Chapter XVI

Recombinant DNA Technology
• Recombinant DNA technology: known as genetic engineering

* It is the science that studies:
  - structure of genes
  - normal physiological function
  - pathological defects
  - gene therapy

* It involves
  - Isolation of the gene
  - Insertion into another organism for multiplication
  - Uses of this gene for protein synthesis
Uses of Molecular biology (biotechnology)

a) Diagnosis of diseases
b) Prediction of genetically-based diseases in offspring of next generation
c) Detection of site of defect, in the genome, in inheritable disorders
d) Gene therapy
e) Forensic purposes as disputed paternity or crime investigations.
Restriction Endonucleases

• These are **bacterial enzymes** that split the double stranded DNA chain at a specific site to obtain what's called *restriction fragments*.

• The *restriction site or the recognition sequences* is recognized as sequence of few nucleotides, usually (4 or 7 bp) that have *palendromic* arrangement; i.e. the sequence of bp in the 5' to 3' in one strand is identical to that of 5'→3' in the other.

• *DNA ligase* is used to join the cut ends of restriction fragments with other DNA molecules according to the technique used.
The cut ends may be

1. **Staggered Cut**: produces *two cohesive ends*: the resulted fragments have single stranded portion complementary to each other at the cutting site.

   
   ![Staggered Cut Example]

   
   **BamH1**

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

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

   

2. **Blunt ends**: both portions are blunted double stranded fragments.

   
   ![Blunt Ends Example]

   
   **SmaI**

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

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

   
   5'-G-G-G-T-C-
   
   3'-C-C-C-A-G-
Nomenclature of the restriction endonucleases

- Also called restriction enzymes
- Occur naturally in bacteria
- Hundreds are purified and available commercially
- Named for bacterial genus, species, strain, and type

Example: \textit{EcoRI}
- Genus: \textit{Escherichia}
- Species: \textit{coli}
- Strain: \textit{R}

- Protect bacteria from bacteriophage infection (Restricts viral replication)
- Bacterium protects it’s own DNA by methylating those specific sequence motifs.
Gel Electrophoresis of restriction site

Restriction Map
DNA Markers:

- **Single nucleotide polymorphism (SNP)**
  - Single base substitution
  - One in 500-1000 bp in noncoding region
  - One in 1000-3000 bp in coding region

- **Restriction fragment length polymorphism (RFLP)**

- **Uses of DNA markers:**
  - RFLP: marker for sickle cell anemia
  - Gene mapping
  - Finger printing
DNA amplification Techniques

- DNA cloning: in-vivo amplification of genes
- PCR: in vivo amplification
It is an in-vivo amplification of genes

• A clone is a large number of:
  – Identical molecules
  – Cells
  – Same source

• The process consists of:
  – Preparation of a gene
  – Insertion of the gene into a vector
  – Introduction of the vector into a host cell for multiplication
  – Separation of the vector from the host cell DNA
  – Isolation of the gene from the vector
DNA to be cloned is inserted into the vector, forming a "chimeric molecule" (molecule containing DNA of 2 different sources)

A vector must contain a suitable restriction site and must be capable of replication in a host cell

Types of vectors:
- Plasmid
-Viruses
- Phage
Plasmid

- Double stranded circular DNA molecule
- Found in certain bacteria
- Resistance to certain antibiotics.
- Smaller than bacterial chromosomal DNA
- Multiply independently from bacterial chromosomal DNA
- A bacterium usually contains multiple copies of the plasmid.
- DNA up to kilo base-pairs (kbp) long can be inserted into a plasmid.
Recombinant DNA
(plasmid containing the human gene)

• The plasmid is separated from the host cell.
• It is then cut by a restriction endonuclease.
• Human DNA is cut by the same RE and the part containing the required gene is isolated.
• The isolated gene is then inserted into the plasmid by the enzyme DNA ligase.
Cloning into a plasmid

Gene for antibiotic resistance

Plasmid

EcoRI

Foreign DNA

EcoRI

EcoRI

Region of interest

Hybridization + DNA ligase

Sticky ends

Recombinant DNA

DNA insertion

Bacteria cell

Bacterial chromosome

Cloning

Clone

Bacteria plated on medium + antibiotic

Only bacteria containing recombinant DNA grow

Culture

DNA purification

DNA
1. Plasmid isolated
2. DNA isolated
3. Gene inserted into plasmid
4. Plasmid put into bacterial cell
5. Cell multiplies with gene of interest

Gene for pest resistance inserted into plants
Gene used to alter bacteria for cleaning up toxic waste
Protein used to make snow form at higher temperature
Protein used to dissolve blood clots in heart attack therapy
II. Polymerase Chain Reaction (PCR)

- PCR is an *in vitro* amplification of a specific target DNA sequence(s). It needs 2 DNA *primers* (15-25 bp).
- DNA or cDNA (synthesized from RNA by reverse transcriptase enzyme) are *templates* used in the reaction.
- Each PCR cycle comprises the following 3 steps:
  - **Denaturation**: Rise of temperature to 90-95°C for separation of the 2 strands of the template.
  - **Annealing**: Temperature is decreased to 50-70°C to allow *hybridization* of the primers with the complementary single stranded template.
  - **Elongation**: The temperature is re-raised to 70-75°C to allow building up of DNA *chain* by the DNA polymerase (*Taq polymerase*; that acts at this high temperature). The four types of dNTPs (dATP, dGTP, dCTP and dTTP) should be existed in satisfactory concentrations.
- The cycle is repeated 20-30 times to allow synthesis of millions copies of the template double strands which was visualized by electrophoresis.
PCR: One cycle of amplification

Temperature

Time

25°

95°

60°

72°
Polymerase Chain Reaction (PCR)

Template (cDNA or DNA)
Primers
NTPs (ATP, GTP, CTP, TTP)
Tag polymerase

Cycle 1
- denature DNA (95°C)
- annealing step (55-60°C)
- extension step (74°C)

Cycle 2

Cycle 3
1 pg template yields
200 to 300 ng

After 30 cycles
$2^{30}$ molecules
1 pg = 1 µg
Polymerase Chain Reaction

T3 Thermocycler
Gel Electrophoresis (1/2)
Gel Electrophoresis (2/2)
Applications of PCR in medical practice

- Diagnosis of infectious diseases.
- Prenatal detection of inherited diseases as:
  - Cystic fibrosis
  - Hemoglobinopathies
  - Inborn metabolic errors.

Cystic fibrosis

- Autosomal recessive disease (Chromosome 7)
- Deletion of triple base representing phenylalanine in CFTR (cystic fibrosis transmembrane regulator)
- DNA is isolated from blood
- PCR is used for amplification of CFTR gene in DNA
- Gel electrophoresis.
- The mutant gene is of shorter length, lesser molecular weight, runs faster than the "wild" gene.
Synthesis of cDNA:
cDNA library

- Genes synthesized from mRNA by the enzyme reverse transcriptase
- mRNA is isolated from a cell involved in protein synthesis
- Complementary DNA (cDNA) is formed by:
  - mRNA mixture
  - Oligo- dT primer (4 dRN)
  - Reverse transcriptase enzyme (RNA- dependent DNA polymerase)
- It includes transcription regions of active genes in the tissue of origin
- It does not include regulatory elements or introns
- It is suitable for expression of protein in host cells
cDNA library construction

1. Anneal oligo(dT) primers of 4 bases in length to 3’ mRNA (all mRNAs in cell).
2. Add reverse transcriptase and dNTPs to initiate cDNA synthesis.
3. Add RNaseH (specific for the RNA strand of an RNA-DNA hybrid) and carry out a partial digestion.
4. Short RNA fragments serve as primers for second strand synthesis using DNA polymerase I.
short RNA fragments serve as primers for second strand synthesis using DNA polymerase I

DNA polymerase I removes the remaining RNA with its 5’ to 3’ exonuclease activity and continues synthesis

DNA ligase seals the gaps

double-stranded cDNA
Probes

• Probe is a labeled short single stranded DNA or RNA of known sequence that is complementary for the base sequence of the target DNA or RNA.

• Preparation of probes:
  - by synthetizer: The probe can be prepared in vitro, the corresponding nucleotide sequence of this probe is based on nucleotide arrangement of the target.
Probe labeling

- **Radioactive $^{32}$P:**
  Labeled probe hybridizes with the complementary sequence of interest. Hybridization is detected by autoradiography after exposure to a radiosensitive X-ray film.

- **Fluorescence.**

- **Biotin:**
  Probe is detected by an enzyme-bound avidin. Detection of the biotinylated probe by an enzyme-catalyzed reaction (alkaline phosphatase, peroxidase).
DNA is extracted from the nucleated cells, digested by the selected restriction endonuclease to find out fragment of a particular sequence, e.g. gene. Fragments are separated by agarose gel electrophoresis.

After electrophoresis, the fragments are transferred to a solid support media, nitrocellulose, by capillary effect of salt solution.
• A labeled probe in solution is added to the medium. It hybridizes with the complementary fragment transferred to the membrane.

• This fragment is identified by a method according to the label and the reporter molecule used in the probe preparation; *i.e.* either by radiosensitive film plate, colorimetric, fluorescent or antibody-labeled fluorescent reaction.
Southern Blotting

1. Restriction fragment preparation
2. Electrophoresis
3. Blotting
4. Hybridization with radioactive probe
5. Autoradiography
B. Northern blot

It is the same as southern blot but with use of RNA fragment instead of DNA.

C. Western blot

It is a technique based on the same principle like southern blot, but a protein is the primary sample, then electrophoresed, transferred to a membrane and identified by a labeled antibody.
Northern Blot Assay 1

1. Electrophoresis

2. Transfer

3. Hybridization (Probing)

4. Detection
Prenatal diagnosis of genetic disorders:

• Sickle cell anemia
Restriction fragment length polymorphism (RFLP):

Ex: MstI RFLP for Sickle-Cell detection

A
Normal
Normal β gene (β^A)
Cleavage sites for MstII

1.1 kb

Sickle-Cell
Sickle-cell gene (β^S)

Missing cleavage site

1.3 kb

Cleavage by MstII
Denaturation to single strands
Hybridization with specific probe

1.1-kb fragment

1.3-kb fragment

Extra 0.2-kb piece

Origin → AS AS AA SS

Parent carriers

Normal Sickle cell
Forensic applications:

Identification of alleged person in a crime by DNA analysis in sample left in the scene of the crime (blood, semen, or hair).

Verification of paternity:

- DNA contains short sequences called *tandem repeats* (unique to the same individual) *molecular fingerprints*.
- DNA in the provided sample is amplified by PCR.
- The products undergo cleavage by restriction endonuclease.
- PCR products are run on agarose-gel electrophoresis.
- Southern blotting may be applied to visualize the restriction bands.
Regions of Chromosome Analyzed for DNA Fingerprinting Often Contain simple tandem repeats (STRs)

Person 1

\[ \text{ACTACT} \quad \text{ACTACT} \]

EcoRI

Person 2

\[ \text{ACTACT} \quad \text{ACTACT} \]

EcoRI

EcoRI fragment from Person 2 is 900bp longer than in Person 1
III - Reverse transcription-PCR (RT-PCR)

• Uses of RT-PCR:
  Evaluation of the expression of certain gene(s) in tissues or cells.
  Diagnosis of RNA viral diseases as HCV and HIV.

• Steps of RT-PCR:
  - Total RNA can be extracted from tissues or cells, and used as a template
  - Reverse transcriptase (RT) and primers (random or oligo dT) convert RNA into complementary DNA (cDNA).
  - A small aliquot of the RT reaction is then added to a PCR reaction containing primers specific to the sequences to be amplified, Taq polymerase and dNTPs.

• The products of the RT-PCR can be then visualized by agarose gel electrophoresis.
IV-Restriction fragment length polymorphism (RFLP) analysis

• Genetic variability at a particular locus (gene) alters the behavior of the enzyme towards the restriction sites of the gene.

• Pathogenic alterations within the gene being analyzed can be due to **deletion**, **insertion** or **point mutation**.

• This alteration creates or deletes a restriction enzyme recognition site. Restriction fragments produced will be variable in length.

• Size variability in detectable fragments within a family pedigree indicates differences in the pattern of restriction sites within and around the gene being analyzed.

• A classic example of a disease detectable by RFLP is sickle cell anemia
RFLP patterns are inherited in Mendelian fashion, allowing their use in genotyping such as in cases of disputed paternity or in criminal investigations.

variable number of tandem repeats (VNTRs) polymorphism. When RE digestion cuts DNA flanking the VNTRs, the lengths of the resultant fragments will be variable depending upon the number of repeats at a given locus.

Many different VNTR loci have been identified and are extremely useful for DNA fingerprint analysis such as in forensic and paternity identity cases.
This method studies mutations and determines the pattern of gene expression.

mRNA from the cells undergoes RT reaction to generate cDNA. The latter is labeled with fluorescent agent.

Mixture of fluorescence-labeled cDNA is applied to a microarray chip containing multiple gene spots.

The expressed genes undergo variable degrees of fluorescence, but the nascent gene spot is still black (no fluorescence).

This technique is helpful to identify the cancer cells among healthy cells. In addition, lack of certain gene expression could be defined.
Chips impregnated with variable labeled antibodies could be used to identify proteins encoded and expressed by a particular gene, known as proteomics.

Proteomics are more informative than genomics in disease detection because it reflects what's going on in the cells.

Proteins derived from both normal control and patient's sample at the same time to find-out the difference(s) in the protein pattern.

This technique detects post-translational modifications that may require the activity of enzymes or chaperon proteins.
Thank you