RECOMBINANT DNA TECHNOLOGY
BBT6TH

How to Analyze results of restriction endonuclease digest

- Why it is required?
  - There should be a way to determine the number and sizes of the fragments if restriction enzymes are to be used
  - If we are using restriction enzymes, it is possible to detect whether a DNA is cut or not?
  - This was the major issue that scientists face in early years of how to analyze DNA based experiments….
  - The problem was solved in early 1970s when a technique of gel electrophoresis was developed..

How to Analyze results of restriction endonuclease digest

- Gel Electrophoresis
  - DNA has net negative charge so it migrates toward positive pole
  - Gel is made from agarose, polyacrylamide making a network of pores through which DNA molecule travel to reach positive pole
  - Separation is based on size
  - Smaller molecule move faster while large slower

- Agarose is a natural polysaccharide made from seaweed.
  - Linear polymer of repeating units of agarobiose (a disaccharide) made up of D-galactose and 3, 6-anhydro-L-galactopyranose.
  - Available as white powder
  - Can be cast easily
  - Samples can also be recovered easily
  - Gel can be stored
  - Mostly gels can be made in between 0.7% (good separation for 5-10 kb DNA) and 2% (good separation for 0.2 to 1 kb fragments)

- A 0.5cm thick slab of 0.5% agarose, which has relatively large pores, would be used for molecules in the size range **1 to 30 kb**, allowing, for example molecules of 10 and 12 kb to be clearly distinguished.
- However, a very thin (0.3 mm) 40% polyacrylamide gel, with extremely small pores, would be used to separate much smaller DNA molecules, in the range **1 to 3000 bp**, and could differentiate molecules in the length by just a single nucleotide.

Visualizing DNA molecule in a gel….

- Staining
  - Easiest way it to stain the gel with a compound that will make DNA visible
    - Ethidium bromide is used as a means of visualizing DNA
    - Bands can be shown clearly under ultraviolet irradiation after EtBr staining, so long as sufficient DNA is present.
  - Ethidium bromide intercalates into DNA and fluoresce with an orange color when exposed to UV light
    - Ethidium Bromide(EtBr) (carcinogenic) , 0.2-0.5ug/ml

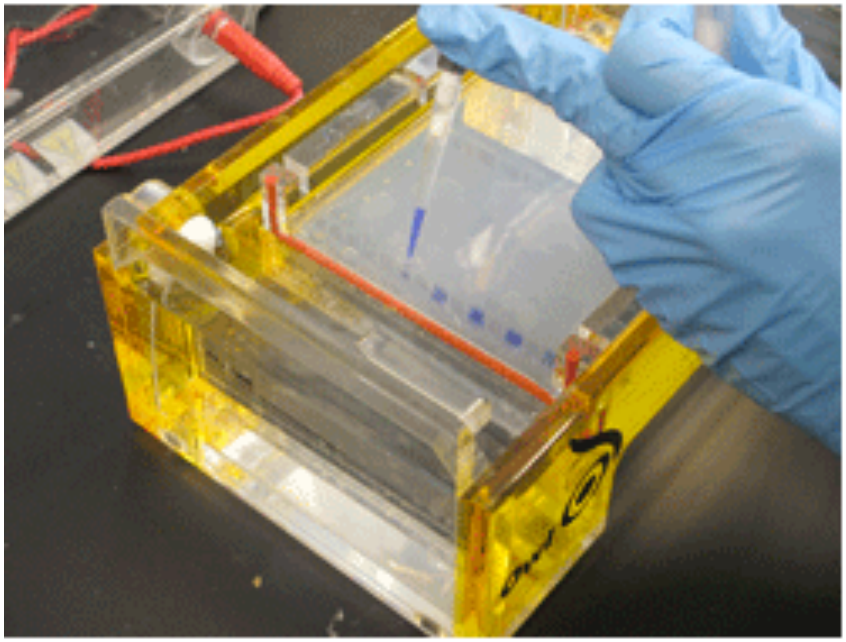
- Autoradiography of radioactively labeled DNA
  - Drawback with EtBr is that it only detects DNA if sufficient amount of DNA is present, so there is limit to its sensitivity
  - If less than 25ng of DNA is present per band then it is unlikely that the results will show up with EtBr staining, therefore more sensitive DNA detection method is required
    - **Autoradiography**

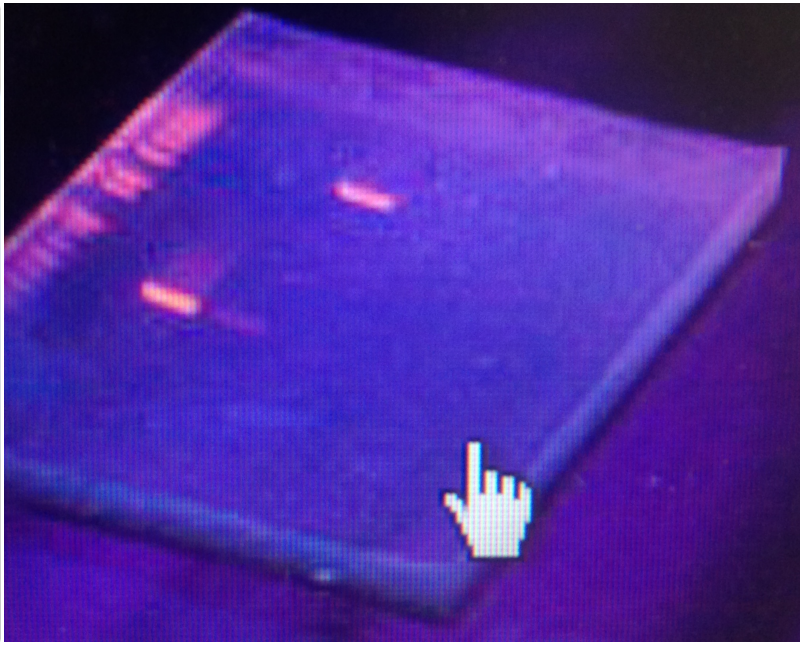
- If DNA is labeled before electrophoresis, by incorporation of a radioactive marker into the individual molecules, then DNA can be visualized by placing an X-ray-sensitive photographic film over the gel. The radioactive DNA exposes the film, revealing the banding pattern.
- DNA molecules can be labelled by incorporating nucleotides that carry a radioactive isotope of phosphorous ^{32}P, some of the methods are
  - Nick Translation
  - End-filling

- Nick Translation
  - 1977, by Rigby and Paul Berg
  - Process involves, treatment of DNA with DNAase to produce single-stranded “nick”, this is followed by replacement in nicked sites by DNA polymerase I, which elongates the 3’-hydroxyl terminus, removing nucleotides by 5’-3’ exo-nuclease activity, replacing them with dNTPs.
  - One of the incorporated nucleotides should be radiolabelled, which can then be detected.

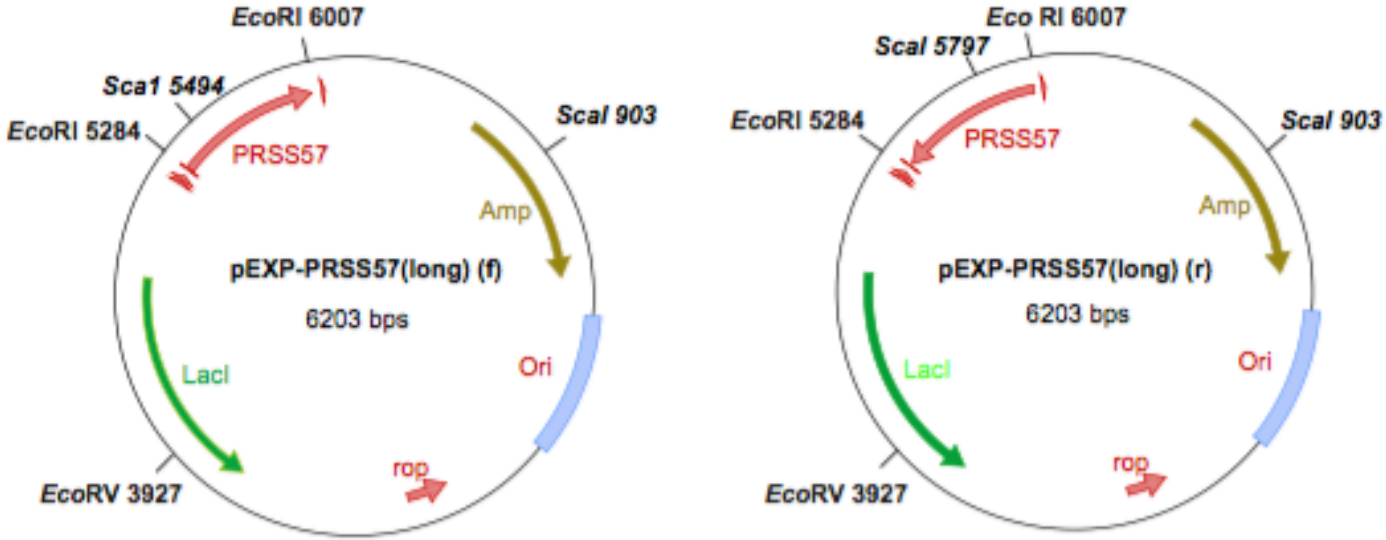
End-filling….

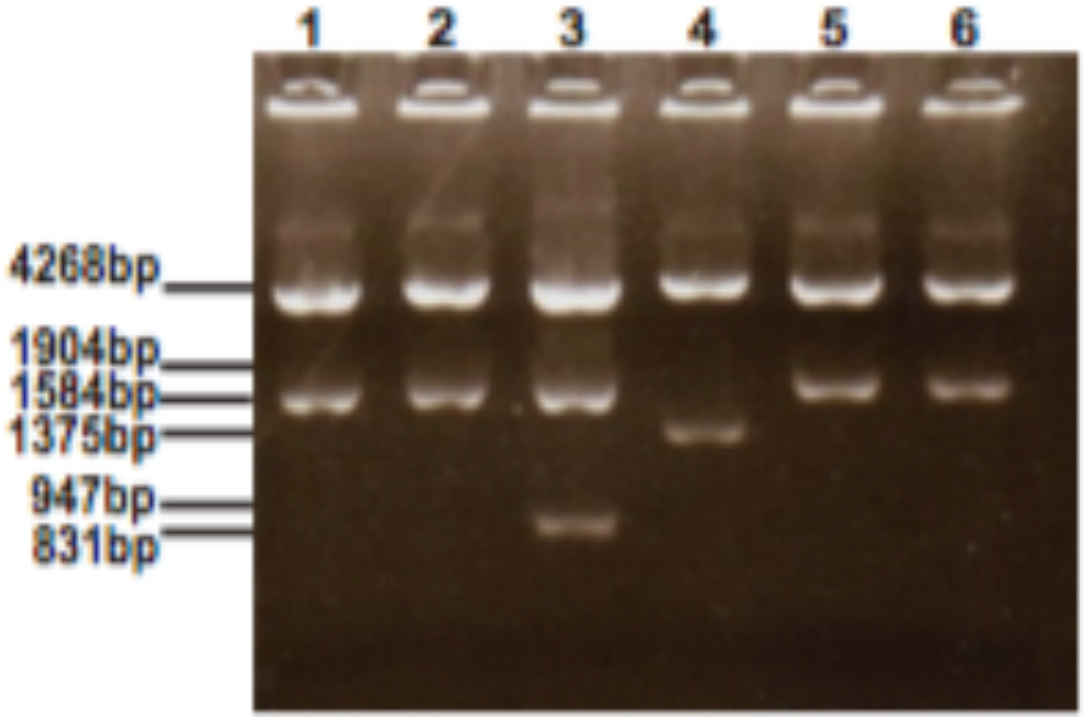
- This technique can only be used, when DNA that needs to be labeled have sticky ends. The enzymes used is the klenow fragment which fills in a sticky ends by synthesizing the complementary strand.
- Both these techniques can label DNA to such an extent that very small quantities can be detected in gels by autoradiography. As little as 2 ng of DNA per band can be visualized under ideal conditions.
- **Loading Dye**
  - Bromophenol blue
    - To make solution visible
    - To make it a bit heavier than water so that it can sit properly at the bottom of the gel





Plasmid Treatment with Restriction Enzymes





Orientational Digest with ***ScaI***

-~1612 and 4591 bp Correct Orientation
-~1306 and 4903 bp Reverse Orientation

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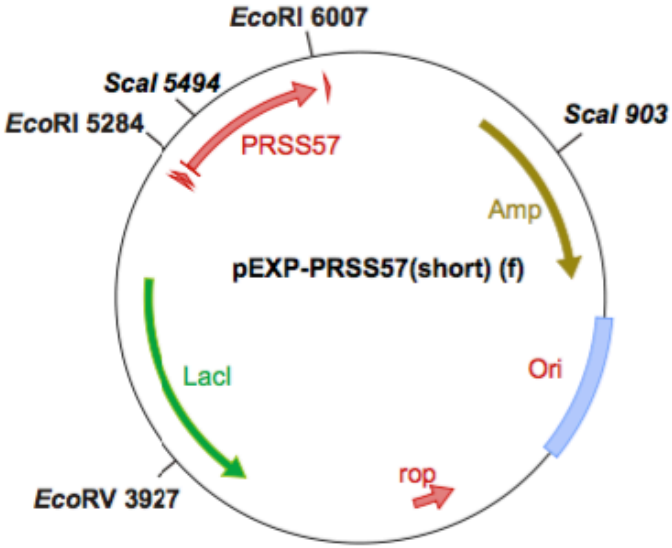
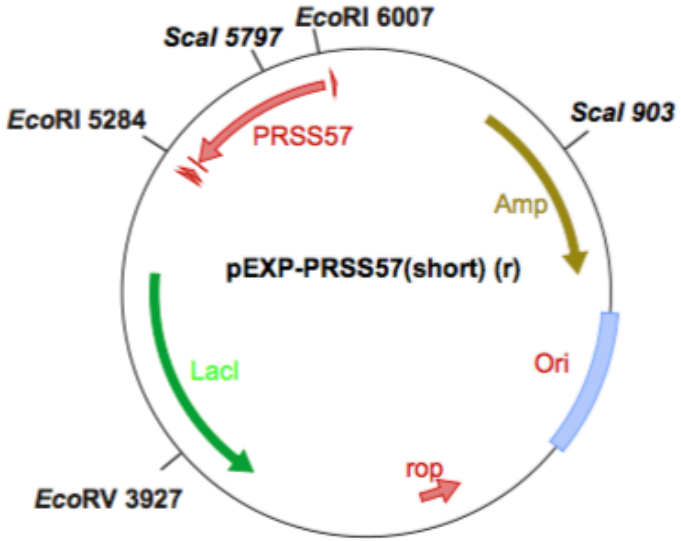




EcoRV &
ScaI

- **Size and Copy number..**
  - **Size**
    - Range from 1.0kb to over 250kb, only few are useful for cloning, less than 10kb is desirable for cloning vehicle
  - **Copy number**
    - Refers to number of molecules of an individual plasmid that are normally found in a single bacterial cell, each plasmid has a characteristic value that may be as low as one (usually for large molecules) or as many as 50 or more. For cloning purpose we need that it should be present in multiple copies so as large quantity of DNA molecule can be obtained

Plasmid Classification

- **Fertility Plasmid (F Plasmid)**
  - Carry only tra genes essential for conjugal transfer of plasmids (e.g. F-plasmid of *E.coli*)
- **Resistance plasmid (R Plasmid)**
  - Carry genes conferring on the host bacterium resistance to one or more antibacterial agents such as chloramphenicol, ampicillin etc.
  - Antibiotic resistance genes
- **Col Plasmid**
  - Code for colicins-proteins that kill other bacteria
    - Col1 of *E.coli*

- **Degradative Plasmid**
  - Allow host bacterium to metabolize unusual molecules such as toluene and salicylic acid e.g. TOL of Pseudomonas putida
- **Virulence Plasmid**
  - Confer pathogenecity on host bacterium
    - Ti Plasmid of ***Agrobacterium tumefaciens*** induces crown gall disease

Bacteriophages

- Are viruses that specifically infect Bacteria
- Simple in structure
  - Consisting of a DNA (occasionally RNA)
  - Contains number of genes essential for replication of phage
  - Surrounded by a protective coat or capsid made up of protein molecules

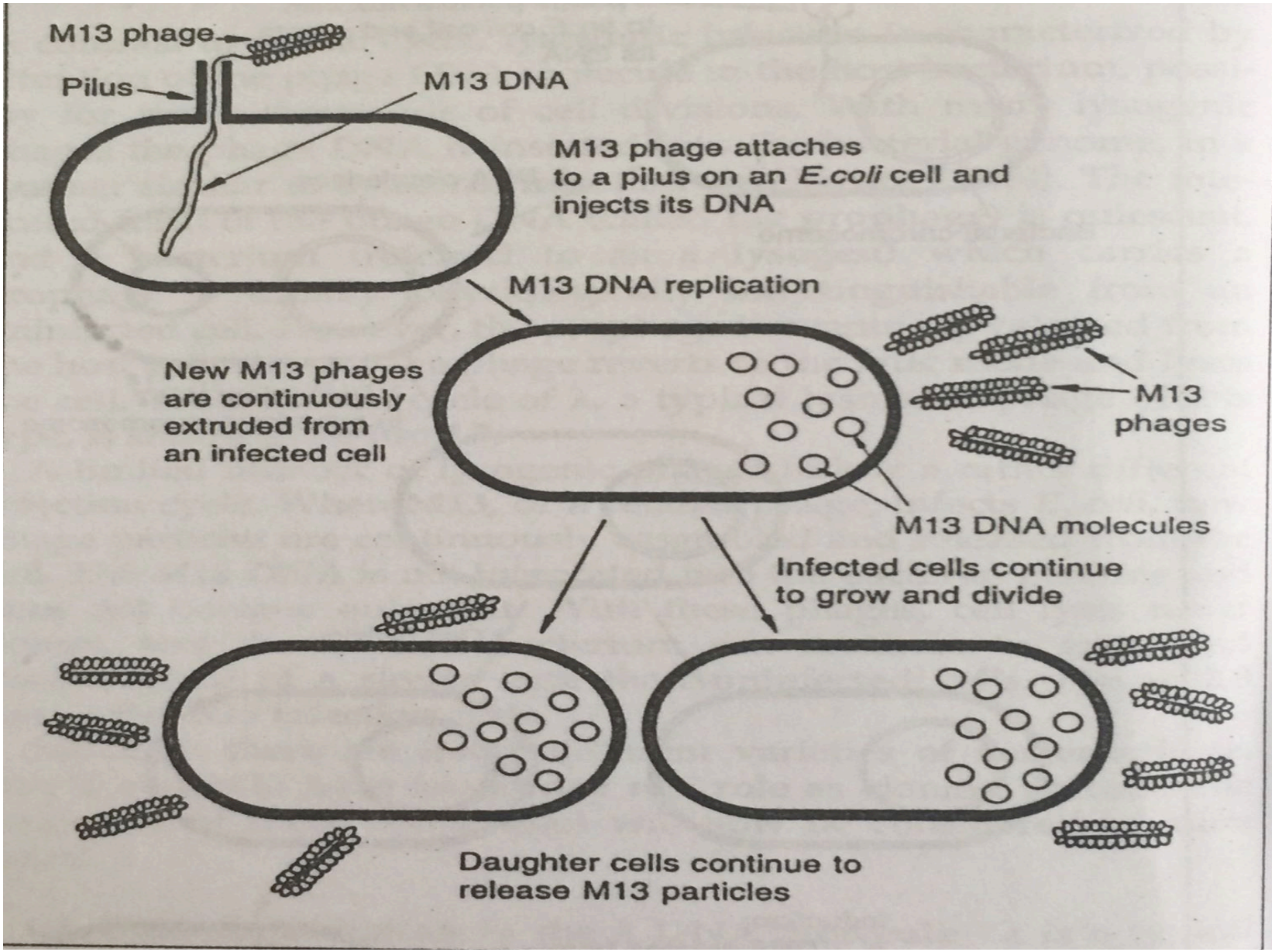
- The general pattern of infection is same for all types of phages and is a three step process:
  - Attachment of phage particle to bacterium and injection of its DNA into the cell
  - Replication of phage DNA molecules, usually by specific phage coded enzymes
  - Other phage genes direct synthesis of the protein components of the capsid and new phage particles are assembled and released from the bacterium

- With some phage types the entire cycle is completed very quickly possibly in less than 20 minutes, this type of rapid cycle is called as **“Lytic cycle”**, as new phage molecules are released with lysis of bacterial cells
- The characteristic feature of that lytic cycle is that phage DNA replication is immediately followed by synthesis of capsid proteins, and the phage DNA molecule is never maintained in a stable condition in the host cell

Lysogenic Phages

- Lysogenic infection is characterized by retention of phage DNA in a host bacterium, for many thousands of cell divisions
- The phage DNA is inserted into bacterial genome. The integrated form of phage DNA is (known as prophage) usually inactive, and the bacterium (referred to as lysogen) which carries a prophage is usually physiologically indistinguishable from an uninfected cell
- However eventually the prophage is released from host genome and phage reverts to the lytic mode and lyse the cell.

- However few lysogenic phages follow rather different infection cycle
  - M13, infects E.coli, new phage particles are continuously assembled and released from the cell without integration of M13 DNA into bacterial genome.
  - With these phages cell lysis never occur and the infected bacterium can continue to grow and divide, although at a slow rate than uninfected cells
  - Some of the properties of these phages are as:


M13 phage
Pilus
M13 DNA
M13 phage attaches to a pilus on an E.coli cell and injects its DNA
M13 DNA replication
New M13 phages are continuously extruded from an infected cell
M13 phages
M13 DNA molecules
Infected cells continue to grow and divide
Daughter cells continue to release M13 particles

Gene organization in the lambda DNA Molecule

- λ is a typical example of a head-and-tail phage
  - DNA is contained in a polyhedral head structure and tail serves to attach the phage to the bacterial surface
  - 49 kb in size and has been intensively studied
  - Genes closely related to functions are clustered together on the genome
    - Like capsid proteins are grouped together on one side of the genome
    - Similarly genes responsible for integration of prophage into the host genome are clustered in the middle
    - Clustering is important as it then allows genes to be switched on and off as a group rather than individually
    - Clustering is important while construction of lambda based cloning vectors

Linear and circular forms of λ DNA

- Second important feature is conformation of the DNA molecule
- It is linear with two free ends, and represents the DNA present in the phage head structure
- This linear molecule consists of two complementary strands of DNA, however at either end of the molecule is a short 12-nucleotide stretch, in which the DNA is single stranded
- These two strands are complementary, and so can base-pair with one another to form a circular, completely double stranded molecule

- Complementary single strands are often referred to as “sticky ends” or “cohesive” ends because base-pairing between them can stick together the two ends of a DNA molecule.

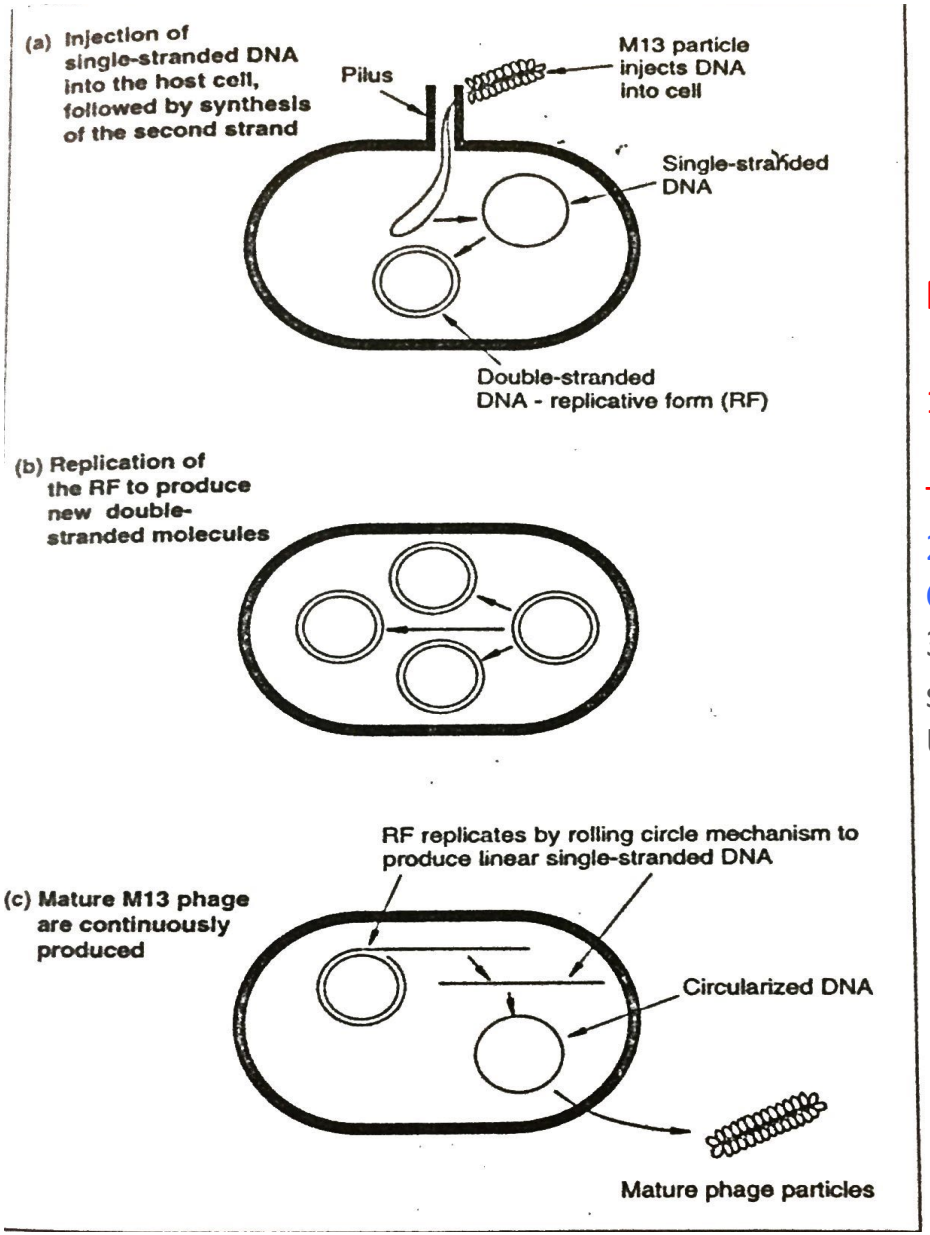
- The **λ** cohesive ends are called the **“cos”** sites and they play two distinct roles during the **λ** infection cycle.

  - They allow linear DNA molecule that is injected into the cells to be circularized, which is a necessary pre-requisite for insertion into the bacterial genome
  - The second role comes into play after the prophage has excised from the host genome. At this stage a large number of new **λ** DNA molecules are produced by the rolling circle mechanism of replication, in which a continuous DNA strand is “rolled off”, of the template molecule. The result is a catenane consisting of a series of linear **λ** genomes joined together at the cos sites. The role of the cos sites is now to act as recognition sequences by an endonuclesaes which cleaves the catenane at the cos sites producing individual **λ** genomes.

    - Endonuclease is a product of gene A on the **λ** DNA molecule creates a single stranded sticky ends, and also acts in conjuction with other proteins to package each λ genome into a phage head structure

M13- A Filamentous Phage

- M13 is an example of a filamentous phage and is different in structure from **λ**
- M13 DNA is much smaller- 6407 nucleotides
- It is circular, and is unusual in that it consists entirely of single-stranded DNA
- As it is small therefore few genes are present in it
  - M13 capsid is constructed from multiple copies of just three proteins (Only three genes) while **λ**-head and tail structure involves over 15 different proteins.
  - In addition, M13 follows a simpler infection cycle than **λ** and doesn't need genes for insertion into the host genome



M13 infection cycle

1-after infection the single stranded
M13 DNA molecule is converted into
The double stranded replicative form(RF)
2-The RF replicates to produce multiple
Copies of itself
3-Single stranded molecules are
synthesized by rolling circle replication and
Used in the assembly of new M13 particles

Protein Purification

- Protein purification is a series of processes intended to isolate a single type of protein from a complex mixture.

- Protein purification is vital for the characterization of the function, structure and interactions of the protein of interest.

Classification....

- On the basis of interaction of solute to the stationary phase
- On the basis of chromatographic bed shape
- Techniques by physical shape of mobile phase

- On the basis of interaction of solute to the stationary phase
  - Adsorption chromatography
  - Partition chromatography
  - Ion exchange chromatography
  - Molecular exclusion/ size exclusion chromatography

- On the basis of chromatographic bed shape
  - Column chromatography
  - Thin layer chromatography
  - Paper chromatography

- Techniques by physical shape/state of mobile phase
  - Gas chromatography
    - GC-MS
  - Liquid chromatography
  - Affinity chromatography

Terms commonly used in chromatography…

- Mobile phase
  - A solvent that flows through the supporting medium
  - The solvent system that carries the mixture to be separated
- Stationary Phase
  - Immobile surface which is particulate in nature, this is the region over which the compound gets separated

- Elution
  - Motion of the mobile phase through the stationary phase
- Elution time
  - The time taken for a solute to pass through the system. A solute with short elution time travels through the stationary phase rapidly – it elutes fast
- Adsorption
  - Interaction of solute molecules with the surface of stationary phase

Applications of Chromatography….

- Used for separation of amino acids, proteins and carbohydrates
- Used for the analysis of drugs, hormones vitamins
- Helpful for the qualitative and quantitative analysis of complex mixtures
- For determination of molecular weight of proteins

Types…..

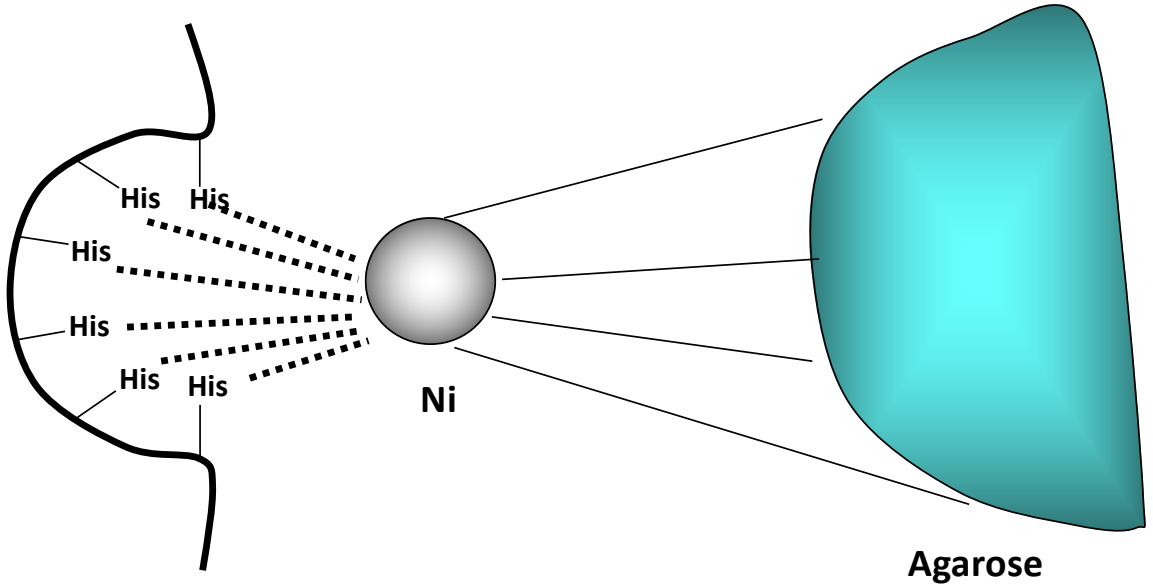
- Paper chromatography
- Thin layer …..
- Column chromatography

Affinity chromatography separates proteins on the basis of a reversible interaction between a protein (or group of proteins) and a specific ligand coupled to a chromatography matrix.

Affinity Chromatography

His. Exhibits the strongest interaction with immobolised metal ion matrices, as electron donor groups on the histidine Immidazole ring readily form coordination bonds with the immobilised transsition metal.

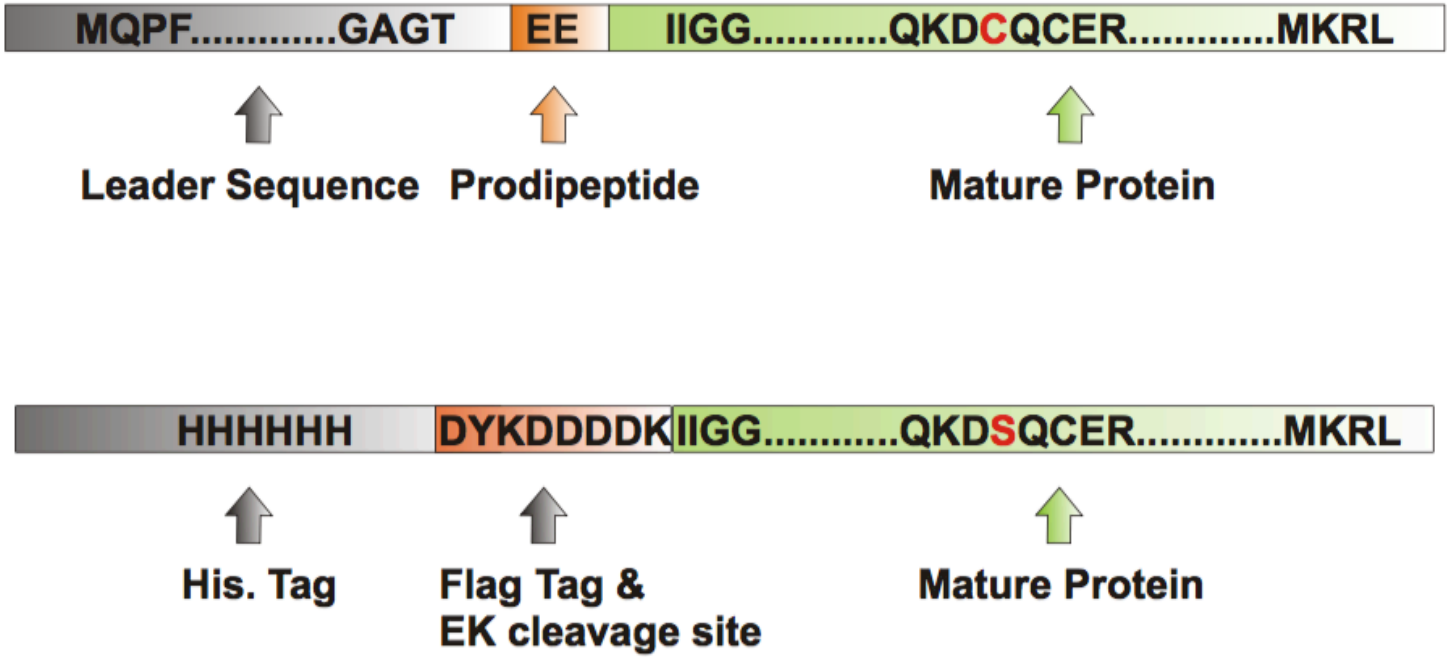
6 Histidine residues in a row.



- Affinity chromatography commonly utilizes **zinc (Zn2+), nickel (Ni2+) or copper (Cu2+)** to form stable complexes with **histidine, tryptophan and cysteine** residues within proteins.

- Once bound, the proteins can be eluted via **pH** or **imidazole** gradients

His Tag with EK cleavage site



Coomassie stain/gel

His-Tag for Purification of Recombinant Proteins

- It has been shown that an amino acid sequence consisting of 6 or more His residues in a row will also act as a metal binding site for a recombinant protein.
- A His-Tag sequence can be placed on the N-terminal of a target protein by using vectors

MetGlySerSer**HisHisHisHisHisHis**SerSerGly**LeuValProArgGlySer**....recombinant protein sequence

Thrombin Cleavage site

Cleavage of His tag

His tag is not part of the protein. It needs to be removed in order to perform structural and biophysical studies on the protein.

- Thrombin is used to remove the His tag.

Thrombin:

It is a protease, an enzyme that cleaves of the protein at a specific recognition sequence.

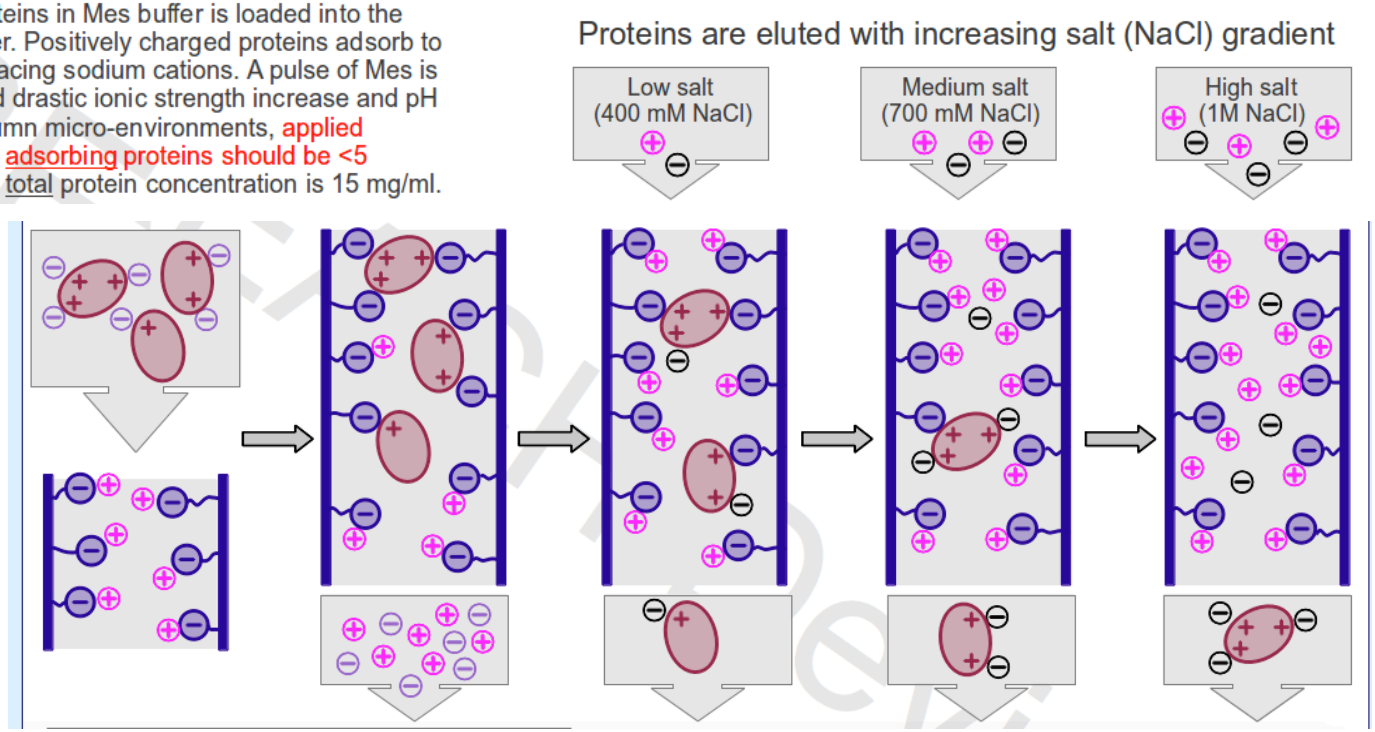
When Thrombin is added in the solution it recognizes the cutting site, located just after the His tag and cleaves The His tag off.

Cleavage of His tag

Now we have Thrombin mixed in with our protein.

- To remove Thrombin we add benzamidine sepharose.
- Benzamidine will bind to the protease (Thrombin).
- Because benzamidine is linked to a resin (sepharose), the benzamidine-Thrombin complex can be separated from our protein by spinning it down.

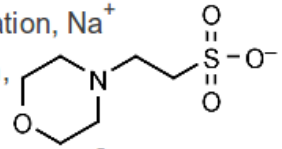
A mixture of proteins in Mes buffer is loaded into the cation exchanger. Positively charged proteins adsorb to the media, displacing sodium cations. A pulse of Mes is formed. To avoid drastic ionic strength increase and pH decrease in column micro-environments, applied concentration of adsorbing proteins should be <5 mg/ml. The max total protein concentration is 15 mg/ml.



Legend:

– Sodium cation, Na^+

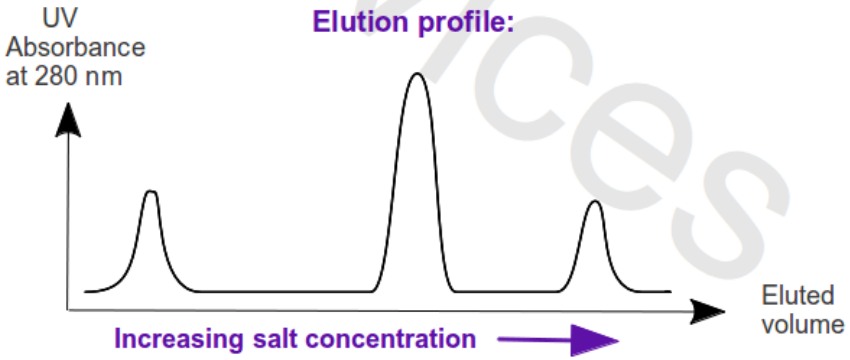
– Mes anion,



– Chloride anion, Cl^-

– Carboxymethyl anion (CM),

– Protein bearing a number of positive charges (as marked)



Cloning Vehicles

Plasmids & Bacteriophages

Plasmids…

- **Basic Features….**
  - Plasmid are circular, extrachromosomal, self-replicating cytoplasmic DNA
  - Carry one or more genes generally gives a characteristic feature to a host bacterium
    - Like antibiotic resistance gene which is generally used as a selectable marker to ensure that bacteria in a culture contain a particular plasmid
    - Origin of replication (at least one), gives the ability to replicate independently of bacterial chromosome
      - Smaller plasmid use host cells own DNA replicative enzyme however larger plasmids carry genes responsible for plasmid replication
      - Few plasmids are also able to replicate by inserting themselves into the bacterial chromosomes. These are known as episomes, may be maintained like that through numerous cell divisions but will at some stage exist as independent elements.
  - **Cryptic Plasmid**
    - Those plasmids to which phenotypic characteristics are not yet ascribed

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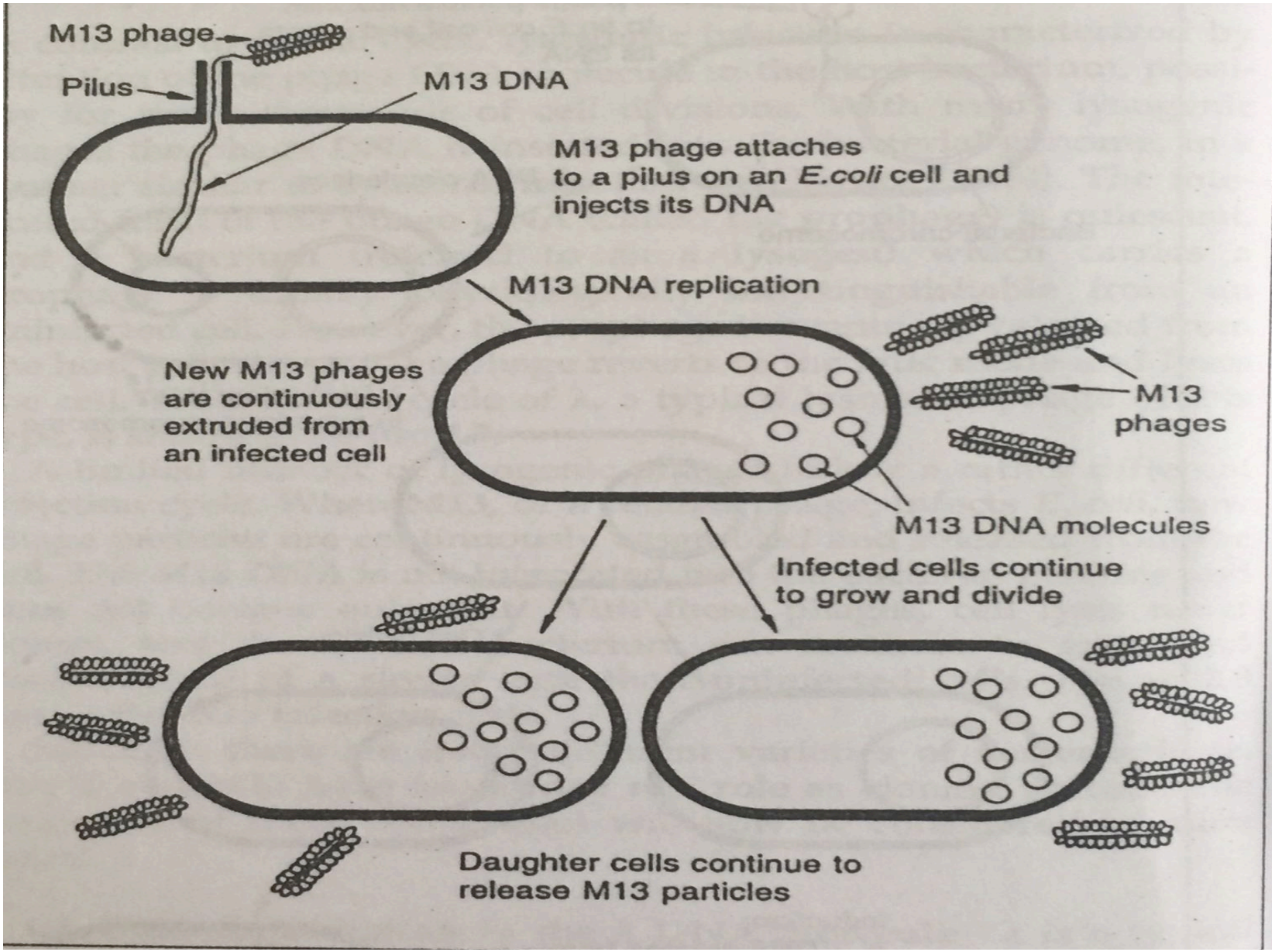
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M13 phages
M13 DNA molecules
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DNA Sequencing
Chemical Method and Termination Method

DNA Sequencing

- The process of determining the order of bases adenine (A), thymine (T), cytosine (C), and guanine (G) along a DNA strand.
- All the information required for the growth and development of an organism is encoded in the DNA of its genome.
- So, DNA sequencing is fundamental to genome analysis and understanding the biological processes in general.

Technical Breakthrough For DNA Sequencing

In 1977, two separate methods for the large-scale sequencing of DNA were devised:

- Chemical cleavage method

  by Allan Maxam and Walter Gilbert
  -most favoured method for sometime

- Enzymatic chain termination method

  by Fred Sanger, but by 1980s it became a preferred one.

-Sanger Method is more popular

-Without changing the underlying concept of both methods, Improvements have been done over the years by applying different strategies, by developing various modifications and by automation.

-This has resulted in a very large scale sequencing e.g.

- *E. coli*,
- Saccharomyces cerevisiae,
- Human Genome Project etc.

Chemical Cleavage Method

- Involves modification of the bases in DNA followed by chemical base-specific cleavage.

- Sequences DNA fragments containing upto ~500 nucleotides in length.

- Involves sequencing of a single stranded DNA by a two step catalytic process using piperidine and two chemicals that selectively attack purines and pyrimidines

- Purines will react with dimethyl sulfate (DMS) and pyrimidines will react with hydrazine in such a way as to break the glycosidic bond between the ribose sugar and the base displacing the base

- Piperidine will then catalyse phosphodiester bond cleavage where the base has been displaced/modified.

- Moreover, DMS and piperidine alone will selectively cleave guanine nucleotides but DMS and piperidine in formic acid will cleave both guanine and adenine nucleotides

- Similarly, hydrazine and piperidine will cleave both thymine and cytosine nucleotides whereas hydrazine and piperidine in 1.5 M NaCl will only cleave cytosine nucleotides

Steps:

1. The double-stranded fragment to be sequenced is isolated and radioactively labeled at the 5'-ends with ^{32}P.

2. The fragment is then cut with restriction enzyme and thus the label is removed from one end.

3. The fragment of DNA with one end labeled is denatured.

4. Four identical samples of these end-labeled DNA fragments are subjected to chemical cleavage at different chemical nucleotides.

5.There are four specific sets of chemical reactions that selectively cut the DNA backbone at G, A+G, C+T, or C residues.

- G only: Dimethyl sulphate(DMS) and piperidine
- A+G : DMS, piperidine, formic acid
- C+T : Hydrazine, piperidine
- C only : Hydrazine, 1.5M, piperidine

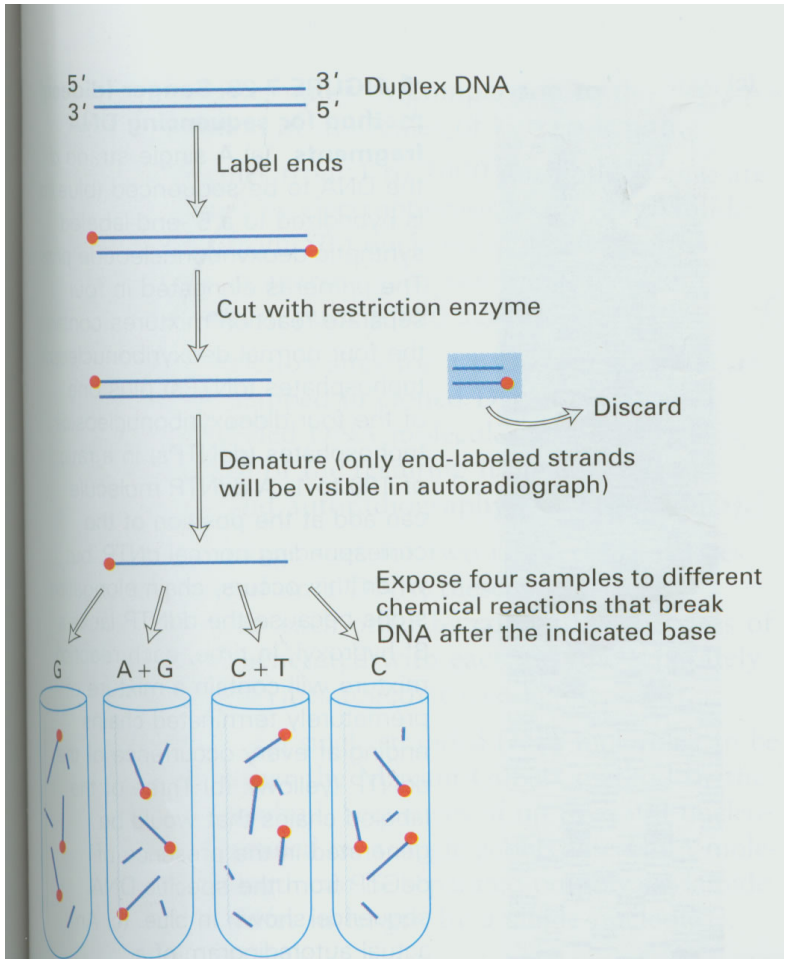


Figure: Maxam-Gilbert method (continued)

Lodish, H.;Berk, A. *et. al.* (4th ed); *Mol. Cell Biol.*; W. H. Freeman and Co. (2000) p: 233

6. For each labeled chain to be broken only once, the reactions are controlled.

7. The labeled subfragments created by the four reactions have

- the ^{32}P label at one end and
- the chemical cleavage point at the other end.

8. The reaction products are separated by polyacrylamide gel electrophoresis which is based on size. Smallest fragment goes fastest.

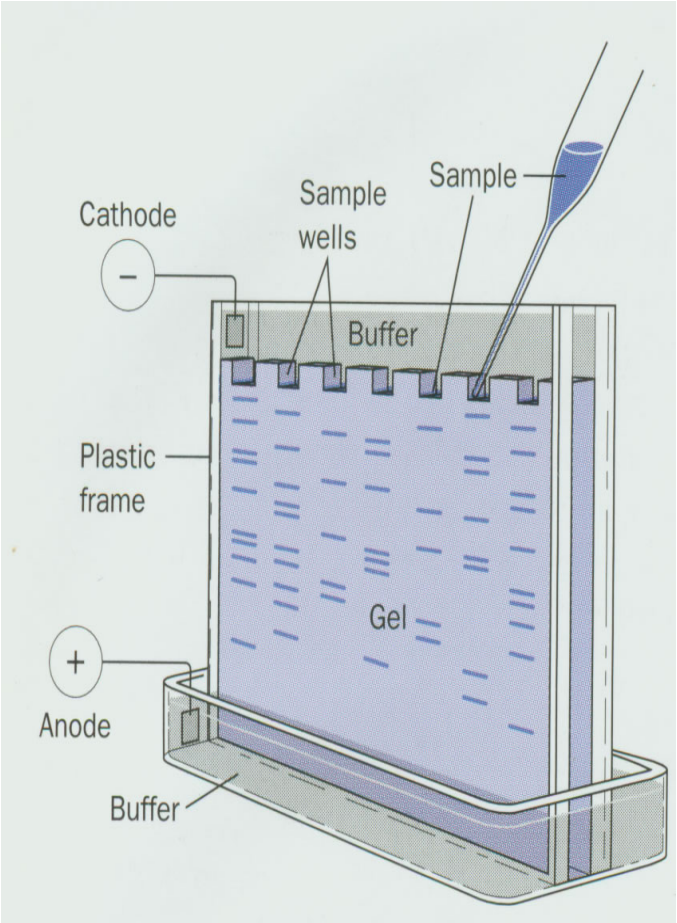


Figure: Apparatus for gel electrophoresis

Voet, D.; Voet, J. and Pratt, C. (upgrade ed) *Fundamentals of Biochemistry*; John Wiley and Sons, Inc (2002); p: 58

9. The labeled fragments in the gel are visualized by autoradiography.
10. The sequence is read from bottom to top of the gel.

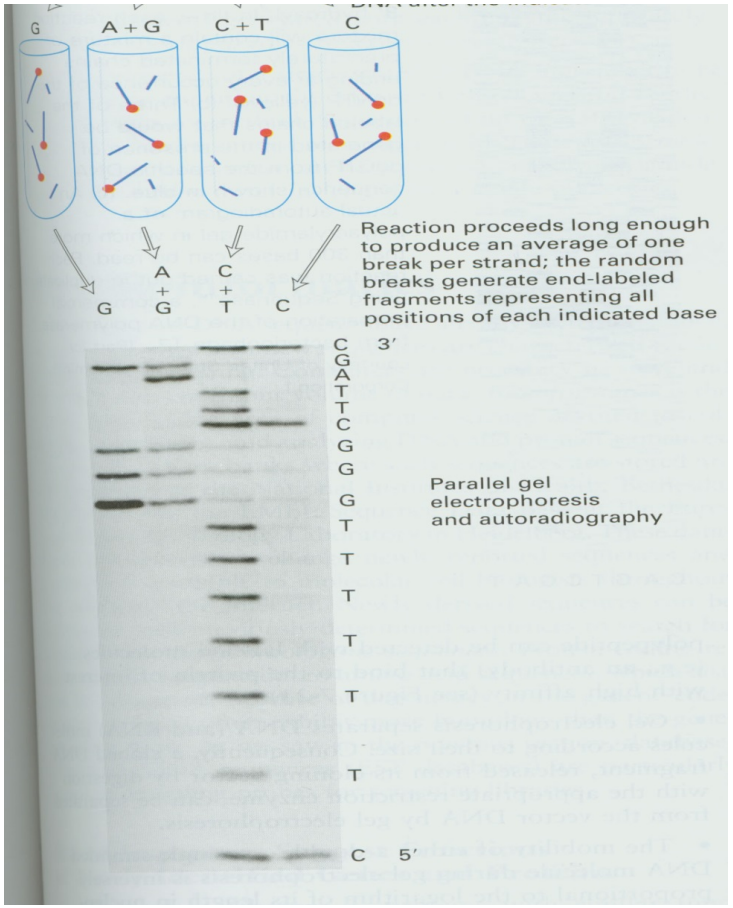
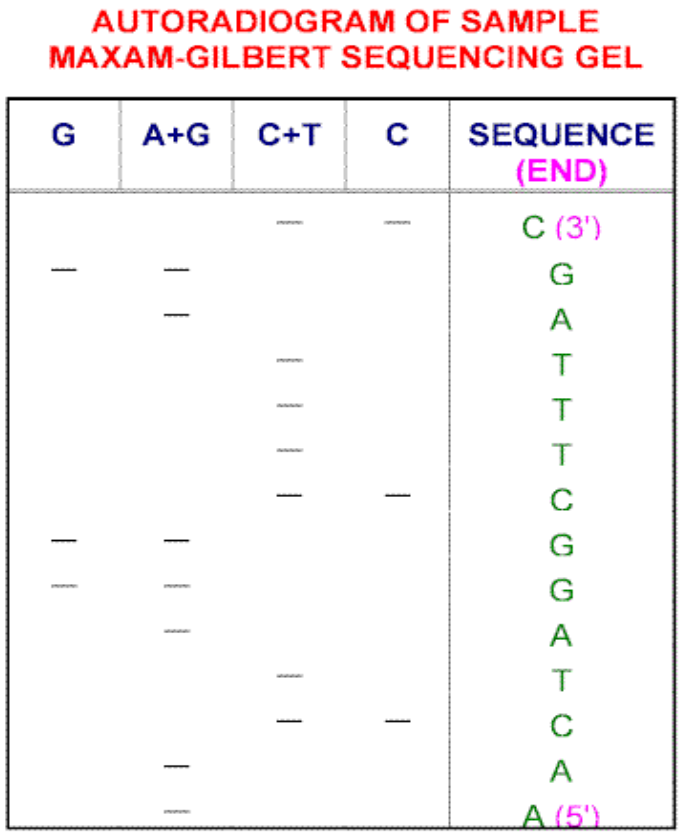


Figure: Maxam-Gilbert method

Lodish, H.;Berk, A. *et. al.* (4th ed); *Mol. Cell Biol.*; W. H. Freeman and Co. (2000) p: 233

Example of DNA Sequencing by Chemical Method



http://users.wmin.ac.uk/~redwayk/lectures/sequence.htm

Chain Termination method

- This method uses single-stranded DNA.
- Also known as **dideoxy sequencing** method because it involves the use of analogue of normal nucleotide 2',3'-dideoxynucleoside triphosphates (ddNTPs). These are chain terminating nucleotides lacking 3'-OH ends.
- This method is based upon the incorporation of ddNTPs into a growing DNA strand to stop chain elongation.

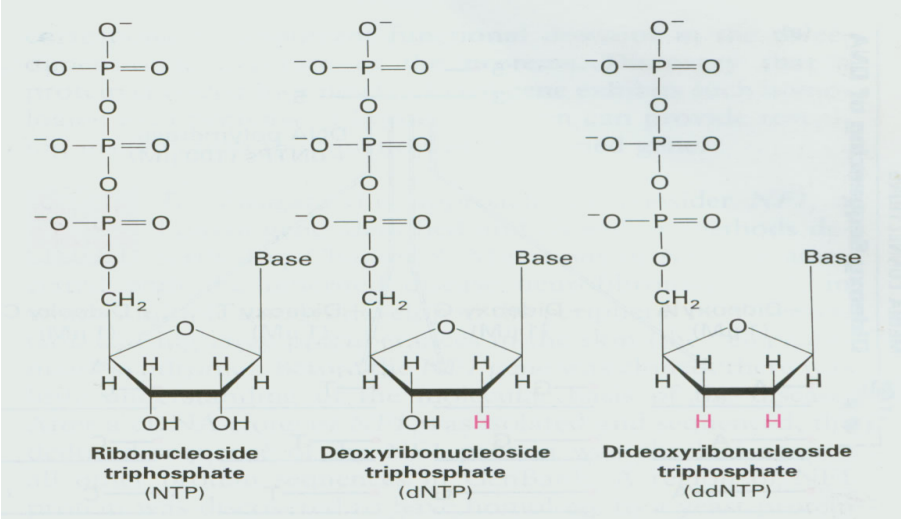


Figure: Structure of NTP, dNTP, and ddNTP

Lodish, H.;Berk, A. *et. al.* (4th ed); *Mol. Cell Biol.*; W. H. Freeman and Co. (2000), p: 233

Stages:

1.The DNA to be sequenced is called the template DNA.

2. A synthetic 5'-end-labeled oligodeoxynucleotide is used as the primer.

3. The template DNA is hybridized to the primer.

4. The primer elongation is performed in four separate polymerization reaction mixtures. Each mixture contains

- 4 normal deoxynucleotides (dNTPs) in higher concentration and
- a low concentration of the each of the 4 ddNTPs.

5. There is initiation of DNA synthesis by adding enzyme DNA polymerase since the enzyme cannot distinguish between the normal nucleotides and their analogues.

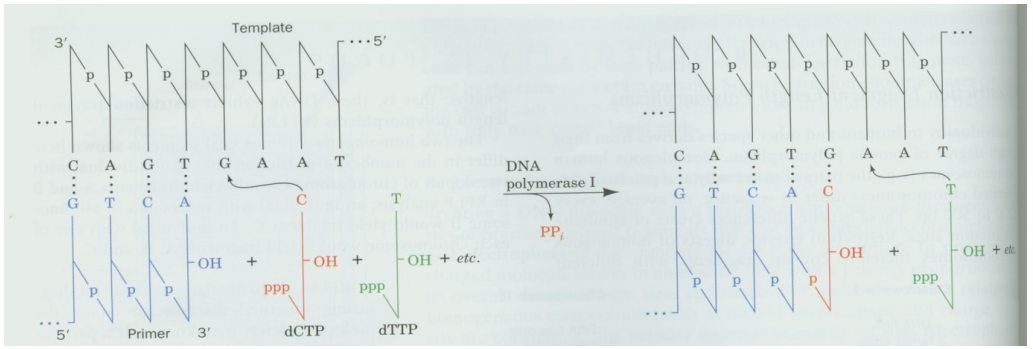


Figure: Action of DNA polymerase I

Voet, D.; Voet, J. and Pratt, C. (upgrade ed)
Fundamentals of Biochemistry; John Wiley and Sons, Inc (2002); p: 60

6. The strand synthesis continues until a ddNTP is added. The chain elongation ceases on the incorporation of a ddNTP because it lacks a 3'-OH group which prevents addition of the next nucleotide.
7. There is a result of mixture of terminated fragments, all of different lengths.
8. Denature DNA fragments.
9. Each of the four mixtures are run together on a polyacrylamide gel for electrophoresis.

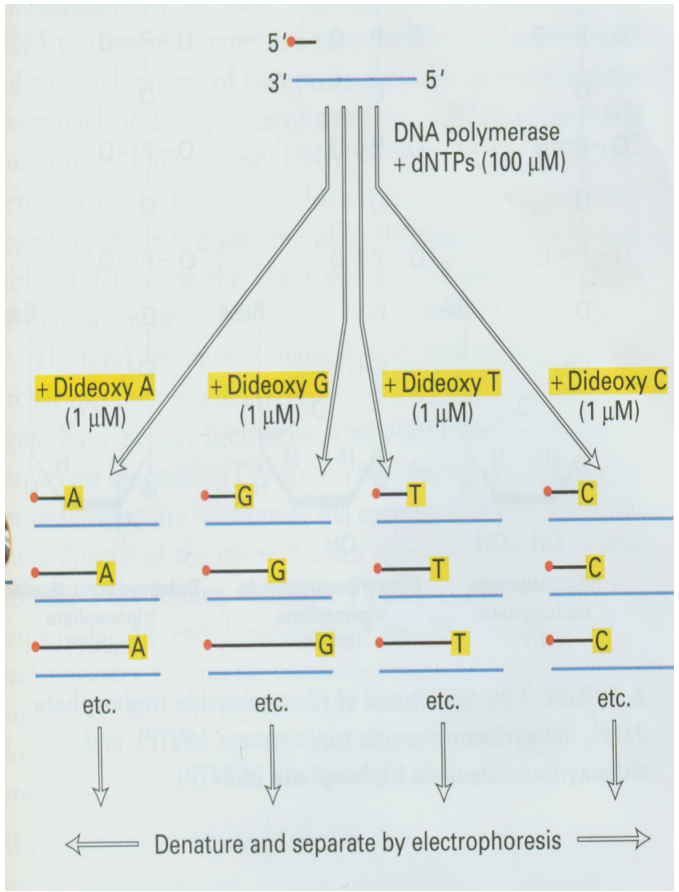


Figure: Sanger method

Lodish, H.;Berk, A. *et. al.* (4th ed); *Mol. Cell Biol.*; W. H. Freeman and Co. (2000) p: 234

10. The separated fragments are then visualized by autoradiography.

11. From the position of the bands of the resulting autoradiogram, the sequence of the original DNA template strand can be read directly.

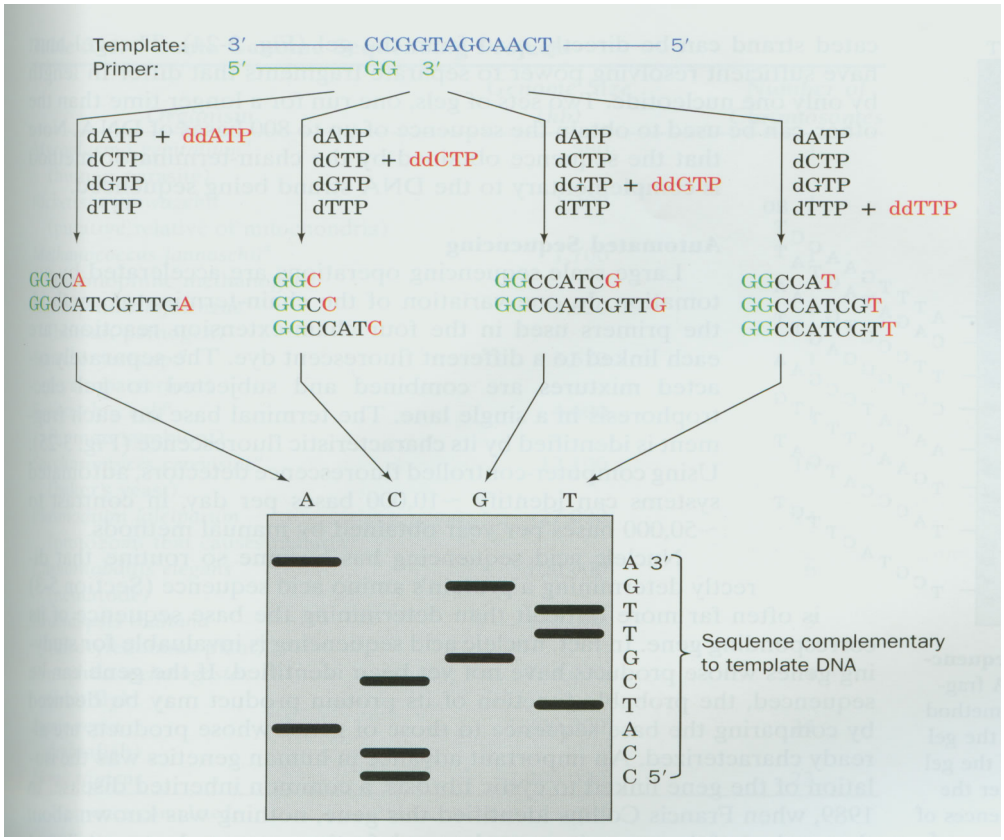


Figure: Chain termination method

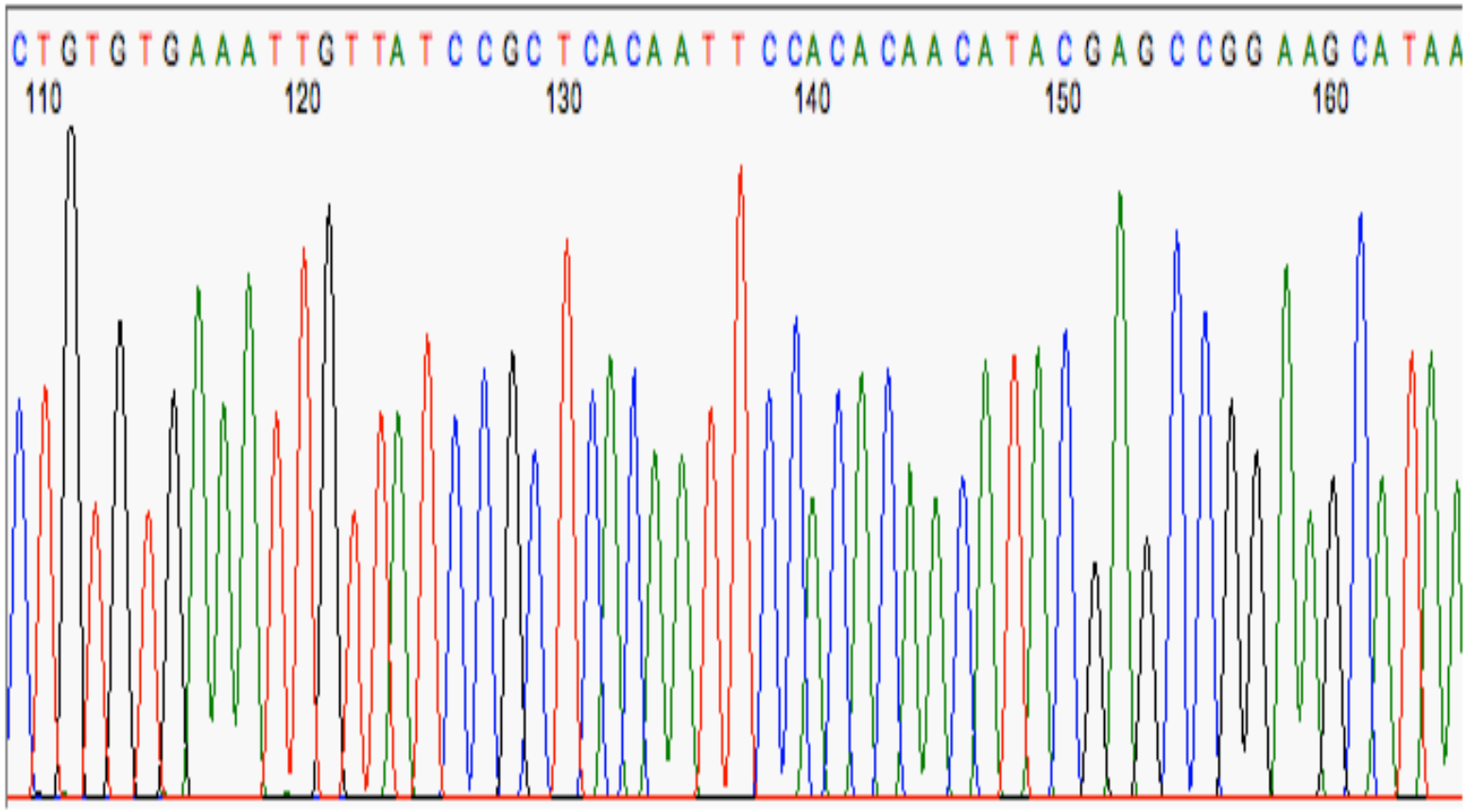
Voet, D.; Voet, J. and Pratt, C. (upgrade ed)
Fundamentals of Biochemistry; John Wiley and Sons, Inc (2002);
p: 61

Automated sequencing

- Commonly used sequencing
- Dye terminator-cycle-sequencing
  - Template DNA is subjected to PCR
- Reaction mix contains
  - BigDye mix (contains Taq DNA polymerase, standard dNTPs, and labeled ddNTPs (different fluorescent dye, one unique color for each), one primer, DNA (PCR product), water
  - Higher concentrations of dNTPs in your tube than ddNTPs
  - BigDye contains dNTPs and ddNTPs in a mixture of approx. 100: 1
- Can be sequenced from either direction
- The PCR product is purified and then sequenced


ABI PRISM
3100 Genetic Analyzer

Here's an example of excellent sequence. Note the evenly-spaced peaks and the lack of baseline 'noise' (see further down for examples of higher baseline noise):



Base at 310 number is not readable