Ministry of Higher Education and Scientific Research
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Practical Molecular biology & Bacterial genetic
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المرحلة الرابعة - الدراستين الصباحية والمسائية
الفصل الدراسي الاول

الكادر التدريسي في المختبر

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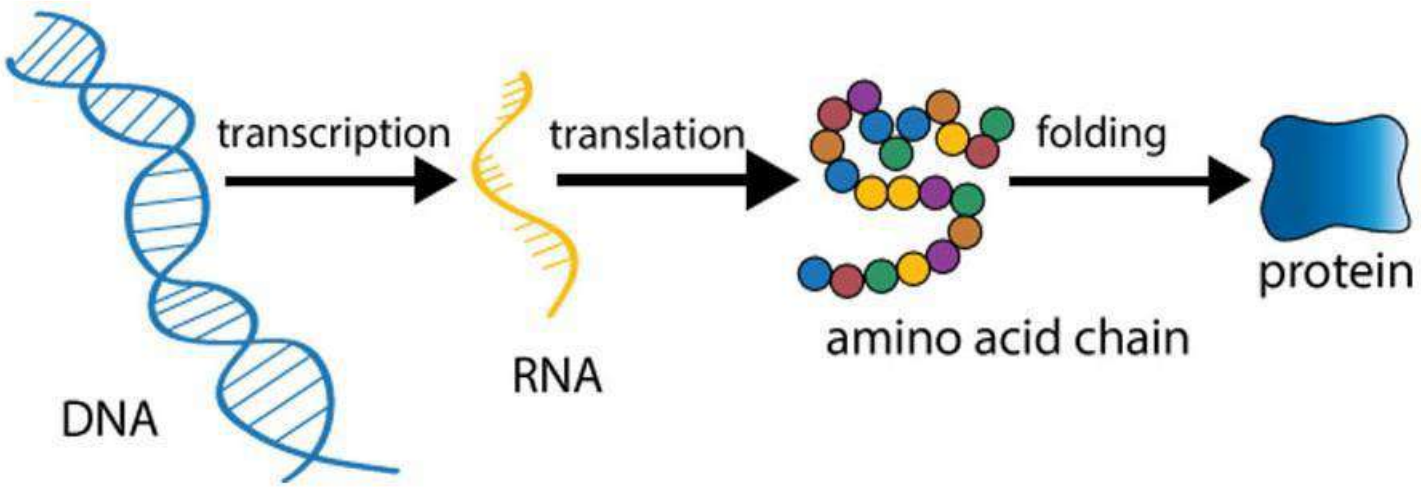


Molecular Structure of Nucleic Acids

- DNA and RNA are marco-molecular structure composed of regular repeating polymers formed from **nucleotides** called **polynucleotides**.

- DNA has two strand of nucleotides that contains the genetic instructions used in the development and functional of all living organisms while RNA has one strand used in protein synthesis.

- The basic building blocks of nucleic acids are **nucleotides**, that composed of:

1) Nitrogenous base

There are two types of nitrogen base:

a) **purines (A, G):** aredouble ring

b) **pyrimidines (C,T in DNA; C, U in RNA):** are single ring

A binds to T or U by two hydrogens bond while G binds to C by three hydrogen bonds

2) Pentose carbon sugar

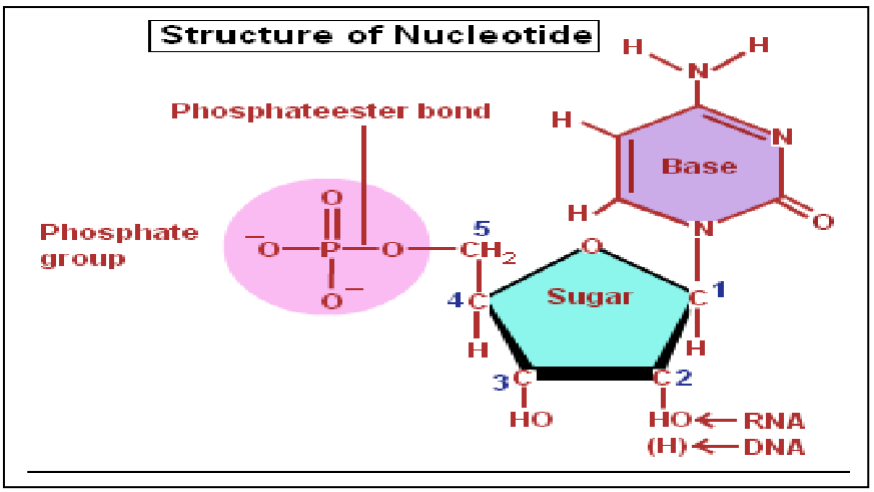
Compose of five carbon atoms; It founds as **2-deoxyribose in DNA & ribose in RNA.** Ribose sugar in RNA has (OH^-) group at 2^- carbon atoms while 2-deoxyribose in DNA lack the (OH^-) group

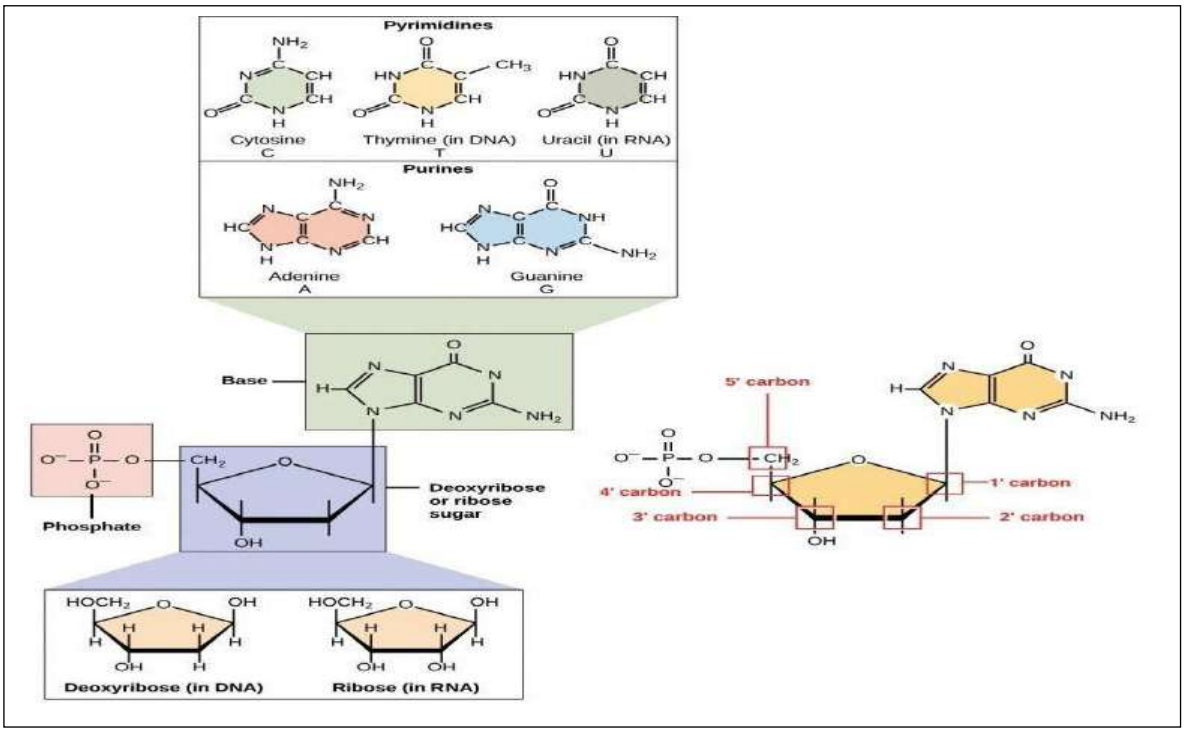
In one strand, pentose sugar bind with the nitrogenous base at 1^- carbon atom by glycocosidic bond forming Nucleoside, which it binds to phosphate group at 5^- carbon atom by an ester bond

3) Phosphate group $(PO_4)^{-3}$

It gives the nucleic acid (DNA or RNA) its strong negative charge.

It binds to the 5^- carbon atom of a 2-deoxyribose sugar in a nucleotide by **ester bond** and to 3^-hydroxyl group of another nucleotide by second ester bond that mention as **phosphodiester bond**; which represented backbone of DNA & RNA.



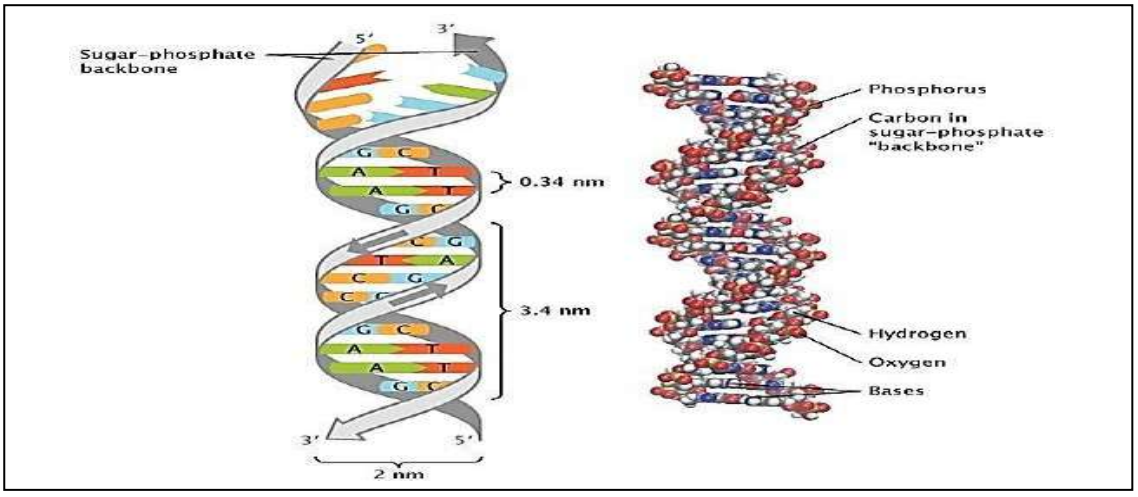


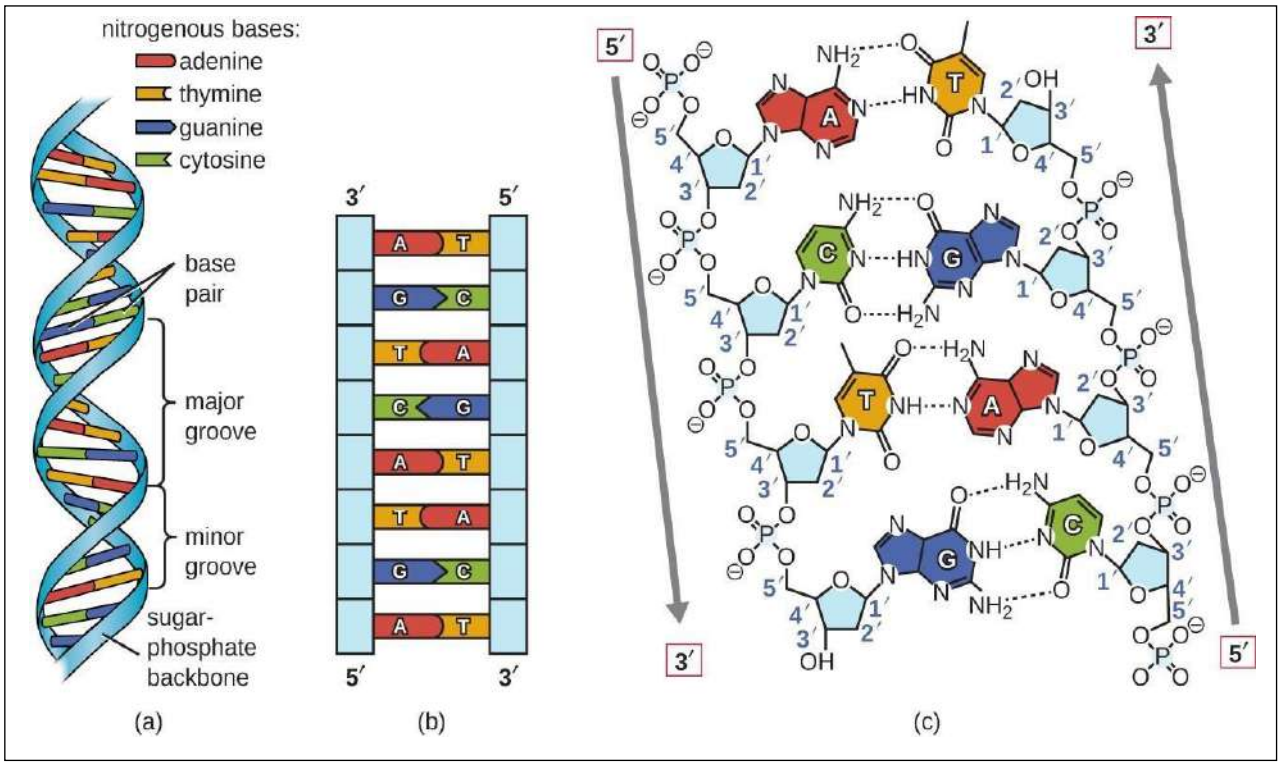
❖ DNA molecule is described as an **antiparallel structure** that is has two polynucleotide strands in opposite direction, one strand has3¯ to 5¯directionand the other one has has 5¯ to 3¯direction.

<u>The antiparallel strands of DNA are held together by:</u>

1) Weak forces of hydrogen bonds between bases.
2) Partly hydrophobic interaction between stacked base pairs.

❖ Furthermore, both strand remain separated by 2 nm distance. The coiling of double helix is right handed (form B) and composed of 10 ten nucleotides, since it occupies 0.34 nm distance between each nucleotide so a complete turn occurs every 3.4 nm.The helix has two external grooves, a deep wide one called major groove and a shallow narrow one, called minor groove; both of these grooves are large enough to allow protein molecules to contact with the bases.





Must be remember:

- Diameter of double helix DNA= 20 A (2 nm)
- Base pair (bp) = double nucleotides in the antiparallel strand of DNA
- Three nucleotides = codon (coded for one Amino Acid)
- The average of M.W to bp (DNA)= 618 dalton
- The average of M.W to ribonucleotide (RNA)= 320 daltons
- The average of M.W to amino acids (protein)= 120 daltons
- Distance between each pb= 0.34 nm …length of one turn = 3.4nm (0.34nm x 10pb)

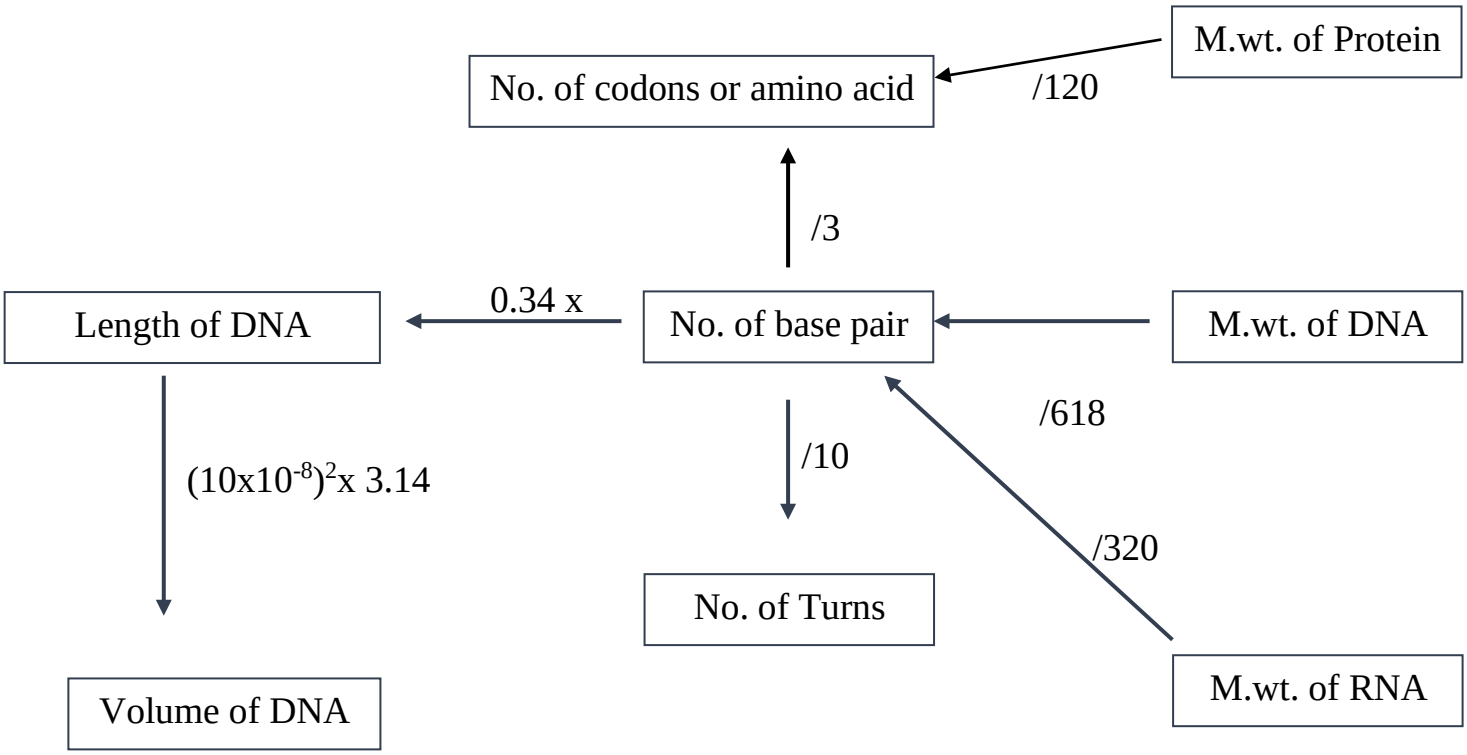
Kilo base pairs = 10^3 bp
Mega Dalton (mega dal.) = 10^6dalton

1 bp = 618 dalton

1000 = 618000 dalton, therefore 1 Kbp = 618000 dalton& 1 Kbp = 0.62 mega dalton

Calculation of molecular structure of DNA:

Important note (move with the direction of the arrow, use operation on arrow otherwise use opposite operation)



Important note (conversion from large quantity to a small quantity multiply by 10^{1}, conversion from small quantity to a large quantity multiply by 10^{-1})

The Powers of 10

10^{+0}	1 Meter
10^{2}	1 Centimeters
10^{3}	1 Millimeter
10^{-6}	1 Micron
10^{-9}	1 Nanometer
10^{10}	1 Angstrom

Calculation of molecular structure of DNA:

Ex. (1): Calculate the **length**, **volume** and **number of turns** of double helix DNA molecule, if the M.Wt of this DNA molecule is 3×10^7 dalton.

Solution: No. of nucleotides pairs= 3×10^7 dalton / 618 dalton = 48544 bp
Length of DNA strands =48544 bp x 0.34nm = 16.5049 nm
16.5049×10^{-7} cm

- The shape of DNA molecule is cylindrical, so ….
  The length of cylinder (DNA) = 16.5049×10^{-7} cm
  (and the diameter is 20A (20×10^{-8}cm)
  The Volume of cylinder = $3.14 \times r^2 \times$ length
  $= 3.14 \times (10 \times 10^{-8}\text{cm})^2 \times 16.5049 \times 10^{-7} = 5.18 \times 10^{-20}$ cm^3

No. of turns = 48544 bp / 10 bp turn = 4854.4 turns

Ex. (2): Calculate the M.wt of mRNA that coded to protein if the M.wt of this protein is equal to 75000 dalton ?

Solution: No. of amino acids = 75000/ 120 = 625 amino acids
No. ribonucleotides of mRNA = 3x 625 = 1875 ribnucleotides
M. wt of mRNA = 1875 x 320 = 6×10^5dalton

Ex. (3): The M.Wt of T4 DNA is 1.3×10^8dalton, calculate:
a) No. of amino acid that coded from this DNA

Solution: No. of bp of T4 DNA 1.3×10^8dalton/ 618 dalton = 2.1×10^5bp
No. of codons of T4 DNA = $2.1 \times 10^5 / 3 = 7 \times 10^4$ codons

Buffers

- **Buffer** is a solution containing either a weak acid and its salts or a weak base and its salts; which resistant to change in pH.

- **Buffer** is a solution which resists change in pH value on dilution or on addition on an acid or alkali solution, such as phosphate buffer, citrate buffer, Tris buffer and biocarborate buffer in the blood.

$CO_2 + H_2O \rightleftharpoons H_2CO_3$ (carbonic acid)

$H_2CO_3 \rightleftharpoons HCO_3^- + H^+$ PH (7.35- 7.45)

Biocarbonate ion

In case of addition $(OH)^-$, OH reacts with H^+ forming H_2O making the reaction goes toward the right direction to replace the amount of (H^+) that loosed through addition of (OH) (keep pH constant)

In case of addition (H^+), it reacts with HCO_3^- forming H_2CO_3 making the reaction goes toward the left direction to get away from the amount of (H^+) that added to the starting of the reaction (keep pH constant).

- **Buffer capacity:** represents the ability of a buffer to resist changes in pH.
- **Buffer agent:** the weak acid or the weak base in a buffer solution.

Properties of buffer solution:

Any buffer solution acidic or basic possesses of following properties:

1. It has defined pH
2. It has pH value does not change with long time or on dilution.
3. It has pH value remains practically constant when small quantities of strong acid or strong base are added to the buffer

The purpose function of biological system requires control of pH, since most metabolic processes are inactivated outside a certain narrow range of concentration of hydrogen ions.

Kinds of buffers that used in molecular biology

TBE buffer: Tris-borate-EDTA buffer

TSE buffer: Tris- sucrose- EDTA buffer

STET buffer: sucrose-Tris-EDTA- TritonX-100

These buffers are often used in procedures of nucleic acid, such as in electrophoresis.

Tris-base:

$(CH_2OH)_3\,CNH_2 \rightleftharpoons 3\,OH + (CH_2)_3\,CNH_2$ ….. pka 8, pH (7-9)

solutions are effective buffers for slightly basic conditions, which keep DNA deprotenated and soluble in water.

EDTA: Ethylene diamine tetra acetic acid ($C_{10}H_{16}N_2O_8$)

is a chelator agent of divalent cations, particularly of Mg^{+2} that act as co-factor for many enzymes including contaminant nucleases, therefore the role of EDTA is protected the nucleic acid from enzymatic degradation.

Mg^{+2} is also acts as a co-factor for many useful DNA-modifying enzymes, such as, restriction enzymes and DNA polymerase.

- The concentration of EDTA in TBE buffer is generally kept low (typically around at 1 mM), **why?**
  This concentration is enough to chelate the divalent cations and inhalation exposure to high concentration of EDTA would produce toxic effect on human and animals.

Methodology:

1) Preparation of TE buffer

TE buffer contains of (10 mM of Tris + 1 mM of EDTA)

- Prepare stock solution of 500 mM of Tris (M.wt= 121) and 500 mM of EDTA (M.wt =372)
- Prepare working solution with (V= 10 ml) from stock solution by dilution of stock solution to get 10 mM of Tris and 1 mM of EDTA.
- Measure pH by pH meter and adjust pH=8.

Solution

1) Stock solution (Tris)

$$M = \frac{Wt}{M.\ wt} \times \frac{1000}{volume}$$

$$0.5 = \frac{Wt}{121} \times \frac{1000}{100} = 6.05\ gm \quad up\ to\ 100ml$$

2) Stock solution (EDTA)

$$M = \frac{Wt}{M.\ wt} \times \frac{1000}{volume}$$

$$0.5 = \frac{Wt}{372} \times \frac{1000}{100} = 18.6\ gm\ up\ to\ 100ml$$

3) Working solution of Tris

C1 V1 = C2 V2

500V1 = 10 x 10 ⟶ 0.2 ml

4) Working solution of EDTA

C1 V1 = C2 V2

500 x V1 = 1 x 10 ⟶ 0.02 ml

To prepare TE buffer add 0.2 ml of **Tris** +0.02 ml of **EDTA) up to 10 ml.** and adjust pH to 8 by pH meter

2) Prepare of STET buffer

STET buffer contains equal volume of [8% sucrose (w/v %) + 10 mM Tris +1 mM EDTA + 3% Triton X-100 (v/v%)]

Preparation of STET:

1) Tris

$$M = \frac{Wt}{M.\ wt} \times \frac{1000}{volume}$$

$$0.01 = \frac{Wt}{121} \times \frac{1000}{100} = 0.121\ gm$$

2) EDTA

$$M = \frac{Wt}{M.\ wt} \times \frac{1000}{volume}$$

$$0.001 = \frac{Wt}{372} \times \frac{1000}{100} = 0.0372\ gm$$

To prepare STET buffer

- Weight 8 g sucrose+0.121 g Tris+ 0.0372 g EDTA
- Add 3 ml of Triton X-100 to the mixture above
- Complete it to 100 ml with D.W
- Measure pH by pH meter and adjust pH=8

Estimation the amount of DNA in *E.coli* by Burton method

1) Grow *E.coli* in nutrient broth and incubate for overnight (18 hrs.) to obtain bacteria in **log phase. Why?**
In log phase, the number of the bacterial cells and the rate of population increase double with each consecutive time period.

2) Take (10 ml) from the growth and inoculate it in (50 ml) of new nutrient broth. This is considered as the **zero time** of growth and then incubated for different periods in **shaker incubator** at 37C. **Why?**
We inoculate a new nutrient broth to support the growth of bacteria while the experiment is on going to estimate the amount of DNA from the culture growth. Furthermore, we used shaker incubator to ensure distribution of all nutrients with bacteria.

3) Take (7.5 ml) of growth and add (2.5 ml) from the **perichloric acid (P.A) 1N. Why?**

(P.A 1N) plays an important role in bacterial cell wall and cell membrane disruption and extensive protein degradation and fragmentation.

4) Put the mixture in **ice bath at 0C for 15 min.** in order to:
a. Reducing the heat that generated from the addition of P.A (1N)
b. Freezing the precipitate cell and DNA after opening the cells

Strong acid generating heat leads to denature the nucleic acid through losing structure of the molecule affecting the estimation of the DNA.

5) Centrifuge for (10 min.) at 3000 rpm, discarded the supernatant and keep the pellet that contain the DNA.

6) Add (2 ml) of **P.A (0.5 N)** to pellet and mix well, put the mixture in **water bath at 70C. Why?**
(P.A 0.5 N) helps in solubilize the DNA and dissociate the DNA from conjugated proteins.

7) Centrifuge for (15 min.) at 3000 rpm, discarded the pellet and keep the supernatant that contain DNA. **Why?**

Centrifugation is carried out to separate the DNA from other components of the cells.

8) Add **Burton reagent (1 volume from mixture + 2 volumes from reagent)**

9) Prepare **Blank** from 2 ml of reagent + 1 ml of P.A (0.5 N). **Why?**

The blank is used to calibrate the colorimeter which means the O.D of analyte of insert will be only taken.

10) Incubate the mixture and the blank for (18 hrs.) at 37C. **Why?**
Incubation period is necessary to allow the component of Burton reagent to dissociate deoxyribose sugar from the DNA contents.

11) Measure the O.D of mixture and blank at (**600 nm) using spectrophotometer**, the colour will change to **greenish blue** and the colour density depends on DNA concentration. **Why?**
The wavelength is adjusted at 600 nm because the optimum wavelength to read sugar deoxyribose is around 600 nm.

Preparation standard curve of DNA:

The aim of this curve is to estimate the amount of DNA, which is extracted from *E.coli* for each hour from the above experiment by measuring the O.D of standard gradient concentration of DNA as the following:

1) Prepare stock solution of DNA with (500 μg/ml) by taking (25 mg) from DNA powder and solve it with (50 ml) of P.A (0.5 N)

   25 mg x 1000 = 25000 μg/ml
   25000 / 50 ml = 500 μg/ml

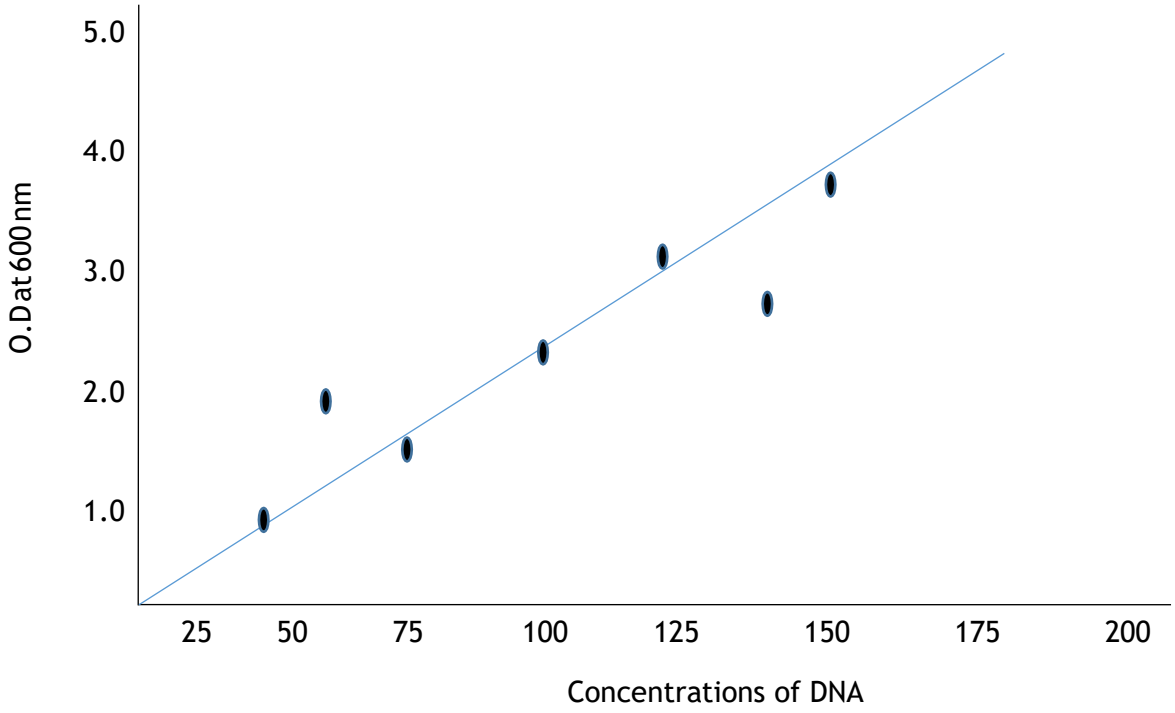
2) Prepare gradient concentration of DNA (25-200 μg/ml) from the original solution, P.A is used to complete the volume to 2 ml.

Concentration	C1V1= C2V2	Take from stock	Complete it with
25	500 x V1= 25 x2	0.1	1.9
50	500 x V1= 50 x2	0.2	1.8
75	500 x V1= 75 x2	0.3	1.7
100	500 x V1= 100 x2	0.4	1.6
125	500 x V1= 125 x2	0.5	1.5
150	500 x V1= 150 x2	0.6	1.4
175	500 x V1= 175 x2	0.7	1.3
200	500 x V1= 200 x2	0.8	1.2

3) Add **4 ml of Burton reagent** to each **2 ml of each final concentration.**
4) Prepare **Blank** as it mentioned above
5) Mix the tubes and put them in **water bath in 100 C for 10 min. <u>Why?</u>**

Incubation period is necessary to allow the components of Burton reagent to dissociate deoxyribose sugar from DNA content.

6) then cool the tubes in order to measure O.D at 600 nm.
7) Draw the curve that show the relationship between O.D and gradient concentration of DNA, then point the values of O.D of test sample in the experiment on the curve to obtain the unknown concentration for each O.D after each hour.



<u>The components of Burton reagent:</u>

1. **Diphenylamine:** react with deoxyribose sugar to produce greenish blue colour
2. **Glycial acetic acid:** breaks down hydrogen bonds between base pairs and glycoside bonds between nitrogenous base and deoxyribose sugar.
3. **H_2SO_4:** breaks down the phosphodiester bonds in the nucleotide among the single strand of DNA
4. **Acetaldehyde:** a co-factor that **increase** the reaction between deoxyribose sugar and reagent; and **fix** the colour of mixture.

The principle of Burton reagent work:

Diphenylamine in the reagent will react with deoxyribose sugar release from broken DNA, which lead to the greenish blue colour.

Methodology:

- **Preparation of Burton reagent:**
  1) Take (1.5 gm) from diphenylamine and solve it in (100 ml) of glycial acetic acid, then (1.5 ml) from concentrated H_2SO_4 is added to mixture.
  2) Store the mixture in the dark (to prevent oxidation by the light).
  3) Added (0.5 ml) acetaldehyde to (100 ml) of the mixture.

- **Preparation of acetaldehyde:**
  Ex.: Prepare (10 ml) of (16 mg/ml) of acetaldehyde, if the original concentration was (0.78 gm/ml).

  C1V1= C2V2
  0.78 gm/ml x 1000 = 780 mg/ml
  780 mg/ml x V1 = 16 mg/ml x10
  V2 = 0.2 ml from stock and completed to 10 ml with D.W

- **Preparation of perichloric acid**
  The percentage of the original concentration is = 70%
  M.wt = 100.64
  Specific gravity = 1.67

$$M = \frac{10 \text{ (percentage con. X S.G}}{\text{M.wt}} \qquad \frac{\text{M= 10 (70 x 1.67)}}{100.46} = 11.6$$

This formula gives the original molarity of the solution, and then the normality can be calculated by:

$$N = \frac{M}{\text{Eq. number}}$$

The chemical formula of perichlorite acid is $HClO_4$, so the equivalent number is 1

$$N = \frac{11.6}{1} = 11.6 \text{ of the orginal solution}$$

$$HClO_4 \rightleftharpoons H + ClO_4$$

Ex.: Prepare (400 ml) of P.A with (0.25 N)?

$N_1 V_1 = N_2 V_2$

$11.6 \times V_1 = 0.25 \times 400$

$V_1 = 8.6$ ml

Take this volume from the original solution and complete it to 400 ml with D.W.

Extraction of DNA molecules

There are many different methods and techniques available for isolation of genomic DNA from prokaryotes (Bacteria) and Eukaryotes (animals & plants).

All methods involve:

disruption and lysis of cell wall and cell membrane, followed by **removal of proteins and other contaminants.**

Two general types of procedure are used **for purification of DNA**:

a) **centrifugation**
b) **chemical extraction.**

The DNA yield and purity are highly variable by using these methods.

Spin is achieved to separate bacterial cells from the media which is carried out at 4 C to increase the amount of precipitation.

First: Opening of different cells:

a) Bacterial cells

Method:

1) Suspend the overnight bacterial cells in (5 ml) of **TE buffer**, mix well by vortex, spin for (6000 rpm for 10 min. at 4 C). vortex is used to homogenize the suspension of bacterial cells with TE buffer.
   - **Tris :** buffer solution, pH=8
   - **EDTA:** chelating agent sequester divalent cations, such as: Mg^{+2} & Ca^{+2}; this stops DNAase enzyme from degrading the DNA.

2) Add (0.2 ml) of **(2.5% SDS)** to lysis Gram negative bacteria, and (10 mg/ml) of **lysozyme + (2.5% SDS)** to lysis Gram positive bacteria; then incubate (30-60 min.) at 37 C
   - **Lysozyme:** is capable of breaking the bonds in the peptidoglycan in the cell wall of bacteria which is thick in Gram positive bacteria comparing with Gram negative bacteria; therefor, lysozyme used to open the Gram positive bacteria.
   - **SDS:** anionic detergent used to soluble the cell membrane to release cell contents.

3) Add (0.6 ml) 0f **(5M Na-perchlorite)** it dissociate DNA from proteins.

b) Cells of animal tissue:

Method:

1) Wash animal tissue with **(40%) formaldehyde**
2) Use blender- glass mortar homogenizer to homogenize the tissue into tiny fragments.
3) Add **(0.25%) trypsin,** incubate (5 min.) at 37C
4) Add **STET buffer**

- **Formaldehyde:** is disinfectant used to kill most of bacteria & fungi
- **Trypsin:** helps in damaging and opening the cell by striping cell surface proteins and kill the cell
- **STET:**
  a) **Sucrose:** it has long been proposed to have osmotic role in animal cells
  b) **TE buffer pH= 8**
  c) **Triton x-100:** non ionic detergent (used for lysis cell membrane).

c) Cells of plants tissue:

Method:

1) Plant tissue must be ground into tiny fragments by used **liquid-N_2** with **metal homogenizer.**
2) Add extraction solution **(CTAB)** (cetyl- trimethyl ammonium bromide)

- **Liquid N_2**: helps in making ice crystals that play a role in scratching and opening the cell.
- **CTAB:** as **a cationic detergent makes complex with polysaccharides.** facilitates the separation of polysaccharides from DNA during purification process therefore it used in plant DNA extraction because of high polysaccharides content in plant tissues.

Seconed: Isolation of DNA from other componants:

Method:

1) Add **[phenol/ chloroform/ isoamylalcohol (25/24/1)]**
   It is an extraction solution that used to remove proteins and other contaminants from nucleic acid sample.
   - Nucleic acids are remained in **the aqueous phase** and
   - Proteins are separated in the **inter phase by phenol**
   - Most of lipids & polysaccharides are separated in the **lower organic phase by chloroform.**
   - **isoamylalcohol** acts as an anti-foam

2) Centrifuge the mixture in (10000 rpm for 10 min. at 4 C). three layers are separated; the first aqueous layer contains DNA.
   Centrifugation at 4 C is **to prevent** the generation of heat through centrifugation that lead to denature the DNA and **to increase** the precipitation.

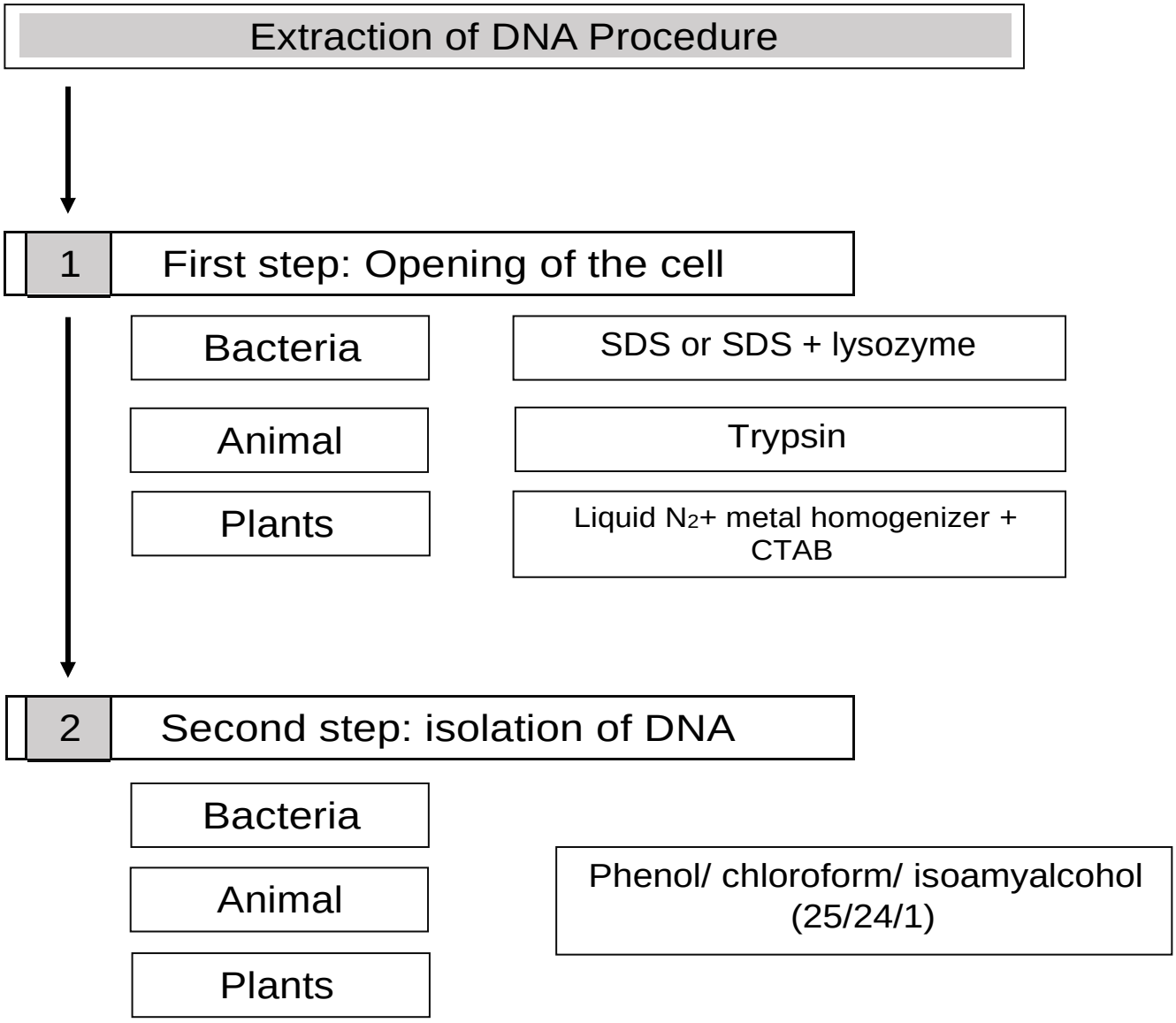
3) Transfer the upper layer to another **plastic tube (**because the DNA has ability to bind to glass tube therefore plastic tube is used**)**, then add twice volume of **cold absolute ethanol**, which makes dehydration and pull out the water molecule from the DNA hence precipitate the DNA.

4) Mix by inversion; after 3 min., DNA will precipitate like spool DNA on a glass rod.

5) Dissolve the DNA in **(50-100 μl)** of **TE buffer.**

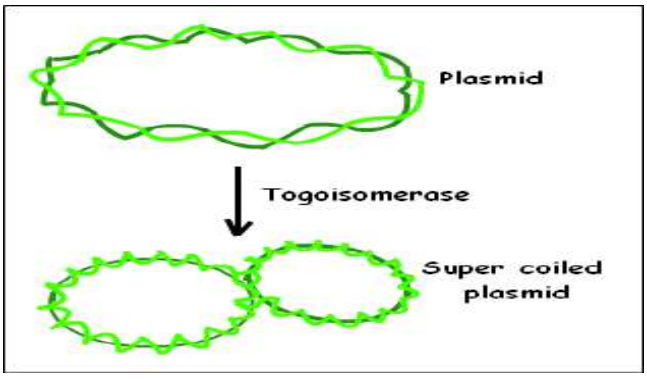
NOTE:

Wear plastic gloves to prevent DNAase enzyme on your hands from cutting DNA into small fragments.


Extraction of DNA Procedure
1
First step: Opening of the cell
Bacteria
SDS or SDS + lysozyme
Animal
Trypsin
Plants
Liquid N2+ metal homogenizer + CTAB
2
Second step: isolation of DNA
Bacteria
Animal
Plants
Phenol/ chloroform/ isoamyalcohol (25/24/1)

Plasmid DNA extraction

A plasmid is a super coiled (covalently closed circular CCC) extra-chromosomal DNA molecule, it is capable of replicating independently from chromosomal DNA.



❖ There are many methods are used for plasmid DNA isolation, such as:

a) Alkaline lysis method
b) Boiling lysis method
c) Phenol method. Some method as phenol-chloroform extraction (DNA extraction)
d) Ethidium bromide- Caesium chloride density gradient centrifugation method
e) Mini- column

Alkaline lysis protocol for plasmid extraction:

- **Harvesting**

1) Inoculate *E. coli* (RRI) carried PBR 322 plasmid into 2 ml of LB medium **(it is used because most plasmid purification are optimized with culture grown in LB (Luria Bertani medium))**, containing the appropriate **ampicillin 100 mg/ml (ampicillin is added to kill the bacteria that do not contain PBR322 plasmid carrying ampicillin resistance gene)** in 15 ml tube volume.
2) Incubate the culture **overnight at 37C in shaking incubator (It is used to make homogenization and to ensure all the bacterial cell will display equally to ampicillin antibiotics)** .
3) Pour 1.5 ml of the culture into a microfuge tube. **Centrifuge for 30 sec. at 12,000 rpm** in a **microfuge at 4 C (centrifugation is carried out to separate the bacterial cells from the media, and carried out at 4C to increase the amount of precipitation)**.
4) Remove the medium, leaving the bacterial pellet as dry as possible.

- **Lysis by alkali:**

1) Resuspend the bacterial pellet (obtained from step 3 above) in **100 µl of ice- cold solution I** by vigorous vortexing.

Solution I	Role of action
50 mM glucose	It is propose to have osmotic role in bacterial cells so the cell don not burst
25 mM Tris –Hcl	Works a buffer
10 mM EDTA	Works as chelating agent for divalent cation

Solution I can be prepared in batches of approximately 100 ml, adjust the pH to 8 with HCl then autoclave for 15 min. at 10 1b/in 2 hr. and store at 4C.

2) Add 200 µl of freshly prepared **solution II**, close the tube tightly and mix the contents by inverting the tube gently **(Do not vortex the samples because genomic DNA is coprecipitated by DNA -bound protein, If you vortex the genomic DNA is sheared and proteins are detached from the genomic DNA which will lead to contamination).** and store the tube on ice.

Solution II	Role of action
0.2 N NaOH (freshly diluted from a 1 N stock)	Helps to break down the cell wall but the most important it disturbs the hydrogen bonding of the DNA in process called denaturation.
1% SDS	Solubilize the cell membrane and also denatures most of the protein in the cell which helps with separation of protein from plasmid

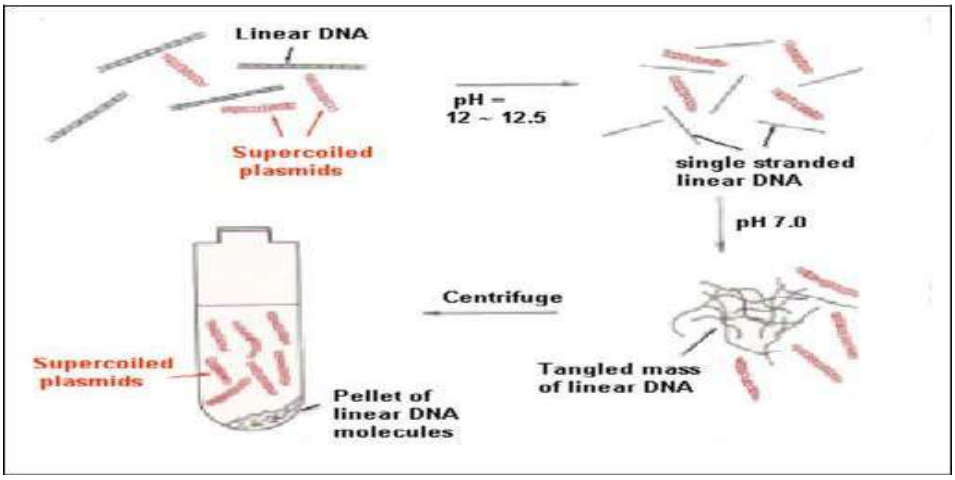
3) Add 150 µl of **ice – cold solution III.** Close the tube and mix gently by inversion (6 times), then disperse solution III through the viscous bacterial lysate and store the tube on ice for 3-5 min.

Solution III	
5 M potassium acetate	60 ml
Glycial acetic acid	11.5 ml
H_2O	28.5 ml

- **5 M potassium acetate**

  Decrease the alkalinity of the mixture, hydrogen bond re-established. **This is selective part,** while it is easy for the small circular plasmid DNA to re- nature, it is impossible to re-nature or anneal huge DNA genome stretches. While the plasmid can dissolve in solution.

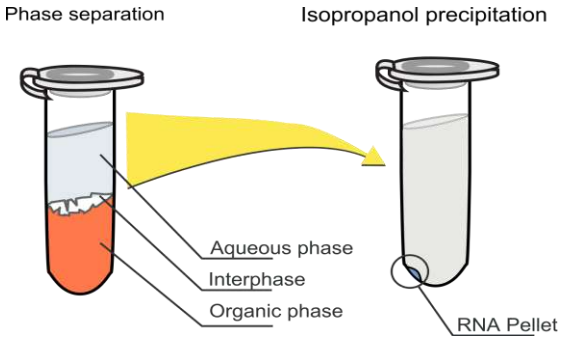
  ssDNA of genome, SDS, denatured protein stick together through hydrophobic interaction to form white precipitate, then the precipitate can easily be separated from the plasmid DNA solution by centrifugation.



4) Centrifuge milky suspension (emulsion) at 12000 rpm for 5 min. in a microfuge Eppendorf tube. Transfer **the supernatant** to a new tube.

5) Add **450 µl of phenol : chloroform (ratio 25/25)**. Mix by vortexing, after centrifugation at 12000 rpm for 2 min. in a microcentrifuge, transfer the aqueous phase to a new tube.

   - Now your plasmid is mixed with salt, EDTA, some residual cellular proteins are debris therefore **it is necessary to use phenol- chloroform extraction to clean your plasmid**



6) Add **(0.7 volume) of ice- cold isopropanol 100%** to supernatant, mix by vortexing, centrifuge at 12000 rpm for 20 min. at 4 C.

- **Isopropanol** is used to neutralize the charge on the nucleic acid backbone, so DNA become less hydrophilic and precipitate out of solution.
- **Ice** to chill the sample, lower temperature promote the flocculation of the nucleic acid so they form large complexes.

7) Rinse the pellet of double strand DNA (super coiled) with **1 ml of 70% ethanol**. Remove the supernatant and allow the pellet of nucleic acid to dry in the air for 10 min.
   - DNA is less soluble in isopropanol so it precipitate faster even at low concentration therefore we used in plasmid extraction but the short come or disadvantage of this salt is that it will precipitate with DNA while ethanol stay soluble, therefore, we need to wash the pellet with 70% ethanol to remove isopropanol salt.

8) Re-suspend the nucleic acids in **50 µl of TE (pH 8)** containing DNAse- free RNAse solution (20 µg/ml). vortex briefly and store the DNA at 4 C.

9) Test the size of plasmid and finding protocol using gel electrophoresis technique.

Extraction of RNA

Main types of RNA are:

1) **mRNA:** transfer the genetic information from DNA to protein synthesis place (ribosome) in cytoplasm.
2) **tRNA:** found in cytoplasm, carrying amino acids for arranging them on the strand of mRNA. There are more than 20 kinds of tRNA in each cell.
3) **rRNA:** it calls ribosomal RNA, because it builds the ribosomal structure with nucleoproteins, have a role in protein synthesis in the cytoplasm.

To isolate RNA molecules, we have to follow the steps below:

A) Lysis the cell

- Treat the suspension of *E. coli* (0.5 O.D at 600 nm) with equal volume of **Diethyl pyrocarbonate (DEPC)**, which work as **inhibiter to RNAse (exogenous RNAse).** Homogenized and incubate at 4 C.

  - **DEPC is unstable in water and hydrolyze to CO_2 & ethanol; therefore, it is not used with Tris buffer but it can be used with phosphate buffer; it makes denaturation through covalent modification of histidine, lysine, cystine and tyrosine.**
  - **RNA is unstable molecule compared with DNA, therefore, it should be incubated with 4 C.**

- Add **(0.4 ml) of 5% SDS** to the cell suspension

  - (**SDS acts on lipid of membrane that allowed access of lysozyme to peptidoglycan, also SDS inhibits RNAse).**

- Use (**2ml**) of **lysozyme (400 μg/ml)** containing buffer per (10 ml) of *E. coli*, homogenized and incubated at room temperature (37C) for 5-20 min.

B) Purification of RNA

- Centrifuge the suspension of lysis cells at 3000 rpm for 10 min.

- Suspend the pellet with equal volume of **guanidinium thiocyanate** which acts as **strong inhibitors of RNAse** and **denaturation of protein.** The pH should not be alkaline due to liability of RNA.

- **Guanidinium thiocyanate is used in RNAase enzyme and protein denaturation through chaotropic agent.**
  Chaotropic means the agents disrupt the hydrogen bonding network between water molecules and protein or RNAase enzyme and this has effect on nctive state of molecule by weaking hydrophobic effect cause denaturation.

❖ DNA can be removed by using **DNAse I** to minimize genomic DNA contamination.

❖ The contaminant proteins are removed by adding a mixture of **phenol: chloroform: isoamyl alcohol (25:24:1)**, and repeat this step many times.

❖ Transfer the upper layer to another tube and add **1 volume of ice cold absolute isopropanol** to precipitate RNA.

❖ Centrifuge 1000 rpm for 20 min. at 4 C

❖ Discard supernatant and wash pellet with **70% ethanol**

❖ Centrifuge 1000 rpm for 5 min. at 4 C

❖ Re-suspend the RNA in **100 µl of TE buffer** and frozen

Calculate the Concentration of RNA:

Concentration of RNA (µg/ml) = O.D 260 nm x 40 x dilution factor

- 1 O.D 260 nm = 40 µg/ml of RNA

Calculate the Purity of RNA:

O.D 260 nm/ O.D 280 nm = should be 2

Spontaneous mutations

The mutation is divided in general into two main categories:

1) **Spontaneous mutation**
2) **Induced mutation**

Spontaneous mutation:

It is an auto mutation which occurs in the nature without participating the human and it occurs because of either physical or chemical reasons.

a) Physical reasons:

It represents by displaying to different types of rays or change in temperature, pH, pressure or other physical effects.

b) Chemical reasons:

It represents by displaying to the products of industry, like pesticide, stream, smock, food preservations…. etc.

Gradient plate method for isolation the spontaneous mutation:

This method is considered as simple and easy for primary detection of spontaneous mutation.

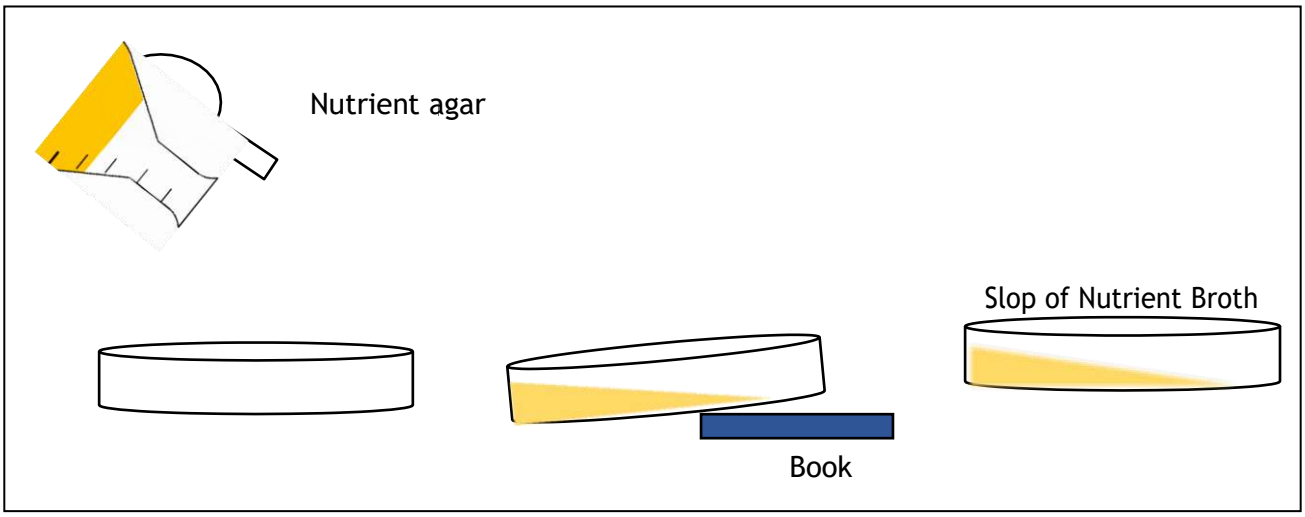
- **Procedure:**

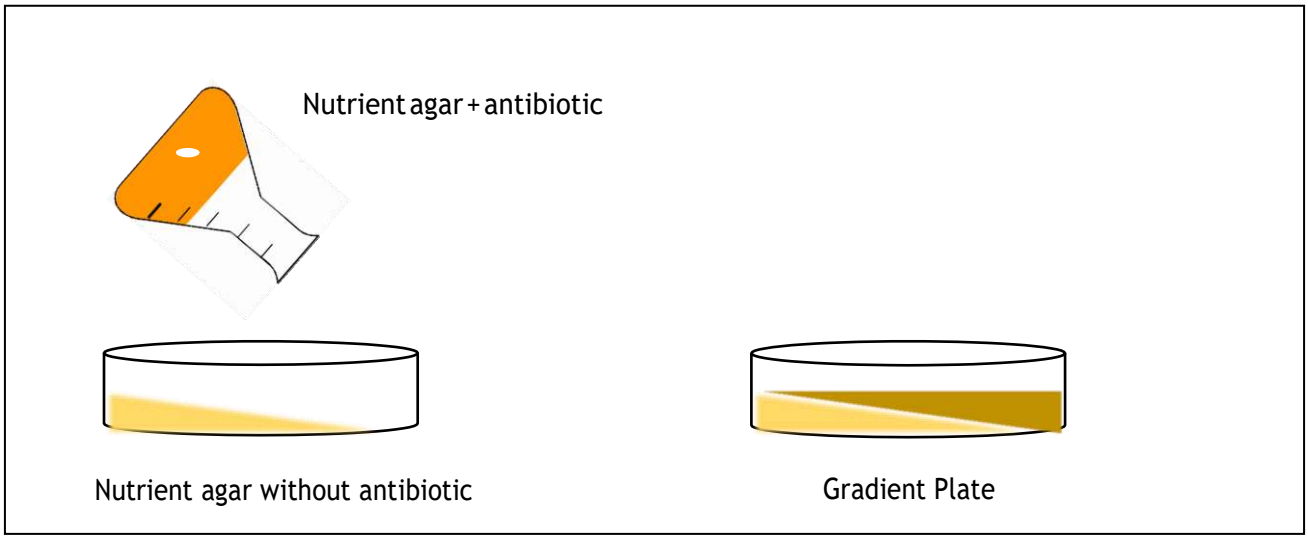
1) Prepare nutrient agar, sterile it and pour it into sterile plates as average 15 ml in each plate (half the plate), after that, the plates are slopped by using any book edge, leave it till it gets solidified.

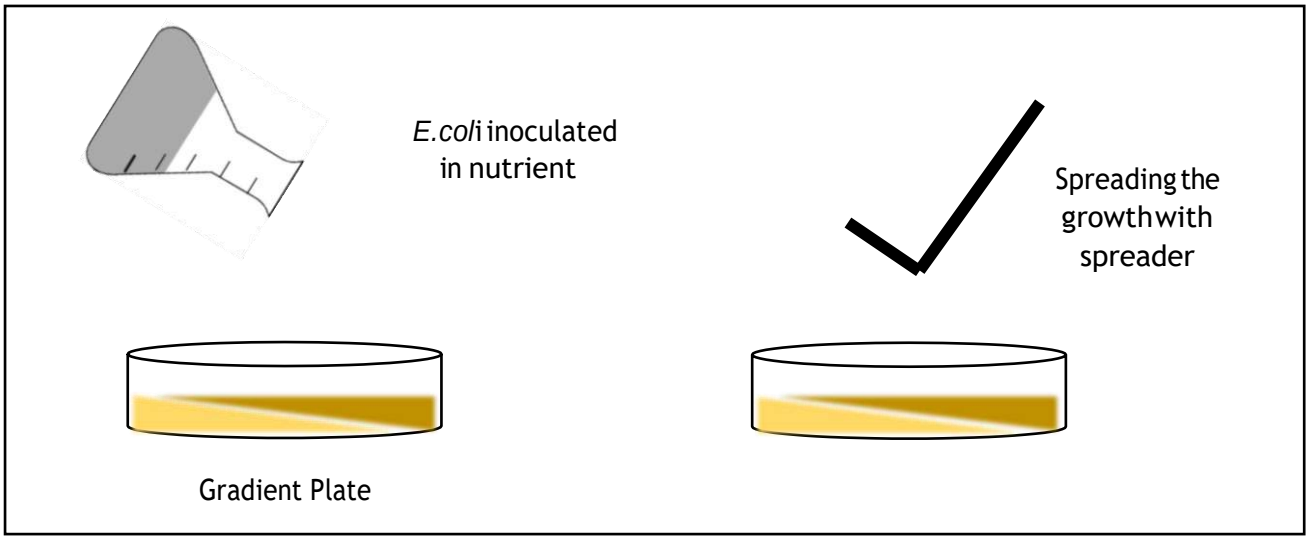
2) Prepare another nutrient agar, after sterilization cool it to 45 C. Add the chosen antibiotic for at concentration of 500 µg/ml to the media and move the flask in a circle way to mix the antibiotic properly with the media. (foam should be avoided).

3) The (nutrient agar + antibiotic) is poured over the previous solidified slopped nutrient agar until the plate is full and the media reaches to a straight line.

4) After the plates are solidified, inoculate the plates with *E. coli* by taking 0.2 ml from the original growth and spread it by using a spreader over the whole surface of the media, then incubate the plates for 24 hrs. at 37 C.







- **Discussion the results:**
  1) **If there is no growth**; this means that the bacteria do not have the ability to resist the antibiotic in this concentration.

  2) **If there is full growth**; this means that bacteria have the ability to resist the antibiotic**.**

  3) **If the growth appears only in the region of low concentration of antibiotic in the plate**, this means that the bacteria can only resist the low concentration of this antibiotic.

- In this case loop is used to touch single colony and move it towards the high concentration region of antibiotics.
- Incubate the plate for 24 hrs., then if there is any growth, the colony is transferred again to the high concentration region of the palate; then watch the results:

A) If there is no further growth, this mutation is called **(single mutation)**

Single Step Mutation: Is a mutation in which the bacteria can resist limited concentration of the antibiotic but they can not be grown on higher concentration.

B) If there is any further growth, this mutation is called **(Multi Step Mutation)**

Multi Step mutation: Is a mutation in which the bacteria can be grown in a higher concentration than the original concentrations in a gradient way.

What are the main benefits of gradient methods?

1) The possibility to identify the approximate concentration for bacterial resistant
2) The possibility to test more than one type of bacteria in the same plate and for the same antibiotic, by growing the bacteria in parallel manner to insure that all types of bacteria will be exposed to the same concentration of antibiotic.
3) We can identify the types of antibiotics and bacterial resistant and types of mutation whether is it a single or multi step mutation.

What are the disadvantages of gradient methods?

1) There is no possibility to get fix and exact mutation, it is always an approximate manner, as an example: the mutation will grow until the half of the plate, we can say it resists half the concentration of the used antibiotic.
2) There is no possibility to account the mutation frequency in this method.

The method of antibiotics solution preparation:

A stock solution of antibiotic should be prepared before adding it to the medium; important notes must be concerned during preparation stock solution:

a) Types of antibiotics; whether it is capsule or an ampule, etc…
b) The final concentration of antibiotics
c) The volume of the used media
d) The amount of antibiotics solution that will be added to the medium

The reason of adding 0.6 ml of antibiotic solution to 50 ml of media not more, to prevent a gained dilution, which delay the solidity of the media

Types of antibiotic:

a) Capsule:

Ex. (1):

Prepare 50 ml nutrient medium that contains **Erythromycin 250 mg** with final concentration 500 µg/ml

250 mg ⟶ 250,000 µg
250,000 /5 = 50000 µg/ml

C1V1= C2V2
50,000 x V1 = 500 x 50
V1 = 0.5 ml
Take this volume from the stock solution and add it to 50 ml of the medium

Ex. (2):

Prepare 50 ml nutrient agar that contains **Ampicillin 150 mg** with final concentration is 400 µg/ml

150 mg ⟶ 150,000 µg
150,000 /5 = 30,000 µg/ml

C1V1= C2V2
30,000 x V1= 400 x 50
V1= 0.66 ml

We can not add this volume because it is more than 0.6 ml, so….

150,000/ 3= 50, 000 µg/ml
C1V1= C2V2
50,000 x V1= 400 x 50
V1= 0.4 ml
Take this volume from the stock solution and add it to 50 ml of the medium

b) Ampoule contain liquid antibiotic

Ex. (3):

Prepare 50 ml nutrient medium that contains **Gentamycin 300 mg/ 3ml** with final concentration 600 µg/ml

300 mg/3 ml ⟶ 100 mg/ml

100 mg/ml ⟶ 100, 000 µg/ml

C1V1= C2V2

100,000 x V1 = 600 x 50

V1= 0.3 ml

Take this volume from the stock solution and add it to 50 ml of the medium

c) Ampoule contain powder antibiotic

Ex. (4):

Prepare 50 ml nutrient medium that contain **Ampicillin 400, 000 IU** with final concentration is 500 µg/ml.

400, 000 IU = 400, 000 µg

400, 000 µg /5 ml ⟶ 80, 000 $\frac{\&^{\bullet}}{\mathrm{O}}$

C1V1 = C2V2

80, 000 x V1= 500 x50

V1= 0.3 ml

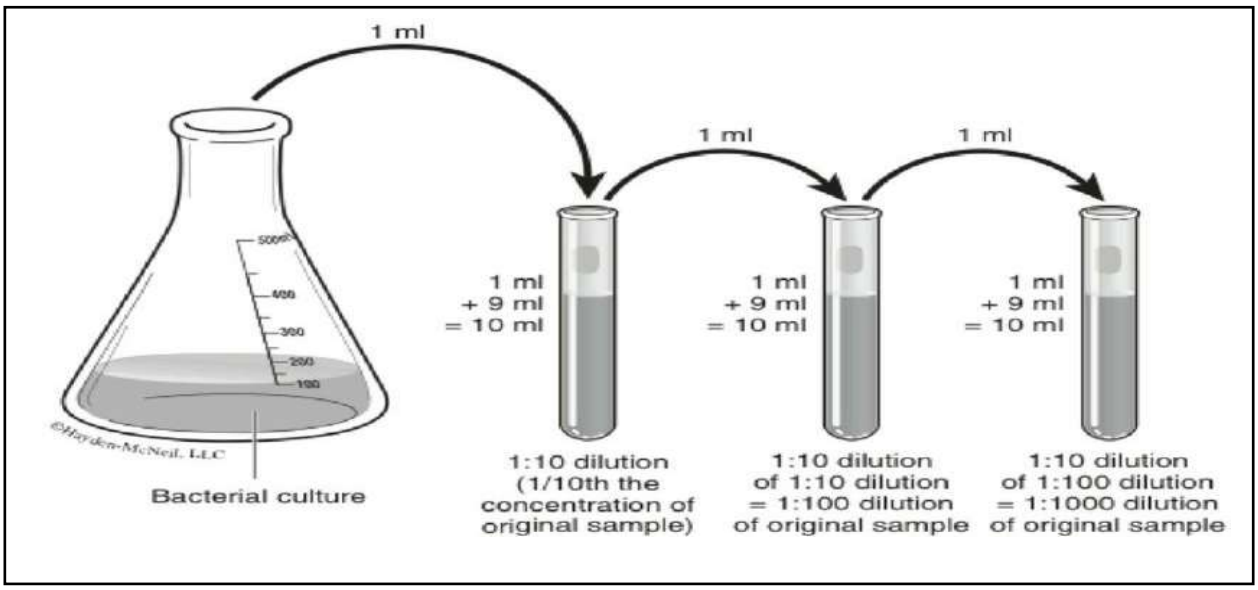
Take this volume from the stock solution and add it to 50 ml of medium

Optimum method for isolation of spontaneous mutation

The method includes that the antibiotics is tested in different concentration and choose the appropriate concentration according to the result of the previous experiment.

Procedure:

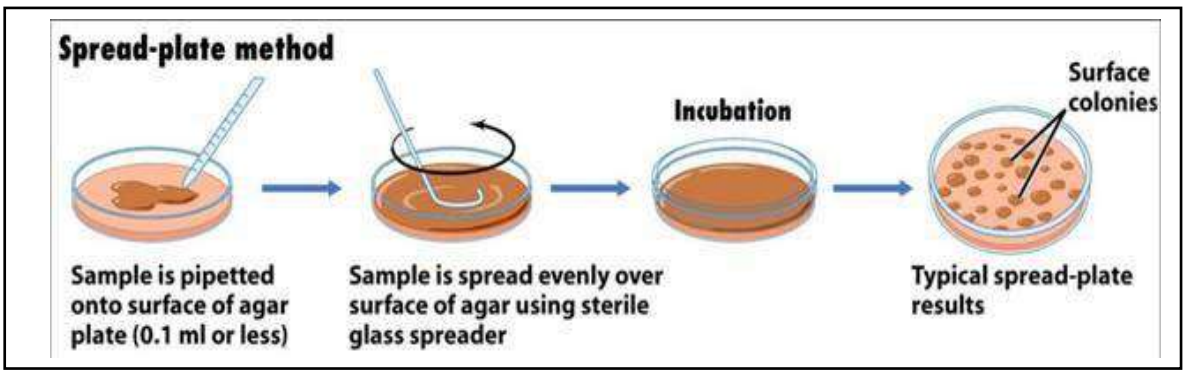
1) The viable count of bacteria is done by making serial dilution (10^{-1}_ 10^{-10}), 1 ml of *E. coli* growth is taken (which has been grown for 18 hours) from first tube and is added to 9 ml of D.W to reach final dilution 10^{-1}, then we will continue in making serial dilution by taking 1 ml from second tube and 9 ml of D.W is added till reach to the final concentration 10^{-10}.



2) 0.1 ml of *E. coli*) which is taken from the last five dilution) is inoculated by spreading method, on different plates of nutrient agar without adding the antibiotic; then the plates are incubated at 37C for 24 hrs. then the viable count is done by using this formula.

The viable count = No. of the colonies x the reverse of dilutions x 10

The formula included that multiplying the equation by 10 to get the result of colonies in 1 ml because the experiment was done in 0.1 ml.



NOTE:

The no. of colonies between 50 -200 is calculated in the consideration of counting the viable count of bacteria to get the most approximate results.

Example:

Calculate the viable count of bacteria, if the no. colonies in the plates is shown in the table below?

The viable count = no. of colonies x reverse dilution x 10

$190 \times 10^8 \times 10 = 190 \times 10^9$ cell/ml

19×10^{10} cell/ ml

No. of colonies	The dilution
1050	10^{-5}
765	10^{-6}
430	10^{-7}
• 190	10^{-8}
50	10^{-9}

3) prepare the plates which contain antibiotic with concentration depend on the previous experiment, thereby each concentration is added to single plate, then the plates are inoculated with 0.2 ml of *E. coli* incubated for 24 hrs. at 37 C then the no. of mutation is calculated by using the formula:

No. of mutation = average of mutation x 5

the formula multiply by 5 because, 0.2 ml was inoculated and the calculations are done in 1 ml.

the average of mutation = equal to the no. of colonies in the plates divided by number of plates for each concentration.

Con. µg/ml	No. of colonies	The average
100	4.6	5
75	8.10	9
50	10.14	11
25	12.18	13

4) the no. of mutation for previous example is:
   a) the no. of mutation = 5 x5 = 25 mutations for 190 x 10^9 cell/ml viable count for 100 µg/ml concentration

   b) the no. of mutation = 9x 5 = 45 mutations for 190 x 10^9 cell/ml viable count for 75 µg/ml concentration

   c) the no. of mutation = 12 x5 = 60 mutations for 190 x 10^9 cell/ml viable count for 50 µg/ml concentration

   d) the no. of mutation = 15 x5 = 75 mutations for 190 x 10^9 cell/ml viable count for 25 µg/ml concentration

Mutation frequency:

The main goal of the experiment is to know in which type of antibiotic and concentration is going to give **(high mutation frequency)**

Example:

Which of the two experiment have high mutation frequency?

• first experiment: one mutation happened in 5 cells

• second experiment: one mutation happened in 10 cells

definitely, mutation frequency in first experiment is higher than second experiment; that means, there is opposite relationship between the viable count of cells and mutation frequency.

To account the **mutation frequency** in the previous example, use the following formula:

$$\frac{\dfrac{\text{No. of mutation}}{\text{No. of mutation}} \text{ For viable count}}{\text{No. of mutation}} = \mu g/ml$$

1) 25/25 mutation for 190×10^9 /25
   1 mutation for 7.6×10^9 viable count and 100 µg/ml

2) 45/45 mutation for 190×10^9 /45
   1 mutation for 4.2×10^9 viable count and 75 µg/ml

3) 60/60 mutation for 190×10^9 /60
   1 mutation for 3.1×10^9 viable count and 50 µg/ml

4) 75/75 mutation for 190×10^9 /75
   1 mutation for 2.5×10^9 viable count and 25 µg/ml

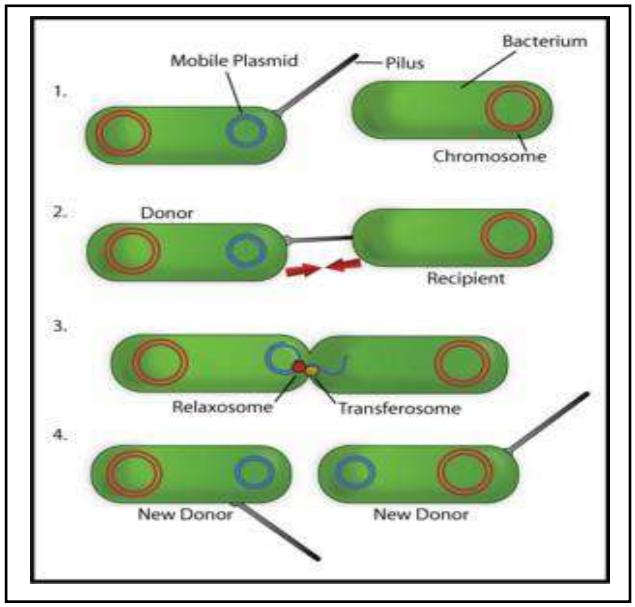
so the high mutation frequency is the con. of 25 µg/ml because it gives one mutation in lowest viable count comparing with the other concentrations.

Bacterial conjugation

Bacterial conjugation is the transfer of genetic material between two bacterial cells by direct cell-to- cell contact or by a bridge – like connection between two cells; this take place through a **pilus**

Bacterial conjugation is often regarded as the bacterial equivalent of sexual production or mating since it involves the exchange of genetic material; However, it is not a sexual reproduction, since no change of gamete occurs and indeed no generation of new organisms.

The genetic information transferred is often beneficial to the recipient, which may include: antibiotic resistance or the ability to use new metabolites.



Mechanism:

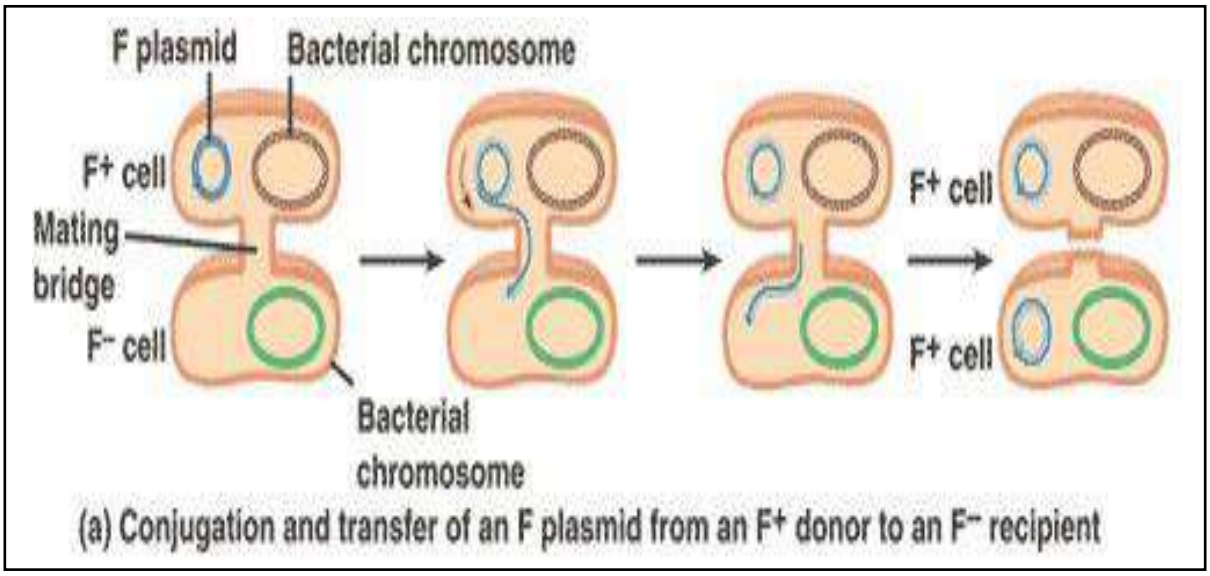
1) Donor cell produce pilus
2) Pilus attaches to recipient cell and brings the two cells together
3) The mobile plasmid (F- plasmid) is nicked and single strand of DAN is transferred to the recipient cell
4) Both cells synthesize a complementary strand to produce a double strand circular plasmid and also reproduce pili
5) Both cells are now viable donor for the F-plasmid

F-factor:

The prototypical conjugation plasmid is the F-plasmid or F-factor. It is can be only one copy of the F-plasmid in a given bacterium, either free or integrated.

Bacterial cells possess a copy that called F- positive or F- donor. Cells that lack F plasmid are called F-negative or recipient cells.

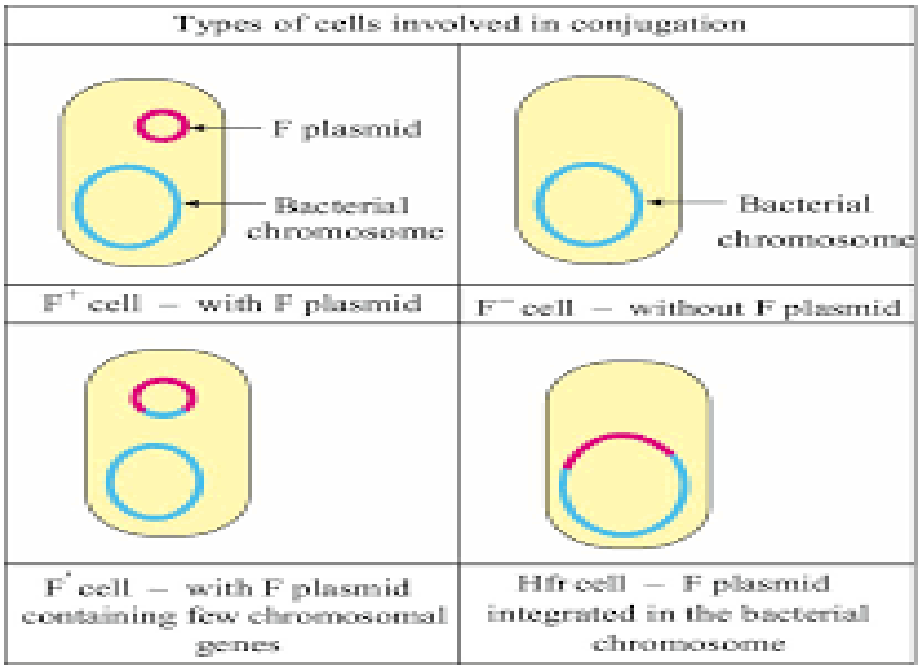
It carries its own origin of replication ori T and consist of about 40 genes. The tra locus includes pilin gene and regulatory genes which together form pili on the cell surface.

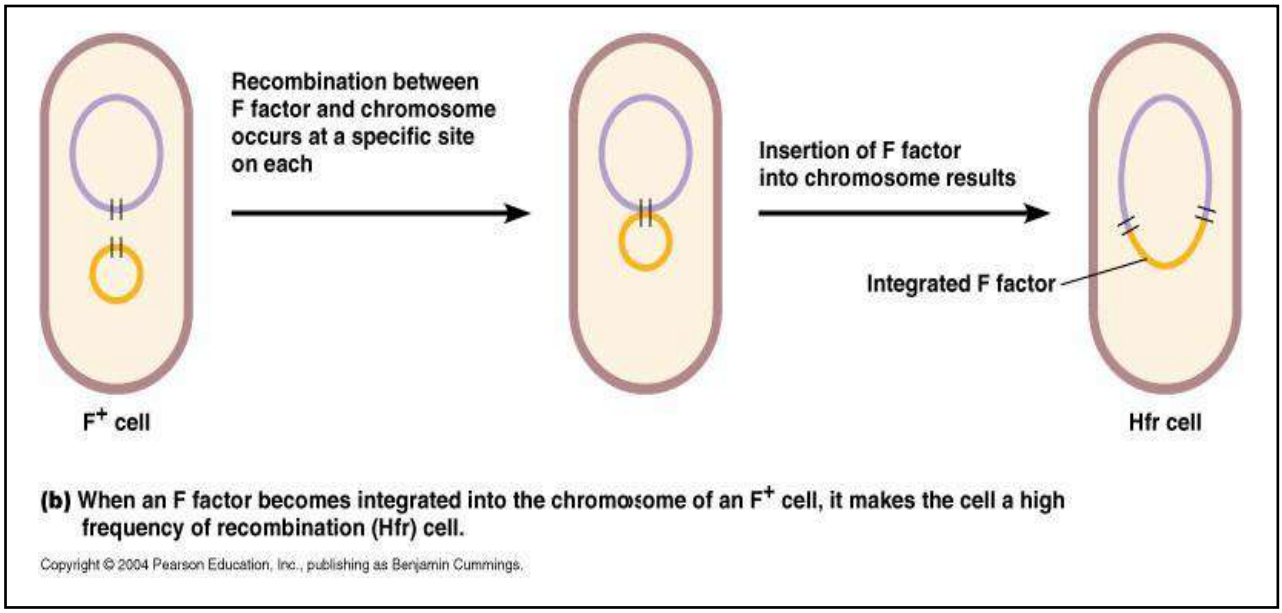


F+ + F- → F+ & F+

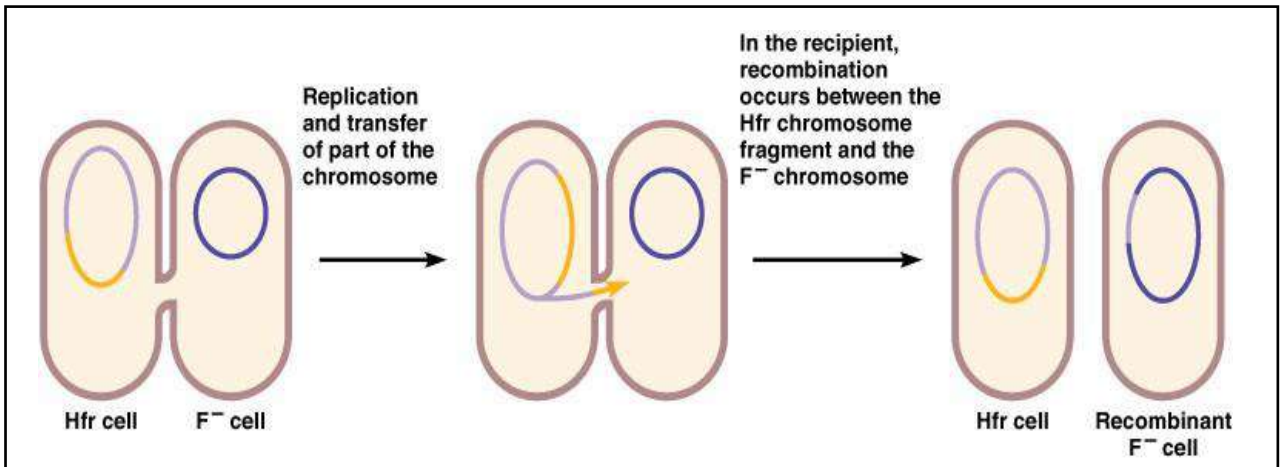
Hfr (high frequency of recombinant:

If the F-plasmid that is transferred has previously been integrated into the donor`s genome some of the donor`s chromosomal DNA may also be transferred with the plasmid DNA, because such strains transfer chromosomal genes very efficiently they are called Hfr (high frequency of recombinant).



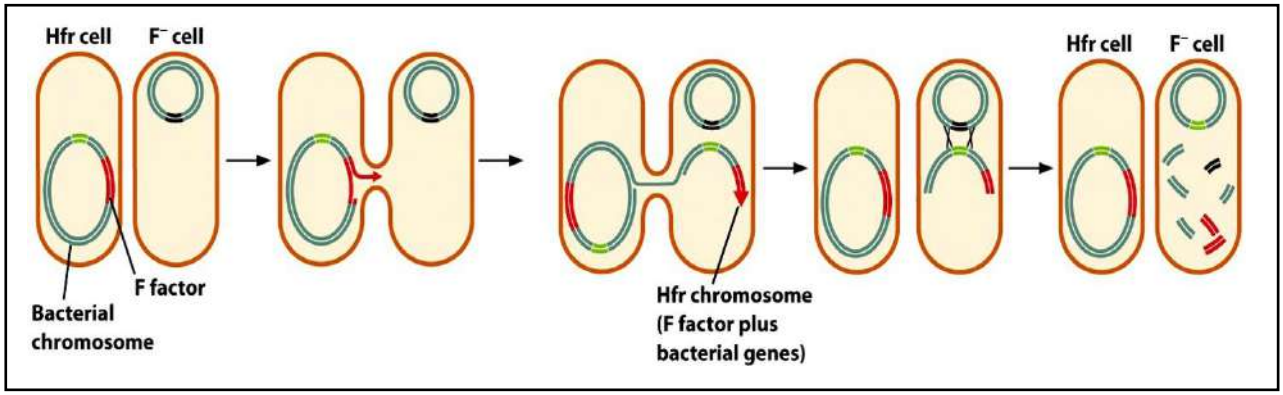


(b) When an F factor becomes integrated into the chromosome of an F^+ cell, it makes the cell a high frequency of recombination (Hfr) cell.



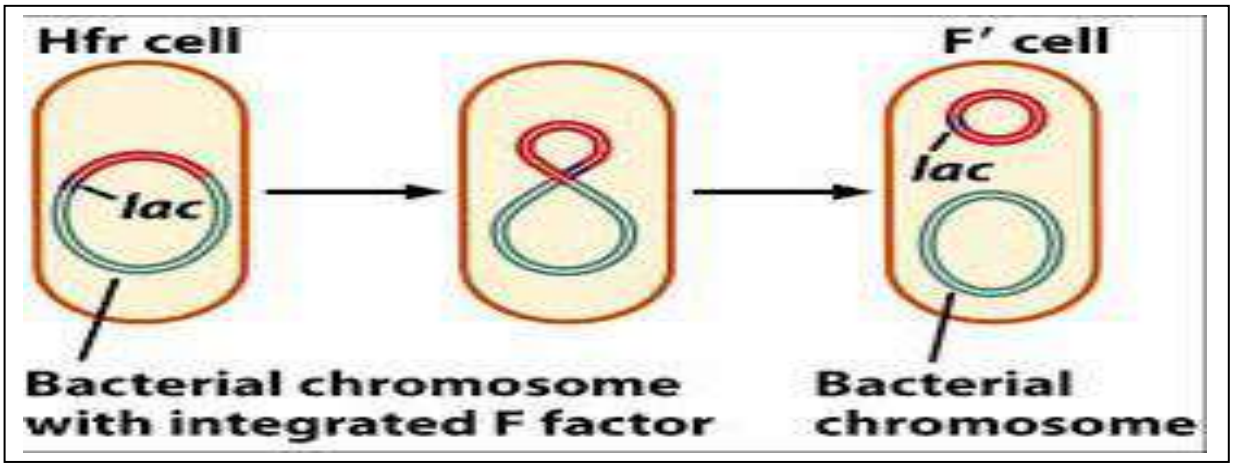
(c) When an Hfr donor passes a portion of its chromosome into an F^- recipient, a recombinant F^- cell results.

Hfr + F- ⟶ Hfr & F-



F′ (F-prime):

Hfr cells, sometimes reverse to F+ by releasing F factor from chromosomal DNA with small fragment of DNA.



F prime + F- ⟶ F + & F+

Different situations of mating among bacterial cells:

- $F^+ + F^-$ ⟶ $F^+ + F^+$
- $Hfr + F^-$ ⟶ $Hfr + F^-$
- $F_{prime} + F^-$ ⟶ $F_{prime} + F_{prime}$

⟹ **Very important**

Why the mating between Hfr & F^- does not produce F^+ cell?

For the F^- to transfer to the F^+ by the mating with Hfr, the whole chromosome with integrated F plasmid of donor cell should be transferred into recipient cell to transfer to F^+ and this does not occur in nature because the time of mating is not enough for transferring whole integrated chromosome of the donor cell.

Lederberg experiment:

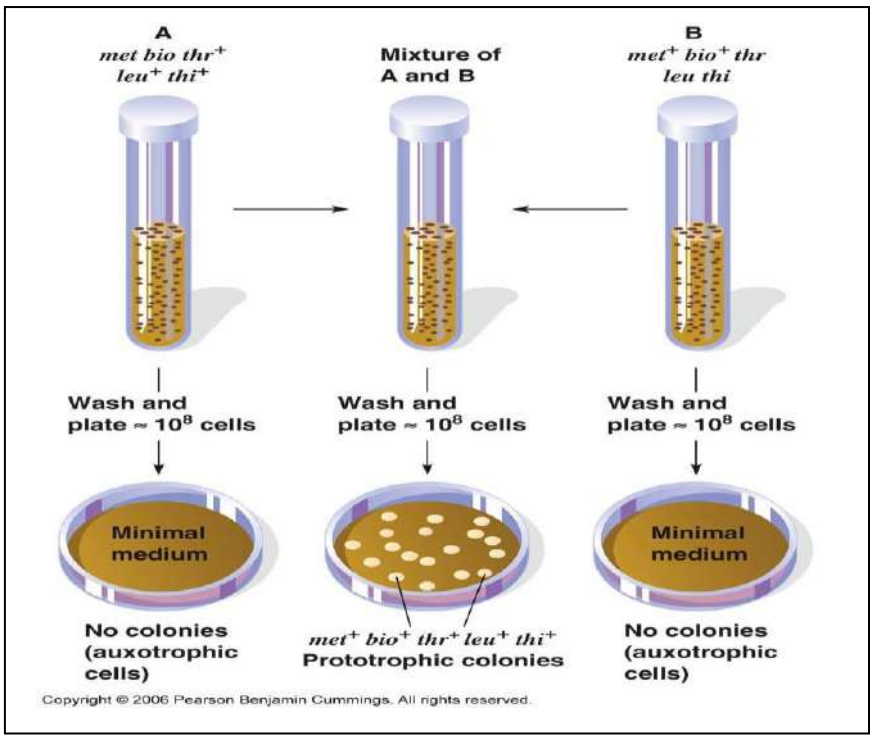
This experiment was designed to demonstrate conjugation as follows:

- Start with 3 cultures of bacteria
  - Strain A (*thr- leu-*) alone
  - Strain B (*bio- mef thi)* alone
  - A mixture of strain A & strain B
- Grow all 3 cultures in minimal media supplement with the 5 nutrients (therionine, leucine, biotin, methionine & thiamine)
- Collect the cells, wash them in minimal media and plate them on minimal media plates without any supplements.

The results were as follow:

- **Plate 1 :** strain A alone --- no colonies were observed
- **Plate 2 :** strain B alone --- no colonies were observed
- **Plate 3 :** mixture of strain A and strain B --- 1 colony/ 10^7 cells plated was observed

The colonies were observed in the mixture because the conjugation occurred between strain A & strain B. Thereby the mixture strain carrying all the gene thr, leu, bio, mef, and were able to growth



Procedure:

1) **Strain A:** *E. coli* strain (**sensitive to ampicillin and resistance to amoxicillin**) was inoculated for 24 hrs. at 37C
2) **Strain** B**:** *E. coli* strain (**resistance to ampicillin and sensitive to amoxicillin**) was inoculated for 24 hrs. at 37C
3) Mix gently (0.1 ml from strain A and 0.1 ml of strain B)
4) Ampicillin and amoxicillin nutrient agar plate (with 100 Mg/ml for each antibiotics) is prepared by using previous calculation for preparation of stock solution of antibiotics
5) Ampicillin and amoxicillin nutrient agar plate (with 100 Mg/ml for each antibiotics) is inoculated with 0.2 ml of mixture (strain A & B) and then is inoculated for 24 hrs. at 37C
6) Check the results, **if there is growth means that conjugation is happened between strain A &B because strain A alone or strain B alone can not resist both ampicillin and amoxicillin with 100 Mg/ml)**

Preparation of stock solution of antibiotics for conjugation experiment:

Q: **In conjugation experiment methodology, prepare 50 ml of media from (ampicillin with 250 mg and gentamycin ampoule 300 mg/3ml) with final concentration 500 μg/ml?**

For ampicillin stock

250 * 1000 = 250, 000 μg

250,000 / 2 = 125, 000 μg/ml

C1 * V1 = C2 * V2

V1 = 500 * 50 / 125,000 = 0.2 ml take it and complete it to 50 ml media

For gentamycin stock

300 mg/ 3 ml ⟶ 100 mg/ml

100 * 1000 = 100, 000 μg/ml

C1 * V1 = C2 * V2

V1 = 500 * 50 / 100,000 = 0.25 ml take it and complete it to 50 ml media

Note: the sum of V1 of ampicillin and V1 of gentamycin stocks should be not more then 0.6 ml to 50 ml of media

Q: **In conjugation experiment methodology, prepare 40 ml of media from (amoxicillin with 500 mg and ampicillin ampoule with 300, 000 IU) with final concentration 500 μg/ml?**

For amoxicillin stock

500 * 1000 = 500, 000 μg

500,000 / 5 = 100, 000 μg/ml

C1 * V1 = C2 * V2

V1 = 600* 40 / 100,000 = 0.24 ml take it and complete it to 40 ml media

For ampicillin ampoule stock

300, 000 IU ⟶ 300, 000 μg

300,000 /2 = 150, 000 μg/ml

C1 * V1 = C2 * V2

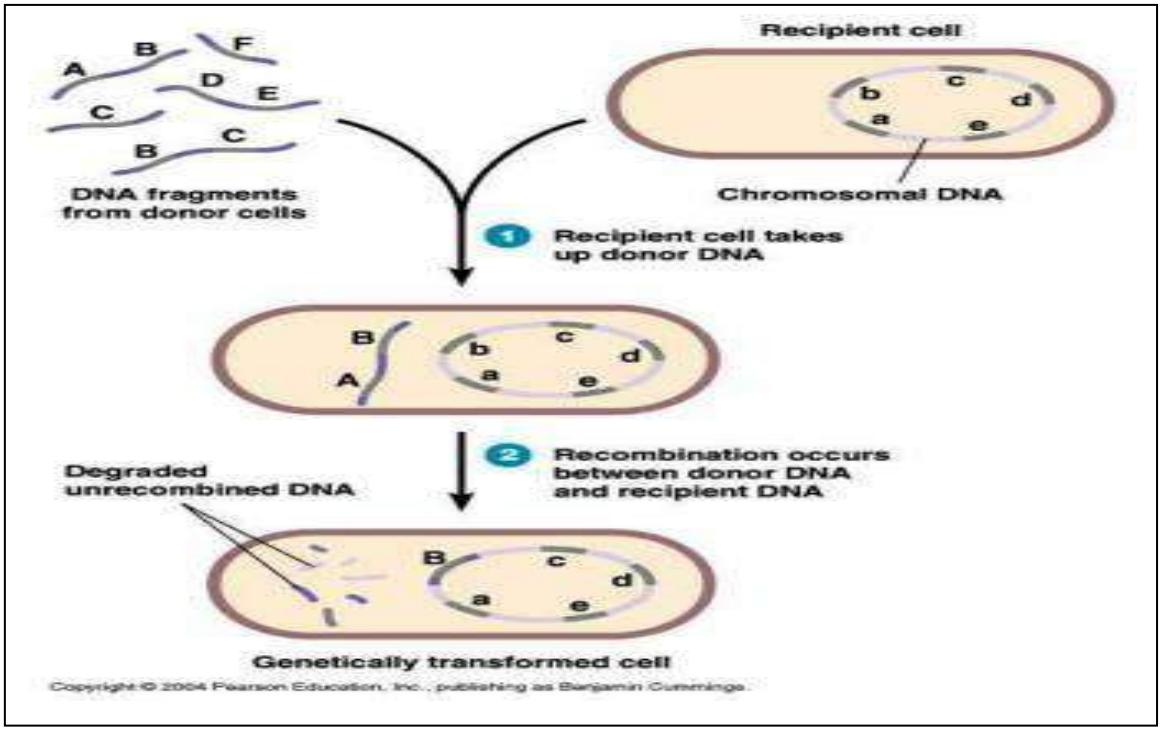
V1 = 600 * 40 / 150,000 = 0.16 ml take it and complete it to 50 ml media

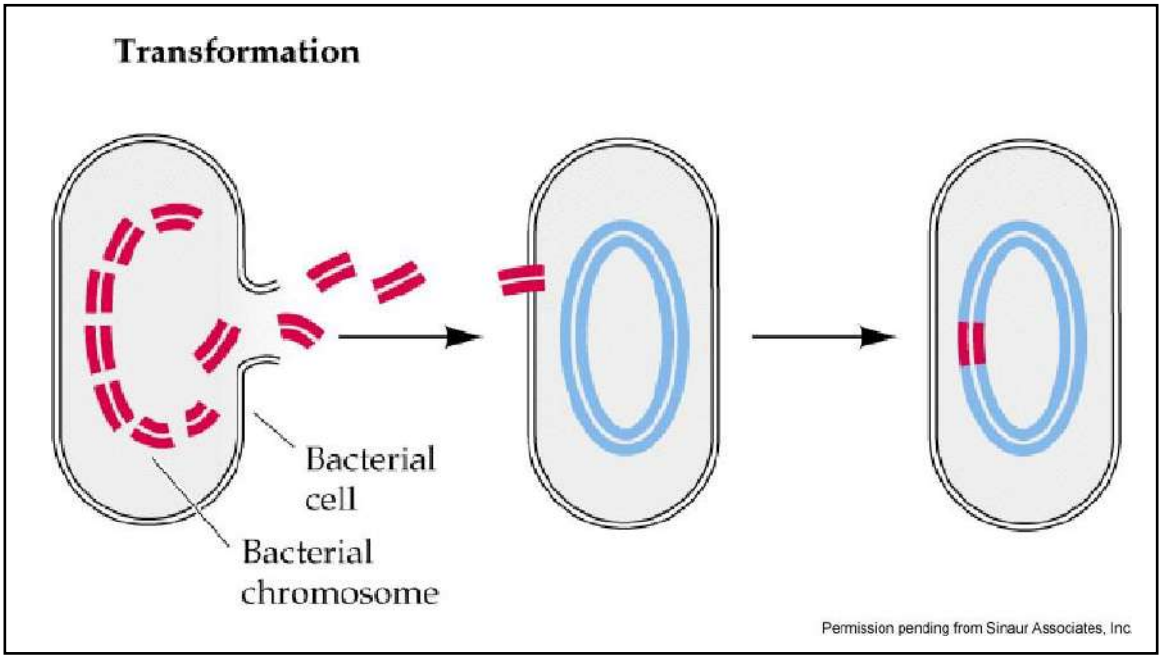
Bacterial Transformation

It is genetic alteration of a cell resulting from the uptake, incorporation and expression of exogenous genetic material (DNA) that is taken up through the cell wall (s).

Transformation occurs most commonly in bacteria and in some species occurs naturally. Bacteria that are capable of being transformed, are called **Competent**.

Transformation is also used to describe the insertion of new genetic material into non bacterial cells including animal and plate cells. However, this process is called transfection.





Mechanism:

Artificial competence

Bacterial transformation may be referred to as **a stable genetic change brought about by the uptake of naked DNA (DNA without associated cells or proteins)** and competence refers to the state of being able to uptake exogenous DNA from the environment.

In artificial competence is induced by laboratory procedures and involves making the cell passively permeable to DNA by exposing it to conditions that do not normally occur in nature.

Calcium Chloride transformation is a method of promoting competence. Chilling cells in the presence of divalent cations, such as: Ca^{+2} (in $CaCl_2$) prepares the cell membrane to become permeable to plasmid DNA. The cells are incubated on ice with DNA and then briefly heat shocked (e.g, 42 C for 30-120 sec.) thus allowing the DNA to enter the cells.

To remember :

- **Calcium chloride partially disturbs the cell membrane, which allows the recombinant DNA to enter the host cell.**
- **The surface of bacteria is negatively charged due to phospholipids and lipopolysaccharides on its cell surface; and the DNA is negatively charged too. One function of the divalent cations therefore, would be to shield the charges thereby allowing a DNA molecules to adhere to the cell surface.**
- **The heat pulse is through to create a thermal imbalance across the cell membrane, which forces the DNA to enter the cells through either pores or damaged cell membrane.**

Procedure:

1) Prepare over night culture from:
   a) *E. coli* sensitive to ampicillin (suspension 1)
   b) *E. coli* resistant to ampicillin (suspension 2)

2) Lyse suspension 2 by incubation for 15 min. at 70C and allow ampicillin resistance gene to be free and up taken by suspension I through transformation

3) Take 3 ml of suspension 1 and centrifuge for 3000 rpm for 10 min.; then add (100 mM $CaCl_2$ with 2.5 ml) to pellet and keep it for 10 min. in ice bath. The suspension is centrifuged again for 300 rpm for 10 min.
This step is required to prepare competent cells.

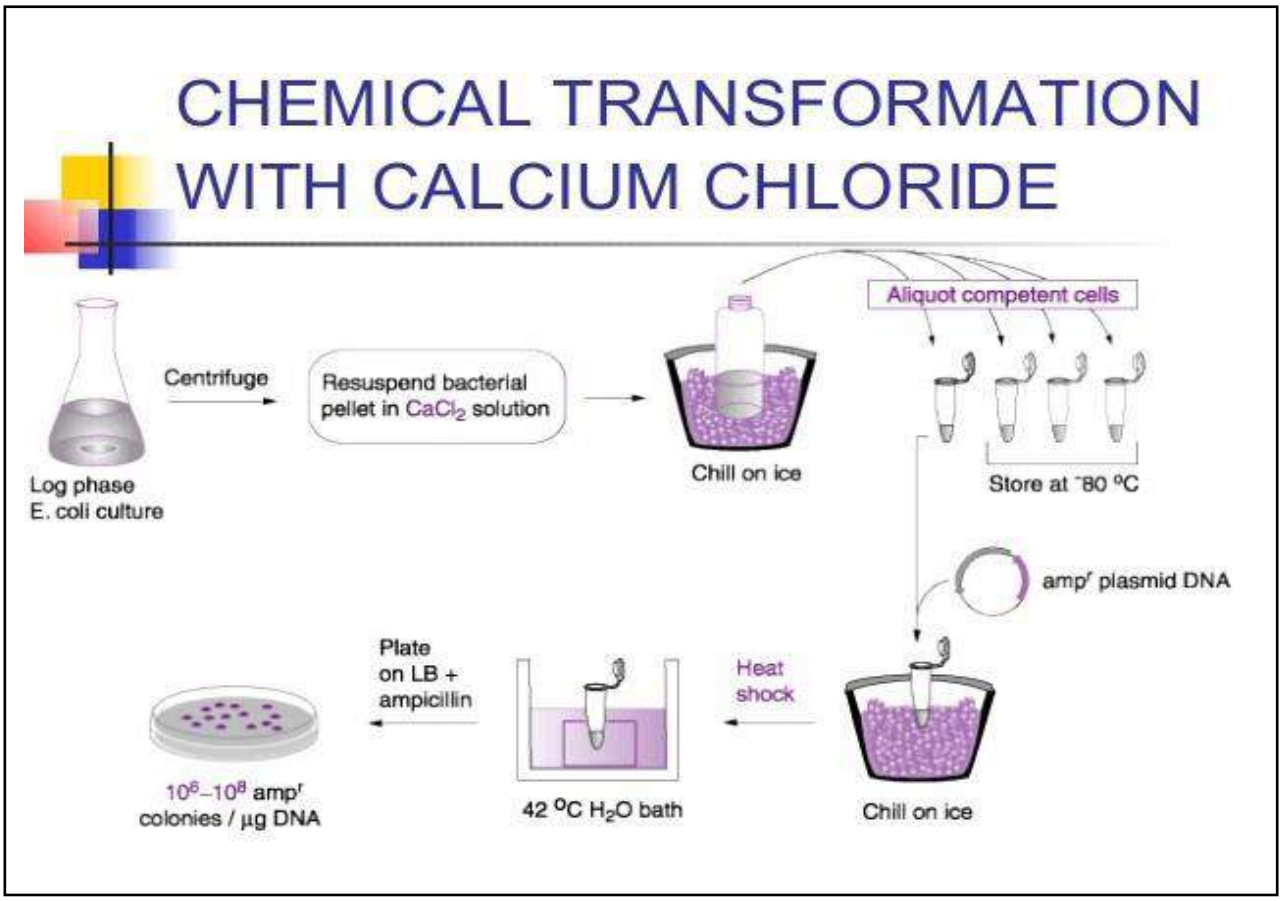
4) Mix (1 ml) from 1 & 2 suspensions, then put the mixture at 0 C for 20 min.

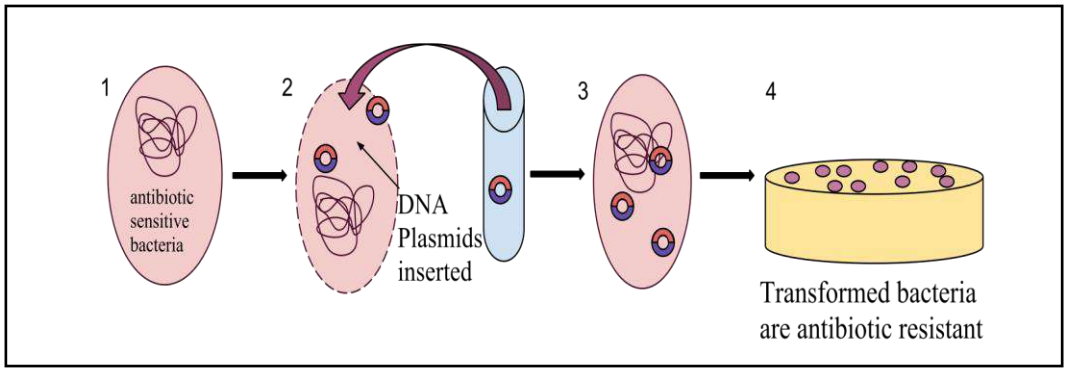
5) Transfer the mixture to water bath at 42 C for 90 sec., then again transfer it to ice bath at 0 C for 2 min. to make a shock in the cell wall of suspension 1 (competent cells) and receive the DNA

6) Incubate the mixture for 15 min. at 37 C, then add it to ampicillin agar plate with 300 µg/ml and incubate for 24 hrs. at 37 C.

- The incubation after heat shock is to enhance and give a period time which could be enough for transformation to occur.

7) Discuss the results





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