Ministry of Higher Education
King Abdul Aziz University
Faculty of Science
Department of Biological science

# Laboratory Manual of

# Principle of Genetic Engineering
373
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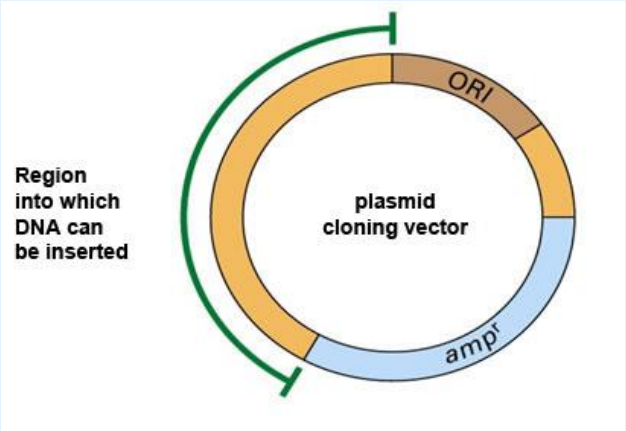
| Lab. # | Date | Exp/Practical title | Reading Assignment | What is Due? |
|---|---|---|---|---|
| 1 | 2/4/1432<br>7/3/2011 | Safety & Regulations | Practical movie | |
| 2 | 9/4/1432<br>14/3/2011 | DNA Technology and Genomes | Lab hand out practical | explain the structure of DNA and Genomes |
| 3 | 16/4/1432<br>21/3/2011 | DNA engineering | Lab hand out practical | Do the experiment of Carrier of genetic information |
| 4 | 23/4/1432<br>28/3/2011 | Cleavage of DNA by Restriction Endonucleases | Lab hand out practical | discussed Restriction Endonucleases tools of DNA technology |
| 5 | 30/5/1432<br>4/4/2011 | Cloning Vectors and techniques | Lab hand out practical | discussed cloning technology |
| 6 | 7/5/1432<br>11/4/2011 | VACATION | | |
| 7 | 14/5/1432<br>18/4/2011 | The Mid Exam | | |
| 8 | 21/5/1432<br>25/4/2011 | cDNA library | Lab hand out practical | Prepare of cDNA library . |
| 9 | 28/6/1432<br>2/5/2011 | RT-PCR | Lab hand out practical | describes the difference of PCR and RT-PCR |
| 10 | 6/6/1432<br>9/5/2011 | Primers and probes preparation | Lab hand out practical | Designing primers and probes |
| 11 | 13/6/1432<br>16/5/2011 | Gene expression<br>And genetic engineering | Lab hand out practical | explain the gene expression<br>and step of genetic engineering |
| 12 | 20/6/1432<br>23/5/2011 | The Final Exam | | |

Lab (1)

## Plasmid isolation from Bacteria

### Plasmid

Plasmid is a double stranded, circular extra chromosomal DNA of bacterium. It is used in recombinant DNA experiments to clone genes from other organisms and make large quantities of their DNA. Plasmid can be transferred between same species or between different species. Size of plasmids range from 1-1000 kilo base pairs. Plasmids are part of mobilomes (total of all mobile genetic elements in a genome) like transposons or prophages and are associated with conjugation. Even the largest plasmids are considerably smaller than the chromosomal DNA of the bacterium, which can contain several million base pairs.




The term 'plasmid' was introduced by American molecular biologist Joshua Lederberg. Plasmids are considered as transferrable genetic elements or 'replicons'. They are actually naked DNA. Plasmids are important tools in genetic and biotechnology labs where they are commonly used to multiply or express particular genes. Plasmids are also used to make large amounts of proteins.
Plasmids encoding Zinc Finger Nucleases are used to deliver therapeutic genes to a preselected chromosomal site with a frequency higher than that of random integration. Mainly there are two types of plasmids: conjugative and non conjugative. Conjugative plasmids have tra-genes (tra-transfer) and can perform conjugation. Non conjugative plasmids cannot perform conjugation. There is an intermediate class of plasmid called mobilizable plasmid. Mobilizable plasmid can

carry only a subset of genes required for transfer. They can parasitize a conjugative plasmid transferring at high frequency only in its presence.

Based on function plasmids can be of five types:

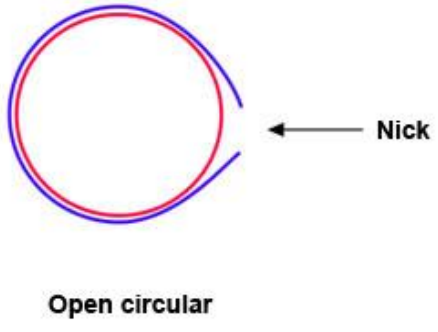
- F/Fertility plasmid for conjugation
- R/Resistant plasmid which contains genes that provides resistance to antibiotics. It also helps bacteria in producing pilus.
- Col plasmid which contain genes that code for bacteriocin (toxins produced by bacteria to inhibit the growth of similar or closely related bacterial strains)
- Degradative plasmid which help in the digestion of unusual substances like toluene
- Virulence plasmid which is responsible for pathogenecity

Bacteria contain one or more plasmids in them. Bacteria have several mechanisms to maintain high copy number of plasmids Different plasmids have different copy numbers in the cell.

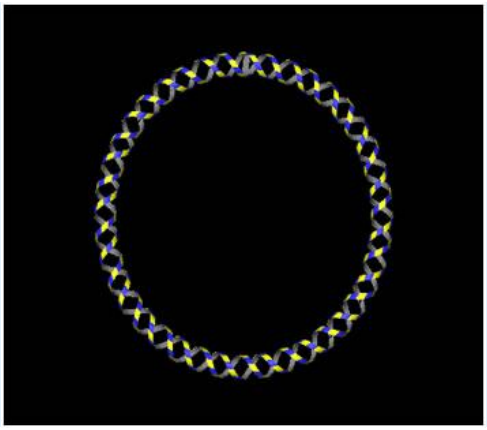
- **relaxed** plasmids are maintained in multiple copies per cell.
- **stringent** plasmids have low copy number

Plasmid DNA may appear in one of five conformations, which run at different speeds in a gel during electrophoresis. The conformations are listed below in order of electrophoretic mobility from slowest to fastest:

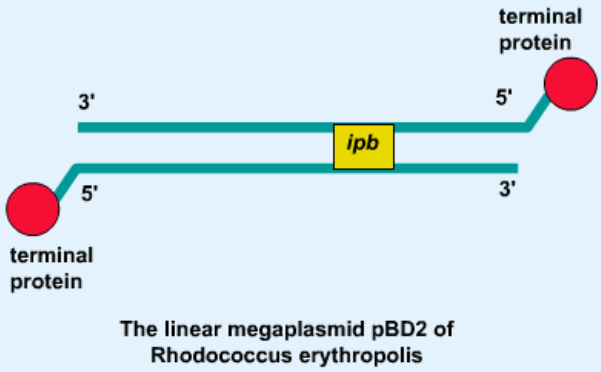
1. **Nicked Open-Circular DNA** has one strand cut.



2. **Relaxed Circular DNA** is fully intact with both strands uncut, but has been enzymatically relaxed.



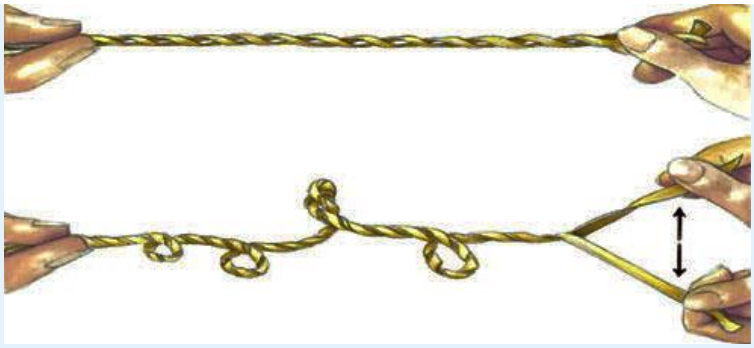
3. **Linear DNA** has free ends, either because both strands have been cut, or because the DNA was linear in vivo.




The linear megaplasmid pBD2 of Rhodococcus erythropolis

4. **Super coiled (or Covalently Closed-Circular) DNA** is fully intact with both strands uncut, and with a twist built in, resulting in a compact form.

5. **Super coiled Denatured DNA** is like **super coiled DNA**, but has unpaired regions that make it slightly less compact; this can result from excessive alkalinity during plasmid preparation.



**Alkaline lysis**

Alkaline lysis is a method used in molecular biology to break cells open to isolate plasmid DNA or other cell components such as proteins. Bacteria containing the plasmid of interest is first grown, and then lysed with a strong alkaline buffer consisting of a detergent sodium dodecyl sulfate (SDS) and a strong base sodium hydroxide. The detergent breaks the membrane's phospholipid bilayer and the alkali denatures proteins involved in maintaining the structure of the cell membrane. Through a series of steps involving agitation, precipitation, centrifugation, and the removal of supernatant, cellular debris is removed and the plasmid is isolated and purified.

## Sodium dodecyl sulfate:

Sodium lauryl sulfate (SLS), sodium laurilsulfate or sodium dodecyl sulfate (SDS or NaDS) ($C_{12}H_{25}SO_4Na$) is an anionic surfactant. The molecule has a tail of 12 carbon atoms, attached to a sulfate group, giving the molecule the amphiphilic properties required of a detergent. SLS is a highly effective surfactant and is used in any task requiring the removal of oily stains and residues. SLS has not been proven to be carcinogenic when either applied directly to skin or consumed. It has however been shown to irritate the skin of the face with prolonged and constant exposure (more than an hour) in young adults. Proteins are contaminating agents in any type of DNA isolation so as in plasmid DNA isolation also. They can interfere with the final product and result in low yield. SDS is used to denature the proteins and facilitate the DNA purification process.

Agarose gel electrophoresis is a powerful separation method frequently used to analyze plasmid DNA. The agarose gel consists of microscopic pores that act as a molecular sieve. Samples of DNA can be loaded into wells made in the gel during molding. When an electric field is applied, the DNA molecules are separated by the pores in the gel according to their size and shape. Generally, smaller molecules pass through the pores more easily than larger ones. Since DNA has a strong negative charge at neutral pH, it will migrate towards the positive electrode in the electrophoresis apparatus. The rate at which a given DNA molecule migrates through the gel depends not only on its size and shape, but also on the type of electrophoresis buffer, the gel concentration and the applied voltage. Under the conditions that will be used for this experiment, the different forms of the same plasmid DNA molecule have the following rates of migration (in decreasing order): Super coiled > linear > Nicked Circles >dimer > trimer > etc.

**Requirements for plasmid isolation:**

- Micro centrifuge
- Water bath (37°C)
- Automatic micropipettes with tips
- 95-100% isopropanol Ice

**Buffers and Solutions**

- Alkaline lysis solution I
- Alkaline lysis solution II
- Alkaline lysis solution III
- Antibiotic for plasmid selection
- Ethanol
- Phenol: chloroform (1:1, v/v)
- STE
- TE (pH 8.0) containing 20 μg/ml RNAse A
-

**Media**

- Rich medium

**Recipes for buffers, solutions and media:**

**Alkaline Lysis Solution I**
50 mM glucose
25 mM Tris-Cl (pH 8.0)
10 mM EDTA (pH 8.0)
Prepare Solution I from standard stocks in batches of approx. 100 ml, autoclave for 15 minutes at 15 psi (1.05 kg/cm2) on liquid cycle, and store at 4°C.
(For plasmid preparation.)

**Alkaline Lysis Solution II**

0.2 N NaOH (freshly diluted from a 10 N stock)
1% (w/v) SDS
Prepare Solution II fresh and use at room temperature.
(For plasmid preparation.)

**Alkaline Lysis Solution III**

5 M potassium acetate, 60.0 ml
Glacial acetic acid, 11.5 ml
H2O, 28.5 ml
The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate. Store the solution at 4°C and transfer it to an ice bucket just before use.
(For plasmid preparation.)

**EDTA**

To prepare EDTA at 0.5 M (pH 8.0): Add 186.1 g of disodium EDTA•$2H_2O$ to 800 ml of H2O. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (approx. 20 g of NaOH pellets). Dispense into aliquots and sterilize by autoclaving. The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to approx. 8.0 by the addition of NaOH.

## Glycerol

To prepare a 10% (v/v) solution: Dilute 1 volume of molecular-biology grade glycerol in 9 volumes of sterile pure $H_2O$. Sterilize the solution by passing it through a prerinsed 0.22-μm filter. Store in 200-ml aliquots at 4°C.

## LB

Deionized $H_2O$, to 950 ml
Tryptone, 10 g
Yeast extract, 5 g
NaCl, 10 g
For solid medium, please see Media Containing Agar or Agarose.
To prepare LB (Luria-Bertani medium), shake until the solutes have dissolved. Adjust the pH to 7.0 with 5 N NaOH (approx. 0.2 ml). Adjust the volume of the solution to 1 liter with deionized $H_2O$. Sterilize by autoclaving for 20 minutes at 15 psi (1.05 kg/cm 2) on liquid cycle.

## NaCl

To prepare a 5 M solution: Dissolve 292 g of NaCl in 800 ml of $H_2O$. Adjust the volume to 1 liter with $H_2O$. Dispense into aliquots and sterilize by autoclaving. Store the NaCl solution at room temperature.

## NaOH

The preparation of 10 N NaOH involves a highly exothermic reaction, which can cause breakage of glass containers. Prepare this solution with extreme care in plastic beakers. To 800 ml of $H_2O$, slowly add 400g of NaOH pellets, stirring continuously. As an added precaution, place the beaker on ice. When the pellets have dissolved completely, adjust the volume to 1 liter with $H_2O$. Store the solution in a plastic container at room temperature. Sterilization is not necessary.

## Potassium Acetate

5 M potassium acetate, 60 ml
Glacial acetic acid, 11.5 ml
$H_2O$, 28.5 ml
The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate. Store the buffer at room temperature.

**Rich medium**

LB
YT
Terrific Broth
For solid medium, please see Media Containing Agar or Agarose.

**SDS**

Also called sodium lauryl sulfate. To prepare a 20% (w/v) solution, dissolve 200 g of electrophoresis-grade SDS in 900 ml of $H_2O$. Heat to 68°C and stir with a magnetic stirrer to assist dissolution. If necessary, adjust the pH to 7.2 by adding a few drops of concentrated HCl. Adjust the volume to 1 liter with $H_2O$. Store at room temperature. Sterilization is not necessary. Do not autoclave.

**STE**

10 mM Tris-Cl (pH 8.0)
0.1 M NaCl
1 mM EDTA (pH 8.0)
Sterilize by autoclaving for 15 minutes at 15 psi (1.05 kg/cm2) on liquid cycle. Store the sterile solution at 4°C.

**TE**

100 mM Tris-Cl (desired pH)
10 mM EDTA (pH 8.0)
(10x Tris EDTA) Sterilize solutions by autoclaving for 20 minutes at 15 psi (1.05 kg/cm 2) on liquid cycle. Store the buffer at room temperature.

**Terrific Broth**

Deionized $H_2O$, to 900 ml
Tryptone, 12 g
Yeast extract, 24 g
Glycerol, 4 ml
For solid medium, please see Media Containing Agar or Agarose.
Shake until the solutes have dissolved and then sterilize by autoclaving for 20 minutes at 15 psi (1.05 kg/cm2) on liquid cycle. Allow the solution to cool to 60°C or less, and then add 100 ml of a sterile solution of 0.17 M $KH_2PO_4$, 0.72 M $K_2HPO_4$. (This solution is made by dissolving 2.31 g of $KH_2PO_4$ and 12.54 g of $K_2HPO_4$ in 90 ml of deionized $H_2O$. After the salts have dissolved, adjust the

volume of the solution to 100 ml with deionized $H_2O$ and sterilize by autoclaving for 20 minutes at 15 psi [1.05 kg/cm2] on liquid cycle.)

**Tris-Cl**

Dissolve 121.1 g of Tris base in 800 ml of $H_2O$. Adjust the pH to the desired value by adding concentrated HCl.
pH HCl
7.4 70 ml
7.6 60 ml
8.0 42 ml
(1 M) Allow the solution to cool to room temperature before making final adjustments to the pH. Adjust the volume of the solution to 1 liter with $H_2O$. Dispense into aliquots and sterilize by autoclaving. If the 1 M solution has a yellow color, discard it and obtain Tris of better quality. The pH of Tris solutions is temperature-dependent and decreases approx. 0.03 pH units for each 1°C increase in temperature. For example, a 0.05 M solution has pH values of 9.5, 8.9, and 8.6 at 5°C, 25°C, and 37°C, respectively.

**YT**

Deionized $H_2O$, to 900 ml
Tryptone, 16 g
Yeast extract, 10 g
NaCl, 5 g
For solid medium, please see Media Containing Agar or Agarose.
To prepare 2x YT medium, shake until the solutes have dissolved. Adjust the pH to 7.0 with 5 N NaOH. Adjust the volume of the solution to 1 liter with deionized $H_2O$. Sterilize by autoclaving for 20 minutes at 15 psi (1.05 kg/cm 2) on liquid cycle.

## Procedure

1. Inoculate 2 ml of rich medium (LB, YT, or Terrific Broth) containing the appropriate antibiotic with a single colony of transformed bacteria. Incubate the culture overnight at 37°C with vigorous shaking.
2. Pour 1.5 ml of the culture into a microfuge tube. Centrifuge at maximum speed for 30 seconds at 4°C in a microfuge. Store the unused portion of the original culture at 4°C.
3. Remove the medium by aspiration, leaving the bacterial pellet as dry as possible.
4. Resuspend the bacterial pellet in 100 µl of ice-cold Alkaline lysis solution I by vigorous vortexing.
5. Add 200 µl of freshly prepared Alkaline lysis solution II to each bacterial suspension. Close the tube tightly, and mix the contents by inverting the tube rapidly five times. Do not vortex! Store the tube on ice.
6. Add 150 µl of ice-cold Alkaline lysis solution III. Close the tube and disperse Alkaline lysis solution III through the viscous bacterial lysate by inverting the tube several times. Store the tube on ice for 3-5 minutes.
7. Centrifuge the bacterial lysate at maximum speed for 5 minutes at 4°C in a microfuge. Transfer the supernatant to a fresh tube.
8. (Optional) Add an equal volume of phenol: chloroform. Mix the organic and aqueous phases by vortexing and then centrifuge the emulsion at maximum speed for 2 minutes at 4°C in a microfuge. Transfer the aqueous upper layer to a fresh tube.
9. Precipitate nucleic acids from the supernatant by adding 2 volumes of ethanol at room temperature. Mix the solution by vortexing and then allow the mixture to stand for 2 minutes at room temperature.
10. Collect the precipitated nucleic acids by centrifugation at maximum speed for 5 minutes at 4°C in a microfuge.
11. Remove the supernatant by gentle aspiration as described in Step 3 above. Stand the tube in an inverted position on a paper towel to allow all of the fluid to drain away. Use a Kim wipe or disposable pipette tip to remove any drops of fluid adhering to the walls of the tube.
12. Add 1 ml of 70% ethanol to the pellet and invert the closed tube several times. Recover the DNA by centrifugation at maximum speed for 2 minutes at 4°C in a microfuge.

13. Remove all of the supernatant by gentle aspiration as described in Step 3. Take care with this step, as the pellet sometimes does not adhere tightly to the tube.
14. Remove any beads of ethanol that form on the sides of the tube. Store the open tube at room temperature until the ethanol has evaporated and no fluid is visible in the tube (5-10 minutes).
15. Dissolve the nucleic acids in 50 µl of TE (pH 8.0) containing 20 µg/ml DNase-free RNase A (pancreatic RNase). Vortex the solution gently for a few seconds. Store the DNA solution at -20°C

**Check whether you have done all the steps listed below**

- Prepare the culture containing the desired plasmid.
- Incubate the culture for 24 hours at 37°C.
- Take the culture from the incubator.
- Transfer 1.5ml of the culture to a microfuge tube.
- Centrifuge the tube for 30seconds at maximum speed (4°C).
- Remove the supernatant.
- Add 100µl alkaline lysis solution I.
- Vortex the sample.
- Add 200µl of alkaline lysis solution II.
- Mix the sample by inverting the tube.
- Store in ice for 1 minute.
- Add alkaline lysis solution III.
- Mix by inverting the tube.
- Store in ice for 3-5 minutes.
- Centrifuge for 5 minutes at maximum speed (4°C).
- Transfer the supernatant to a fresh tube.
- Precipitate the nucleic acid by adding 2 volumes of ethanol.
- Mix by vortexing.
- Stand the tubes for 2 minutes.
- Centrifuge for 5 minutes.
- Collect the precipitated DNA.
- Remove the supernatant by aspiration.
- Stand the tube as inverted to drain the fluid away.
- Add 1ml 70% ethanol.
- Mix by inverting.
- Centrifuge for 2 minutes.
- Remove the supernatant by aspiration.

- Air dry for 3-5 minutes.
- Add TE buffer with RNAse.
- Mix by flickering.

Detect the plasmid by doing agarose gel electrophoresis.

**Lab (2)**

## AGAROSE GEL ELECTROPHORESIS OF DNA

Nucleic acids can be conveniently separated on the basis of size and density using electrophoresis. DNA is a repeating polymer and thus has a constant charge-to-mass ratio. Hence, its migration in an electric field is proportional to its mass. In gel electrophoresis, a supporting medium is used to retain the nucleic acid sample. Several supporting matrices are available, but agarose is the most commonly used due to its relative ease and safety of preparation. Because DNA is soluble in water, we first add a loading dye to the sample which will prevent the DNA from floating out of the gel. In this exercise, you will prepare and run an agarose gel and fractionate plasmid DNA samples. Several different agarose gel concentrations will be compared.

### Purpose

To gain expertise in the preparation of agarose gels for the separation of DNA samples. Each group will run the same DNA samples, but will use varying agarose concentrations.

To become familiar with photodocumentation of ethidium bromide-stained gels using the Eagle Eye system.

### *Reagents and Supplies*

Plasmid DNA samples
MW standards (1 Kb ladder; GIBCO-BRL)
10X loading dye
1X TAE buffer
Agarose
Distilled water
10 mg/ml ethidium bromide staining solution
Electrophoresis apparatus and power supply (EmbiTec, GIBCO-BRL, and Bio-Rad)
MW standards, linear digest control of pBlueScript vector (Stratagene)

## Procedures

### A. Preparation of the gel

1. Wearing gloves, insert a clean comb into a casting plate and set the mold on a level bench.

2. Add the correct amount of agarose to a measured quantity of 1X TAE buffer in a glass bottle. You will need approximately 30 ml per gel. For a 0.8% gel use 0.24 g agarose.

3. Place the cap *loosely* on the bottle, and heat the bottle in the microwave until the mixture just boils. Swirl the bottle to suspend all the agarose and reheat briefly to ensure that all granules have melted. *Leave the lid of the bottle very loose and to not boil over the agarose*.

4. Cool to 60_C (so you can hold the bottle comfortably in your hand), then pour 15-25 ml into the casting tray from the end opposite the comb. The gel should be 3-5 mm thick.

5. While the agarose is still liquid, remove any air bubbles to the end of the tray with a pipet tip.

6. After the gel is set (10-20 min) and appears translucent, remove the tape and place the gel on the platform in the gel box with the comb toward the black electrode.

7. Fill the box with 1X TAE to a level that just covers the gel. *Carefully* remove the comb as to not tear the sample well.

8. Attach the electrical leads to the gel box such that the positive electrode (*Run to red!*) is at bottom of the gel.

B. Electrophoresis

1. DNA samples already contain gel mix and are ready to load.

2. *Carefully* add 8-10 µl of a single DNA sample to a well. Dip the pipet tip through the surface of the buffer, center it over the well, and gently depress the pipet plunger slowly. Be careful not to puncture the wells or to agitate the buffer solution.

3. Attach the red electrode to the bottom of the gel and turn on the power supply. DNA is negatively charged and will migrate towards the positive (*red*) electrode.

4. Set the power to 100 V. Bubbles should slowly appear along the wire leads in the gel tank and the dye should begin to migrate toward the far end of the gel.

5. Electrophorese for about 30 min. Good separation will occur when the xylene cyanol and bromophenol blue bands have divided the gel into 3 equal parts.

6. Turn the voltage to 0, turn off the power, disconnect the leads, and remove the casting tray.

7. Stain the gel in 50-100 ml distilled water containing 0.5 µg/ml ethidium bromide (5 µl of a 10 mg/ml stock) for 5-10 minutes. ***Caution! Ethidium bromide is a powerful mutagen; wash skin immediately should you contact any solution.*** Transfer the gel to distilled water for 5 min to remove excess ethidium bromide. Seal the container of ethidium bromide staining solution for subsequent reuse.

8. View the gel on the UV transilluminator. ***Wear eye/face protection and never look at the UV light source with the naked eye*.**

**9.** Photograph the gel.

10. Compare the migration distance to the standards, and compare the migration patterns between groups using different concentrations of agarose.

11. Determine approximate DNA band sizes based on migration of the MW standards.

lab 3

**DNA Isolation**

1) **Phenol- chloroform based or Liquid Phase Organic Extraction:**

in this method the cells are lyse with high salt or NaOH and digest with protinase K , extract with Phenol : $CHCl_3$ , and ethanol precipitate.

Disadvantages: slow, labor-intensive, toxic (phenol, chloroform), fume hood required, disposal of hazardous materials required

**2) Liquid Phase Nonorganic Salt Precipitation :**

Cell membranes are lysed and proteins are denatured by detergent (such as SDS).

- RNA is removed with Rnase.
- Proteins are precipitated with salt solution.
- DNA is precipitated with alcohol and rehydrated.
- **Advantages**: fast and easy method, uses nontoxic materials, no fume hood required, no hazardous materials disposal issues, produces high-quality DNA.

**3) Solid Phase Procedures or affinity based method :**

following lysis of cell , method exploits binding of DNA to a solid support , with washing off cellular proteins, DNA is then eluted with low salt buffer.

- Uses solid support columns, magnetic beads, or chelating agents
- Solid support columns: Fibrous or silica matrices bind DNA allowing separation from other contaminants.

- Magnetic beads: DNA binds to beads; beads are separated from other contaminants with magnet.
- Chelating resins
- **Advantages**: fast and easy, no precipitation required

lab 4

**Restriction enzymes**

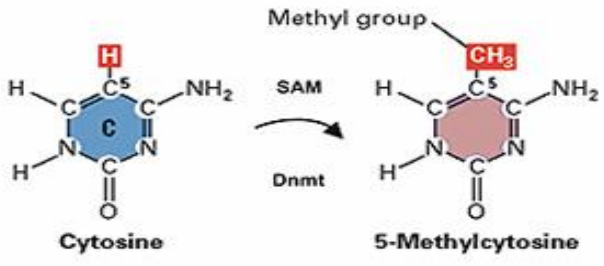
**Restriction endonucleases** are a class of enzyme that cut DNA molecules. Each enzyme recognizes a unique  sequence of nucleotides in the DNA strand, usually about 4-6 - 8  base-pairs long.

Some history :

in (1968) H.O. Smith, K.W. Wilcox, and T.J. Kelley isolated and characterized the first sequence specific restriction nuclease.

Phage growth restriction :

It had been known since the 1950's that phage particles that grow well and efficiently infect one strain of bacteria are often unable to grow well and infect other strains of the same bacterial species. In addition, phage particles that do succeed in infecting a second strain often show the opposite pattern: they are able to efficiently infect the second strain while growing only poorly in the original strain. A series of studies showed that phage particles that efficiently grow and infect host cells have DNA molecules that have been chemically modified by the addition of methyl groups to some of their adenine and/or cytosine bases, while the DNA of poorly infecting phage particles does not show this pattern of chemical modification or "methylation.“ Phage particles with unmethylated DNA do not grow and infect efficiently because their DNA molecules are cleaved and degraded by enzymes of the host cell, while methylated DNA is protected from this degradation. This phenomenon of degrading unmethylated DNA destroys the growth ability of the phage, and is responsible for the pattern of growth restriction described above. In the late1960's, scientists Stewart Linn and Werner Arber isolated examples of the two types of enzymes responsible for phage growth restriction in Escherichia coli (E. coli) bacteria. One of these enzymes methylated DNA, while the other cleaved unmethylated DNA at a wide variety of locations along the length of the molecule.



Working with Haemophilus influenzae bacteria, H.O. Smith, K.W. Wilcox, and T.J. Kelley isolated an enzyme, called HindII, that always cut DNA molecules at a particular point within a specific sequence of six base pairs. This sequence is:

5' G T ( pyrimidine: T or C) ( purine: A or G) A C 3'
3' C A ( purine: A or G) ( pyrimidine: T or C) T G 5'

5' G T T G A C 3'
3' C A A C T G 5'


- ❖ They found that the HindII enzyme always cuts directly in the center of this sequence. Wherever this particular sequence of six base pairs occurs unmodified in a DNA molecule, HindII will cleave both DNA backbones between the 3rd and 4th base pairs of the sequence
- ❖ Moreover, HindII will only cleave a DNA molecule at this particular site.
- ❖ For this reason, this specific base sequence is known as the
- ❖ "recognition sequence" for HindII.
- ✓ more than 900 restriction enzymes, some sequence specific and some not, have been isolated from over 230 strains of bacteria since the initial discovery of HindII.

**Naming :**

- ❖ reflect their origin—
- ❖ The first letter of the name comes from the genus
- ❖ The second two letters come from the species of the prokaryotic cell from which they were isolated.
- ❖ For example **EcoRI** comes from **E**scherichia **co**li **R**Y13 bacteria, while **HindII** comes from **H**aemophilus **in**fluenzae strain R**d**.
- ❖ Numbers following the nuclease names indicate the order in which the enzymes were isolated from single strains of bacteria.
- ❖ Nucleases are further described by addition of the prefix "endo" or "exo" to the name: The term "endonuclease" applies to sequence specific nucleases that break nucleic acid chains somewhere in the interior, rather than at the ends, of

the molecule. Nucleases that function by removing nucleotides from the ends of the molecule are called "exonucleases."

**Blunt ends**

Digestions of double-stranded DNA by many restriction enzymes (e.g. EcoR V) generate ends without any single-stranded sequences. Such ends are called **blunt ends**.

***sticky ends***

A fragment of DNA (often produced by a staggered cut on the DNA using restriction enzymes) in which the terminal portion has a stretch of unpaired nucleotides, and the strands are not of the same length.

Not all restriction endonucleases cut symmetrically and leave blunt ends like HindII described above. Many endonucleases cleave the DNA backbones in positions that are not directly opposite each other. For example, the nuclease EcoRI has the following recognition sequence:

5' G A A T T C 3' , 3' C T T A A G 5'When the enzyme encounters this sequence, it cleaves each backbone between the G and the closest A base residues. Once the cuts have been made, the resulting fragments are held together only by the relatively weak hydrogen bonds that hold the complementary bases to each other. The weakness of these bonds allows the DNA fragments to separate from one each other. Each resulting fragment has a protruding 5' end composed of unpaired bases. Other enzymes create cuts in the DNA backbone which result in protruding 3' ends. Protruding ends--both 3' and 5'-- are sometimes called "sticky ends" because they tend to bond with complementary sequences of bases. In other words, if an unpaired length of bases (5' A A T T 3') encounters another unpaired length with the sequence (3' T T A A5') they will bond to each other--they are "sticky" for each other. Ligase enzyme is then used to join the phosphate backbones of the two molecules. The cellular origin, or even the species origin, of the sticky ends does not affect their stickiness. Any pair of complementary sequences will tend to bond, even if one of the sequences comes from a length of human DNA, and the other comes from a length of bacterial DNA. In fact, it is this quality of stickiness that allows production of recombinant DNA molecules, molecules which are composed of DNA from different sources, and which has given birth to an industry!

lab 5

**Cloning Vectors and techniques**

Definition : **DNA cloning** – also known as molecular cloning, gene cloning and recombinant DNA technology - refers to the process of creating multiple copies of an isolated DNA fragment or fragments by in vitro or in vivo methods. It is possible to clone entire gene fragments, random portions of DNA fragments or specific DNA sequences.

Apart from DNA cloning, two other main cloning types are reproductive cloning, which is concerned with human and animal cloning, and therapeutic cloning, concerned with embryonic cloning to harvest stem cells for research and potential medical treatment purposes.

**Why :**

DNA cloning is usually performed for one of two reasons: either to produce a lot of identical DNA for further study, or to use the DNA in an intact organism to produce useful proteins**.**

**procedures for DNA cloning:**

- some steps are constant for all.
- The process of DNA cloning begins with isolating a DNA fragment or fragments of interest from the chromosomal DNA using restriction enzymes or chemically synthesized oligonucleotides.

**Isolation of insert:**

- polymerase chain reaction .
- Gel electrophorisis .
- restriction enzyme digestion -> sticky-end ligations into vector
- DNA sonication -> Klenow -> blunt-end ligation into vector.

Chemically synthesized oligonucleotides can also be used if the target sequence size does not exceed the limit of chemical synthesis.

**Cloning Vectors:**

- Plasmid;
- Phage;
- Cosmid;
- Shuttle;
- Yeast Artifical Chromosomes (YACs)
- Bacterial Artificial Chromosomes (BACs)

*Table 4.6* Insert capacities of some commonly used vector systems

| Vector system | Host cell | Insert capacity (kb) |
|---|---|---|
| Plasmid | *E. coli* | 0.1–10 |
| Bacteriophage λ | λ/*E. coli* | 10–20 |
| Cosmid | *E. coli* | 35–45 |
| Bacteriophage P1 | *E. coli* | 80–100 |
| BAC | *E. coli* | 50–300 |
| P1 bacteriophage-derived artificial chromosome | *E. coli* | 100–300 |
| Yeast artificial chromosome | Yeast | 100–2,000 |
| Human artificial chromosome | Cultured human cells | >2,000 |

Phage;

Has a chromosome with a ‘left’ arm and a ‘right’ arm, that contain all the genes needed for lysis. Between two arms, there is a disposable segment since it does not contain any lytic cycle genes. These two regions, the arms and the disposable region is separated by EcoRI sites. The lambda chromosome central region is replaced with the insert DNA (~15kb), using RE digestion and ligation.

Cosmid :

Cosmids can function asboth a plasmid in E. coli andpackaged and replicated in Phage

Shuttle Vectors :

A cloning vector capable of replicating in two or more types of organism (e.g., E.coli and yeast) is called a shuttle vector. They replicate autonomously in both hosts or integrate into them.

Yeast Artificial Chromosomes (YACs):

- Function as artificial chromosomes in yeast.
  - Linear structure with a yeast telomere (TEL) at each end.
  - A yeast centromere sequence (CEN)
  - A marker gene on each arm that is selectable in yeast .
  - Unique restriction sites for inserting foreign DNA that can be up to 500kb long.
  - An origin of replication sequence that allows the vector to replicate in yeast.
- Several hundred kb of insert DNA can be cloned in a YAC. YAC clones are made by:
  - Generating YAC arms by restriction digest
  - Ligating with insert fragments up to 500 kb in length
  - Transforming into yeast
  - Selecting for markers .

How to introduce the insert:

- Transfection with the phage lytic cycle
- Transfection with biolistic particle delivery system.
- Bacterial Transformation.

BIOLISTICS

Biolistics (other wise known as Particle Bombardment) involves directly "shooting" a piece of DNA into the recipient plant tissue. This is carried out using a gene gun. Tungsten or gold beads (which are smaller than the plant cells themselves) are coated in the gene of interest and fired through a stopping screen, accelerated by Helium, into the plant tissue. The particles pass through the plant cells, leaving the DNA inside.

This method can be used on both monocotyledonous and dicotyledonous species successfully. It is again a relatively simple laboratory proceedure. The transformed tissue is selected using marker genes such as those that code for antibiotic resistance. Whole plants are then regenerated from the totipotent transformed cells in culture, containing a copy of the transgene in every single cell (Nottingham, 1998).

**.** The particles are fired through the gas acceleration tube, through the rupture disk and into the target tissue.

Bacterial Transformation:

1) **Heat shock:** ice-cold $CaCl_2$ treated cells. Transformation frequency 1 in 1000. Transformation efficiency $10^7$ to $10^8$ transformants/ug of DNA.

2) **Electroporation:** 50ul, 2500 volts, 200 ohms, 25uFarads, 4.6 milliseconds. Transformation efficiency $10^9$ for ~3kb DNA and $10^6$ for ~136 kb DNA.

3) **Conjugation:** Transformation for organisms that are hard to transform.

**How to detect if the gene of interest was transformed ?**

**1) Colony Lift Hybridization to Find a Cloned Sequence in a Plasmid (or Cosmid) Library**

- The presence of a clone containing a specific sequence can be determined by making a “lift” of the colonies, lysing the cells on the surface of a “membrane”, and hybridizing a labeled (radioactive) “probe” of the sequence being searched for.

## 2) Finding a Cloned Sequence: Plaque Lift Hybridization in a Lambda Library :

If a library is made in phage l, the desired sequence can be found by hybridizing a probe to a "lift" of the plaques. This is detected with a probe, as in the colony lift.

## 3) **Antibodies**

- Antibodies can be (in theory, at least) be produced which react with any molecule.
- If a protein is injected into a rabbit (or goat, or sheep, etc.) the blood isolated from the injected animal will have antibodies against the injected protein.
- Mono-clonal antibodies are produced from cells grown in tissue culture, and can be "made" to have antibodies to any protein sequence.
- Sometimes the goal of cloning is to express protein for the production of antibodies.

Induce the protein expression of the plasmid library in bacteria, lyse cells and attach proteins to membrane.

Use primary antibody, secondary antibody linked HRP or AP, and fluorescent substrate

Detection by colorimetric stain or X-ray film

Or use an enzymatic or protein activity assay to screen

## 4) Screening gDNA library for enzymatic activity