DNA sequencing

- Sequencing means finding the order of nucleotides on a piece of DNA .
- Nucleotide order determines amino acid order, and by extension, protein structure and function (proteomics) .
- An alteration in a DNA sequence can lead to an altered or non functional protein, and hence to a genetic disorder .
- DNA sequence is important to detect the type of mutations in genetic diseases and offer hope for the eventual development of treatment DNA .

Methods of sequencing

- there are two main methods of DNA sequencing:

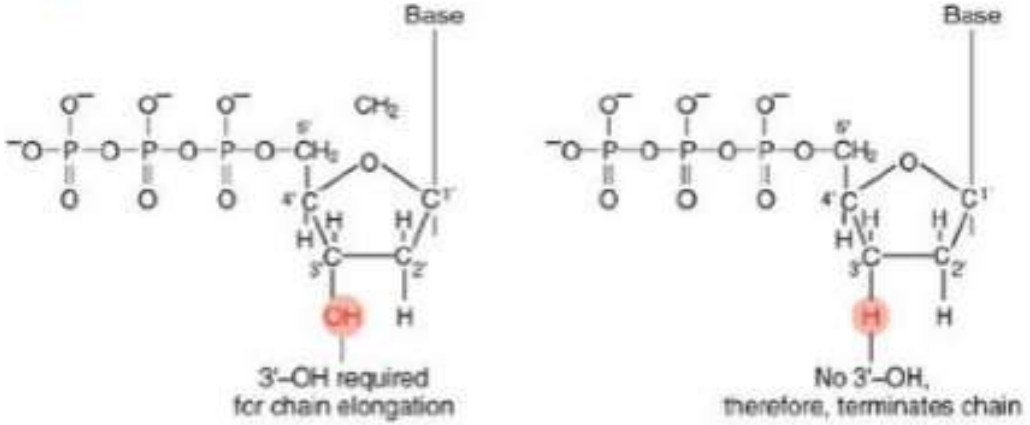
1-Sanger dideoxy (primer extension/chain-termination) method: most popular protocol for sequencing, very adaptable, scalable to large sequencing projects

2-Maxam-Gilbert chemical cleavage method: DNA is labelled and then chemically cleaved in a sequence-dependent manner. This method is not easily scaled and is rather tedious .

- Modern sequencing equipment uses the principles of the Sanger technique.

History of Sequencing

- "Sanger Sequencing" developed by Fred Sanger *et al* in the mid 1970's
- **Uses dideoxynucleotides** for "chain termination", generating fragments of different lengths ending in ddATP, ddGTP, ddCTP or ddTTP.



The 3' hydroxyl has been changed to a hydrogen in ddNTP's, which terminates a DNA chain because a phosphodiester bond cannot form at this 3' location

The Sanger Technique Principle ::

- The Sanger Technique uses dideoxynucleotides (dideoxyadenine, dideoxyguanine, etc) These are molecules that resemble normal nucleotides but lack the normal -OH group.

- Because they lack the -OH (which allows nucleotides to join a growing DNA strand), replication stops.

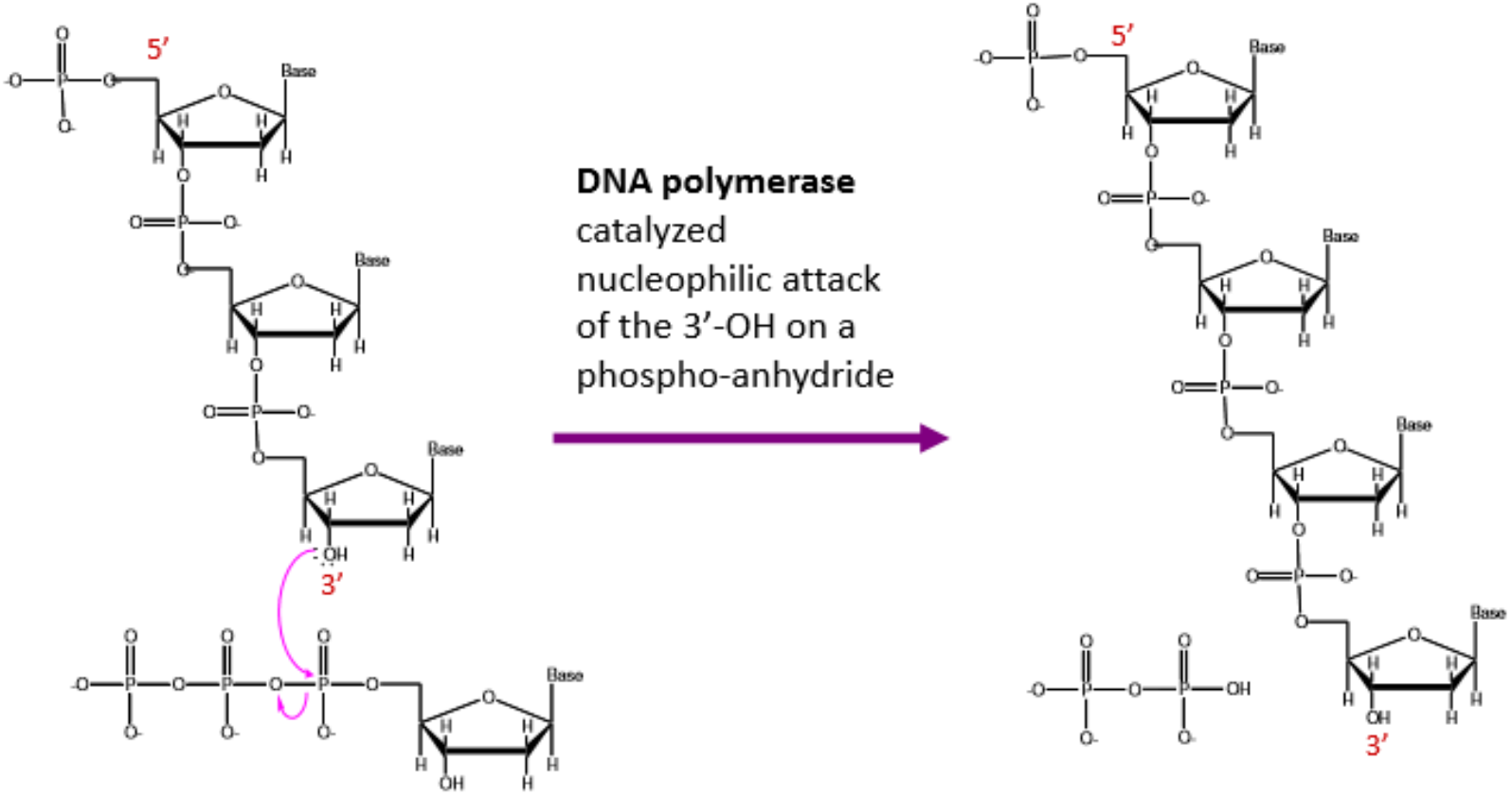
Requirements for Sanger Method

- DNA to be sequenced must be in single strand form.
- The region to be sequenced must be 3' flanked by known sequence.

Reagents needed are:

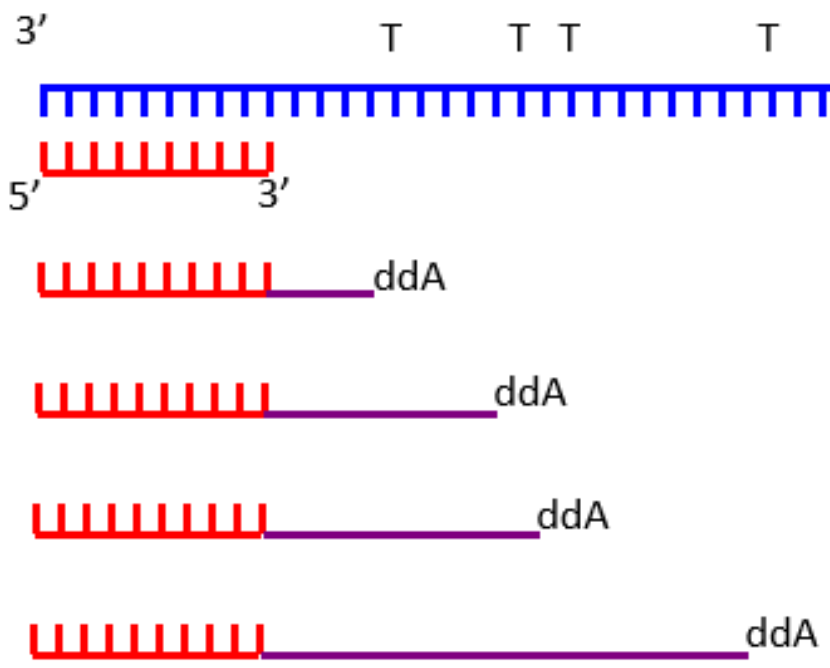
- **A primer complementary to the known region to start and direct chain synthesis. (15-30 nucleotides in length)**
- **DNA polymerase.**
- **4 deoxynucleotide triphosphates (dNTPs).**
- **4 dideoxynucleotide triphosphates (ddNTPs) (** small proportion **) .**

Mechanism of DNA polymerization



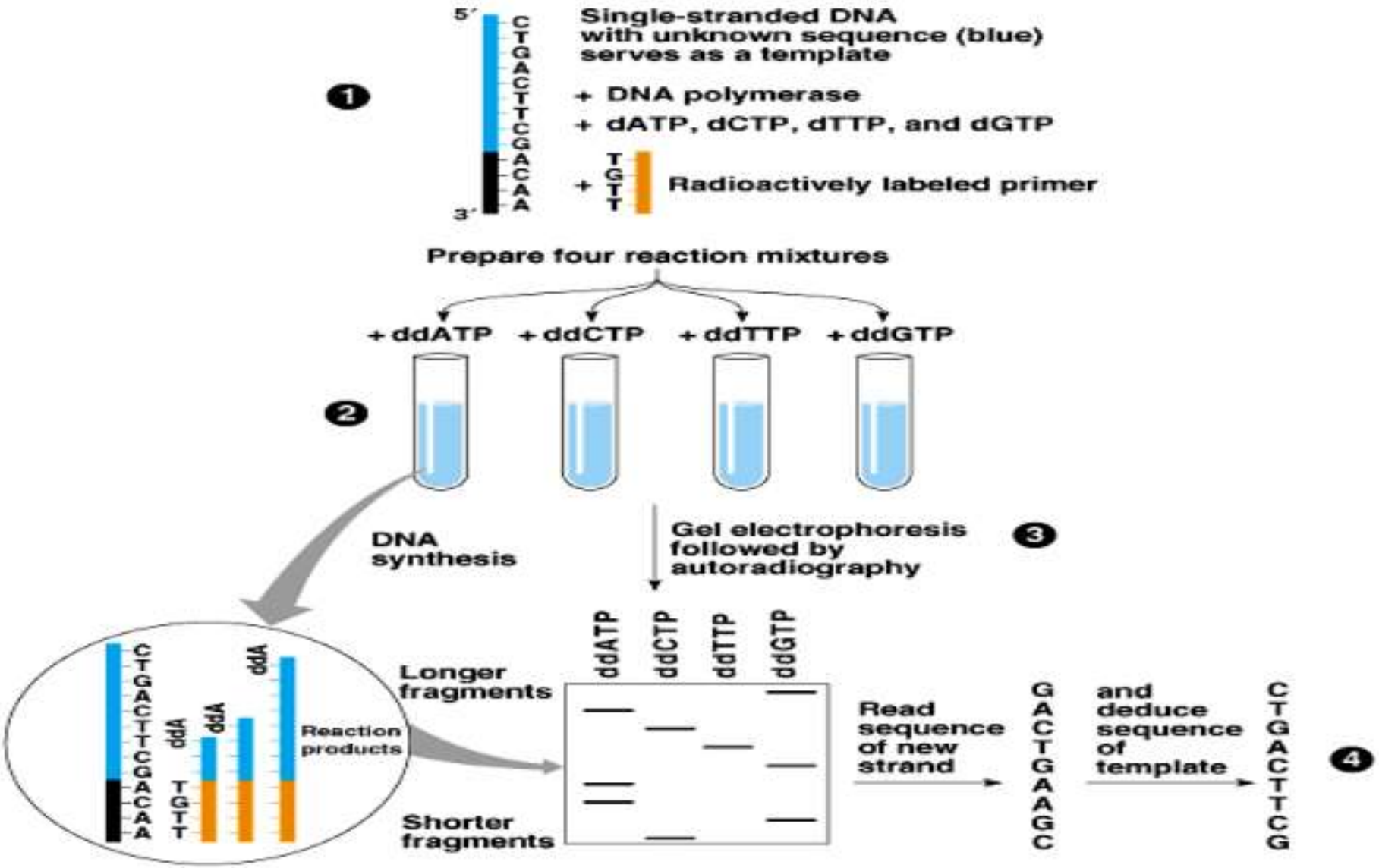
**** Since the 3′ –OH is changed to a –H in ddNTPs, it is unable to form a phosphodiester bond by nucleophilic attack on the phosphate, and it will cause a termination in the DNA chain**

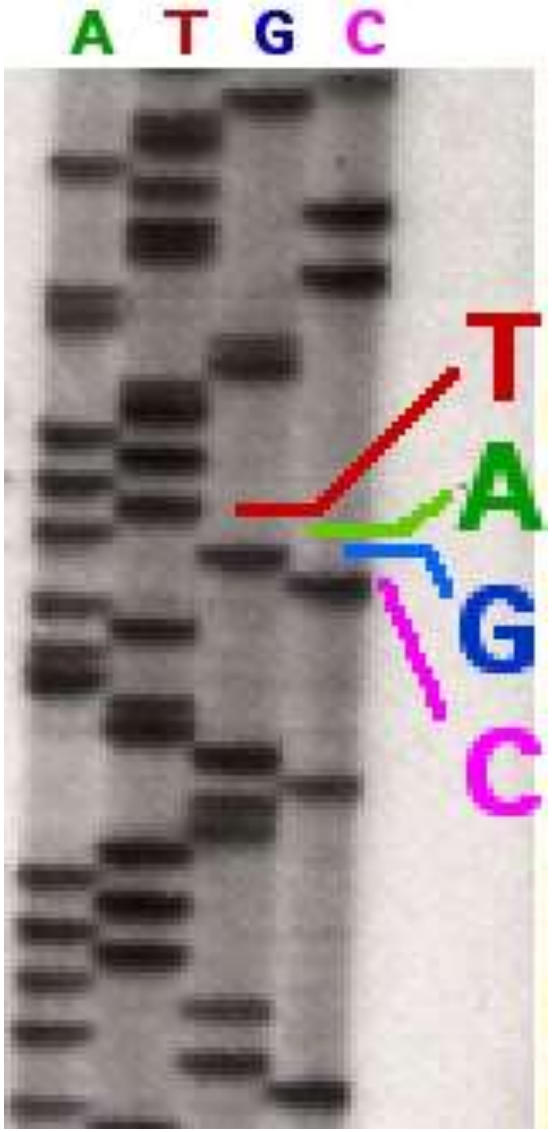
Sanger dideoxy sequencing: basic method

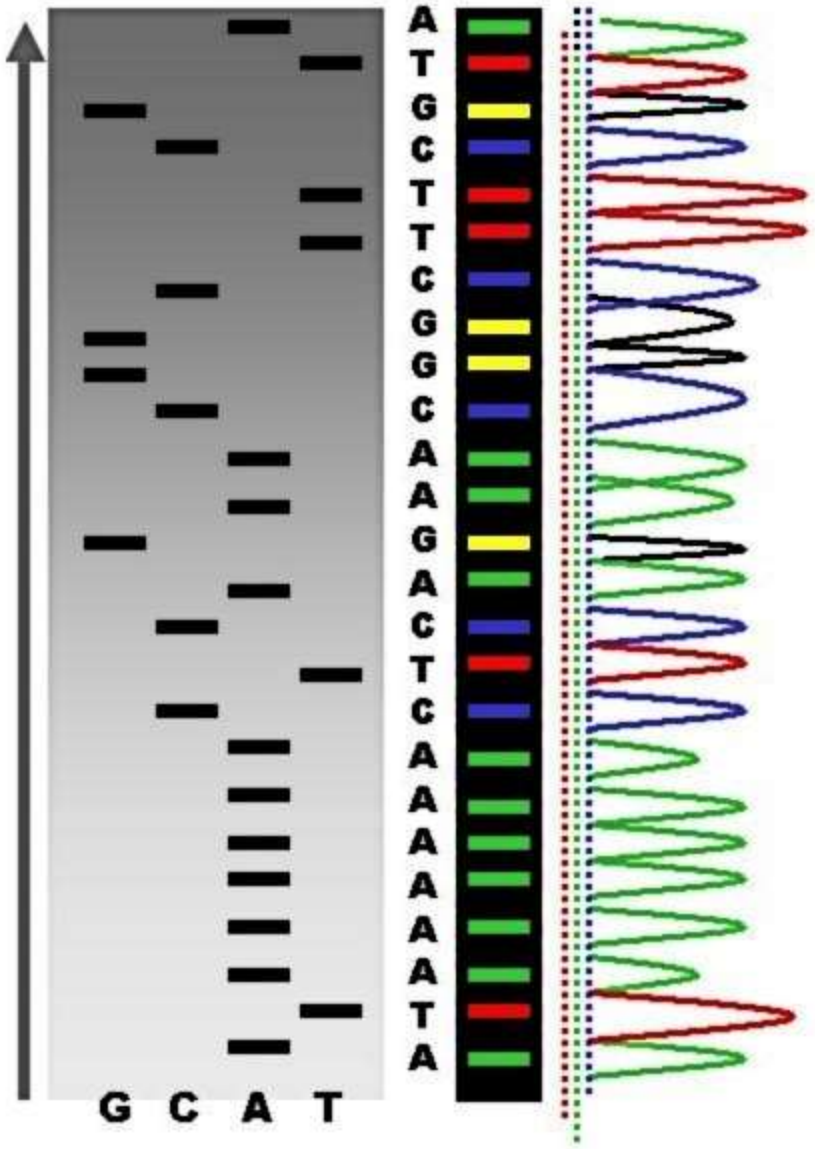


ddATP in the reaction: anywhere there’s a T in the template strand, occasionally a ddA will be added to the growing strand

Sequencing of DNA by the Sanger method

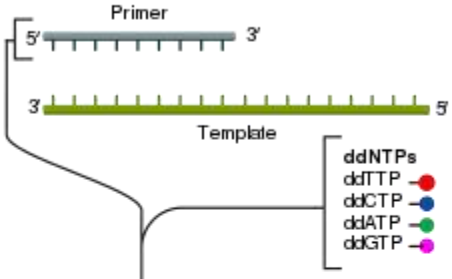



A T G C
T
A
G
C

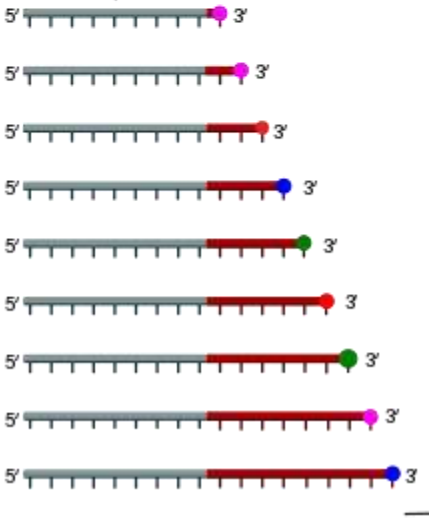

G C A T
A T G C T T C G G C A A G A C T C A A A A A A T A

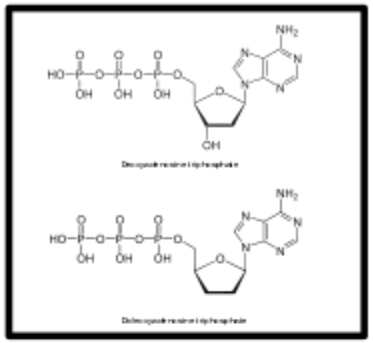
① Reaction mixture

- Primer and DNA template
- DNA polymerase
- ddNTPs with flourochromes
- dNTPs (dATP, dCTP, dGTP, and dTTP)

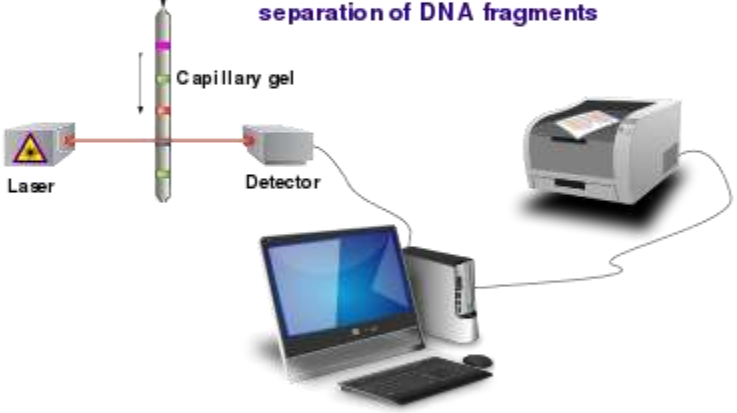


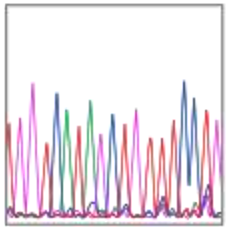
② Primer elongation and chain termination





③ Capillary gel electrophoresis separation of DNA fragments

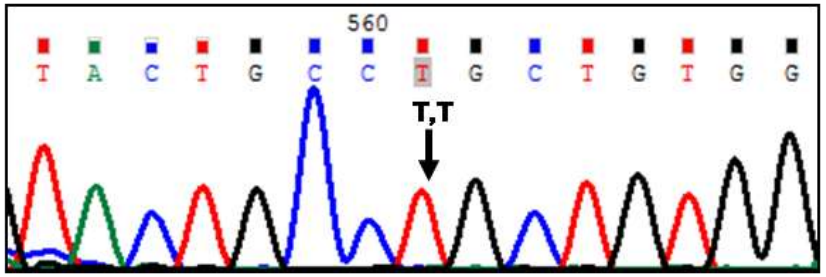




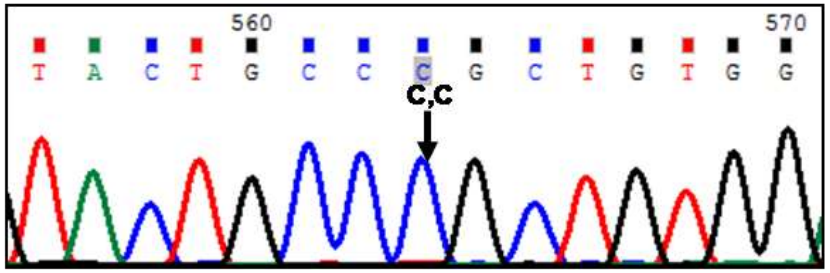
Chromatograph

④ Laser detection of flourochromes and computational sequence analysis

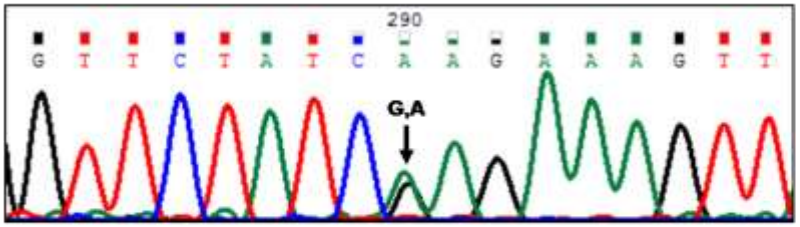
Chromatogram



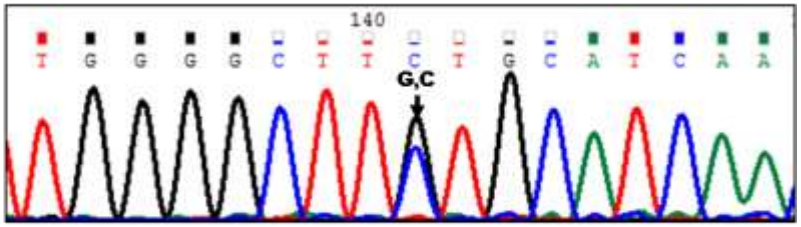
c.26T (p.Lys9, p.L9) (Wild type)



c.26T>C (p.Leu9Pro, p.L9P)



c.814G>A (p.Glu272Lys, p.E272K) (het)



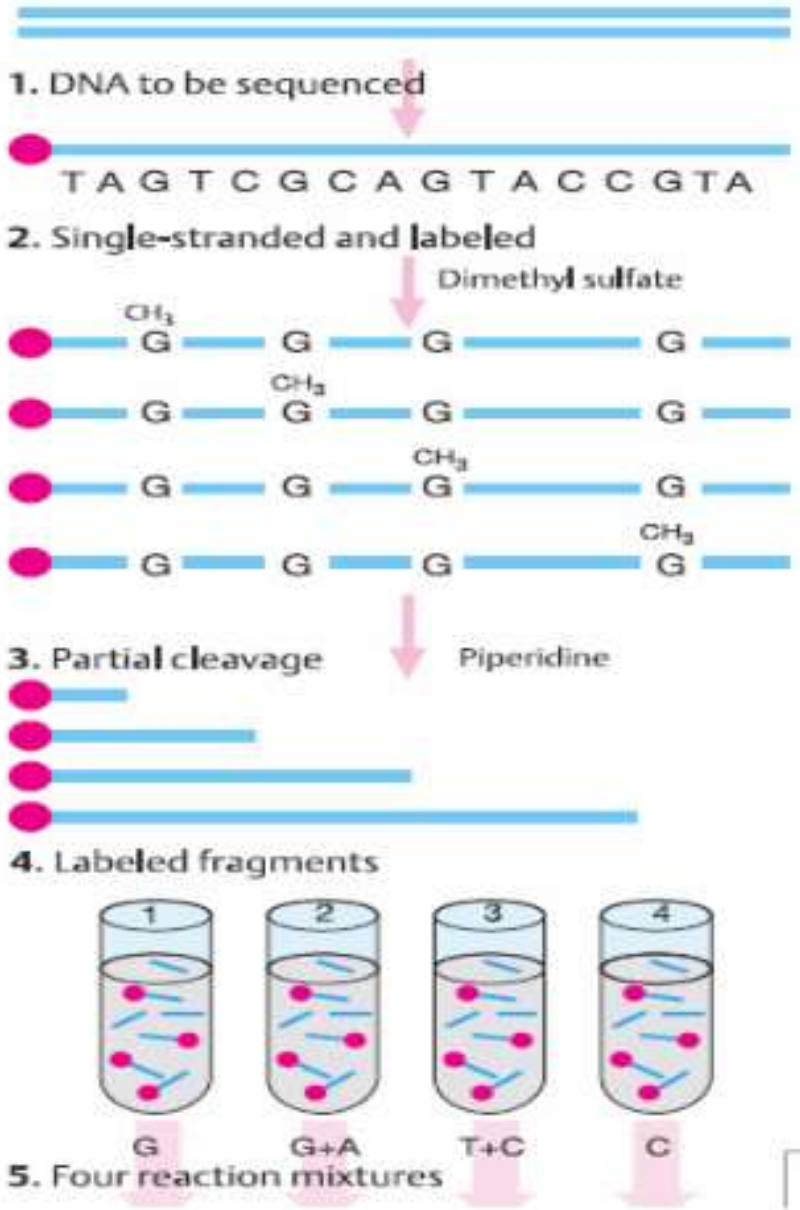
c.1122G>C (p.Leu374Phe, p.L374F) (het)

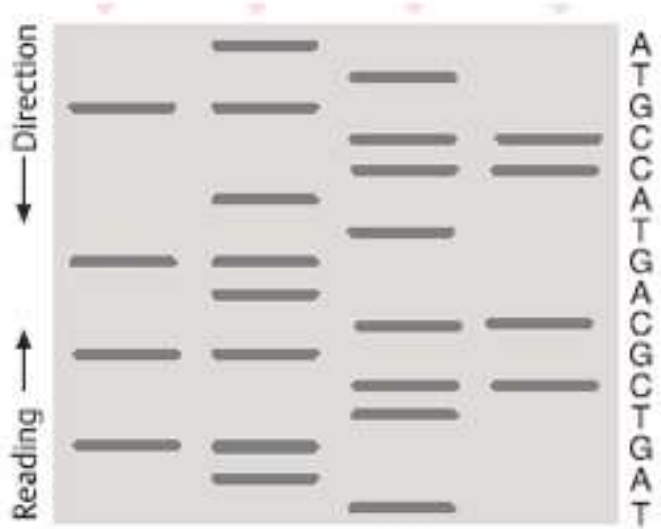
Chemical Degradation Method

- **Maxam-Gilbert method**
  - The single stranded DNA fragment to be sequenced is labeled with radioactive phosphate at 5’ end .
  - The labeled DNA fragment is then divided into four aliquots, each of which is treated with a reagent which modifies a specific base .
  - This will results in strands of varying lengths.
  - Strands separated out with electrophoresis.
  - Gels read with radioautography.

Reagents for cleaving DNA

- Aliquot 1 Cleavage at only G
  - DNA treated with Dimethyl sulfate (DMS)
  - Methylation of G residues at the N7 position
  - the glycoside bond of the methylated G residue is hydrolyzed and the G residue is eliminated.
  - Piperidine is added which reacts with hydrolyzed sugar residue, cleavage of the backbone results
- Aliquot 2 cleavage at G and A
  - Use acid instead of DMS
  - Position of A revealed
- Aliquot 3: cleavage at C and T
  - Treat with hydrazine, then piperidine
- Aliquot 4: cleavage at C only
  - Treat with hydrazine in the presence of 1.5 M NaCl
  - Position of T revealed
- The four are incubated with piperidine which cleaves the sugar phosphate backbone of DNA next to the residue that has been modified



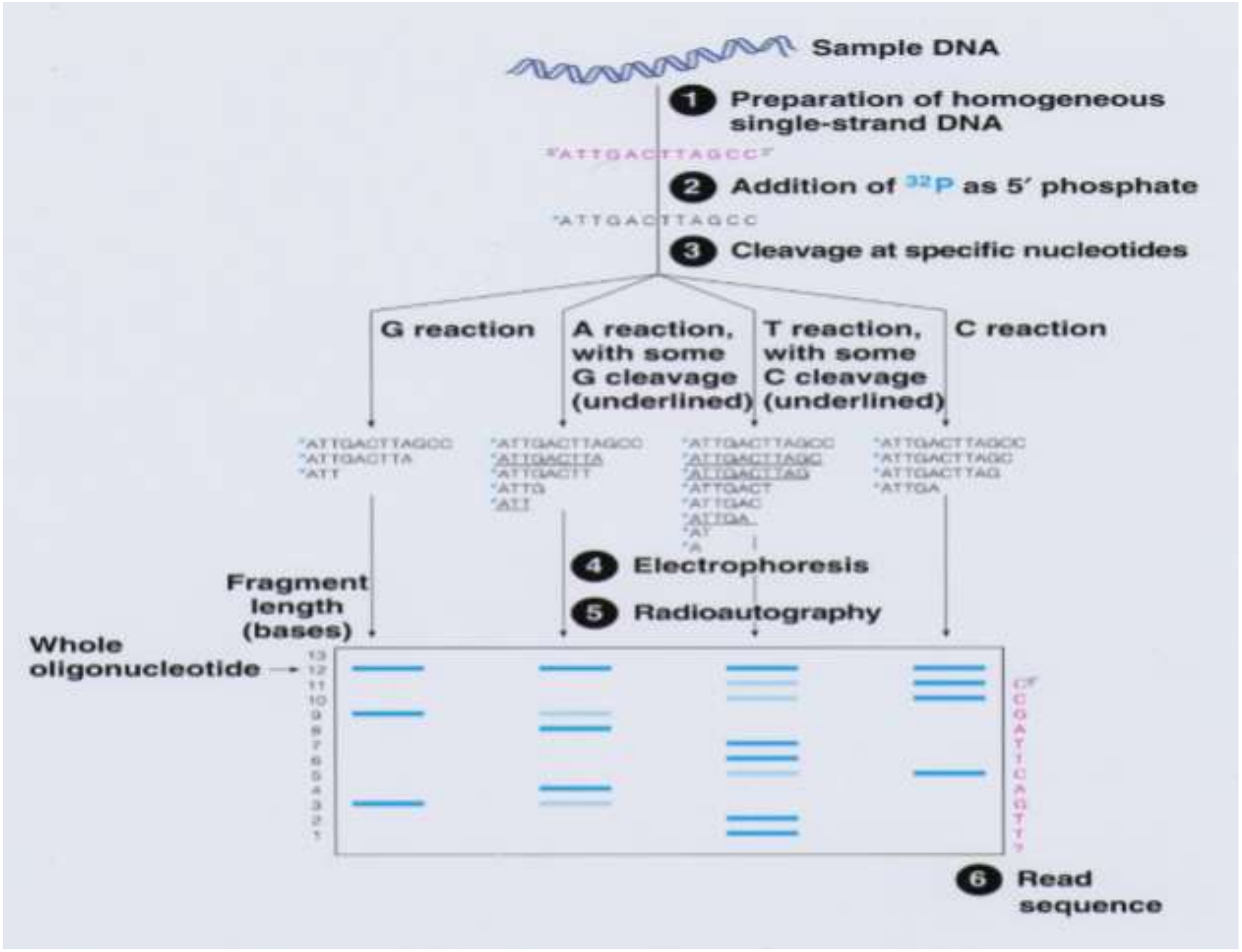


6. Gel electrophoresis (sequencing gel)

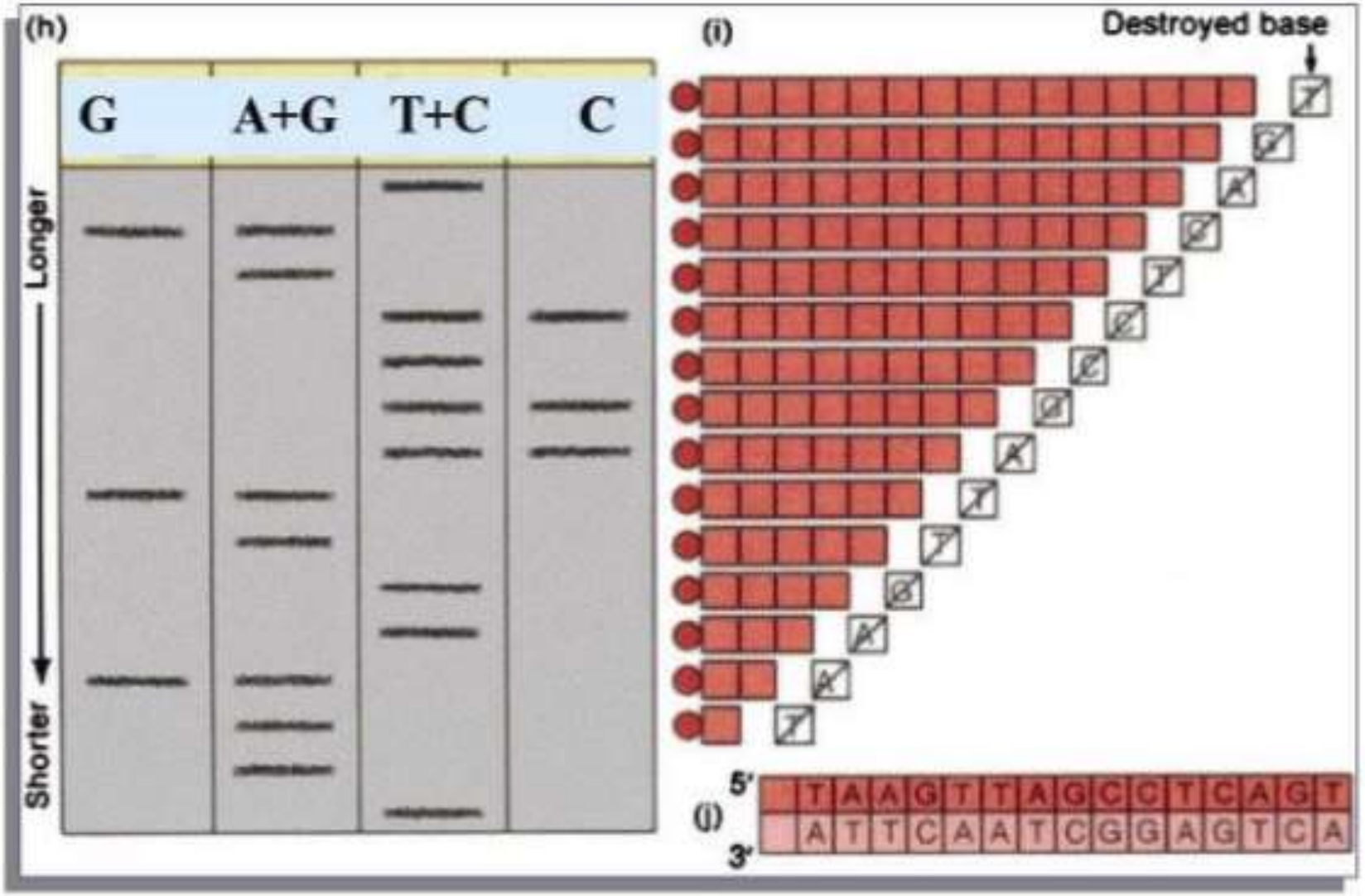
T A G T C G C A G T A C C G T A

7. Determined sequence

A. Sequencing by chemical degradation


Sample DNA
1 Preparation of homogeneous single-strand DNA
*ATTGACTTAGCC
2 Addition of 32P as 5′ phosphate
*ATTGACTTAGCC
3 Cleavage at specific nucleotides
G reaction
A reaction, with some G cleavage (underlined)
T reaction, with some C cleavage (underlined)
C reaction
*ATTGACTTAGCC
*ATTGACTTA
*ATT
*ATTGACTTAGCC
*ATTGACTTA
*ATTGACTT
*ATTG
*ATT
*ATTGACTTAGCC
*ATTGACTTAGC
*ATTGACTTAG
*ATTGACT
*ATTGAC
*ATTGA
*AT
*A
*ATTGACTTAGCC
*ATTGACTTAGC
*ATTGACTTAG
*ATTGA
4 Electrophoresis
5 Radioautography
Fragment length (bases)
Whole oligonucleotide
13
12
11
10
9
8
7
6
5
4
3
2
1
CGATTCAGTTT
6 Read sequence

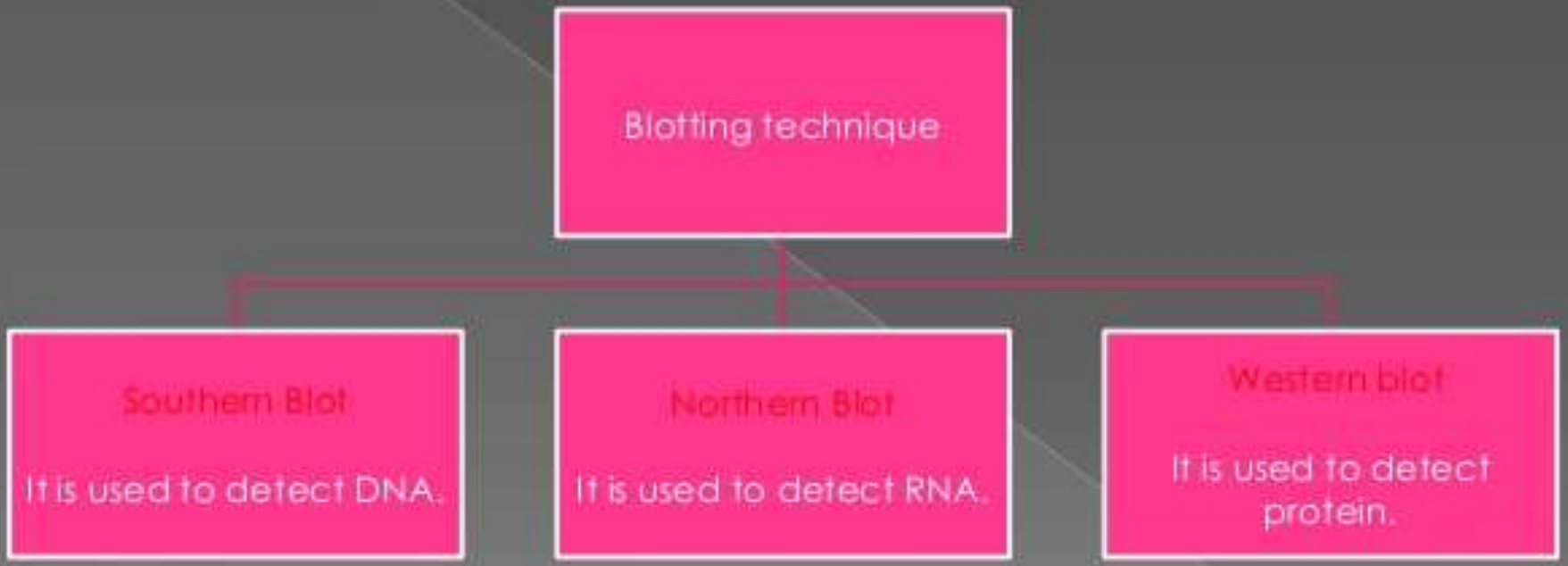
Chemical Degradation Method



What is blotting?

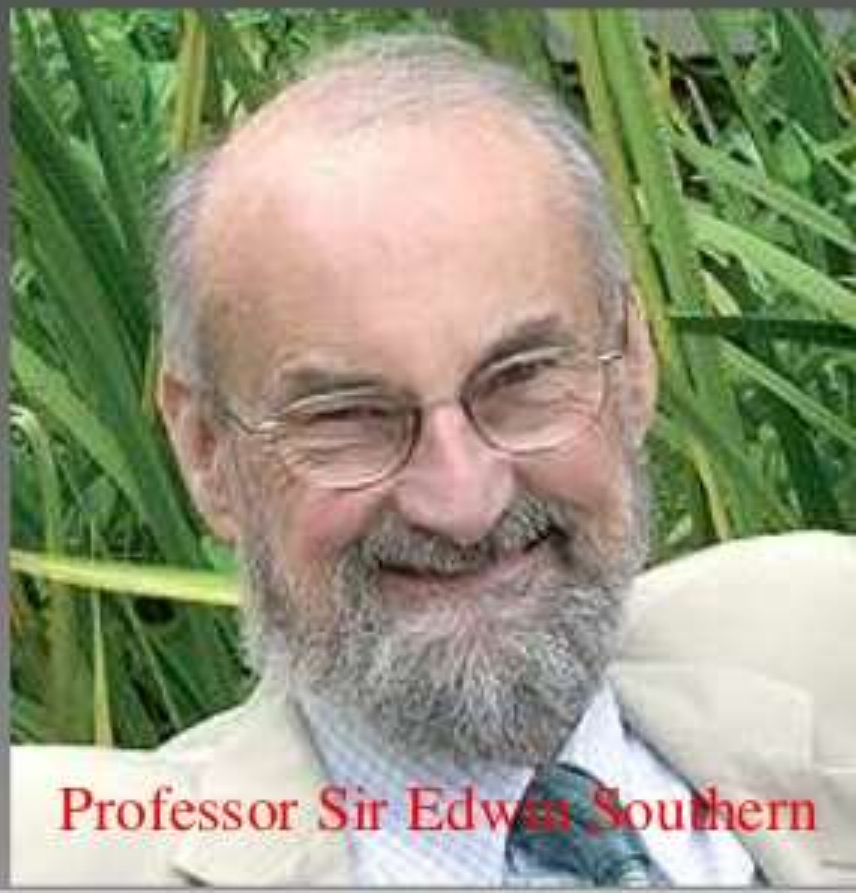
- Blots are techniques for transferring DNA , RNA and proteins onto a carrier so they can be separated, and follows the use of a gel electrophoresis. The Southern blot is used for transferring DNA, the Northern blot for RNA and the western blot for PROTEIN.

TYPES OF BLOTTING TECHNIQUES



SOUTHERN BLOTTING

- Professor Sir Edwin Southern, Professor of Biochemistry and Fellow of Trinity developed this method in 1975.
- Southern won the Lasker Award for Clinical Medical Research prize for the method of finding specific DNA sequences he developed this procedure at Edinburgh University more than 30 years ago. The technique is known as DNA transfer or 'Southern blotting'


Professor Sir Edwin Southern

Cont....

- This method Involves separation, transfer and hybridization.
- It is a method routinely used in molecular biology for detection of a specific DNA sequence in DNA samples.
- The DNA detected can be a single gene, or it can be part of a larger piece of DNA such as a viral genome.

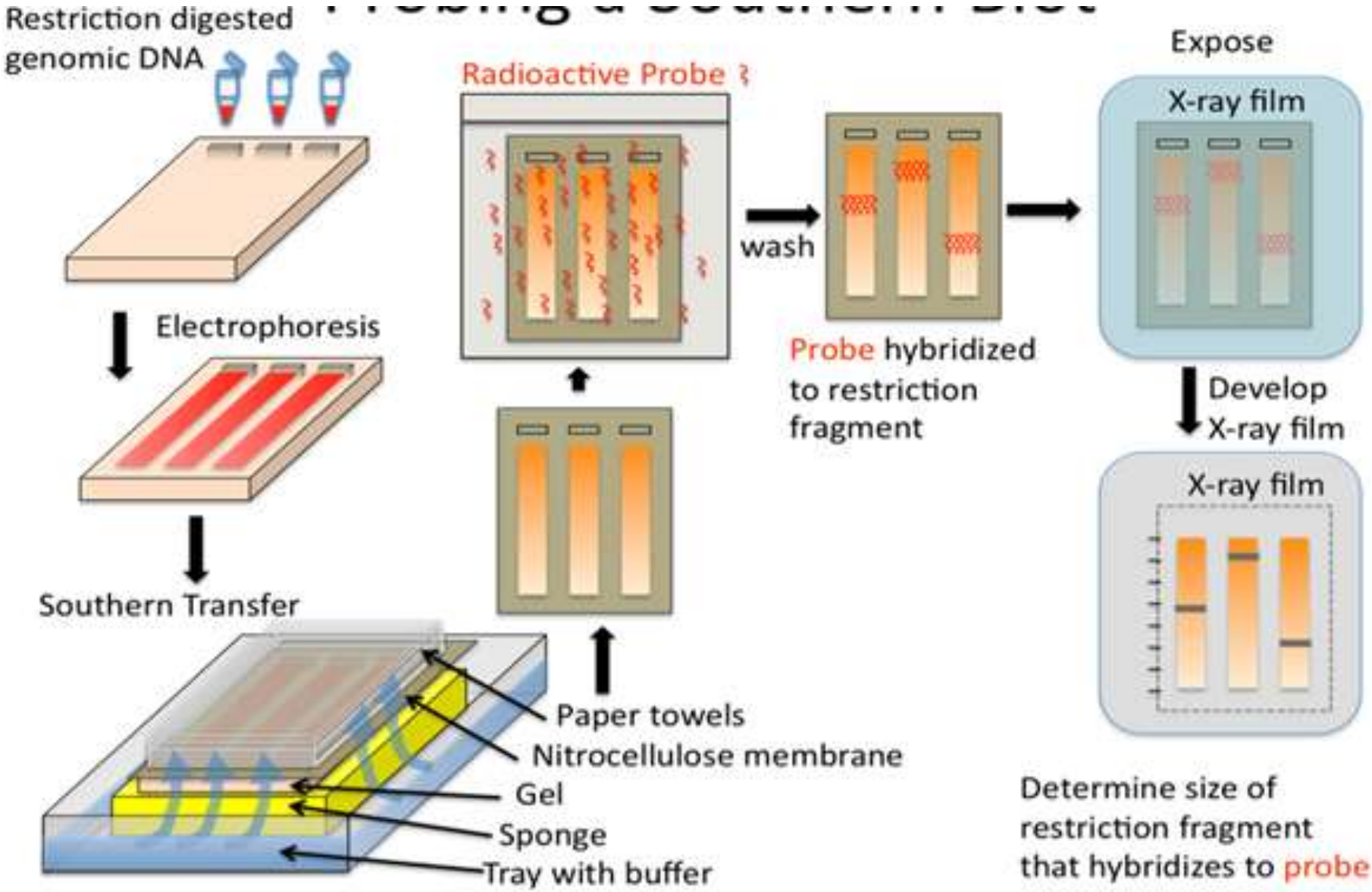
Cont....

- Southern blotting combines agarose gel electrophoresis for size separation of DNA with methods to transfer the size separated DNA to a filter membrane for probe hybridization.
- The key to this method is Hybridization.
- **Hybridization** - Process of forming a double-stranded DNA molecule between a single-stranded DNA probe and a single-stranded target patient DNA.

Southern blotting was introduced by Edwin Southern in 1975 as a method to detect specific sequences of DNA in DNA samples.

The other blotting techniques emerged from this method have been termed as Northern (for RNA), Western (for proteins), Eastern (for post-translational protein modifications) and South-western (for DNA-protein interactions) blotting.

Southern hybridization



APPLICATIONS

- Southern blots are used in gene discovery , mapping, evolution and development studies, diagnostics and forensics (It is used for DNA fingerprinting, preparation of RFLP maps)
- identification of the transferred genes in transgenic individuals, etc.

APPLICATIONS

- Southern blots allow investigators to determine the molecular weight of a restriction fragment and to measure relative amounts in different samples.
- Southern blot is used to detect the presence of a particular bit of DNA in a sample
- analyze the genetic patterns which appear in a person's DNA.

Northern Blotting

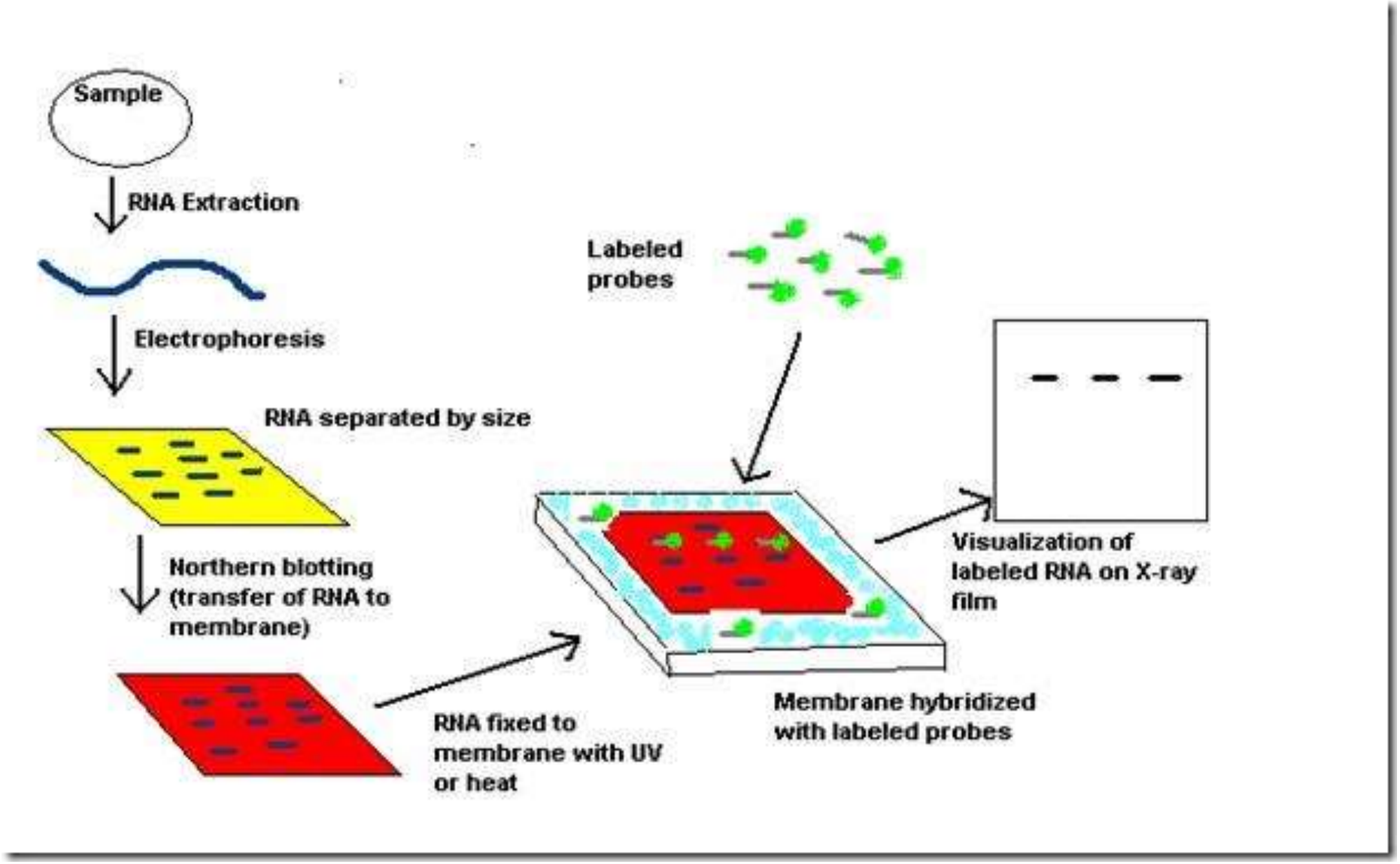
Northern blotting is a technique for detection of specific RNA sequences.

Northern blotting was developed by James Alwine and George Stark at Stanford University (1979) and was named such by analogy to Southern blotting

WHY MEASURE AN mRNA?

- The first is **to determine which tissues express a particular gene**, and this can give some indication of the physiological function of the encoded protein.

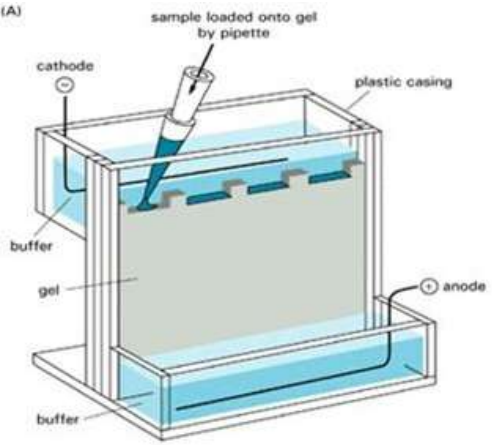
- The second principal reason for measuring an mRNA is **to determine the factors which regulate the expression of a given gene, be they nutritional, hormonal, or environmental**.

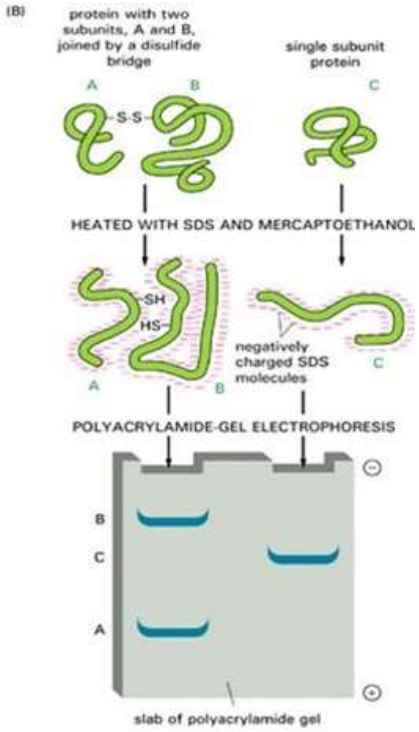

Sample
RNA Extraction
Electrophoresis
RNA separated by size
Northern blotting
(transfer of RNA to
membrane)
RNA fixed to
membrane with UV
or heat
Labeled
probes
Membrane hybridized
with labeled probes
Visualization of
labeled RNA on X-ray
film

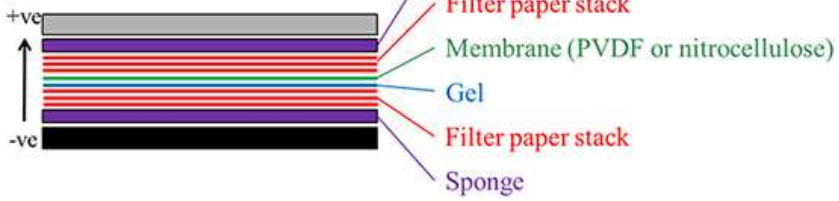
APPLICATIONS

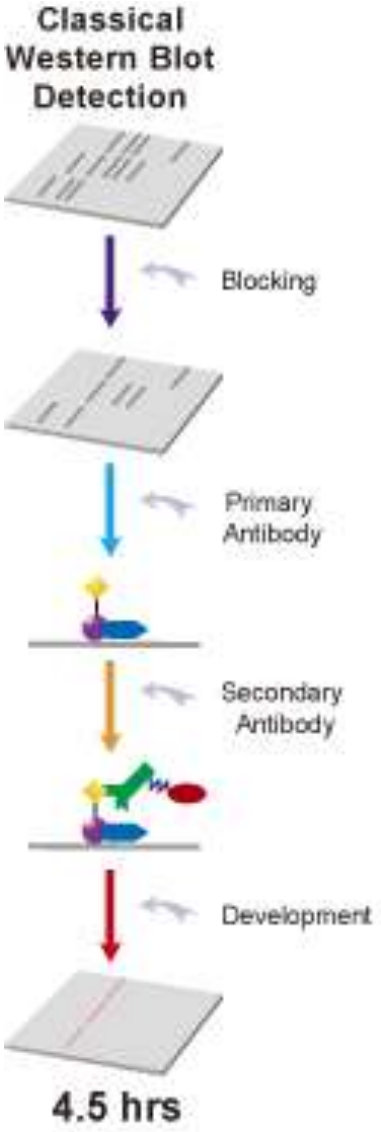
- A standard for the study of gene expression at the level of mRNA (messenger RNA transcripts)
- Detection of mRNA transcript size
- Study RNA degradation
- Study RNA splicing
- Study RNA half-life
- Often used to confirm and check transgenic / knockout mice (animals)

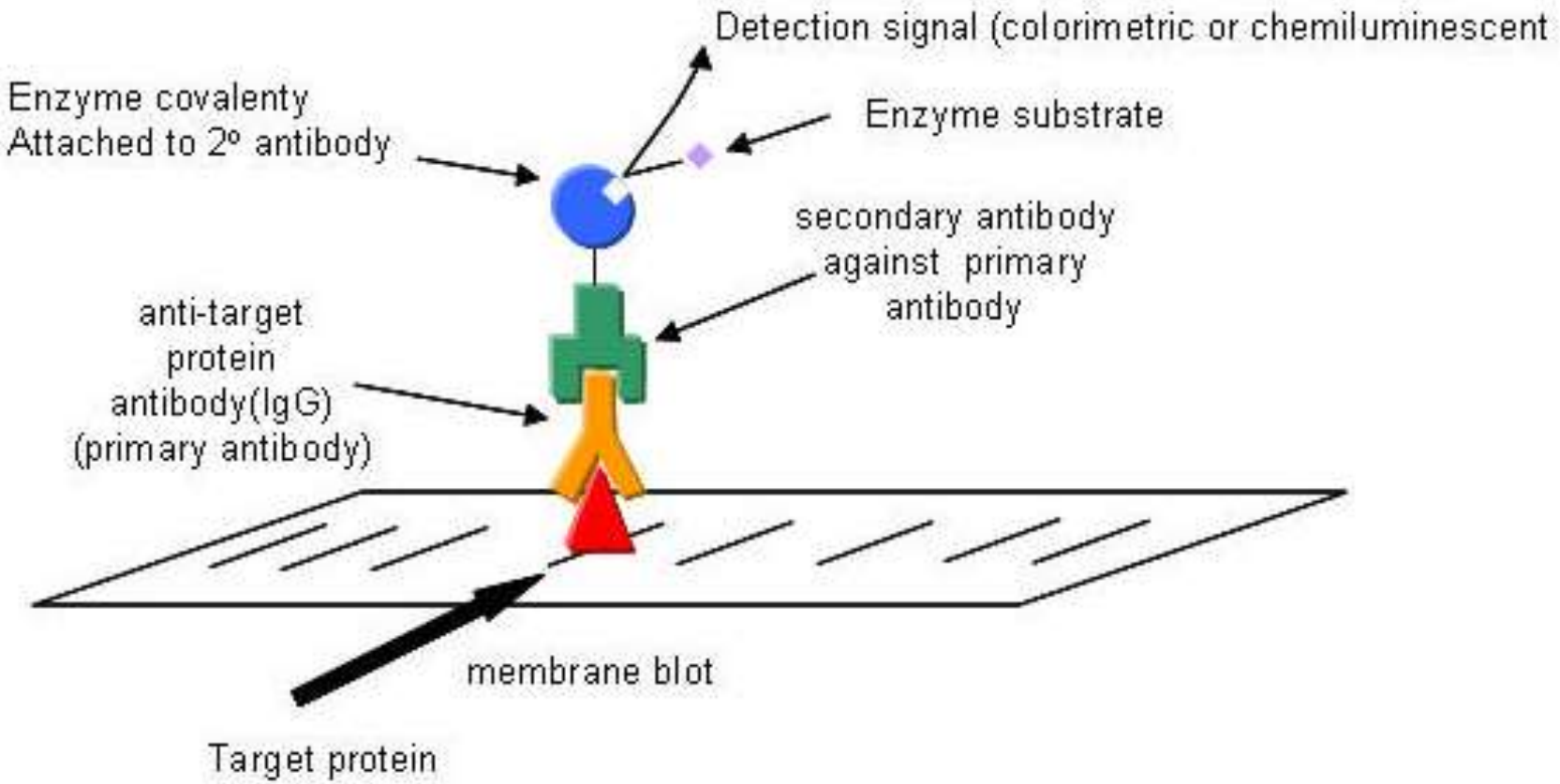
Western hybridization



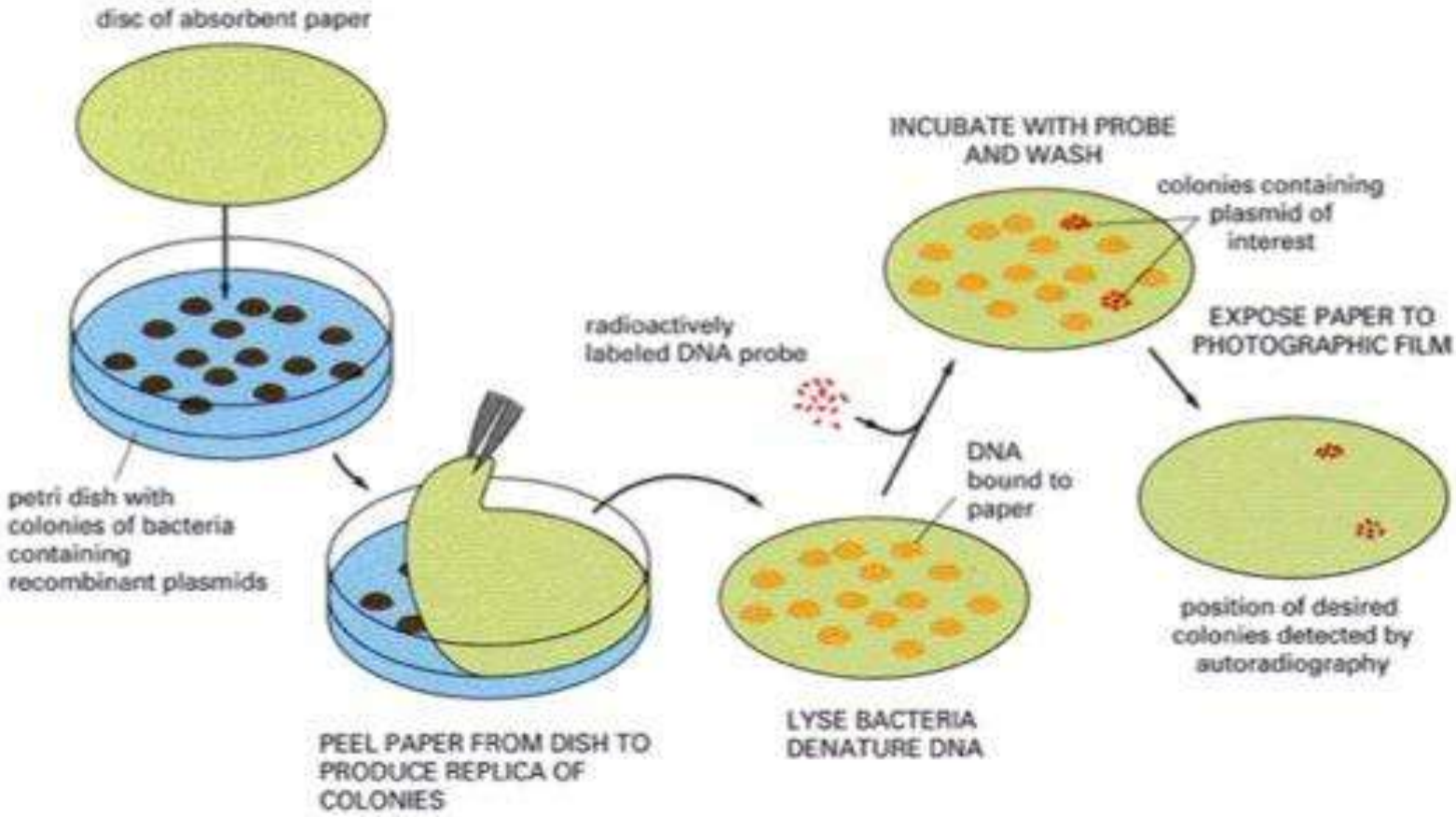




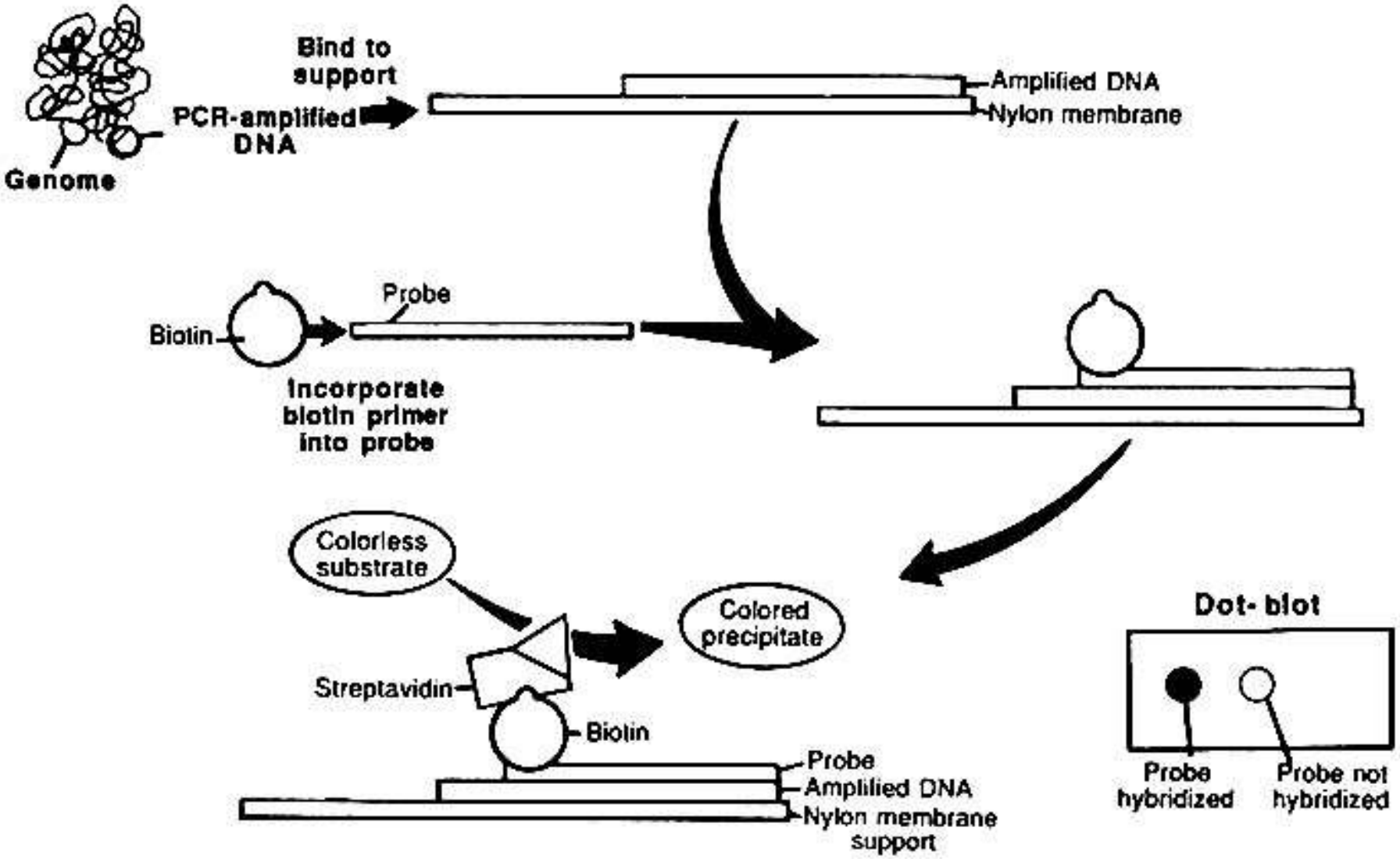



Detection signal (colorimetric or chemiluminescent
Enzyme covalenty
Attached to 2º antibody
Enzyme substrate
secondary antibody
against primary
antibody
anti-target
protein
antibody(IgG)
(primary antibody)
membrane blot
Target protein

Colony hybridization



Dot Blot


Genome
PCR-amplified DNA
Bind to support
Amplified DNA
Nylon membrane
Biotin
Probe
Incorporate biotin primer into probe
Colorless substrate
Colored precipitate
Streptavidin
Biotin
Probe
Amplified DNA
Nylon membrane support
Dot- blot
Probe hybridized
Probe not hybridized