Ministry of Higher Education and Scientific Research
University of Baghdad
College of Science
Department of Biology

Practical Genetic Engineering

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المرحلة الرابعة ـ الدراستين الصباحية والمسائية

الفصل الدراسي الثاني

الكادر التدريسي في المختبر **المحاضرين**

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Lab 1

Lab (1) **Genetic Engineering /Practical labs**

Introduction

Genetic Engineering

It also called **Recombinant DNA technology** or **gene manipulation**. It is refer to the process of direct manipulation of an organism`s genome by manually transfer new DNA gene to an organism across species (prokaryotes & eukaryotes) to change one or more of the character .The goal is to add one or more new traits that are not already found in that Example of genetically engineering (transgenic) organisms including:

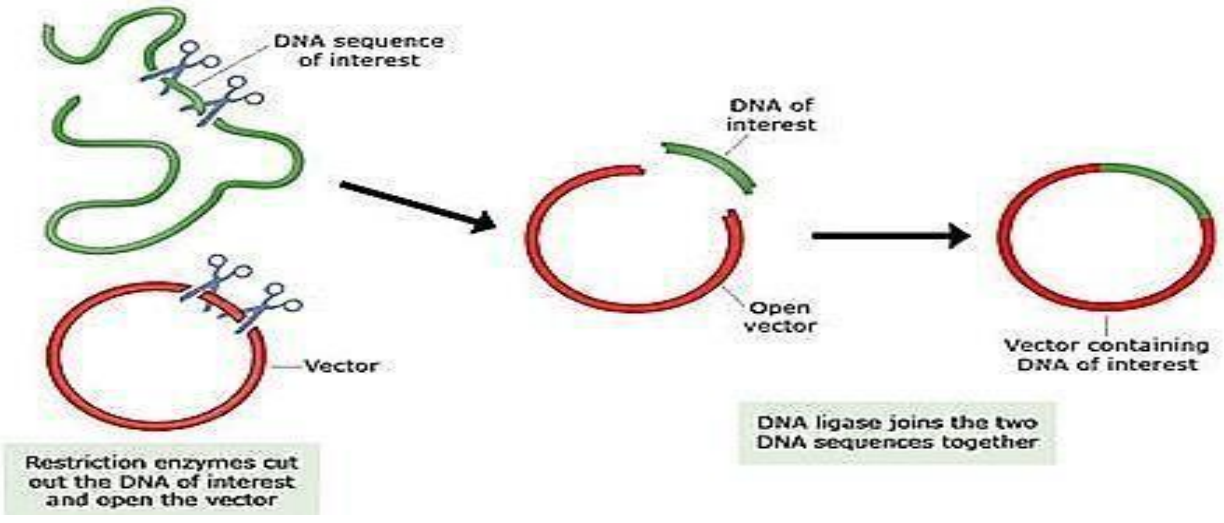
1- One example of a transgenic microorganism is the bacterial strain that produces human insulin.

2- Plants with resistance to some insects, plants that can tolerate herbicides, and corps with modified oil content.

Gene cloning steps

Cutting and ligation

1- Determine the specific gene/s encoded to specific trait/s belong to some an organism and cutting (Isolating) by using **Restriction Enzymes.**
2- Amplification of genetic material (specific gene) for many copies by using **PolymeraseChain Reaction (PCR).**
3- Gene has ligated with vector's genome by **ligase** enzyme to produce new constructed vector (plasmid that contains naked gene) called **recombinant molecule**. The vectors must be restricted by same restriction enzyme.



1- **Bacterial transformation:**

The constructed vector must **transformed** into competent host cells such as *E.coli* BL21 or *E.coli* DH5α that prepared previously by the chemical method $CaCl_2$.

The competent cells exposed to heat shock (at 42 °C for 90 seconds exactly with gentle shaking and rapidly transferred to ice-bath incubated for 4-5 minutes without shaking) and grow up in suitable medium to encourages bacterial cells totake up foreign DNA.

During bacterial reproduce, the plasmid copies increased and pass into offspring.

2- **Finally,** identification the positive clones that carrying specific genes by different methods such as hybridization of DNA or RNA with chemical or radiochemical probs.

Gene expression in host: the successfully transformed colonies grow up in suitable medium again and induced by adding Isopropyl β-D-1-thiogalactopyranoside (IPTG) to expression the specific trait (protein/s) then harvested, checked and purified.

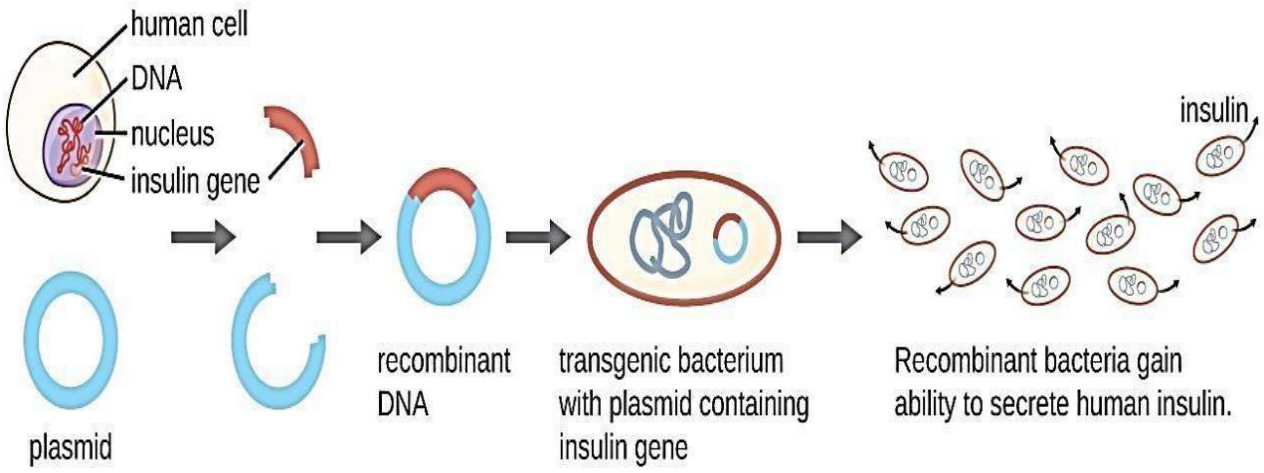


Figure:- Recombinant DNA technology is the artificial recombination of DNA from two organisms. In this example, the human insulin gene is inserted into a bacterial plasmid. This recombinant plasmid can then be used to transform bacteria, which gain the ability to produce the insulin protein.

Benefits of recombinant DNA

1) Development of drug synthesis
2) Vaccines synthesis
3) Improve nutrition & food safety improvement
4) Stronger plants & higher crop yield
5) Healthier farm animals
6) Environmental benefits

Types of vectors:

The most commonly used vectors are:

Plasmids (They are most commonly found in bacteria as small circular, double-stranded DNA molecules separated from a chromosomal DNA and can replicate independently. Plasmids often carry genes that may benefit the survival of the organism such as antibiotics resistance, toxin production, producing pigments…etc. The Plasmids are most common used as vector because they are small enough to manipulate experimentally and furthermore they will carry extra that integrated into them.

1) Viruses
2) Yeast cells.

DNA Extraction:

Although the order of nitrogen bases is what determines DNA's instructions, or genetic code, so the DNA sequences contain the information (gene or genes) required for building organism. During molecular techniques, the extraction of DNA is the first step to preformed cloning, DNA must be isolated from the organism and purified in order to prevent interaction with other molecules such as RNA, proteins, polysaccharides…etc.. DNA can extracted by different methods.

Boiling method:

Boiling method used for extraction of genomic DNA from bacteria (G-ve) as follow:

1) The overnight cultured bacteria harvested in 1 ml of Tris-EDTA (TE) buffer andcentrifuged at 12000 g for 2-5min.
2) The Pellet was re-suspended in 100 µl of sterile distilled water.
3) The mixture boiled at 100ºC for 10 min and then cooled on ice for 10 min.
4) The mixture re- centrifuged at 10000 g for 5 min., and then the supernatant isstored (contain bacterial DNA) at - 20 ºC until use.

Lab 2

Lab(2)

Determination of G+C percentage rate & Melting temperature

Although the ratio of G to C and A to T in an organism's DNA is fixed, the GC content (percentage of G+C) can vary considerably from one DNA to another. This character is very important in determination the genetic similarity among living organisms and widely used in molecular biology techniques and genetic analysis to estimate the genetic distance between two species, Ex: PCR, DNA/DNA and DNA/RNA hybridization.

As molecules, DNA is considered a thermo-stable molecule up to 70°C due to many structural factors although when a DNA solution is heated enough, the non-covalent forces (hydrogen bonds) that hold the two strands together weaken and finally break. When this happens, the two strands come apart in a process known as **DNA denaturation** or DNA melting. The temperature at which 50% of the DNA exists as a double and 50% is single strands (half) is called the **Melting temperature (Tm).**

On further increasing the temperature, both the strand becomes completely separated. However, this separation is not permanent and the complementary strands seal again as soon as the temperature is decreased, the condition called as **Renaturation**. The melting point of a DNA sample hence gives information about

$$AT\ \% + GC\ \% = 100\ \%$$

$$\frac{T + C}{A + G} = 1$$

Marmur formula for calculate Tm in primer sequence design is :-

Tm = 4 x (G+C) + 2 x (A+T)

its base composition (G/C content). In double helix of DNA, the nucleotides are complementary in another word, G=C, A=T; therefore:

The **denaturation of DNA can calculated by the following equation:**

$$\text{Denaturation}\ (\%) = \left(\frac{\text{Final } A_{260}\text{-Blank } A_{260}}{\text{Initial } A_{260}}\text{-}1\right) \times 200$$

Note:- Where, Initial and Final A260 are the absorbance of the DNA at 260 nm before and after any denaturation treatments, respectively. Blank A_{260} is the absorbance of the free water or reagents after physical or chemical treatments.

Measurement of DNA GC% ratio by melting curve analysis:

The melting temperature (Tm - value) of DNA can be easily detected by measuring the absorption at 260 nm. The dissociation process of the base pairs brings about an increase in adsorption. This is called the **hyper chromatic effect**. Alternation of secondary DNA structure upon rising of the temperature results in change of light adsorption.

Example : if a solution of double- stranded DNA has a value of A260= 1.00, a solution of single- stranded DNA at the same concentration has a value of A260 = 1.37.This relation is often described by stating that a solution of double- stranded DNA becomes **hyper chromic** when heated.

These differences related to the facts that DNA helix is stacked. When a DNA sample is gradually cooled the separated stands are renaturated so the absorbance of this sample will reduce due to **hypo chromatic shift.**

For determination of Tm the adsorption values are plotted against the corresponding

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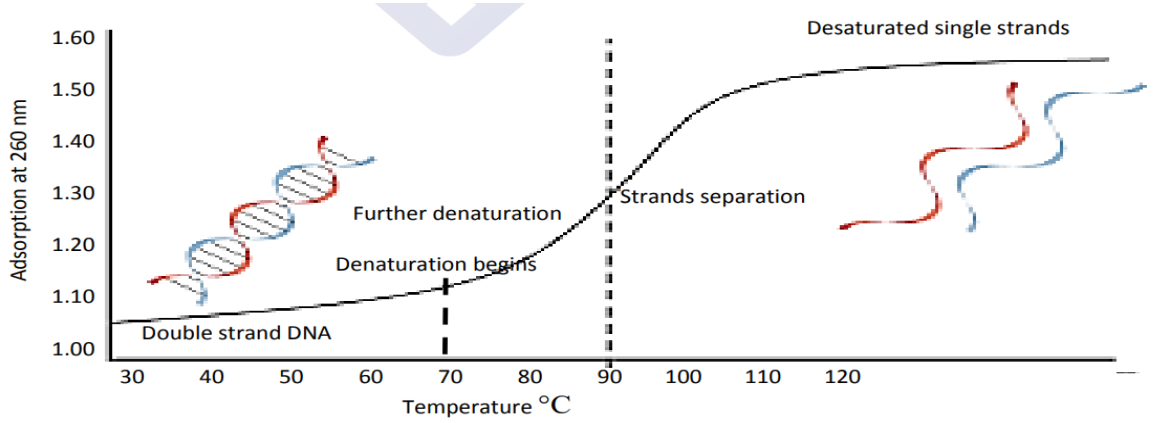
temperature. The inflection points of the **sigmoid curve** gives the determination of melting point for tested DNAsample.



Methodology

1) Suspend DNA sample in TE buffer to obtain 20 µg/ml working concentration.
2) Reset (zero) the spectrophotometer device on 260 nm by TE buffer as blank.
3) Gradually heat the DNA sample in water bath and measure the absorbance of sample at 50,60, 70, 80, 90, 100 °C.
4) Draw the DNA melting curve to determination the Tm value.
   - ❖ Calculate the mol. G+C % by using the following formula:

Note: Tm in solution is a function of:

Length of DNA, GC content (%GC), salt concentration (M) and TE buffer concentration.For a aqueous solution of DNA (no salt used) the formula Tm is

$$Tm = 70 + 0.41 \times (\%G+C)\ ^{\circ}C \qquad \text{——— 1}$$

G+C % of a DNA sample = (Tm – 70) x 2.5

- the optimum temperature of Renaturation (TOR) was calculated by using the followingformula:

$$\mathbf{TOR = 52 + (0.41 \times \%G+C)} \longrightarrow \mathbf{2}$$

From equation 1 & equation 2; equation 3 is derived, as the following:

$$Tm = TOR + 18 \longrightarrow \mathbf{3}$$

There is another equation to calculate the renaturation is:

$$\text{Renaturation}\ (\%) = 100\text{-}\left(\frac{\text{R_Final}\ A_{260}\text{-R_Blank}\ A_{260}}{\text{R_Initial}\ A_{260}}\text{-}1\right) \times 200$$

Note:- Where, the R_Initial A260 is the absorbance of the DNA at 260 nm without any denaturation or renaturation treatment. The R_ Final A260 is the absorbance measured after the denatured DNA being mixed with a buffer for a prolonged time. The R_Blank A260, which serves as a negative control.

Problem: Calculate the Tm and TOR of a DNA sample contain 10% of A.

From all above The melting temperature (Tm) of a DNA sample depends on manyfactors, including:

1) Proportion of GC pairs, because the three hydrogen bonds between GC pairs are morestable than the two between AT pairs.
2) The hydrogen bonds within the structure.

Determination of DNA & RNA purity:

After a DNA purification procedure, the quantity (concentration) of DNA sample should be determined in order to evaluate its purity. Both quantity and quality can be determined by usingspectrophotometer. DNA absorbs light in the UV range A 260 nm, therefore when light of thiswavelength shines through a sample of DNA, the amount of light absorbed is proportional amount of DNA is the solute. The absorbance of light is called **optical density (O.D)** the concentration of DNA can be calculated using the following formula:

$O.D_{260}$ value of 1 unit = 50 µg/ml dsDNA
$O.D_{260}$ value of 1 unit = 37 µg/ml ssDNA
$O.D_{260}$ value of 1unit = 40 µg/ml ssRNA

Actually most interested in the amount of protein that might have been inadvertently purified with DNA. Proteins absorbs light at a different wave length 280 nm. A convenient measure of DNA purity is determined by measuring the **O.D $_{260}$/ O.D $_{280}$ ratio.**

1. Measure the nucleic acid purity **(Pure DNA)** that mean free of protein contamination using **O.D $_{260}$/ O.D $_{280}$** ratio 1.8-2.0 in 10 mM Tris, pH 8.5
    - If phenol or protein contamination is present in the DNA preparations, the **O.D $_{260}$/ O.D $_{280}$ ratio will be less than 1.8.**
    - If RNA is present in the DNA preparation, **O.D $_{260}$/ O.D $_{280}$ ratio may be greater than 1.8.**
2. Use low salt buffers as they provide a more accurate measurement. Purity is influenced by pH and lower pH solutions lower the **O.D $_{260}$/ O.D $_{280}$** ratio and reduce the sensitivity to protein contamination.
3. Pure RNA has an **O.D $_{260}$/ O.D $_{280}$** ratio of 1.9-2.1 in 10 mM Tris, pH 7.5.

Example:

Measuring DNA concentration

Volume of DNA = 50 µl

Dilution: 10 µl DNA sample + 490 µl distilled H2O (1/50 dilution)A260 of diluted sample (1 cm length) = 0.75

DNA Concentration = 50 µg/ml x A260 x dilution factor

= 50 µg/ml x 0.75 x 50

= 1875 µg/ml

Total Amount of DNA = concentration x volume of sample in ml

= 1875 µg/ml x 0.050 ml

= 93.75 µg

also , can determine the amount of contaminated protein by using **Kalcar equation:**

Contaminated protein (µg/ml) = (1.55 x O.D 280) – (0.76 x O.D 260)

To detecting other contamination by:

1. Absorbance at 230 nm and 270 nm indicates the presence of phenol or urea
2. Absorbance at 325 nm indicates contamination by particulates and/or dirty cuvettes.

There are several factors that may affect **O.D $_{260}$/ O.D $_{280}$ ratios.** The 260 nm measurements aremade very near the peak of the absorbance spectrum for nucleic acids, while the 280 nm measurements is located in a portion of the spectrum that has a very steep slops; as a result,very small differences in the wavelength in and around 280 nm will effect greater changes inthe **O.D $_{260}$/ O.D $_{280}$** ratio than small differences at 260 nm.

1. Different instruments
2. Concentration of nucleic sample
3. The type (s) of protein present in a mixture.

Lab 3

Lab(3)

Gel Electrophoresis

Principle of gel electrophoresis

It is one of the most technique method that is used in biochemistry & molecular biologyto separate and analysis macromolecules (such as: DNA, RNA & proteins) and their fragments, based on their mass, size and charge.

When these molecules are placed in matrix of gel and an electrical current is applied, they will move through the pores of the gel towards either the anode or cathode according to the net charge of molecules.

Migration: The movement of charged molecules through the pores of the gel from oneelectrode to another.

Sieving: Smaller molecules are considered less impeded by the gel matrix and hencewill move faster than larger molecules.

Ex.:

1. DNA and RNA are negatively charged molecules due to the presence of a phosphategroup (PO43–) on each nucleotide; they will be moved toward the anode (positivelypole) of the gel.
2. Proteins, may be folded into a variety of shapes (affecting size) and have positive and negative regions (no clear charge), for that, it must first be treated with an anionic detergent such as sodium dodecyl sulfate (SDS) in order to linearized and coats them with a negative charge, which allows them to migrate toward the anodeof the gel .

   Finally, after the DNA, RNA, or protein molecules have been separated by using gelelectrophoresis, bands representing molecules of different sizes can be detected.

Material of gel electrophoresis:

There are several materials that must be used to accomplished an electrophoresis process,such as:

1) Gel:

- It the matrix that molecules are migrated through. There are different types of gel usedaccording to the mixture of molecules that want be to separate depending on pore size of the gel, such as agarose and polyacrylamide (which is used for proteins electrophoresis).

Types of Gel:

1) **Agarose:** It is a polysaccharide extracted from seaweed, which comes as dry, powdered flakes. It is easy to prepare by using electrophoresis buffers and typically used at concentration of 0.5 - 3% for sieving migrated DNA & RNA. This gel is a matrix of agarose molecules that are held together by hydrogen bonds and form tiny pores.

2) **Polyacrylamide:** Polyacrylamide gel electrophoresis (PAGE) is used for separating proteins ranging in size from 5 to 2,000 kDa due to the uniform pore sizeprovided by the polyacrylamide gel. Pore size is controlled by modulating the concentrations of acrylamide and bis-acrylamide powder used in creating a gel .The monomers react with each other by cross-linked. Thelength of the polymer chains is dictated by the concentration of acrylamide used, which is typically between 3- 30% .It prepared by mixed acrylamide and bis-acrylamide powder in electrophoresis buffer. Actually, sometime adding free radicals when associated with water to activate polymerization and must avoiding air bubbles by vacuum because they prevent forming of free radicals and polymerization.

Ex: The following solutions used to preparation of SDS-PAGE.

1-(Resolving gel) Separation gel 12%(1mmthickness):it was prepared fromacryl amide bis acryl amide, Tris-HCl pH=8.8, ammonium persulphate (APS) , Glycerol,and TEMED. The ingredients were mixed well in Distilled water and poured in to glasse plate sandwich for electrophoresis and n-butyl alcohol or distilled water wasadded on the surface of mixture and left to polymerize for at least 30-45 minutes.

2- Stacking gel % 4 (1 mm thickness): it was prepared from same separation gel butwith different concentration. it prepared from acryl amide bis -acrylamide, Tris-HCl pH=6.8 , ammonium persulphate (APS), TEMED, The n-butyl alcohol or distilled water was removed from the surface of separating gel; the comb was fixed well thenthe stacking gel was pipetted on the separation gel until the sandwich space was filled,the gel was left to polymerize for at least10-20 minute.

2) Electrophoresis Buffers:

Buffers in gel electrophoresis are used to provide ions that carry a current and to maintain the pH at a relatively constant value. These buffers have plenty of ions in them, which isnecessary for the passage of electricity through them.
These buffers are preparing as 10X and diluted to 1X for preparing gel and electrophoresis process.

The composition & ionic strength of the electrophoresis buffer affects the electrophoretic mobility of DNA. This is important because the structure and charge of a protein or nucleic acid will change if pH changes, thus preventing proper separation. Therefore, solutions such a: distilled water or benzene that contains few are not ideal for the use inelectrophoresis because it make molecules migrates slowly.

There are many buffers used for electrophoresis such as:

1- Tris acetate EDTA (TAE)
2- Tris borate EDTA (TBE)

3) Loading Buffers:

This buffer is consisting of several martials,as the following:

1) Stop mix:

one has specific role in this process,

it is a compound, which may be glycerol, sucrose, or Ficoll. These compounds increase the weight of DNA sample and prevent its floating because of the low density of DNA. If the DNA sample contains residual ethanol after its preparation,it may float out of the well.

2) Tracking dye:

used as indicator of DNA sample migration, and it is allowing visual mentoring the

molecule in the gel; such as: bromophenol blue and xylene cyanol

❖ To preparation of loading buffer, take 25 mg bromophenol blue & 4 gm sucrose andmixed with 10 ml of D.W, then stored at 4C.

4) Staining and visualization

1. **For DNA and RNA**

   they are staining by ethidium bromide, which intercalates into the major grooves of the DNA and fluoresces under UV light. The intercalation depends on the concentration of DNA and thus, a band with high intensity will indicate a higher amount of DNA compared to a band of less intensity. However, this dye, it is a known mutagen material and should be handle as a hazardous chemical, which required wearing gloves during handling.

2. **For protein**:

   the Staining of gel by Coomassie brilliant blue R-250 for 4 hours with shaking, but toclearly the proteins band need to remove the excise dye by De- staining solution thatconsist acetic acid and 95% methanol .

5) A molecular-weight size marker

Also known as, a size standard the marker are of a known length so can be used to help approximate the size of the unknown fragments in the samples. The principle that molecular weight is inversely proportional to migration rate through a gel matrix. Whenused in gel electrophoresis, markers effectively provide a logarithmic scale by which toestimate the size of standard.

a) **For DNA:** the ladder Mixture of DNA molecules of known fragment sizes resulted fromcutting of lambda phage chromosome by restriction enzymes.

b) **For proteins:** the standard proteins solution containing different proteins such as : ß- galactosidase, Bovine serum albumen (BSA), Ov-albumen, Lactate dehydrogenase, RE ase Bsp981, ß- lactoglobulin and Lysozyme.

Lab 4

Lab (4)

DNA gel electrophoresis procedures:

1) Agarose powder is mixed with an electrophoresis buffer to get the desired concentration.

2) Heated in microwave oven until completely melted.

3) Ethidium bromide is added to the gel (final concentration 0.5 ug/ml) after cooling the solution to about 60C, **why?**

4) It is poured into a tray containing a sample comb & allowed to solidify at room temperature.

5) After the gel has solidified, the comb is removed, place the gel in the electrophoresis tank at correct position & add enough electrophoresis buffer to the tank until the gel is covered.

6) Samples containing DNA (5µ*l*) mixed with loading buffer (2µ*l*) are then pipette into thewells. Load (5µ*l*) of DNA marker in the first well.

7) Close the lid of the tank and applied the desired voltage (3-5 v/cm) and time through connected to power supply.

8) Visualized under UV light.

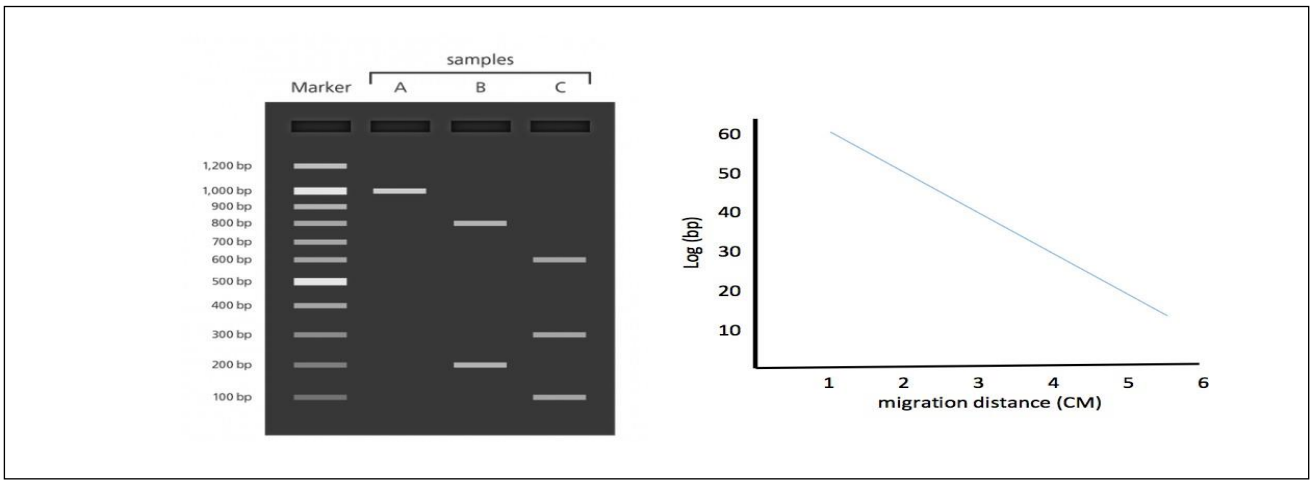
9) The size of unknown sample was measured by draw the standard curve for marker that electrophored with sample which represent the relationship between the migration distance (cm) of the marker and $\log_{10}$ of molecular weight.

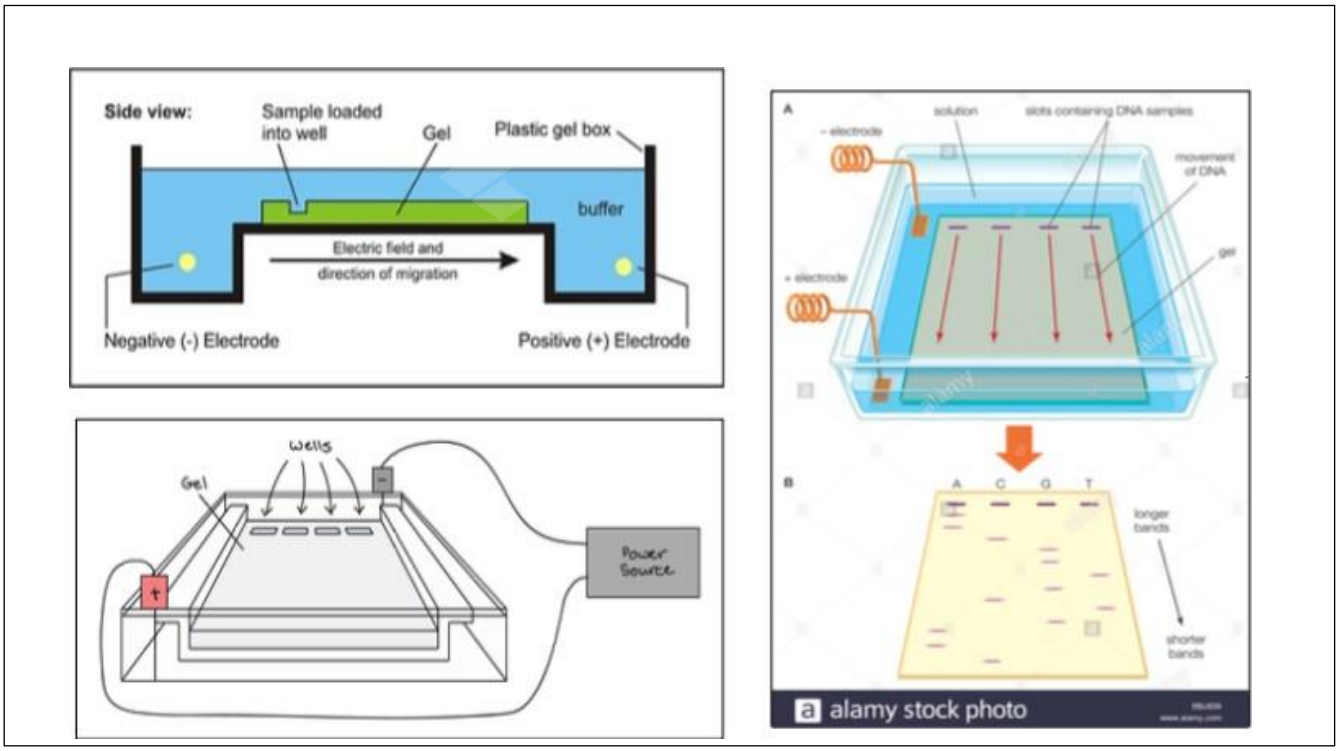
Differences between nucleic acid and protein gel electrophoresis:

1) The nucleic acid visualized under UV light in the presence of ethidium bromide while protein will be seen by naked eye after staining with bromophenol blue dye.
2) Bromophenol blue dye used in nucleic acid electrophoresis as indicator to migrate and allow visual monitoring the molecule in the gel whereas in

protein electrophoresis its used for staining the bands of protein.

3) Protein sample treated with SDS & heated at 100 C for 5 min. to make the protein have negative charge & traveled to anode under electric field.
4) Lymbda DNA used as marker for nucleic acid while different proteins with known molecular size used as markers for protein; such as: phosphorylase B & Bovine Serum Albumin.





	Nucleic Acid electrophoresis	Protein electrophoresis
Molecules	DNA or RNA	Proteins
Gel	Agarose	Polyacrylamide gel
Charge	Native negative charge	Protein sample treated with SDS & heated at 100 C for 5 min. to make the protein have negative charge.
Marker	Lymbda DNA used as marker for nucleic acid	different proteins with known molecular size used as markers for protein; such as: phosphorylase B & Bovine Serum Albumin.
Stain	Ethidium bromide dye	Bromophenol blue dye
Electrophoresis gel type	Horizontal	Vertical
Visualization	visualized under UV light in the presence of ethidium bromide	naked eye after staining with bromophenol blue dye
indicator to migrate & visual monitoring the molecule	Bromophenol blue dye as indicator to migrate and allow visual monitoring the molecule in the gel	Bromophenol blue dye as indicator to migrate and staining the bands of protein

Wondershare PDFelement

Lab5

Lab (5)

The polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) :-

is a scientific technique in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

PCR principles and procedure:-

PCR technique is used to amplify a specific region of a DNA strand (the DNA target). Most PCR methods typically amplify DNA fragments of up to ~10 kilo base pairs (kb), although some techniques allow for amplification of fragments up to 40 kb in size.

PCR steps:-

Denaturation step: This step is the first regular cycling event and consists of heating 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 for allowing annealing of the primers to the single-stranded DNA template.

Extension/elongation step: The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary (opposite) to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction.

PCR amplification protocol :-

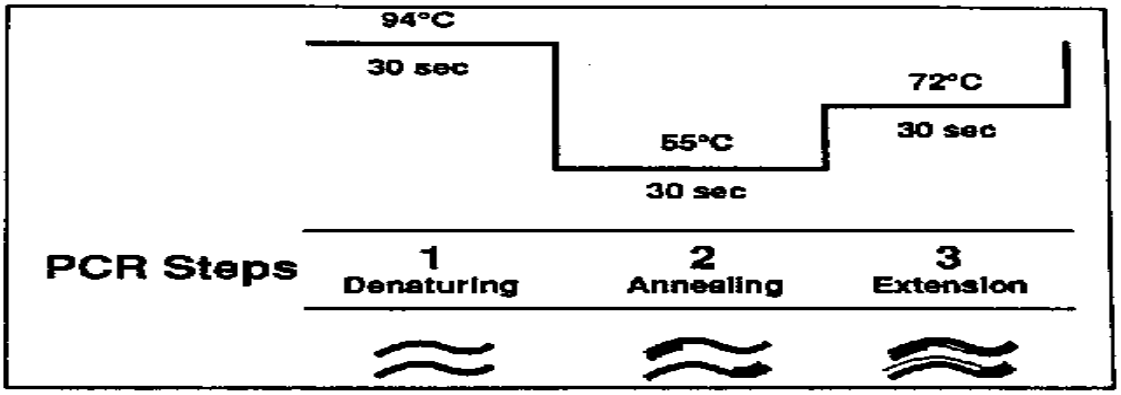
1- Preparation of PCR reaction mix (10µl) consists of the components below:-

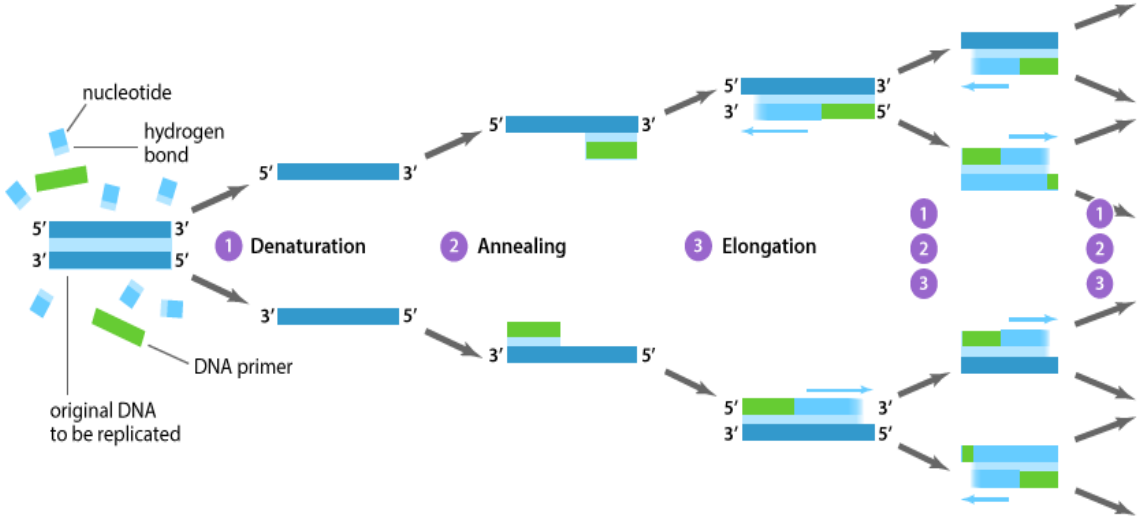
Components	**Amount added (1X)**	**final concentration**
Template DNA (20 ng/ µl)	1.0 µl	2 ng/ µl
Forward primer (10µM)	0.5 µl	0.5 µM
Fevers primer (10µM)	0.5 µl	0.5 µM
dNTPs (2µM)	1.0 µl	0.2 mM
Buffer (10X)	1.0 µl	1X
$MgCl_2$(25mM)	0.6 µl	1.2 mM
Taq polymerase (5U/µl)	0.15 µl	0.076 U/ µl

2- Program of PCR cycles:-

Step	**Temp.**	**Time**
Initial denaturation	94 °C	4min
Denaturation	94 °C	1min
Annealing	58 °C	1min
Elongation	75 °C	1min
Final elongation	72 °C	5min

In general 25 to 35 cycles are used for a PCR reaction. The applications from approximately 34 million to 34 billion copies of desired sequence using 25 cycles and 35 cycles respectively. Additional cycle numbers can be used if there is a small amount of target DNA available for the reaction. However, reaction in excess of 45 cycles is quite rare. Also increasing the number of cycles for larger amount of starting material is counter productive because the presence of very high concentrations of PCR product may be inhibitory.


94°C
30 sec
55°C
30 sec
72°C
30 sec
PCR Steps
1
Denaturing
2
Annealing
3
Extension


nucleotide
hydrogen bond
5′
3′
3′
5′
1 Denaturation
2 Annealing
3 Elongation
DNA primer
original DNA to be replicated

Lab6

Lab (6)

Recombinant DNA technology

Recombinant DNA technology:-

is the basic techniques involved in molecular cloning this involve a gene or other wanted fragment. This fragment is then cloned by inserting it into another DNA molecule, know as a vector .After cloning the recombinant DNA is normally transfer to into an appropriate host cell by transformation.

Steps of recombining DNA technology :-

1- Isolate the foreign DNA fragment that will be inserted using restriction enzymes at a specific nucleotide sequence.

2- Attach the DNA fragment (Desire gene) to carrier (vector).

3-After foreign DNA has been inserted in a suitable vector, the recombined DNA is transfer into a bacterial cell.

Purification of plasmid by alkaline lysis method

Plasmid DNA can be isolated from bacteria by using variety methods which depend on the differential denaturation and reannealing of plasmid DNA compared to chromosomal DNA. One commonly used technique involves alkaline lysis. This method essentially relies on bacterial lysis with sodium hydroxide and sodium dodecyl sulfate (SDS) then is followed neutralization with high concentration of low pH potassium acetate. This gives selective precipitation of bacterial chromosomal DNA and other high molecular- weight cellular components. The plasmid DNA remains in suspension and precipitated with isopropanol.

Cesium chloride gradient ultracentrifugation method for separating DNA

To separate DNA based on density, DNA mixed with CsCl and centrifuged at very high speeds in an ultracentrifuge for many hours, linear gradient of CsCl with the lightest density at the top and the heaviest density at bottom. **CsCl is used because (at a concentration of 1.6 to 1.8 g/ml) it is similar to the density of DNA**. Different types of nucleic acids will also be separated into bands, RNA is denser than supercoiled plasmid DNA, which is denser than liner chromosomal DNA.

Methodology:-

1- Dissolve the DNA pellet in 2.5ml in TE buffer + RNAse (20 µg/ml) then incubates for 20 min at 37C˚.

2- Add 3.2 g of CsCl to DNA solution and invert gently to dissolve the CsCl, add 240 µL EtBr (10 mg/mL) (EtBr will interfere between hydrogen bond that lead to raise up DNA density.

3- The mixture in step 1 will transfer to polypropylene tube.

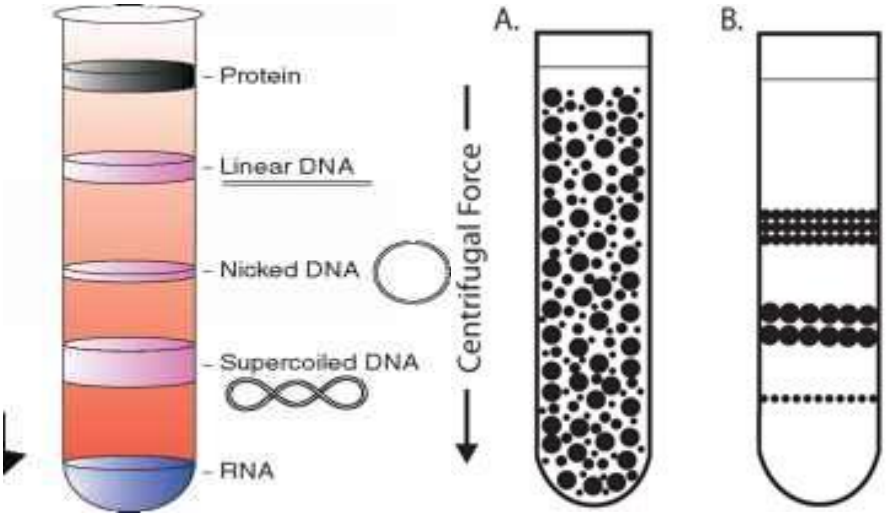
4- Closing the tubes using heat to make ultracentrifugation.

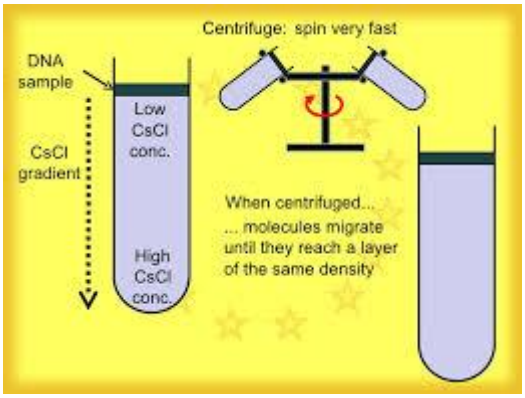
5- Spin the tube to generate cesium gradient at (100000) rpm for 2hr.

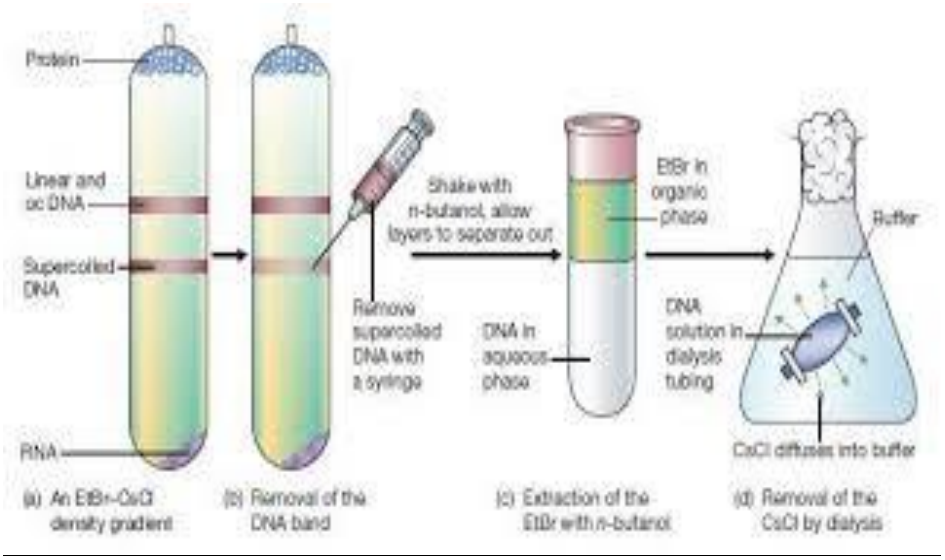
6- Extract DNA as described above using syringe transfer to the 15 ml polypropylene tube.

7- Extract the Etbr with n-butanol.

8- Use dialysis to remove CsCl.





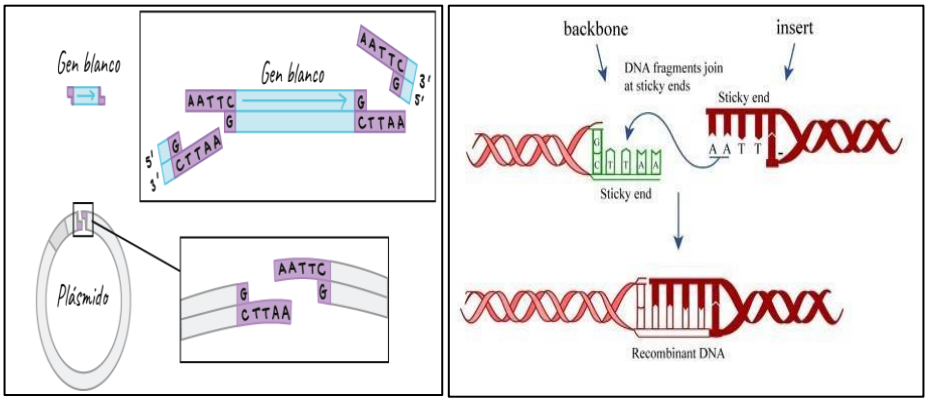


Protocol for DNA Digestion with two different restriction enzymes

Add components to a clean tube in the order shown:

1 µL DNA (concentration 1 µg/µL)
2 µL 10x buffer
1 µL each restriction enzyme
15 µL sterile water

Incubate the reaction at digestion temperature (usually 37°C) for 1 hour.
Stop the digestion by heat inactivation (65°C for 15 minutes) or addition of 10mM final concentration EDTA.
The digested DNA is ready for use in ligation



Ligation

Before use *E.coli* for transformation we must focus on ligation of recombinant vector with desire gene which has sticky ends of exposed nucleotides to gather using ligase enzyme.

Methodology:-

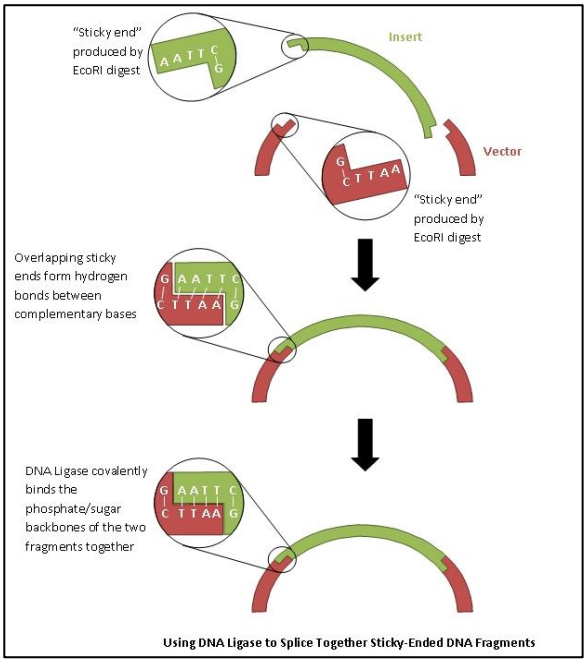
1- Prepare ligation buffer 250mM Tris-HCl (pH 7.5), 50mM $MgCl_2$, 100mM lithothritol, 10mM ATP and 250mg/ml BSA.

2- Add 1mlT4 DNA Ligase.

3-Add 200µl DNA sample (vector with desire gene)

4-Incubate for 2-4 hr at room temperature.

5-Electrophoreses 5 µl of mixture on agarose gel to confirm the ligation



Using DNA Ligase to Splice Together Sticky-Ended DNA Fragments

Lab7

Lab(7)

Transformation of *E.coli* with recombinant DNA

- Bacterial transformation

  is a process by which bacterial cells take up naked DNA molecules or new genetic information fragment from bacterial environment.

- Some bacteria are able to uptake DNA naturally are called "competent cells" other do not have this ability .

  the competent bacteria are prepared by treating *E. coli* MM strain with calcium chloride in the early log phase of growth media, to enhance the bacterial ability to take up selected recombinant DNA molecules into cells. For transformation to occur, either heat shock or electroporation used to stimulate the bacterial cell membrane to open pores to receive DNA molecules.

Note

Note:- Selection of transformed cells by resistance to antibiotics conferred by recombinant plasmid

- **Transduction**

  is the process of transferring genetic material (genes) by bacteriophage.

- **Transfection**

  is the process of introducing transferring foreign DNA into eukaryotic cells.

Transformation Methodology

I. Competent cells preparation

1- Inoculate 5ml of SOC medium with *E.coli* MM (free from plasmid).

2- Harvest the cells by centrifugation 6000 rpm for 15 min at 4˚C.

3- Suspend the cells with 1 ml of 100mM CaCl2 to get competent cells.

3- Resuspend and grow on SOC medium.

ll. Transformation *E.coli* MM with recombinant PBR 322 plasmid DNA

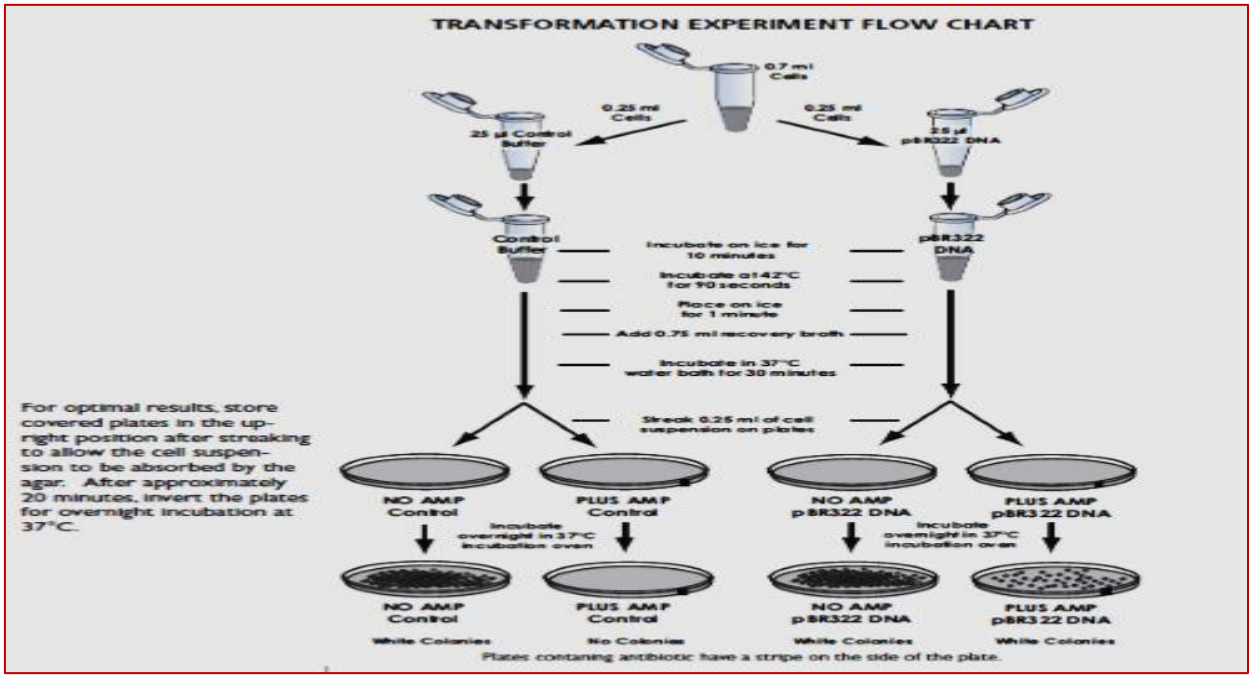
1. Add **0.25 ml of Competent *E.coli* MM cells** into **25 µl recombinant PBR 322 plasmid DNA**.

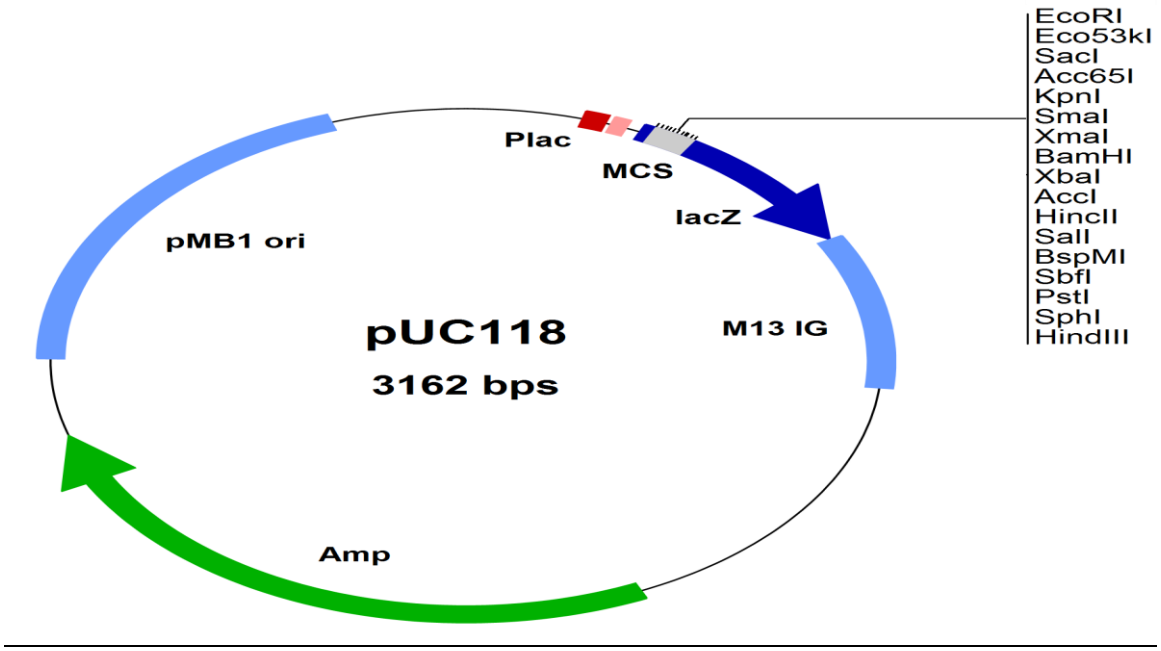
2. Incubate 10 min on ice bath then place the samples on water bath for 90 sec at 42 C° then place on ice again for 1 min **(Heat shock)**.

3. Add 0.75 ml of recovery SOC medium and incubate at 37oC for 30 min in shaking incubator.

4. Streak 0.1-0.25 ml of cell suspension on Amp selective media as shown in the figure below.

(Note: use a buffer instead of DNA as a control)





Yeast Artificial Chromosomes (YACs)

fragment size proximal 100- 1000 Kb

Bacterial Artificial Chromosomes (BACs)

fragment size proximal 75- 350 Kb.

Cosmids

- fragment size proximal 33- 47 Kb.
- DNA molecule that combines features of col E1 plasmids and lambda phage and has cohesive end (cos site).

Bacterial phage

- fragment size proximal 9-22 Kb.
- lambda & M13phage

Plasmids

- fragment size less than 10 Kb.
- pBR322

Lab8

Lab (8)

Hybridization technique

Restriction enzymes:- One of the important tools of hybridization technique is restriction or called **restriction endonuclease** can be used for cleaving DNA molecules double strand at specific recognize nucleotide sequences that **know restriction sites** . In order to be able to sequence DNA , it is first necessary to cut the strand into smaller fragments, this produces a heterogeneous collection of fragments of varying size.

For example, the bacterium *Haemophilus aegypticus* produce enzyme was named Hae III That cuts DNA wherever it encounters sequence . There are two types from restriction enzymes divided according to their DNA cutting were:

- HaeIII that cut straight across the double helix producing" blunt ends".

- BamHI is another kind of enzymes cutting DNA produce " sticky ends" they are able to form base pairs with any DNA molecules (vectors , wanted gene) that contains the complementary sticky end, can be joined with other fragments of other DNA strand using enzyme called **ligase** and these resulting fragments is a **recombinant DNA rDNA .**

Types of restriction enzymes

There are four types of restriction endonucleases are (I, II, III and IV):-

- Type I enzymes cleave at sites remote from a recognition site; require both ATP andS-adenosyl-L-methionine (SAM) and Mg^{2+} to function.
- Type II enzymes cleave within or at specific recognition site and requires only Mg^{2+} .
- Type III enzymes cleave at sites a short distance from a recognition site; require ATP (butdo not hydrolysis it); S-adenosyl-L-methionine and Mg^{2+} function.
- Type IV enzymes Requires Mg^{2+} and SAM and cleaving both DNA strand on both sides ofits recognition site.

Methodology

Protocol for DNA Digestion with a single restriction enzyme at suitable conditions(temperature, pH, ionic strength)

1. Add components to a clean tube in the order shown: 4 µL DNA 2 µL 10x buffer 10 U/ µL HindIII 4 µL sterile water

   Note:- In DNA digestion the quantity must be 20%

2. Incubate the reaction at digestion temperature (usually 37°C) for 1 hour.

   Note :- The restriction enzyme unit define is the quantity of enzyme needed to digest 1 µg

DNA dissolve at buffer for 1 hr.(total volume reaction is 20 µL)

3. Stop the digestion by addition of 0.5 M (final concentration) of EDTA.
4. The digested DNA is ready for use in research applications and on gel electrophoresis.

- **DNA electrophoresis** is an analytical technique used to separate DNA fragments by molecular size of DNA molecules. The electric field induces the DNA chain. The separation of these fragments is accomplished by exploiting to motilities with which different sized molecules are able to pass through the gel; larger molecules migrate more slowly because they experience more resistance within the gel. Because the size of the molecule affects its mobility, smaller fragments end nearer to the anode than larger onein given period.

Southern Blot

is a method routinely used for detection of specific DNA sequence in DNA samples . Southern blotting combines transfer of electrophoresis –separated DNA fragments to a nitrocellulose membrane and subsequent fragments detection by probe hybridization.

Southern Blot Protocol

1- Digestion of DNA with restriction enzyme.

2- Electrophoresis for digested DNA fragment in agarose gel construction.

3- Treat the gel with an acid, such as dilute HCl, which breaking the DNA in smaller fragments.

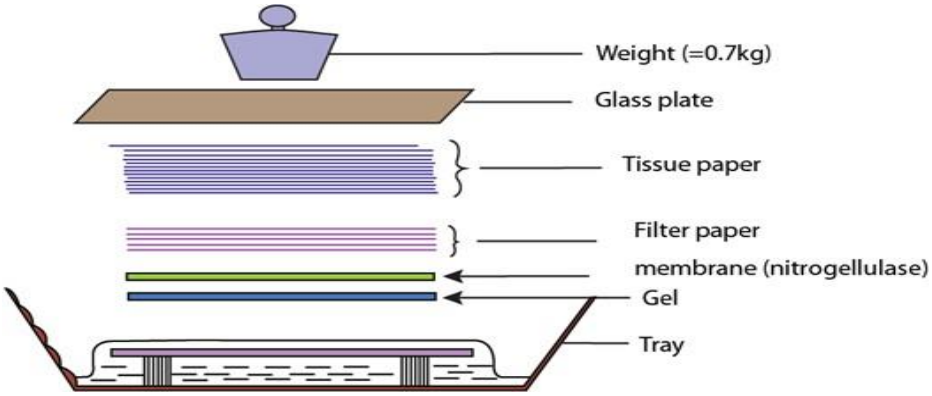
4- Place the DNA gel into alkaline solution (such as NaOH) to denature the double – strandedDNA, that may improve binding of negatively charged DNA to a positively charged membrane, separating it into single DNA strand for later hybridization to the probe, and

destroys any residual RNA that may still be present in the DNA.

5- Membrane Preparation

a- Place a sheet o nitrocellulose (or alternatively nylon) membrane on top of the gel. Pressure is applied evenly to gel **(using stack of paper towels and a weight on top of membrane and gel to ensure good and even contact between gel and membrane)**. Place blot in 2x SSC buffer which transfer by **capillary action** from a region of high water potential to a region of low water potential (usually filter paper and paper tissues) is then used to move the DNA fromthe gel on the gel on the membrane.

b- The membrane is then baked in vacuum or regular oven at 80C˚ for 2 hr (standard condition, nitrocellulose or nylon membrane) or exposed to ultraviolet radiation (nylon membrane) to permanently attach the transferred DNA to membrane.



6- The hybridization step

Expose the membrane to a hybridization probe **(hybridization probe is a single labeled DNA fragment with a specific sequence whose presence in the target DNA is to be determined**

) The probe DNA is labeled so that it can be detected , usually by incorporating radioactivity or tagging the molecule with fluorescent or chromatographic dye . In some cases use deionizedformamide and detergents such as SDS to reduce non specific binding of the probe

Note:- Hybridization of the probe to a specific DNA fragment on the filter membrane indicates that this fragment contain DNA sequence that is complementary to the probe.

Note:- Modification of the hybridization conditions (for example increasing the hybridization temperature) may be used to increase specificity.

7- Washing buffer step

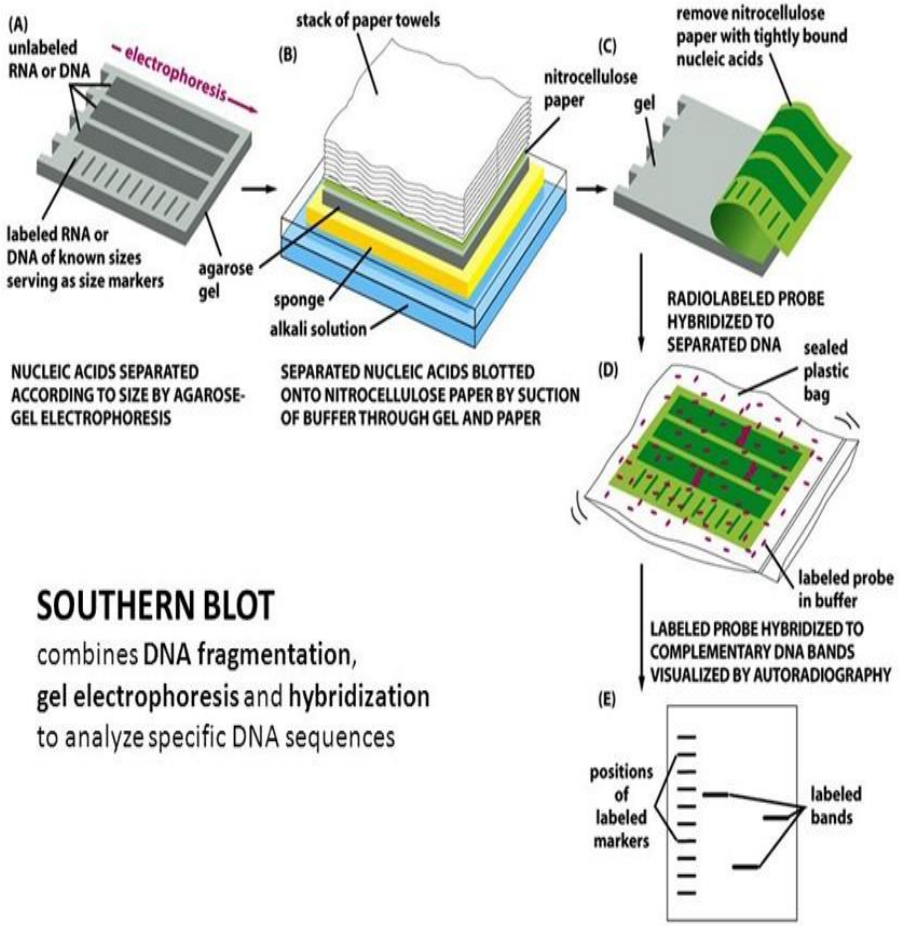
After hybridization, using concentration amount of stock solution to wash labeled membrane with 10 X SSC buffer.

8- Pattern of hybridization is visualized on X-ray film by autoradiography in the case off a radioactive of fluorescent probe to color development on the membrane **as chromatographicdetection method**.

Southern Blot principal is determined the number of sequences (gene copies) in a genome. Aprobe that hybridizes only to a single band on southern blot, where as multiple bands will likely be observed when the probe hybridizes to several highly similar sequences (those may be the result of sequence duplication).

Southern Blotting Technique

MOLECULAR BIOLOGY – Molecular biology techniques



SOUTHERN BLOT

combines **DNA fragmentation**,
gel electrophoresis and **hybridization**
to analyze specific DNA sequences

Lab 9

Lab (9)

Genetic Finger Printing

Genetic finger printing is a way of identifying a specific individual, rather than simply identifying a species or some particular trait. It is also known as genetic finger printing. It is currently used for both **1-** Identifying paternity or maternity and **2-** Identifying criminals or victims ,this process was depending on RFLP technique .

Restriction fragment length polymorphism (RFLP)

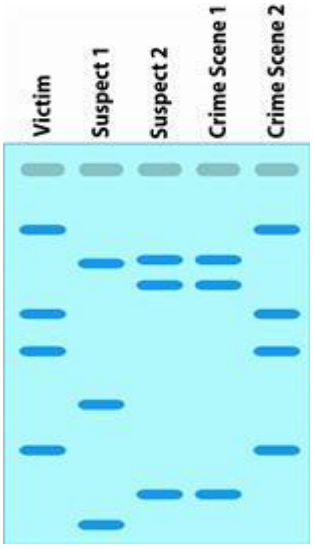
It is a molecular biological technique used to compare DNA from two samples. Special enzymes that cleave the DNA in specific locations are used to digest strands of DNA. The DNA results in strands of different lengths. Electrophoresis is then used to separate the strands according to their length. RFLP is used as part of DNA fingerprinting, to detect genetic diseases and to determine genetic relationships between species.

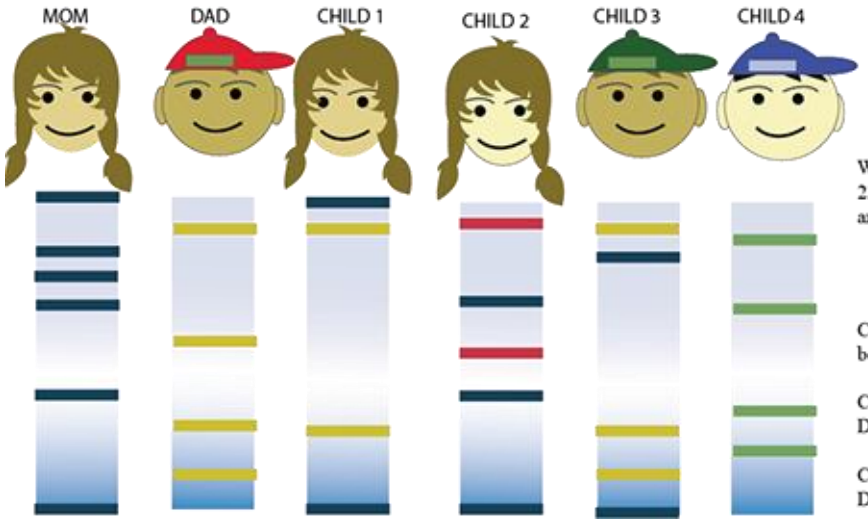
RFLP (Protocol)

- The sample of DNA was extracted then digested by using restriction enzyme .The restriction digest is electrophored The smear of fragment is transferred to nitrocellulose by southern blotting .
- The hybridization analysis is carried out by using a specific probe clone that spread the region of interest. If RFLP is present then it will be clearly visible **when the banding pattern is compared with that obtained from two different individuals are unlikely to be the same, because of the hyper variability of each repeat array and recognizable RFLP present in vicinity of the defective gene .**

DNA fragments

Separate fragments by agarose





When a child is born, they inherate 23 chromosones from the mother and 23 chromosones from the father.

Child 1 and 3 are the childrent of both Mom and Dad.

Child 2 is the child of Mom, but not Dad.

Child 4 is not the child of Mom or Dad.

Lab 10

Lab (10)

DNA sequencing technology:-

Sanger Sequencing forDNA sequencing

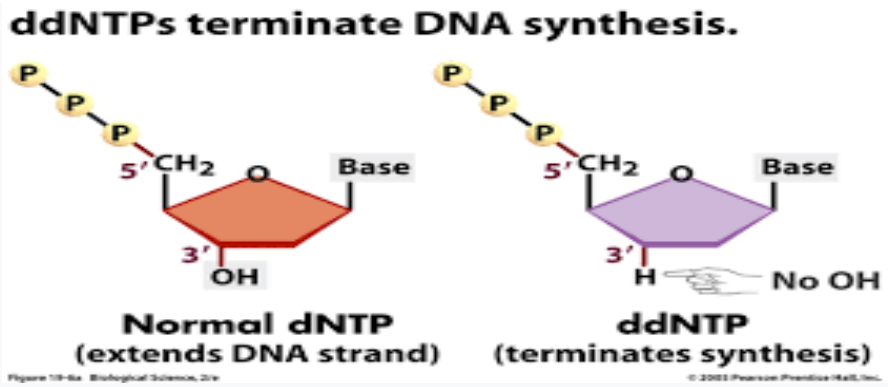
DNA sequencing is the process of determining the precise order of nucleotides within a DNA molecule. It includes any method or technology that is used to determine the order of the four bases—adenine, guanine, cytosine, and thymine—in a strand of DNA. The advent of rapid DNA sequencing methods has greatly accelerated biological and medical research and discovery.

The rapid speed of sequencing attained with modern DNA sequencing technology has been instrumental in the sequencing of complete DNA sequences, or genomes of numerous types and species of life, including the human genome and other complete DNA sequences of many animal, plant, and microbial species.

Not:- the sequencing of complete DNA was developed by Frederick Sanger and colleagues in 1977.

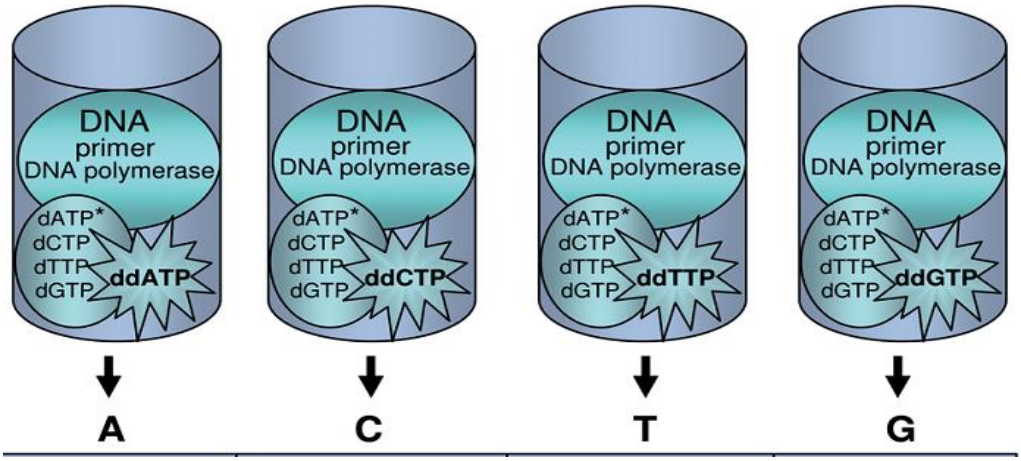
Sanger method of sequencing principle is the determination of the precise sequence of nucleotides in a sample of DNA **that based on The getting dideoxy nucleotide method** , its name from the critical role played by **synthetic** nucleotides that **lack** the -OH at the 3′ carbon atom . A dideoxynucleotide (dideoxythymidine triphosphate — ddTTP —) can be **added** to the growing DNA strand but it cause stopping **chain**

elongation because there is no 3′ -OH for the next nucleotide to be attached to. For this reason, the dideoxy method is also called the **chain termination method**.



1- Classical DNA sequencing :- The classical sager sequencing relies on base-specific chain terminations in four separate reactions (A, G, C, and T) corresponding to the four different nucleotides in the DNA makeup. In the reaction presence of all four (normal) deoxynucleotide triphosphates (dNTPs), a specific -dideoxynucleotide triphosphate (ddNTP) is added to every reaction; DNA templet sample , primer and polymerase for extension of a synthesized DNA strand in PCR .

The extension step in PCR reaction when add nucleotides **the stopping extension occur in present ddNTP that called chain termination.**



DNA classical sequencing protocol:-

1. The region of DNA to be sequenced is amplified in some way and then denatured by heat to produce single stranded DNA.

2. A sequencing primer is annealed to the single stranded DNA templet .

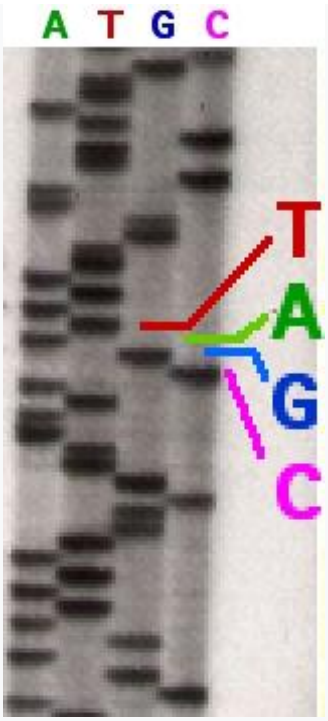
3. The solution divided into four separate reaction , each containing:-
    - The DNA single strand attached with primer
    - DNA polymerase.
    - A small amount from one of four dideoxynucleotides for each reaction.
    - All four deoxynucleotide.

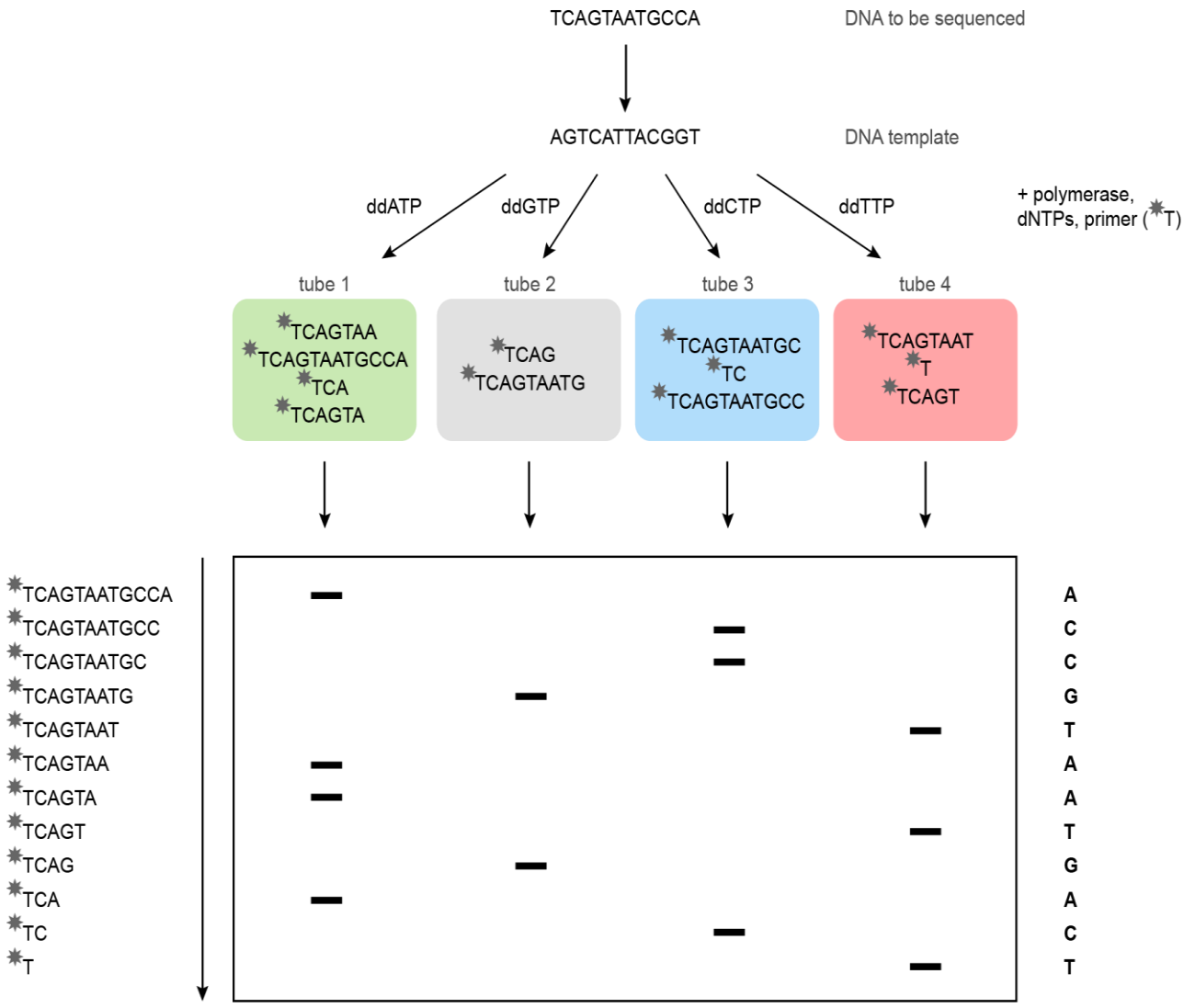
4- Now the four separate sets of chain – terminated fragments can be produce.

Not:- Dideoxynucleotide chain termination DNA sequencing then takes advantage of the fact that a growing chain of nucleotides, extending in the 5’ to 3’ direction, will terminate if, instead of a conventional deoxynucleotide, a 2’3’ dideoxynucleotide becomes incorporated.

5- Following the replication/termination step, these chain terminated fragments will remain bound to the single stranded DNA molecule which has acted as a template. By heating these partially double stranded molecules and adding a denaturing agent such as **formamide**, the single stranded chain termination molecules can be released from their template and separated using high resolution denaturing gel electrophoresis.

6- The sequence of the original region of DNA is then finally deduced by examining the relative positions of the dideoxynucleotide chain termination products in the four lanes of the denaturing gel.

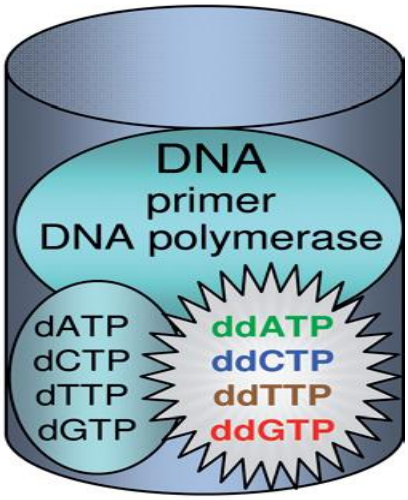

A T G C
T
A
G
C

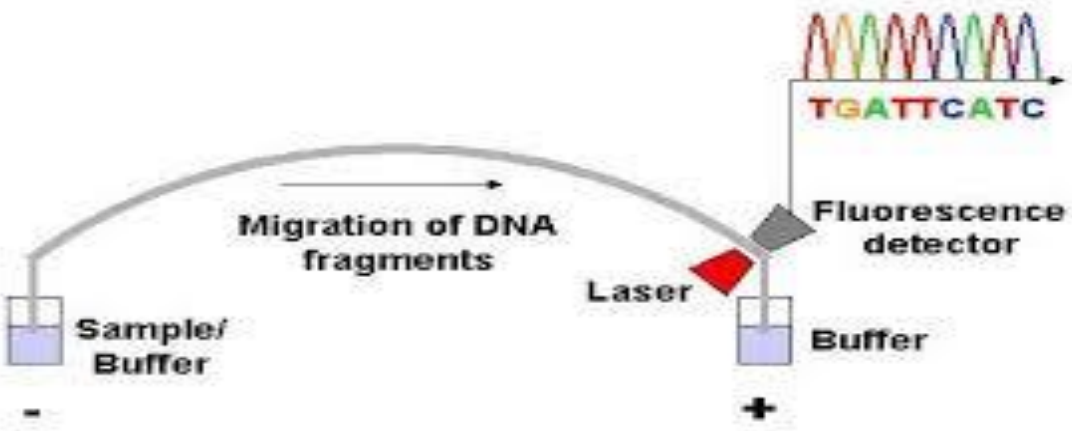


2-Automated chain-termination DNA sequencing.

Most DNA sequencing is now automated. The Sanger method chain termination reactions are still used, but pouring, running, & reading gel electrophoresis has been replaced by automated methods. Instead of labeling the products of all 4 sequencing reactions the same (with a radioactive deoxynucleotide), each dideoxynucleotide is labeled with a different fluorescent marker. When excited with a laser, the 4 different kinds of products are detected and the fluorescence

intensity translated into a data "peak". Thus all four chain termination reactions can be performed in the same tube, and run on a single lane on a gel. A machine scans the lane with a laser. **The wavelength of fluorescence from the label conjugated to the ddNTPs can be interpreted by the machine as an indication of which reaction (ddG, ddA, ddT, or ddC) a particular DNA band came from of a chromatogram.**





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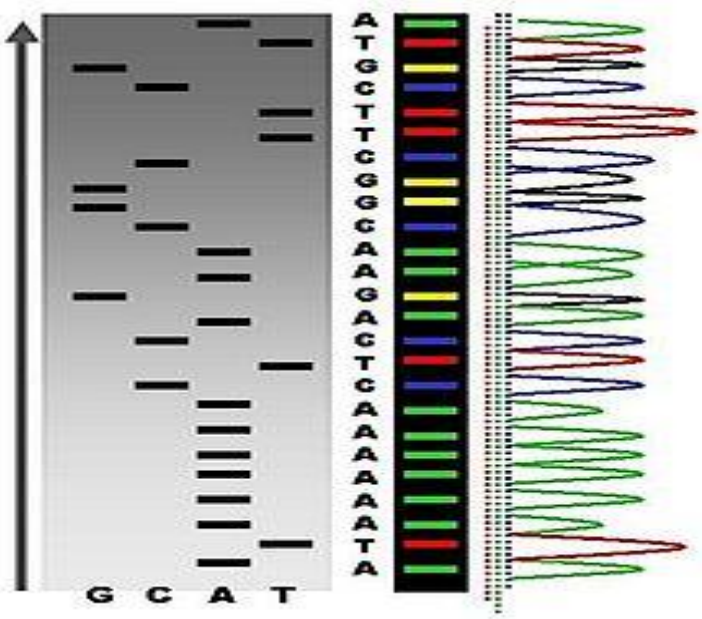
G C A T
A T G C T T C G G C A A G A C T C A A A A A A T A