50mL tubes. For each 10mL of supernatant in a test tube, six mL of cold isopropanol (-20°C) was added and covered tightly before mixing by gently inverting the tube several times. A white, stringy precipitate consisting of DNA and RNA should be visible at this point.

- Spool out nucleic acids with a glass hook. (The hook is a pasteur pipette that has been bent at the end) and transferred to 4mL of a washing buffer for 10-20 minutes.
- Spool out nucleic acids with a glass hook from the washing buffer and were dried at room temperature.
- Re-suspend a nucleic acid pellet in 200-300μL of a TE buffer, and incubated at 65°C for ~20-30 minutes or until the pellet was dissolved and stored at -80°C until use.

Extraction Buffer (CTAB buffer)

It was prepared by dissolving 2g of CTAB (2%), 8.1816g of NaCl (1.4 M), 0.7444g of Na2EDTA (0.02 M)and 1.2114g of Tris–base (0.1 M) in D.W, pH was adjusted to 8.0, volume completed with D.W to 100mL, sterilized by autoclaving for 15 min at 121 °C and stored at 4°C .

Washing Buffer

It was prepared by dissolving 0.140g of ammonium acetate in 76mL ethanol, volume completed with D.W to 100mL.

Tris-EDTA (TE) Buffer

It was prepared by dissolving 0.1211g of Tris-base (10mM), 0.0372g of Na_2EDTA (1mM) in D.W, pH was adjusted to 8.0, volume completed with D.W to 100mL, sterilized by autoclaving and stored at 4°C.

Molecular biology lab (8)

Determining DNA concentration and purity.

The most comprehensive way to evaluate DNA concentration and purity is to use both UV spectrophotometric measurements and agarose gel electrophoresis.

UV spectrophotometric measurement of DNA concentration and purity.

DNA itself, and most of the common contaminants found in DNA preps, have absorbance in the region 230nm to 320nm so measurement of the absorbance in this region allows measurement of the DNA concentration and provides information about the contaminant levels. The most important wavelengths to note are:

- **230nm**: Guanidium salts (used to facilitate DNA binding to silica columns) and phenol (used in phenol/chloroform extractions) absorb strongly at 230nm, therefore high absorbance at this wavelength can be indicative of carry-over of either of these compounds into the sample.
- **260nm:** DNA absorbs light most strongly at 260nm so the absorbance value at this wavelength (called A_{260}) can be used to estimate the DNA concentration. Nucleic acids absorb in the ultraviolet region of the spectrum due to the conjugated double bond and ring systems of the constituent purines and pyrimidines.
- **280nm:** Since tyrosine and tryptophan residues absorb strongly at this wavelength, the absorbance at 280nm is used as an indicator of protein contamination.

- **320nm:** A_{320} provides a general measurement of the turbidity of the sample and is normally subtracted from the A_{260} value as a background reading for the calculation of DNA concentration, but excessive values may indicate non-specific contamination.

DNA concentration can be determined by:

1. 1 OD_{260} unit = 50 µg/ml or

   50 ng/µl

2. Unknown diluted µg/ml = 50 µg/ml x Measured A260 x dilution factor.

Nucleic acid	Concentration µg/ml per A 260
DS DNA	50
SS DNA	33
SS RNA	40

In most DNA preparation, the final step is the separation of DNA from proteins. Carryover proteins during DNA prep could led to problems with subsequent operations, such as cutting with restriction endonuclease. Assessment of DNA purity is therefore important.

The most commonly used assay for DNA purity is:

$$\text{DNA purity} = A_{260} / A_{280}$$
$$= 1.8$$

The most commonly used assay for RNA purity is:

$$\text{RNA purity} = A_{260} / A_{280}$$
$$= 2$$

Measurement of DNA concentration and purity using agarose gel electrophoresis

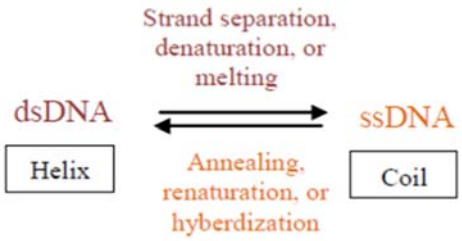
A drawback to spectrophotometric measurements is that contaminants such as genomic DNA (a contaminant in plasmid preps), RNA, guanidium and proteins all display some absorbance at 260nm, so if they are present at high levels in the DNA prep they will contribute to an increased A_{260} reading and lead to an overestimation of the DNA concentration. Agarose gel electrophoresis, using a quantitative dye such as ethidium bromide, can be used as an alternative approach to measure sample DNA concentration with is not affected by these contaminants. The DNA concentration of a sample can be roughly calculated by comparison of the sample band intensity with that of a molecular weight marker band whose DNA content is known. Contaminating RNA or genomic DNA can be detected on an agarose gel, since RNA will run as a low molecular weight smear and genomic DNA as a high molecular band

DNA hyperchromic and hypochromic effect:

The absorption of single strand DNA (ssDNA) is higher than the absorbance of double strand DNA (ds DNA) this is known as a hyperchromic effect. The hydrogen bonds between the paired bases in the double helix limits the resonance behavior of the aromatic ring of the bases which results in decrease in the UV absorbance of ds DNA (hypochromic effect). While in ssDNA the bases are in free form and don't form hydrogen bonds with complementary bases which results in 40% higher absorbance in ssDNA (hyperchromic) at the same concentration.

DNA denaturation:

DNA denaturation, or DNA melting, is the process by which double-strand DNA unwinds and separates into single-stranded strands through the breaking of hydrogen bonds between the bases. Disruption occurs in lab by different methods such as heating to high degree, change salt concentration, Adding alkali or change pH.



DNA denaturation by heating:

When DNA is heated, the temperature at which half of helix structure is lost is known as melting temperature (Tm). The melting temperature depends on both the **length of the DNA** and the **nucleotide sequence composition**. Higher GC content higher Tm. This is because the triple hydrogen bonds between G and C need more energy to disrupt than the double bonds between A and T.When a solution of double-stranded DNA is slowly heated, the absorbance increases rapidly to a higher value. Which is not significantly changed by further heating.

Home work:

A series of tests were performed and the following measurements were obtained using a spectrophotometer at OD 260

O.D at 260	µg DNA in the samples
0.2	10
0.415	20.8
0.694	35
1	50
1.4	70
1.8	90
2	100

Determine the DNA concentrations of the following unknown diluted samples if you know that the dilution factor is 200

O.D at 260
0.0009
0.0028
0.008
0.012