# Biochemical and Gene Techniques تقنيات كيموحيوية و جينية

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المادة تقنيات كيموحيوية و جينية / ( المنهج النظري) الخاص ب
تقنيات الجينات (جزء الـDNA)
المرحلة الثالثة صباحي
الكورس الاول
قسم التقنيات الاحيائية
عدد المحاضرات / (١٠) عشر محاضرات

## Overview

- An overview of the course.
- A review of functions and the basic biochemical principles of DNA.

- Gene Technology
  - Optimization of cloned genes expression
  - Control the cloned gene expression under transcription.
  - Control the cloned gene expression under translation.
- Translation in heterologous backgrounds.

### Overview of the course

In this course: **biochemical and gene techniques** we will study all the Techniques deals with the DNA, then all the Techniques deals with Protein of the cell.

## Lecture One:

- **The Structure and Function of DNA**

### Overview:

### The Molecules of Life:

Most macromolecules are polymers, built from **monomers**. The monomers may be the same or slightly different.

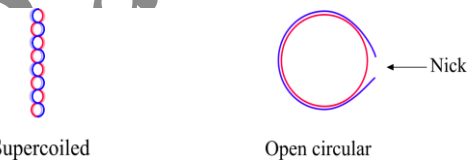
- A **polymer**: is a long molecule consisting of many similar or identical building blocks linked by covalent bonds. The repeated units are small molecules called monomers.

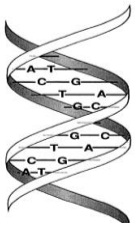
Eukaryotes: Organisms other than the bacteria, such as the plants, insects, and animals. They have a cell nucleus and other membrane-bound organelles.

Prokaryotes: A group of organisms lack a cell nucleus or any other membrane-bound organelles. Prokaryotes belong to two taxonomic domains: the bacteria and the archae. The genomes of prokaryotic organisms are generally composed of a DNA molecule. That is, rather than having free 5′- and 3′- ends, and a number of extra-chromosomal DNA molecules, called plasmids.

- The viruses have a genome composed of either double stranded DNA, single-stranded DNA or RNA, depending on the type of virus.

Linear L, Supercoiled SC, Covalently Closed Circular CCC, Open Circular OC



Although the DNA found in chromosomes (type B) is the most popular type of double-helix structure scribed by Watson and Crick in 1953, there are several other forms of DNA.

## Lecture Two:

**Nomenclature:**

nucleoside - purine or pyrimidine base linked to pentose sugar

ex: Nucleoside- adenosine, guanosine, thymidine, and cytidine- are the terms given to the combination of base and sugar

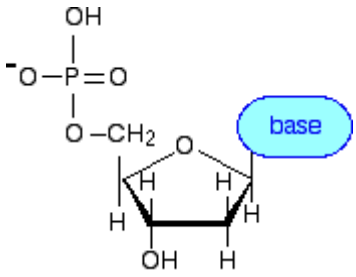
nucleotide - phosphate ester of nucleoside

- Ncleotide has three parts:

Nitrogenous base - pyrimidine (cytosine, uracil, thymine) or purine (adenine or guanine)

- Ribose sugar is found in RNA, Deoxyribos sugar is found in DNA.

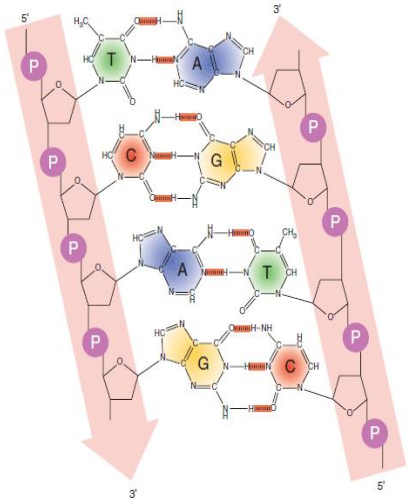
In DNA and RNA the individual nucleotides are joined by a 3"-5" phosphodiester bond. Also Watson and Crick model of DNA structure predicts that the two polynucleotide chains are held together by non-covalent hydrogen bonds.




nucleotide

**"Chargaff's Rules."** In the early 1950s, Chargaff pointed out that the amount of adenine always equaled the amount of cytosine. This was true for DNA purified from a wide variety of organisms, and true regardless of the total G+C (or A+T) content. This condition is met by having two strands of DNA in which the bases are hydrogen bonded with strict complementary base pairing. Specifically, A only pairs with T, and G only pairs with C.

Ex. **These two sequences are complementary to each other:**




**Strand one:** 5′-TCAGATGATCAGTACG-3′

**Strand two:** 3′-AGTCTACTAGTCATGC-5′

## Reversible Denaturing of DNA

- Double-stranded DNA is an immensely stable molecule.
- A major consequence of the non-covalent forces that hold the double helix together is that the two constituent strands of DNA may be separated (denatured) simply by heating.

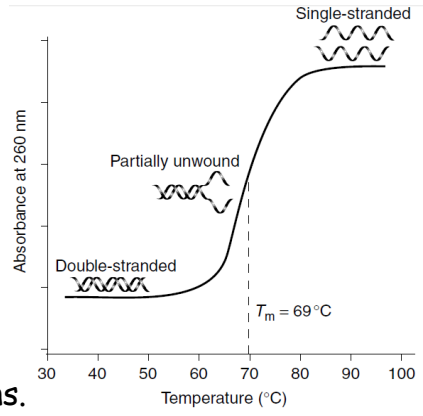
The thermal energy provided by heating a DNA sample will break the relatively weak hydrogen bonds connecting the two strand of the helix, but will not affect the covalent linkages that hold each strand together.
The separation of the two DNA strands in a helix is accompanied by **changes in the physical properties of DNA**.

**One of these changes**: is the way in which DNA absorbs UV light. DNA absorbs light at a wavelength of 260 nm due to the presence of alternating single and double bonds in the DNA bases.

A solution of double-stranded, native DNA, with a concentration of 0.05 mg/mL, has an absorbance (or optical density, OD) of about 1.0 at the 260 nm peak.
When a DNA helix is denatured to become single strands, e.g. by heating, the **absorbance increases**.

1.0 A260 nm double-stranded DNA = 50 µg/mL
1.0 A260 nm single-stranded DNA = 33 µg/mL

The increase in absorbance as double-stranded DNA becomes single stranded, called the **hyperchromic** effect.




The melting temperature **(Tm)**: is the temperature at which half the DNA strands are separated.

The melting temperature of all DNA molecules is not the same, depends upon
*1)* the length of DNA, and
*2)* the proportion of GC and AT base pairs that it contains.

$$Tm = 2(AT) + 4(GC)$$

## Lecture Three:

- **Gene Technology**
  - **Optimization of cloned genes expression**
  - **Control the cloned gene expression under transcription and translation.**

**Nomenclature:**

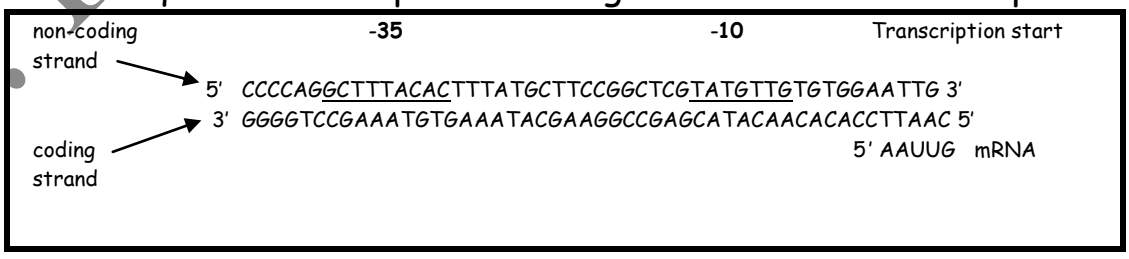
- In order to maximize production of proteins from cloned genes it is essential that both transcription and translation are optimized for an appropriate microbial host.
- Genetic manipulations necessary to circumvent post-translational barriers to high level expression of genes in heterologous backgrounds and to assist in the purification of desired proteins from microbial cells are also considered.

### 1-TRANSCRIPTION OF CLONED GENES

### Promoters and the regulation of gene expression:

**Promoters**: are DNA sequences that direct the binding of RNA polymerase to DNA for the initiation of mRNA synthesis. **Measurements** of the amount of mRNA and the protein it encodes indicate that there is considerable variation in the strength of promoters. Sequencing of a number of *E. coli* promoters has revealed structure similarities that are now regarded as characteristic features:

DNA sequence in the promoter region of the *E. coli lac* opero



There are two highly conserved regions, one at about 35 bases upstream (the -35 region) and one at about 10 bases upstream (the -10 region or Pribnow box) of the start of transcription.

- The spacing between the -35 and -10 regions, which ranges in natural promoters from 16 to 19 nucleotides, also seems to be critical in determining promoter strength. A spacing of 17 nucleotides would appear to be ideal.

The RNA polymerase homoenzymes of both species comprise several subunits designated and, although the sizes of the components and their functions vary. Recognition and selection of promoters in *E. coli* are dependent upon the direct interaction of the sigma ( 6 ) factor with specific DNA sequences.

The sigma subunit of *B. subtilis* RNA polymerase is smaller than that of *E. coli* . Vegetative and sporulating cells of *B. subtilis* contain forms of polymerase that differ with respect to their promoter recognition specificity.

**some controllable promoters for use in expression vectors**

| | Promoter | Source | Operational control | |
|---|---|---|---|---|
| | | | Off | On |
| *E. coli* | *lac* | *E. coli lac* operon | - | IPTG in medium |
| | *trp* | *E. coli trp* operon | Tryptophan in medium | Indoleacetic acid in medium |
| *B. subtilis* | *bla* | *Bacillus licheniformis* β-lactamase gene | - | β-lactamase in medium |

## General solution to the problem of controlling potentially lethal gene expression

May be to exploit the regulatory properties of anti-sense mRNA, are produced by convergent, overlapping transcription of a gene sequence. It seems equally likely that base pairing of the mRNA from the gene sequence with its complementary anti-mRNA species might inhibit subsequent translation.

## Lecture Four:

**Increases in transcription efficiency can be achieved by:**

1. Expression of genes can be controlled by placing genes downstream of known strong promoter.
2. Deleting regulatory sequences from control regions. for example removal of the attenuator region of the *trp* operon leads to a 7- to 8- fold increase in transcription efficiency from the *trp* promoter.
3. The amount of transcription of cloned genes may be increased further by placing the desired sequence downstream of tandem sequences.

**Note:"** Short tandem repeats (**STRs**), which are sometimes referred to as micro-satellites or simple sequence repeats (**SSRs**), are accordion-like stretches of DNA containing core repeat units of between two and seven nucleotides in length that are tandemly repeated from approximately a half dozen to several dozen times"

- **Transcription terminators vary considerably in strength**.
  The termination of transcription is an important factor in optimizing gene expression. Precise termination at the end of a cloned gene prevents long and wasteful transcripts being made.

Transcriptional termination signals in bacterial DNA typically contain GC-rich sequences of variable length (from 3 to 11 consecutive GC bases) and an inverted repeat sequence immediately preceding the actual point of termination.

**Lecture Five:**

## 2-TRANSLATION OF CLONED GENES:

- **Control of translation**

  - In *E. coli* the ribosome-binding site consists of an initiation codon (AUG or less commonly GUG) ,and
  - A sequence typically of 3 to 9 nucleotides called the Shine-Dalgarno (S-D) sequence

- **Coding usage**

With notable exceptions, the same triplet codons encode the same amino acids in different organisms.

The genetic code provides two or more options for all the amino acids except **methionine** and **tryptophan**, which each have a single codon.

It is evident from sequence studies of many prokaryotic, eukaryotic and viral mRNA species that there are marked preferences in the use of alternative codons.

Codon usage depends both upon: 1) the genome from which the message originates and, 2) the degree of expressivity of that message.

For example, CCG (as opposed to CCA, CCC or CCU) is the favoured codon for proline in bacteria, and CUG (as opposed to CUA, CUC, CUU, UUA or UUG) is strongly favoured as the codon for leucine in both bacteria and animal cells. Furthermore, preference for both CCG and CUG is stronger in mRNA species from genes that are normally highly expressed. Codon preferences are believed to reflect molecular optimization of codon-anticodon pairing energies.

- **Termination of translation**

Translation originating upstream of ribosome-binding site used for the expression of cloned genes may lead to undesired gene fusion products or interfere with translation of the target sequence itself.

## Lecture 6:

# Nucleic acid purification, then detect the DNA damage

Learning Goals

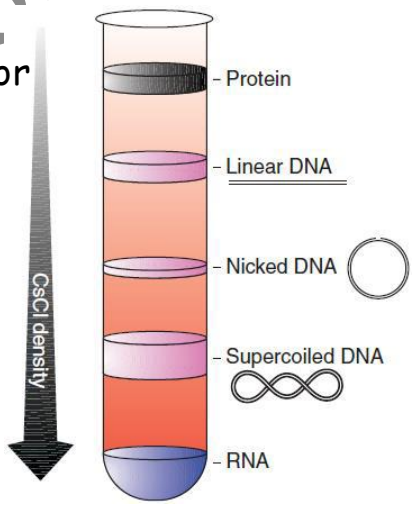
1) Learn how to treat bacteria to take up plasmid DNA.
2) Understand the quantification method for detect the DNA damage (ex. Comet assay technique)

## Overview

purification or isolation of nucleic acids is the first step in most molecular biology studies and all recombinant DNA techniques.

## 1) CsCl (Cesium Chloride) Centrifugation of DNA:

CsCl (cesium chloride) centrifugation is a method for separating DNA based on density.
Nucleic acids can be concentrated by high speed centrifugation for 48 h in an EtBr-CsCl gradient after alcohol precipitation and resuspension.




- Intercalation of EtBr alters the swimming density of the molecule in high molar.
- If DNA of only one density is present, the result will be a single band of DNA.
- If two DNAs are present with different densities, the result will be two bands of DNA, Covalently closed circular molecules will accumulate at lower densities in the CsCl gradient because they incorporate less EtBr per base pair compared to linear molecules.
- The hydrophobic EtBr is then removed with appropriate hydrophobic solvents after extraction.
- The purified nucleic acid will be re-precipitated with alcohol.

## 2) Solid-phase Nucleic Acid Extraction:

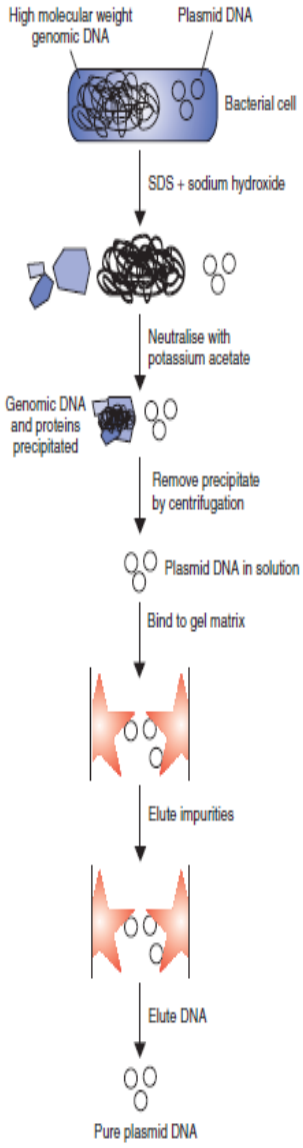
Solid phase system will absorb nucleic acid in the extraction process depending on the pH and salt content of the buffer.

The absorption in solid-phase extraction method based on (The principle) :

hydrogen-binding interaction
with a hydrophilic matrix ionic exchange
under aqueous conditions
by means of an anion exchanger.

Four key steps involved in solid-phase extraction are:

cell lysis, nucleic acids adsorption, washing, and elution.




- The initial step in a solid phase extraction process is to condition the column for sample adsorption.
- Column conditioning can be done by using a buffer at a particular pH to convert the surface or functional groups on the solid into a particular chemical form.
- Next, the sample which has been degraded by using lysis buffer is applied to the column.The desired nucleic will absorb to the column with the aid of high pH and salt concentration of the binding solution.

Other compounds, such as protein may have strong specific bond with the column surface as well.

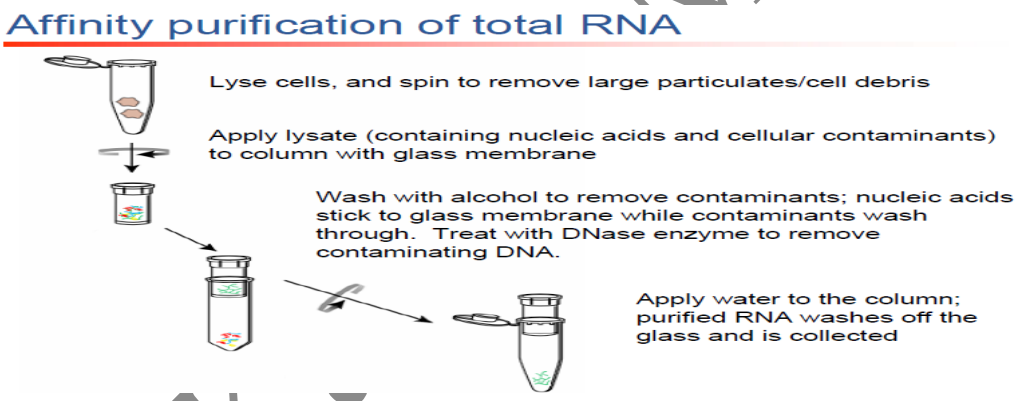
- These contaminants can be removed in the washing ste using washing buffer containing a competitive agent.
- For the elution step, TE buffer or water is introduced release the desired nucleic acid from the column, so that it can be collected in a purified state.

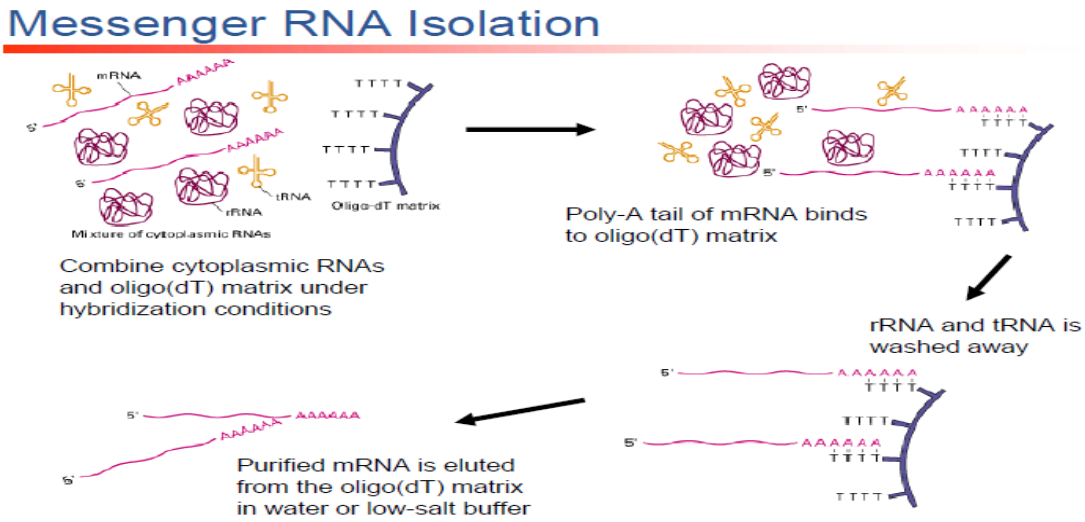
**Note:** Normally, rapid centrifugation is required during the washing and elution steps of purification process.

## 3) oligo(dT) affinity chromatography to isolate mRNA:

Total RNA from eukaryotic sources includes: ribosomal RNA(rRNA), mRNA, tRNA, and other smaller RNAs.
The majority of cellular RNA (about 80%) is ribosomal; represents mRNA just (1 – 5 %) of cellular RNA.
Total RNA can be used in most applications, particularly if oligo (dT) primers or other molecules are used to select poly(A) + mRNA.

- mRNA molecules have a tail of A's at the 3' end (polyA tail)
- Oligo(dT) probes can be used to purify mRNA from other RNAs
- mRNA can be eluted from oligo(dT) matrix using water or low-salt buffer.



## Lecture Seven:

### Overview

The quantification method for detect the DNA damage (ex. Comet assay technique ) which described by Ostling and Johanson as a method of DNA strand break analysis.

**Comet assay technique** Also known as single-cell gel electrophoresis (**SCGE**): Efficient method to detect DNA damage quantification including single and double stranded DNA breaks. Its simplicity, sensitivity, speed and economy make it a prime choice in genotoxicity testing, ecogenotoxicity, human epidemiology studies as well as mechanistic studies of DNA damage and repair.

**Principle action**: the gel electrophoresis of single cell (SCGE) has provide information about DNA damage. DNA migration in an electic field, cell with increased DNA damage display increased migration of chromosomal DNA from the nucleus toward the anode, which resemble the shape of a comet, then staining and acquiring image analysis.

**Potential applications**

**Applications:** in vitro, in vivo and clinical use:

One of the primary uses of the comet assay is in genotoxicity testing. It is provides a set of information about safety and toxicology on newly developed pharmaceuticals and chemicals. Since some drugs are prodrugs and therefore become active only after hepatic metabolism, metabolites can be assessed for their genotoxicity potential. The comet assay is not only useful in the assessment of chemical toxicities but it can also allow the study of the protective effect that some phytochemicals have on genes when exposed to genotoxic agent. Since dietary factors have been estimated to contribute up to 30-40% factor, human nutrition effect on cancer development is an important element to consider. In these studies, the comet assay is a simple cheap a rapid method to assess DNA damage and repair.

## Lecture Eight:

# Nucleic Acids Detection Techniques

Learning Goals

1) Learn how to detection of genetically modified organisms (GMO)
2) Technique for Quantitative detaction of specific DNA sequences.
3) Identify which of genes are differentially expressed using Microarray techniques.

## I. Blotting:

- **Southern blotting technique** : This technique used to detection of a specific DNA sequence within a mixture of DNAs present in different plant species, or in different microorganisms species.
- **Northern blotting technique** : This technique used to detection of specific RNA sequence within a mixture of RNAs, used to find gene expression, and regulation of specific gene.

### Advantages:

- A blot can prove: 1)whether that one species of RNA or DNA is present, 2)how much is there, 3) and its approximate size.
- Identify infectious agents present in the sample.
- Identify inherited disease.

### Disadvantages:

- The process is a complex, cumbersome and time consuming one.
- It requires electrophoretic separation.
- Only one gene or RNA can be analysed at a time.
- Gives information about presence of DNA, RNA or proteins but does not give information about gene interaction.

**General principle:**

The blotting methods are fairly simple and usually consist of four separate steps:

- electrophoretic separation of nucleic acid fragments in the sample;
- transfer to and immobilization on paper support; (typically nitrocellulose or activated nylon)
- binding of analytical probe(radioactive probe) to target molecule on paper; and incubation.
- visualization of bound probe by exposing the membrane to X-ray film produces darkening at a spot correlating with the position of the DNA or RNA of interest. When the darker↑ in the spot, means the ↑nucleic acid present there.

**Molecules in a sample are first separated by electrophoresis:**

Restriction endonucleases are used to cut high molecular weight DNA strands into smaller fragments, which are then electrophoresed on an agarose gel to separate them by size. Although RNA is single-stranded, RNA molecules often have small regions that can form base-paired secondary structures. To prevent this, the RNA is pre-treated with formaldehyde.

**A. ] DNA Gel Electrophoresis**

**Principle:**

- Gel electrophoresis is used to separate DNA fragments according to their size and charge.
- DNA is negatively charged because of the phosphate back bone.
- Different sizes of these fragments will cause variation in migration through the gel, allowing smaller fragments to move faster than the larger ones.

- Two types of gels can be used for gel electrophoresis; Agarose gel and acrylamide.

**B. ] Transfer to Solid Support**

After the DNA, RNA, or has been separated by molecular weight, it must be transferred to a solid support before hybridization. (Hybridization does not work well in a gel) This transfer process is called blotting and is why these hybridization techniques are called blots. Usually, the solid support is a **sheet of nitrocellulose paper** (sometimes called a filter because the sheets of nitrocellulose were originally used as filter paper) DNA, RNA stick well to nitrocellulose.

**The DNA, RNA can be transferred to nitrocellulose in one of two ways:**

1) Electrophoresis, which takes advantage of the molecules' negative charge.

2) Capillary blotting, where the molecules are transferred in a flow of buffer from wet filter paper to dry filter paper.

**Note:** In a Southern Blot, the DNA molecules in the gel are double-stranded, so they must be made single stranded in order for the probe to hybridize to them. To do this, the DNA is transferred using a strongly alkaline buffer, which causes the DNA strands to separate - this process is called denaturation - and bind to the filter as single-stranded molecules. RNA bind to filter without this pre-treatment.

**C.] Hybridization stages:**

- **Hybridization principle:**

The nucleic acid hybridization is the process where in two DNA or RNA single chains from different biological sources, make the double catenary configuration, based on nucleotide complementarity and of contingent sequence homology of the

two sources, resulting DNA-DNA, RNA-RNA or DNA-RNA hybrids.

- In most cases, the purpose of the hybridization techniques is identification or localization of certain nucleic acid sequences in the genome of some species. **Two basic notions are used:** *1)*the target molecule representing the DNA, RNA sequence that should be identified and *2)*the probe molecule who identify the target, by hybridization.
- When hybridization takes place on a solid carrier is named blotting.

**4. Blocking**

At this point, the surface of the filter has the separated molecules on it, as well as many spaces between the lanes, etc., where no molecules have yet bound. If we added the probe directly to the filter now, the probe would stick to these blank parts of the filter. This coats the filter and prevents the probe from sticking to the filter itself. During hybridization, we want the probe to bind only to the target molecule.

So in all blots, the labeled probe is added to the blocked filter in buffer and incubated for several hours to allow the probe molecules to find their targets.

- **Washing**

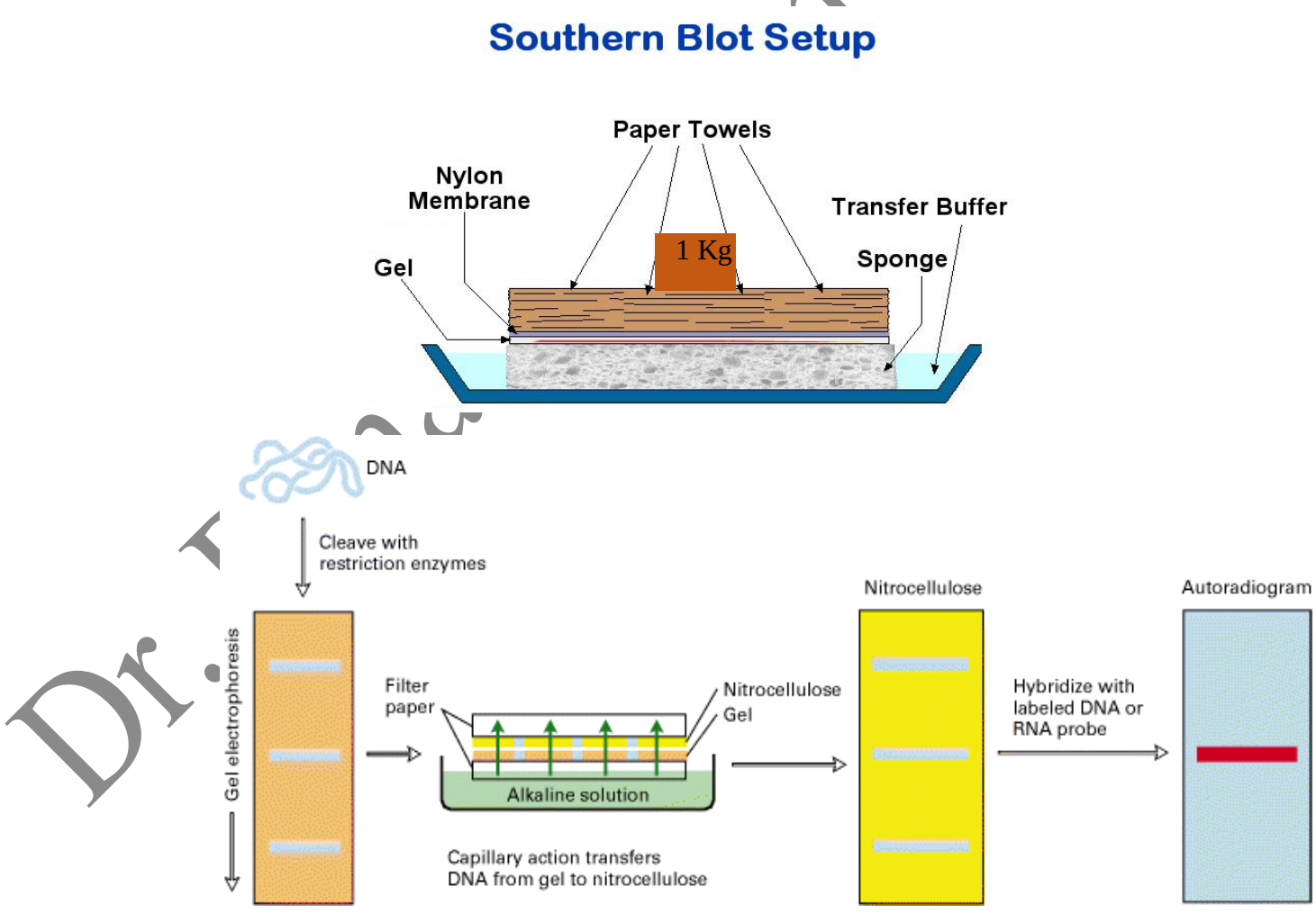
After hybrids have formed between the probe and target DNA sequence, it is necessary to remove any probe that is on the filter that is not stuck to the target molecules.

To do this, the filter is rinsed repeatedly in several changes of buffer to wash off any un-hybridized probe.

- **Autoradiography Detecting the Probe-Target Hybrids**

  If the probe is radioactive, the radioactive particles that it emits can expose X-ray film the film will be exposed wherever the probe bound to the filter will be dark spots on the film wherever the probe bound to the target DNA sequence.

| **Southern blot (DNA)** | **Northern blot (RNA)** |
|---|---|
| DNA extraction | RNA extraction |
| restriction | denaturation |
| gel electrophoresis | gel electrophoresis |
| denaturation | filter transfer |
| filter transfer | labelled probe hybridisation |
| labelled probe hybridisation | DNA |
| DNA or RNA | detection |



## II. PCR (polymerase chain reaction):

PCR technique used in molecular biology to amplify a single copy or a few copies of a piece of DNA generating thousands to millions of copies of a particular DNA sequence in vitro using themostable DNA polymerases.

It is diagnosis and monitoring of genetic diseases.

This technique was Developed in 1983 by Kary Mullis,

## PCR reaction continued

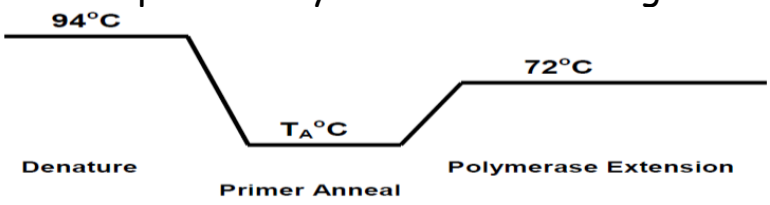
1. **Buffer**
2. **Template DNA to amplify**
3. **dNTPs**
4. **DNA Polymerase**
5. **H2O**
5. **primers**

**Synthesis PCR cycle is composed of three steps:**

**1) Denaturation**. During the denaturation step, the reaction cocktail is exposed to high temperature, usually 95°C. This high temperature will denature the DNA- meaning the two complementary strands of the DNA molecule unravel, exposing the nucleotide bases.

**2) Primer Annealing**. During the second step of each cycle, the temperature is lowered to an annealing temperature allow annealing of the primers to their complemantary targets on the DNA template (one for each DNA strand). Annealing temperature=Tm-5

**3) Extension.** The optimum reaction temperature for *Taq* polymerase (68 to 72°C). During this step, the *Taq* will bind to each DNA strand and "extend" from the priming sites (synthesize a complementary strand of the targeted DNA).



## Lecture Nine:

### III. Microarray technique:

A high throughput and versatile technology used for parallel gene expression analysis for thousands of genes of known and unknown function, or DNA homology analysis for detecting polymorphisms and mutations in both prokaryotic and eukaryotic genomic DNA.

### Types of DNA microarrays

There are currently two platforms/types of DNA microarrays that are commercially available.

1. Glass DNA microarrays which involves the micro spotting of pre- fabricated DNA fragments on a glass slide.

2. High-density oligonucleotide microarrays often referred to as a "chip" (Affymetrix) which involves *in situ* oligonucleotide synthesis.

Usually a single DNA microarray slide/chip may contain thousands of spots each representing a single gene and collectively the entire genome of an organism.

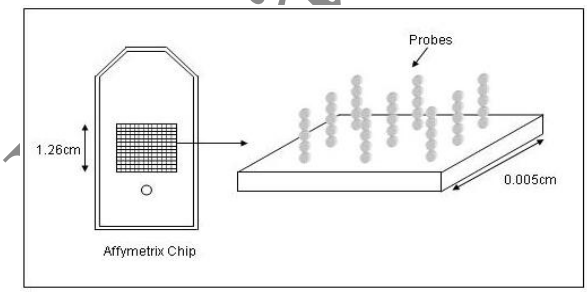



**Figure .DNA GeneChip (Affymetrix).**

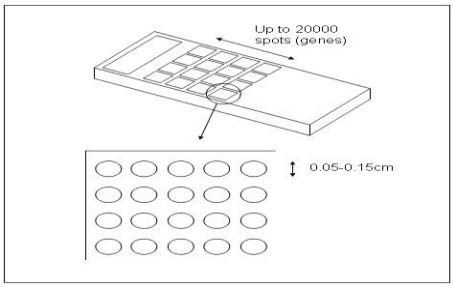



**Figure . Glass microarray.**

## Principles of DNA Microarray experiments

The principle of DNA microarray technology is based on the fact that complementary sequences of DNA can be used to hybridise immobilised DNA molecules.

This involves three major multi-stage steps; to performing a typical microarray experiment

1- Manufacturing of microarrays: This step involves the availability of a chip or a glass slide with its special surface chemistry, the robotics used for producing microarrays by spotting the DNA (targets) onto the chip or for their *in situ* synthesis.

2- Sample preparation and array hybridisation step: This step involves mRNA or DNA isolation followed by fluorescent labelling of DNA probes and hybridisation of the sample to the immobilised target DNA.

3- Image acquisition and data analysis: Finally, this step involves microarray scanning, and image analysis using sophisticated software programs that allows us to quantify and interpret the data.

## Lecture Ten:

# DNA Exchanged

Learning Goals
1) Learn how to treat bacteria to take up plasmid DNA.
2) Understand the process of transformation techniques.

**DNA can be exchanged among bacteria by three methods:**
Transformation,
Transduction
Conjugation.

Transformation: is one of the most popular techniques of molecular genetics because it is often the best way to reintroduce experimentally altered DNA into cells.

This technique was first discovered in bacteria, but other ways have been designed to transform many types of animal and plant cell.

Before 1970, there had been many attempts to transform *E. coli* cells with foreign DNA.

During the process of transformation, genes are transferred from one bacterium to another as 'naked' DNA solution. In nature, some bacteria, perhaps after death and cell lysis, release their DNA into environment. Other bacteria can then encounter the DNA, and depending on the particular species and growth conditions, take up fragments of DNA and integrate them into their own chromosomes by recombination. Transformation works best when the donor and recipient cells are very closely related. But the efficiency of transformation is very low.There are several reasons for this:

**(a)** Getting naked DNA into cells is not a trivial problem. DNA is highly charged and will not easily pass through the membranes that surround the bacterium.

**(b) What is the fate of the foreign DNA once it enters the cell?**

For the foreign DNA to be maintained and replicated with the bacterium, it must either be integrated into the bacterial chromosome, so that it will be subsequently propagated as part of the bacterial genome, or be independently replicated.

**There are two elements are required in a transformation system:**

- **Suitable host bacterium(competent cells).**
  **competent cells**: cells made permeable to take up DNA by particular chemical treatment or electrical treatment. Therefore, both linear and double stranded circular plasmids can be efficiently introduce into cell.

- The **Plasmid**: Plasmid encodes some enzymes and antibiotic resistant markers which are expressed in the bacterium after transformation.

**There are two Types of transformation:**

**Natural** and **artificial**, each process depends on the ability of the organism to transform the DNA into the host cells.

- **Natural Transformation**: bacteria are capable of DNA naturally, which means they can take up DNA from their environment directly. That kind of bacteria is called as naturally transformable.

- **Artificial transformation**: bacteria are not naturally transformable which they do not take up DNA from the environment. Bacterial cells have been exposed to particular *1)*chemical or *2)*electrical treatments to make them more permeable and then only the cells can take up DNA efficiently.

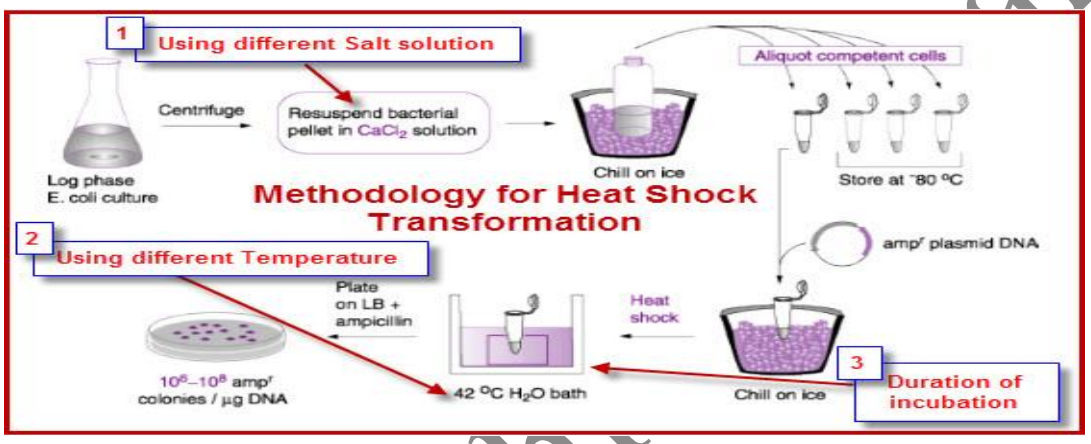
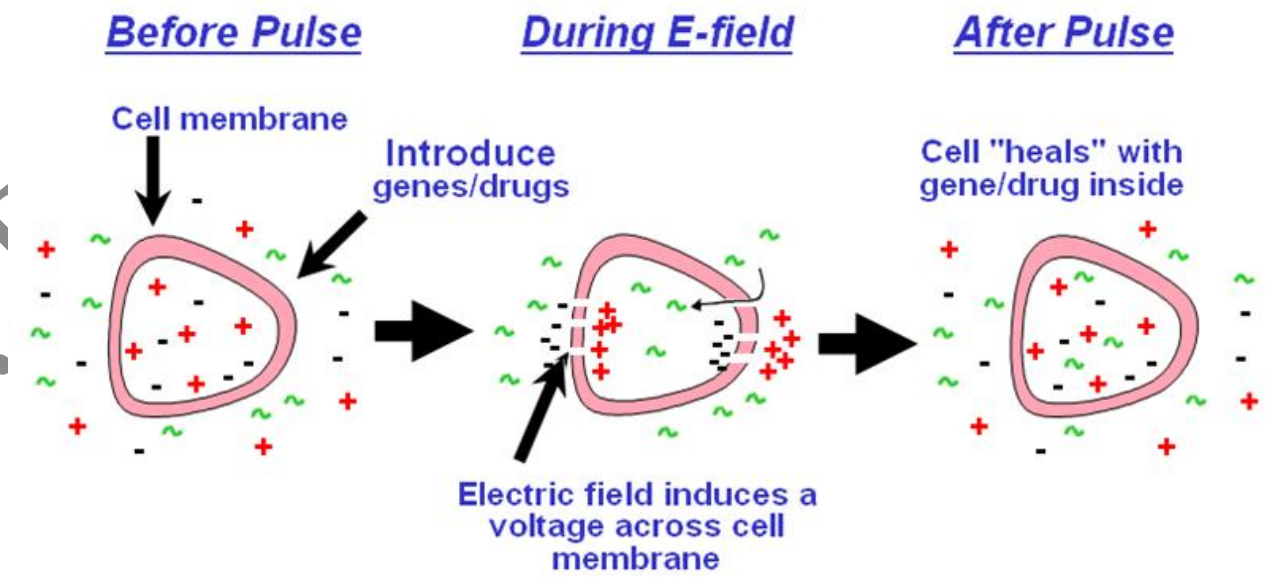



**Figure** : Overview of competence and heat shock.

2] **Electrical method for Transformation by (electroporation).**



By Electroporation technique. Cells are treated with an electrical pulse, which mediates the formation of pores. DNA can enter the cell before the pores spontaneously reseal.