DNA Sequencing

- **DNA sequencing** includes several methods and technologies that are used for determining the order of the nucleotide bases adenine, guanine, cytosine, and thymine in a molecule of DNA.

- In 1977 two separate methods for sequencing DNA were developed:

The chain termination method or cycle sequencing (Sanger and Coulson)

and the chemical degradation method or Maxam-Gilbert sequencing (Maxam and Gilbert)

Maxam–Gilbert method

- In 1976–1977, Allan Maxam and walter Gilbert devised the first method for sequencing DNA fragments containing up to ~ 500 nucleotides.

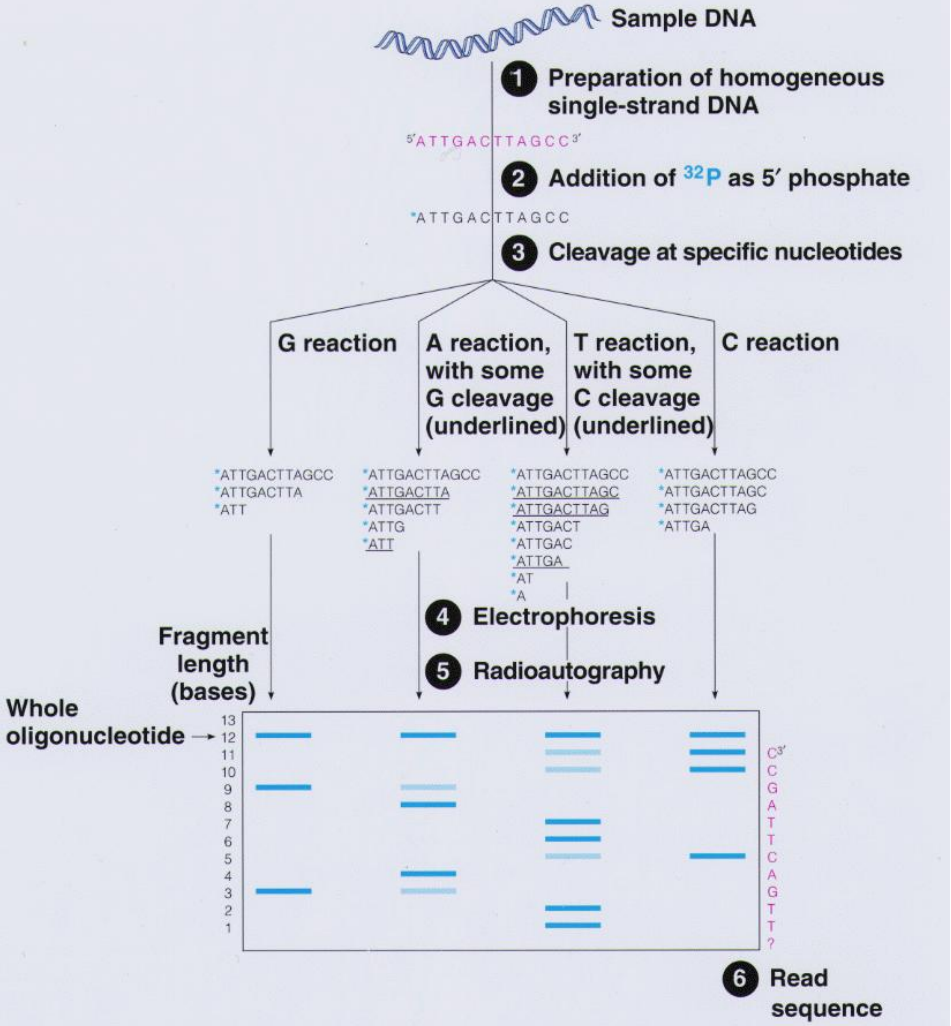
- The sequence of a double-stranded DNA molecule is determined by treatment with chemicals that cut the molecule at specific nucleotide positions.

- It is the early method involving base-specific chemical modification and subsequent cleavage of DNA.

- Maxam-Gilbert sequencing is performed by chain breakage at specific nucleotides.

Procedure

Figure 4A.4 Sequencing an oligonucleotide by the Maxam-Gilbert method



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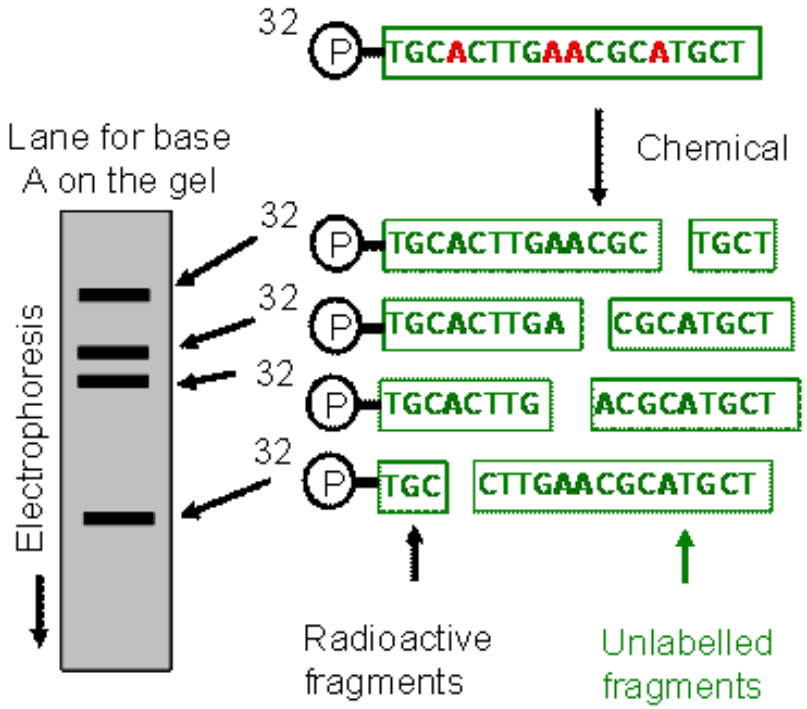


Figure 1. Principle of Maxam-Gilbert procedure. The figure shows an example for only one base (A).

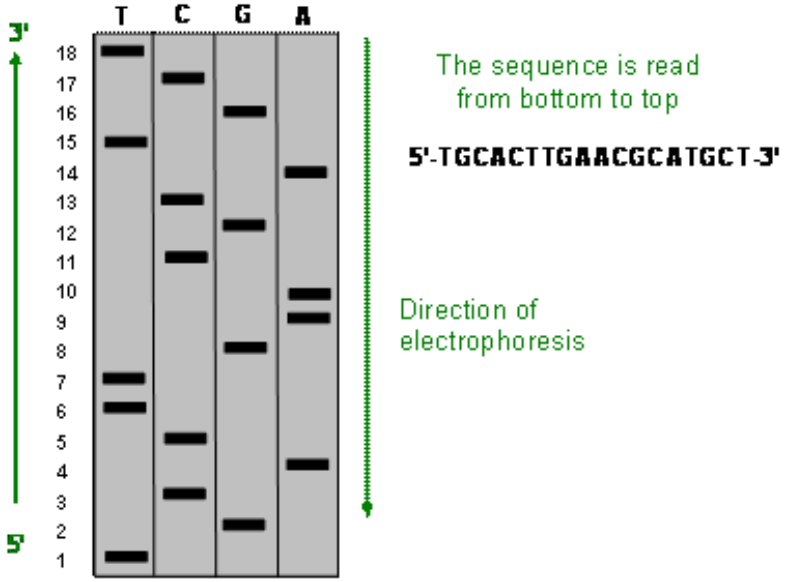
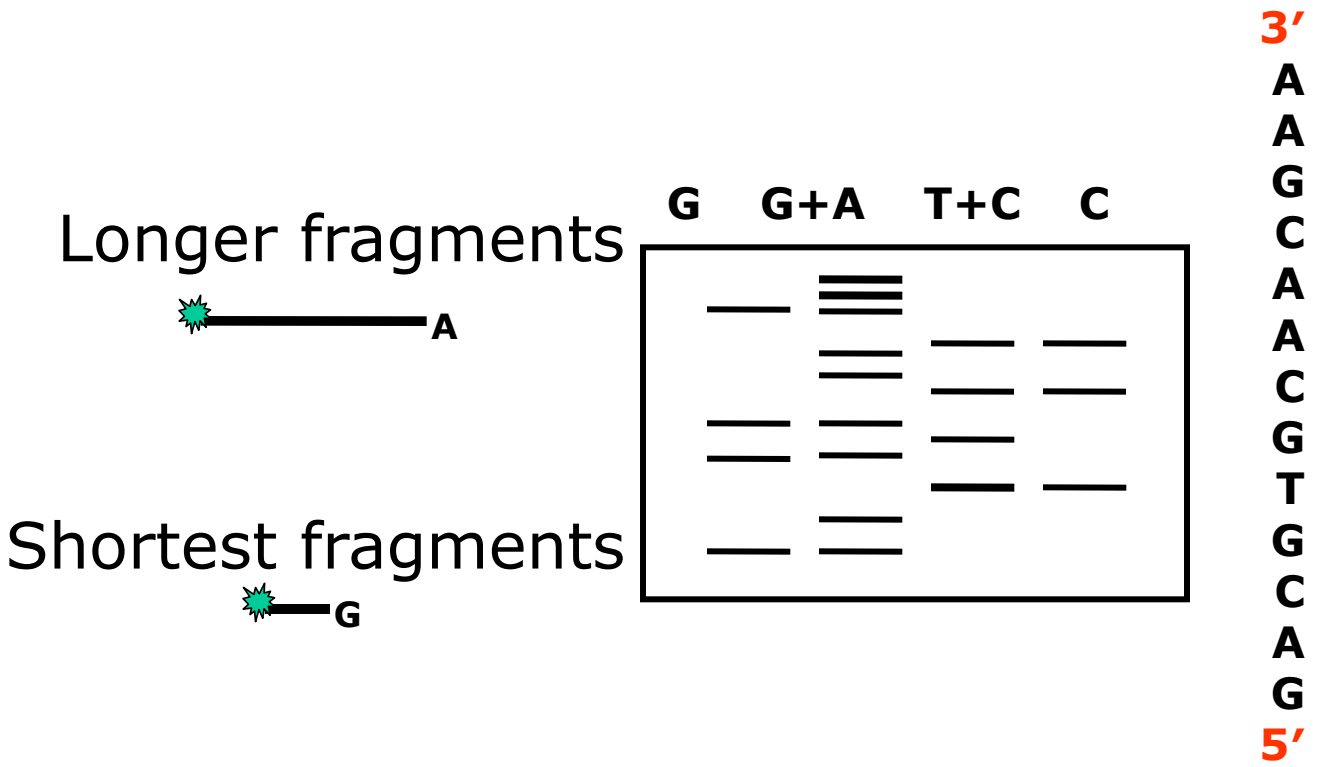


Figure 2. Reading the sequence from the gel.

Maxam-Gilbert Sequencing



Sequencing gels are read from bottom to top (5′ to 3′).

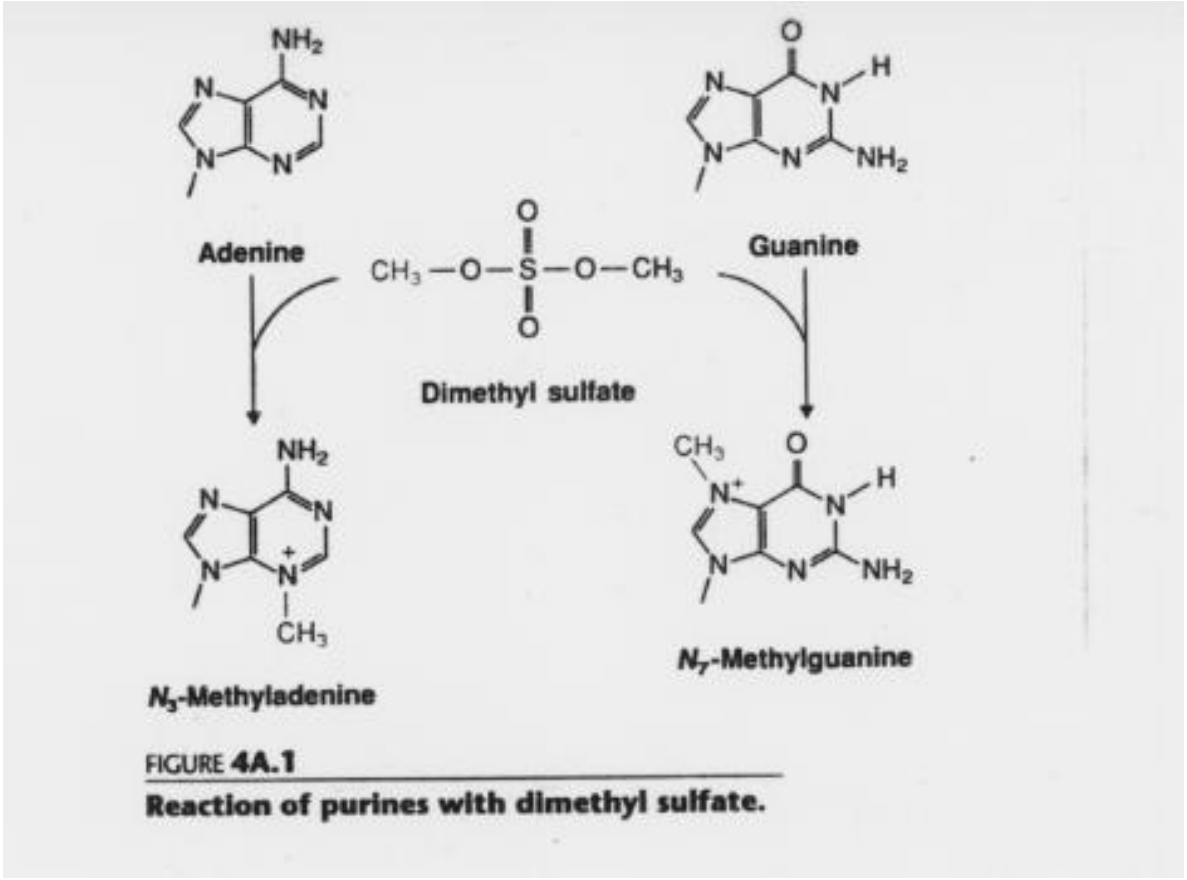
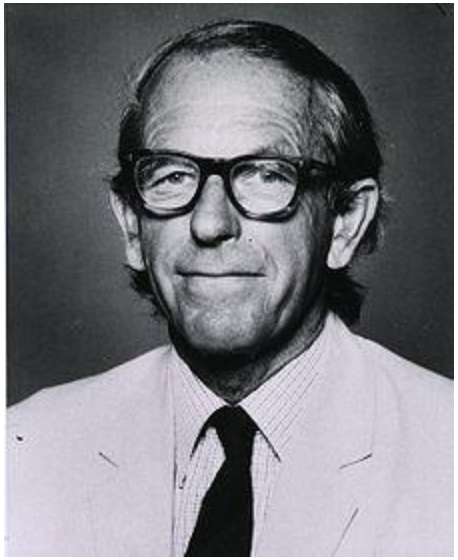


FIGURE **4A.1**

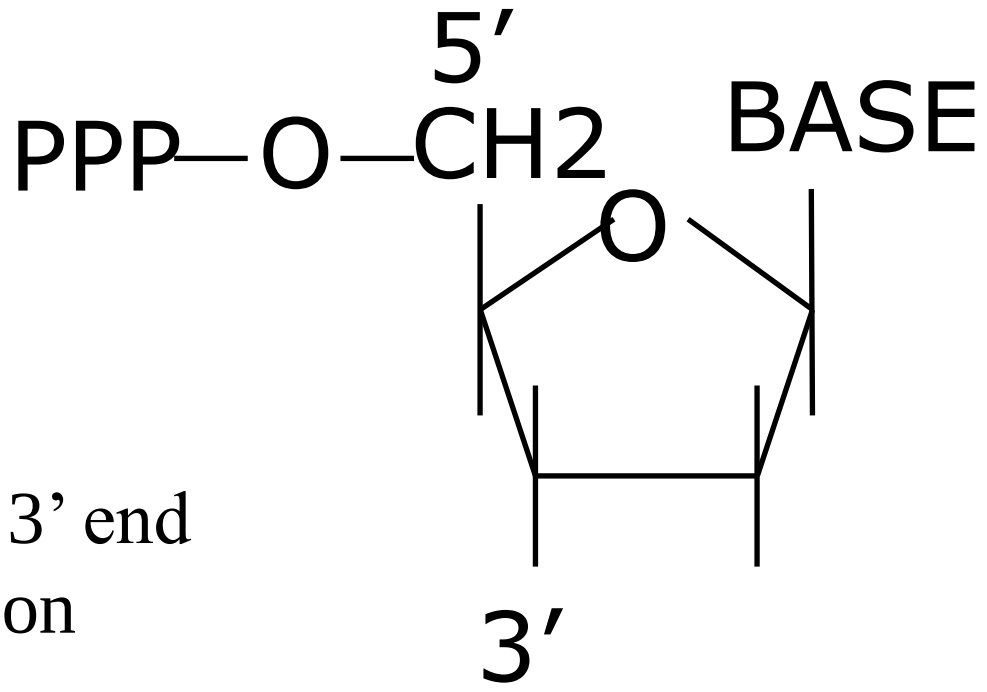
Reaction of purines with dimethyl sulfate.

Chain Termination (Sanger) Sequencing

-The key principle of the Sanger method was the use of dideoxynucleotide triphosphates (ddNTPs) as DNA chain terminators.

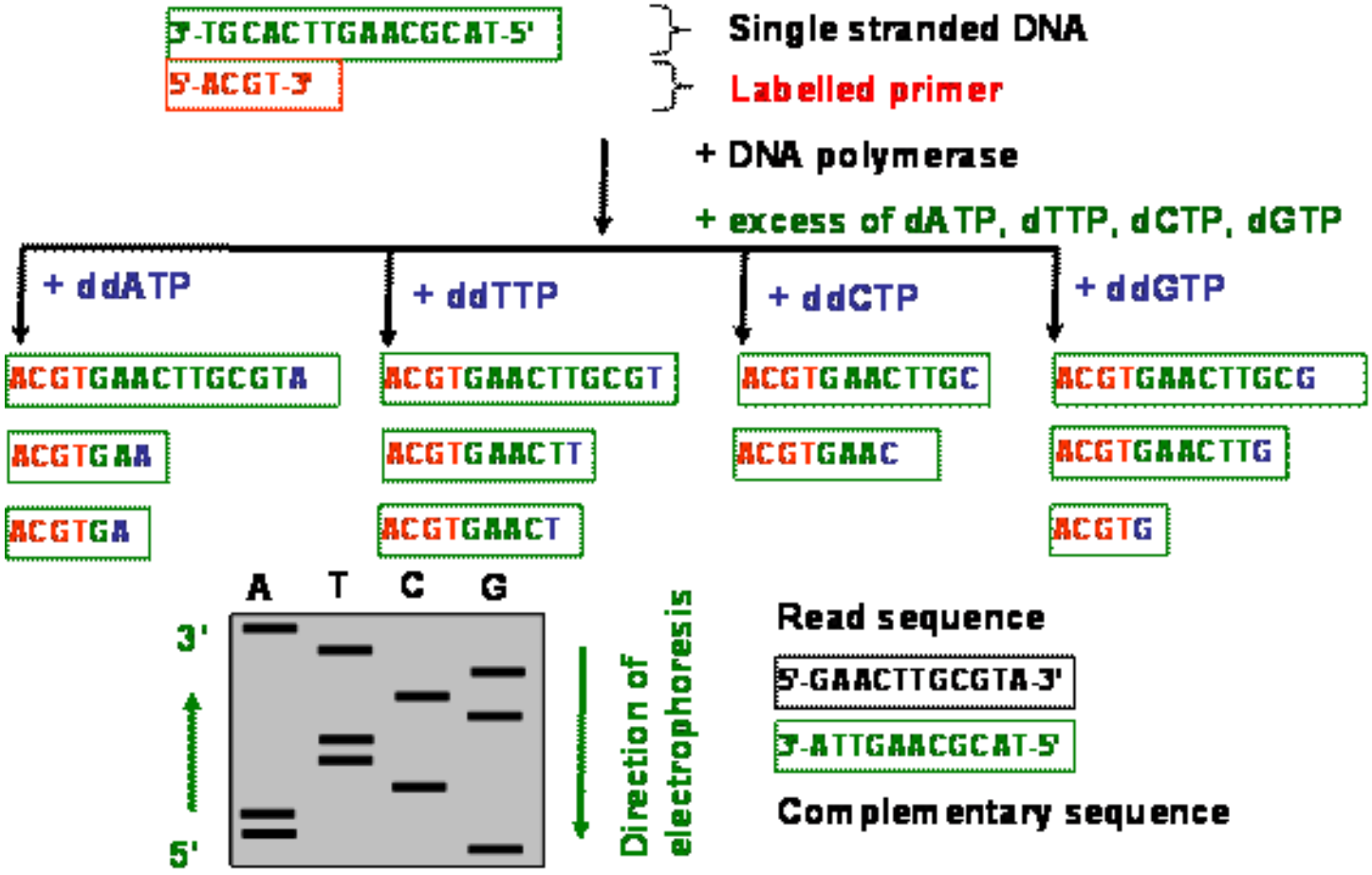


Dideoxynucleotide



- no hydroxyl group at 3’ end
prevents strand extension

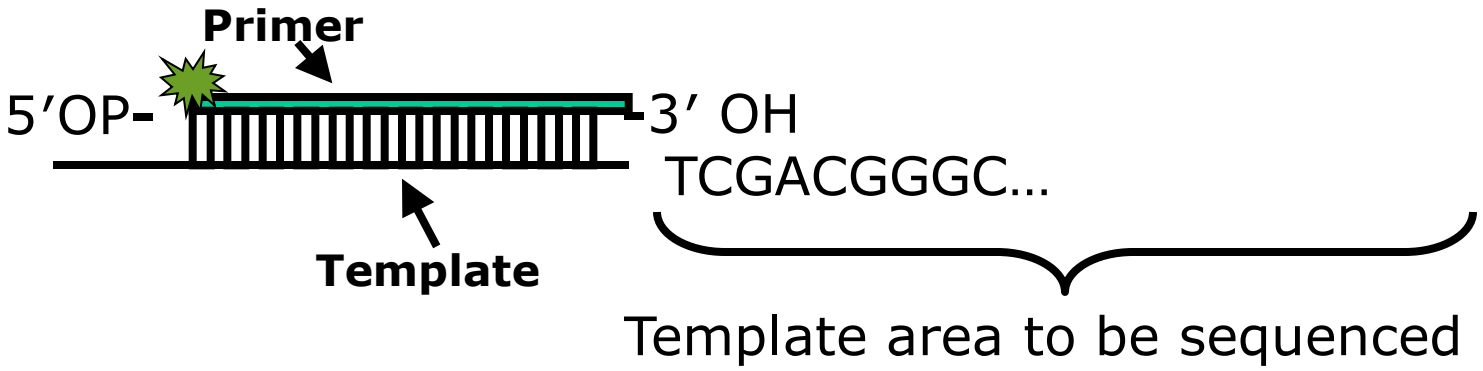
- The 3′-OH group necessary for formation of the phosphodiester bond is missing in ddNTPs.



Principle of Sanger dideoxy sequencing

Sanger Method procedure

1- A sequencing reaction mix includes labeled primer and template.



2- four dNTPs.

3- Dideoxynucleotides are added separately to each of the four tubes.

Sanger Method

ddATP + ddA
fourdNTPs dAdGdCdTdGdCdCdCdG

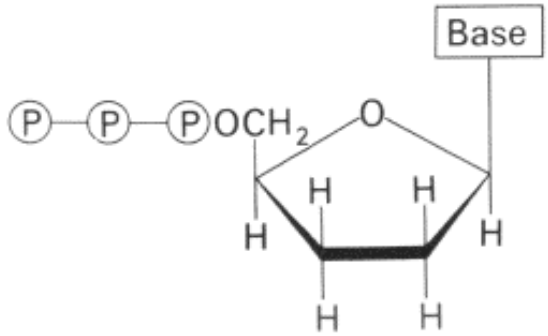
ddCTP + dAdGddC
fourdNTPs dAdGdCdTdGddC
dAdGdCdTdGdCddC
dAdGdCdTdGdCdCddC

ddGTP + dAddG
fourdNTPs dAdGdCdTddG
dAdGdCdTdGdCdCdCddG

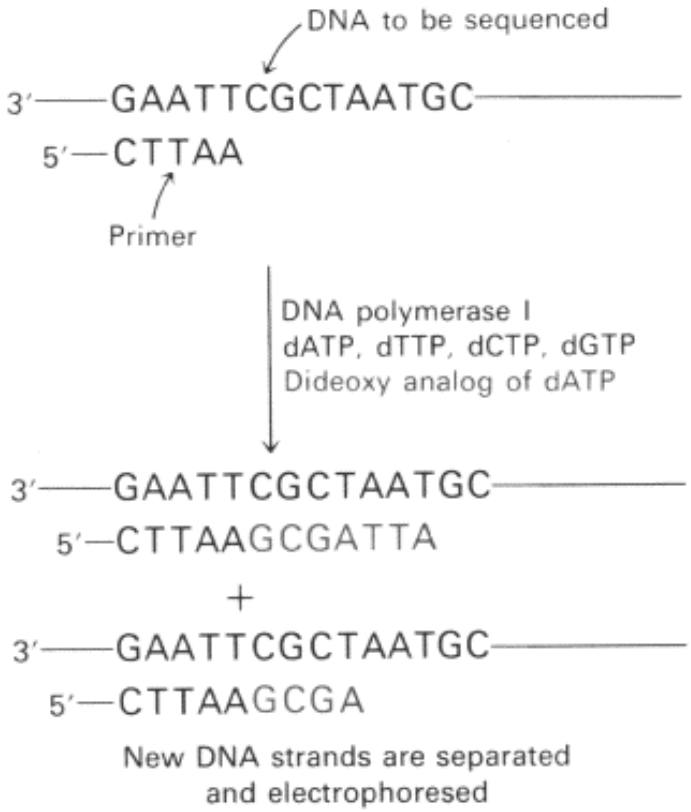
ddTTP + dAdGdCddT
fourdNTPs dAdGdCdTdGdCdCdCdG

- With addition of enzyme (DNA polymerase), the primer is extended until a ddNTP is encountered.
- The chain will end with the incorporation of the ddNTP.
- With the proper dNTP:ddNTP ratio, the chain will terminate throughout the length of the template.
- All terminated chains will end in the ddNTP added to that reaction.

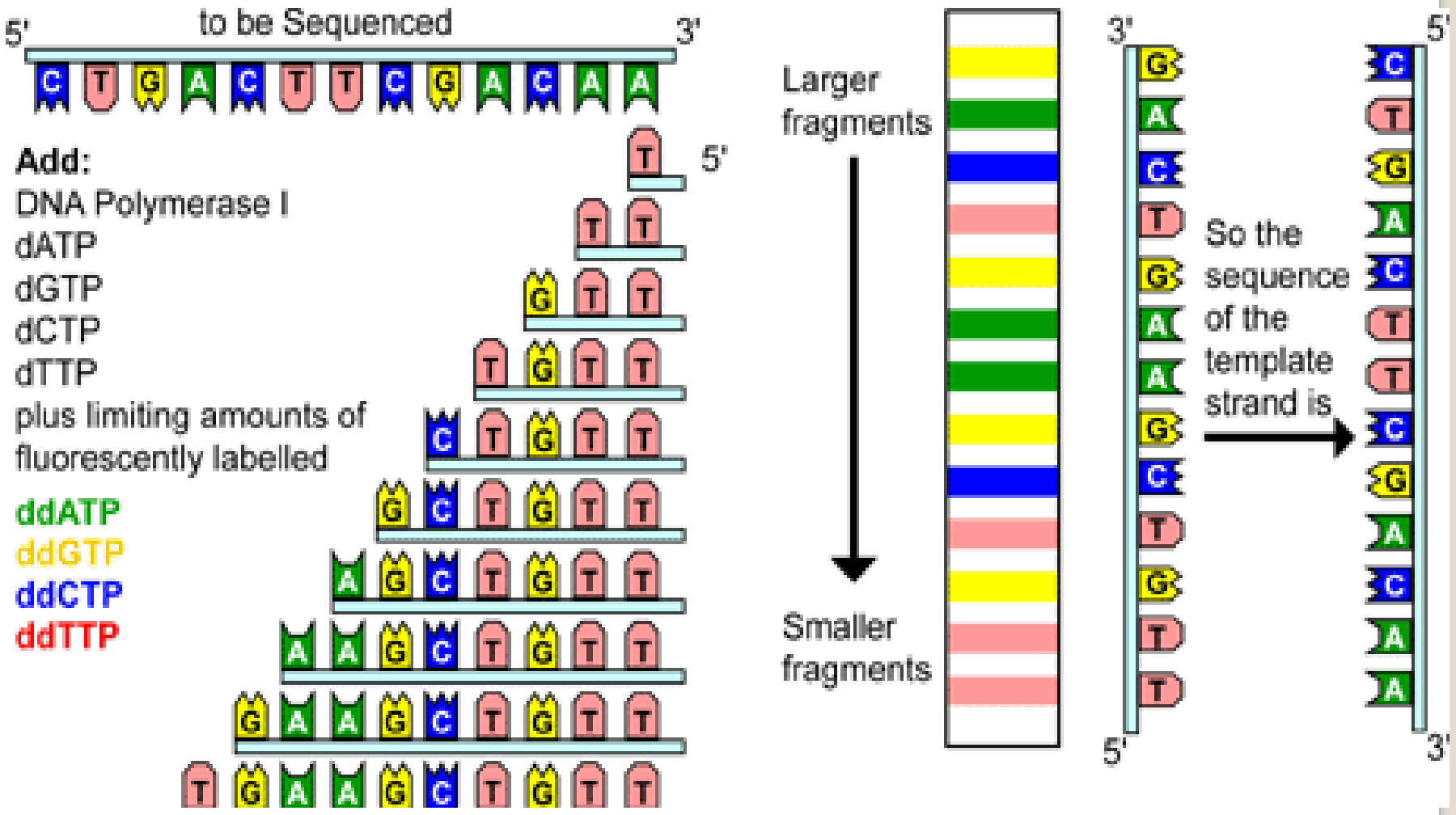
- The collection of fragments is a sequencing ladder.
- The resulting terminated chains are resolved by electrophoresis.
- Fragments from each of the four tubes are placed in four separate gel lanes.
- X-ray film
- Read DNA sequence from bottom to top (5′ to 3′).



2′,3′-Dideoxy analog



New DNA strands are separated and electrophoresed



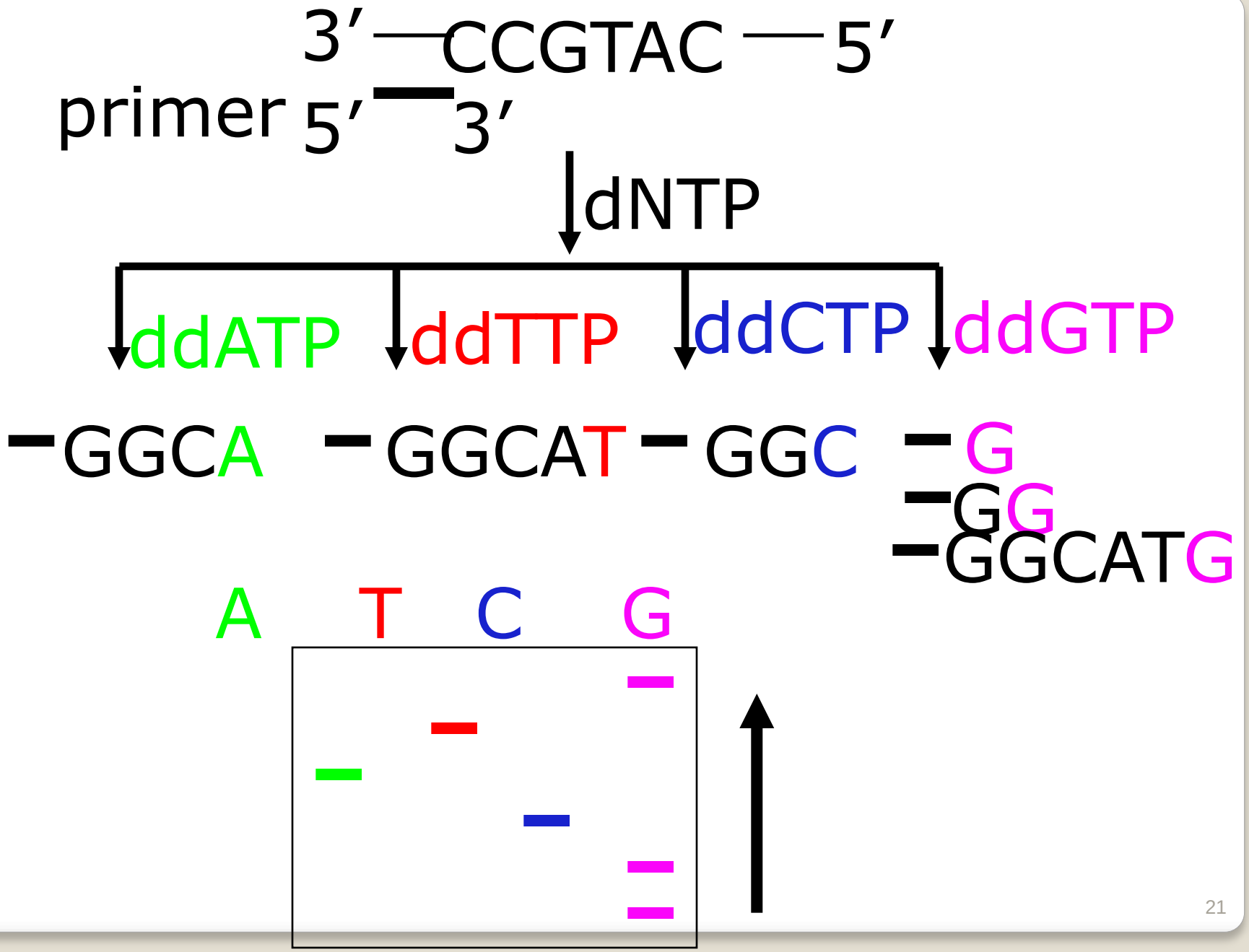
Sequencing gels are read from bottom to top (5′ to 3′).

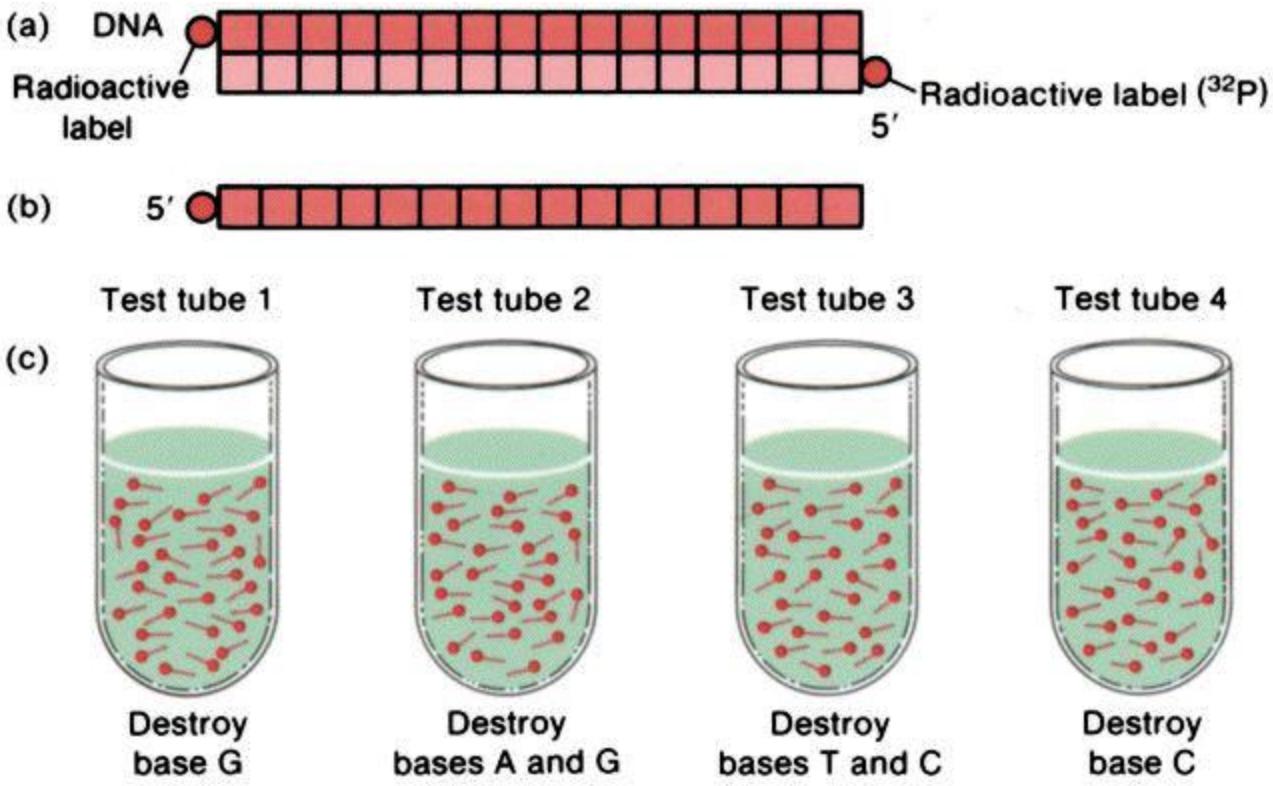
Sanger Method

- in-vitro DNA synthesis using ‘terminators’, use of dideoxi-nucleotides that do not permit chain elongation after their integration
- DNA synthesis using deoxy- and dideoxynucleotides that results in termination of synthesis at specific nucleotides
- Requires a primer, DNA polymerase, a template, a mixture of nucleotides, and detection system
- Incorporation of di-deoxynucleotides into growing strand terminates synthesis
- Synthesized strand sizes are determined for each di-deoxynucleotide by using gel or capillary electrophoresis
- Enzymatic methods

The principles

- Partial copies of DNA fragments made with DNA polymerase
- Collection of DNA fragments that terminate with A,C,G or T using ddNTP
- Separate by gel electrophoresis
- Read DNA sequence

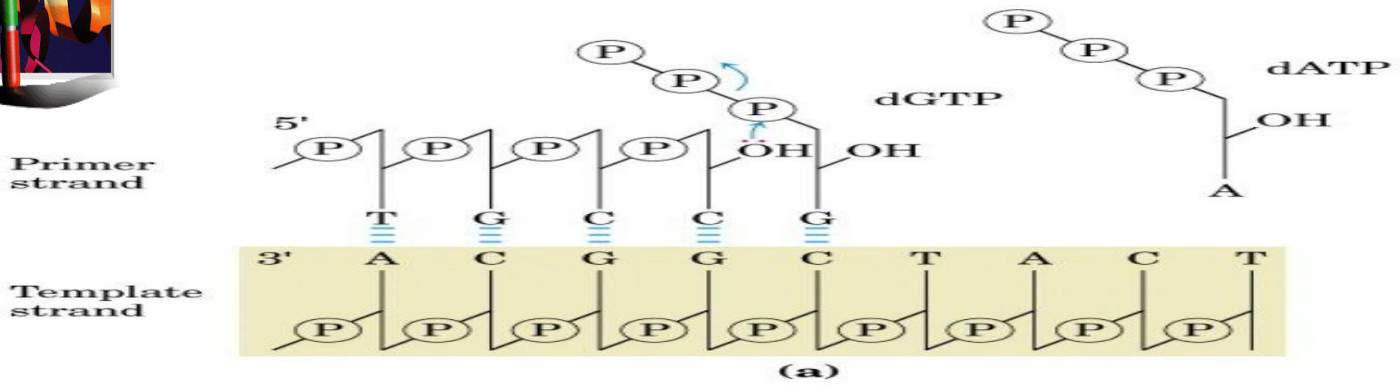

3′ CCGTAC 5′
primer 5′ 3′
dNTP
ddATP
ddTTP
ddCTP
ddGTP
GGCA
GGCAT
GGC
G
GG
GGCATG
A
T
C
G

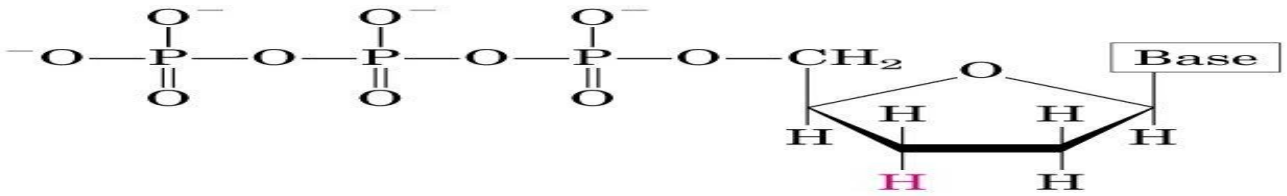

(a) DNA
Radioactive label
Radioactive label (^{32}P)
5′
(b) 5′
Test tube 1
Test tube 2
Test tube 3
Test tube 4
(c)
Destroy base G
Destroy bases A and G
Destroy bases T and C
Destroy base C

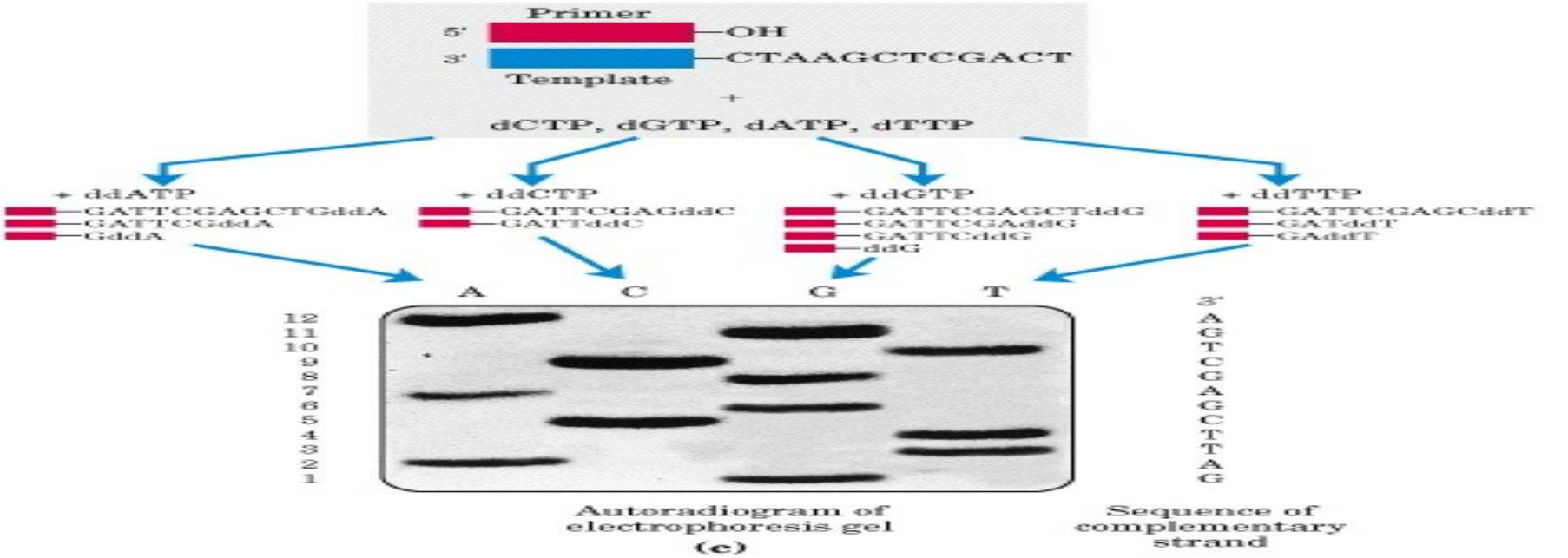
Materials needed DNA sequencing to process

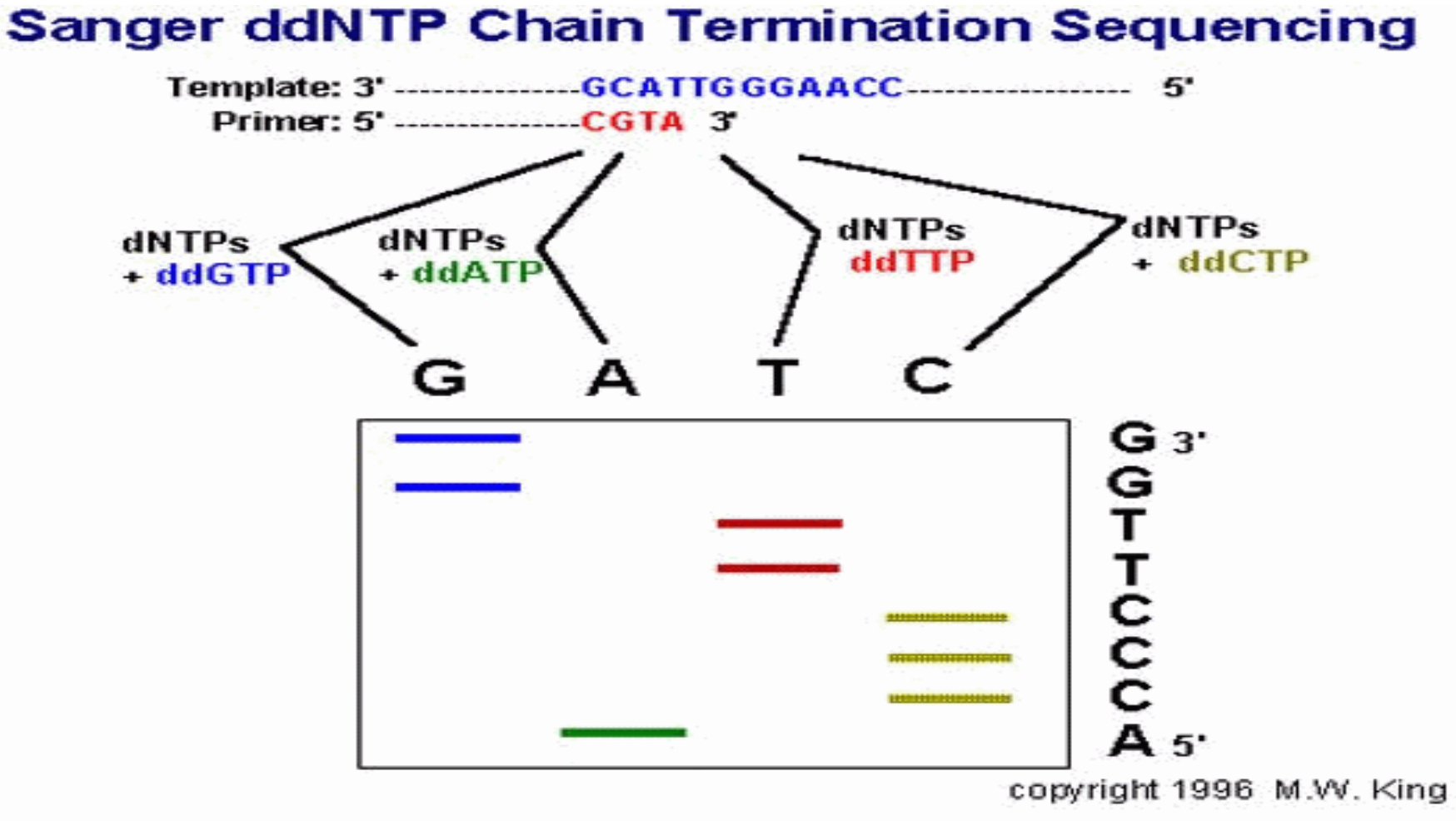
- A **primer** tagged with a mildly radioactive molecule or a light-emitting chemical .
- **DNA polymerase--**an enzyme that drives the synthesis of DNA.
- Four **deoxynucleotides** (G, A, C, T);
- One **dideoxynucleotide**, either ddG, ddA, ddC, or ddT.

DNA Sequencing

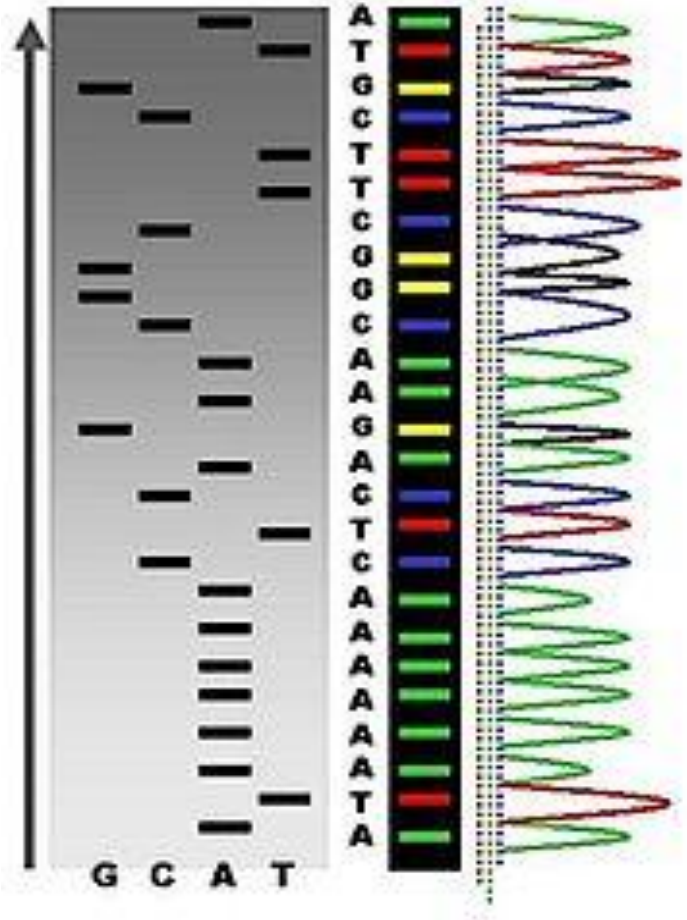


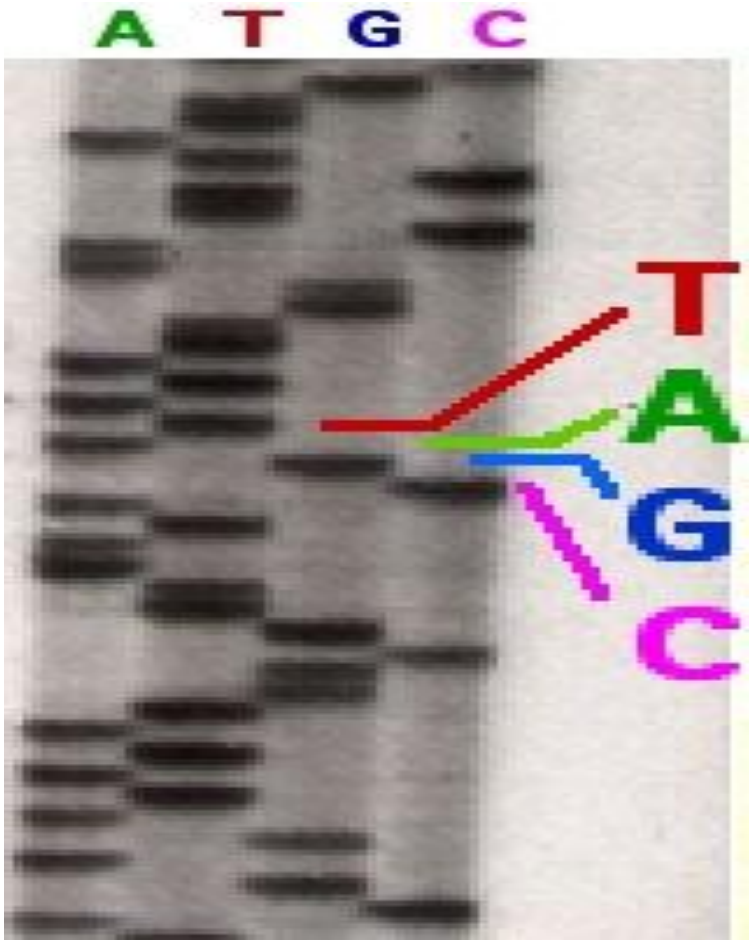






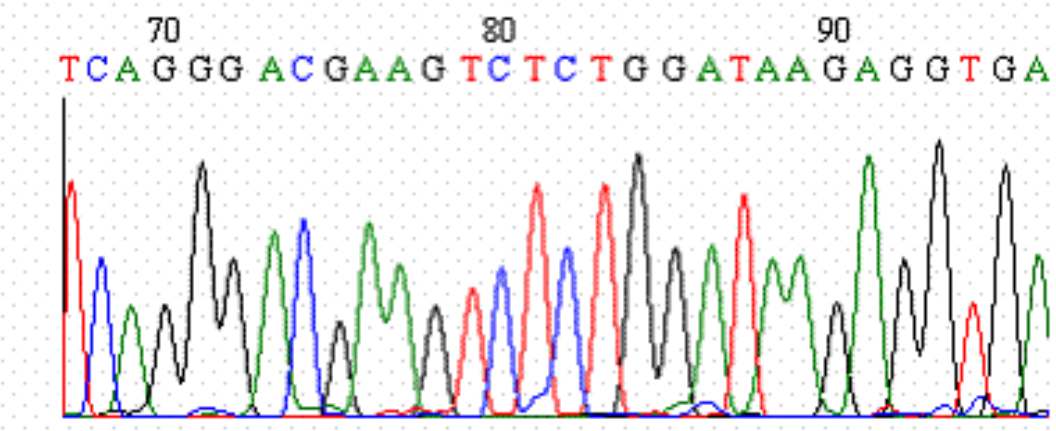
DNA sequencing


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


A T G C
T
A
G
C

Automated Sequencing

Automated sequencing is based on the dideoxy methodology, but four different fluorescent dye-labelled ddNTPs are used. Thus each fluorescent label can be detected by its characteristic spectrum. The products are separated by automated electrophoresis and the bands detected by fluorescence spectroscopy.

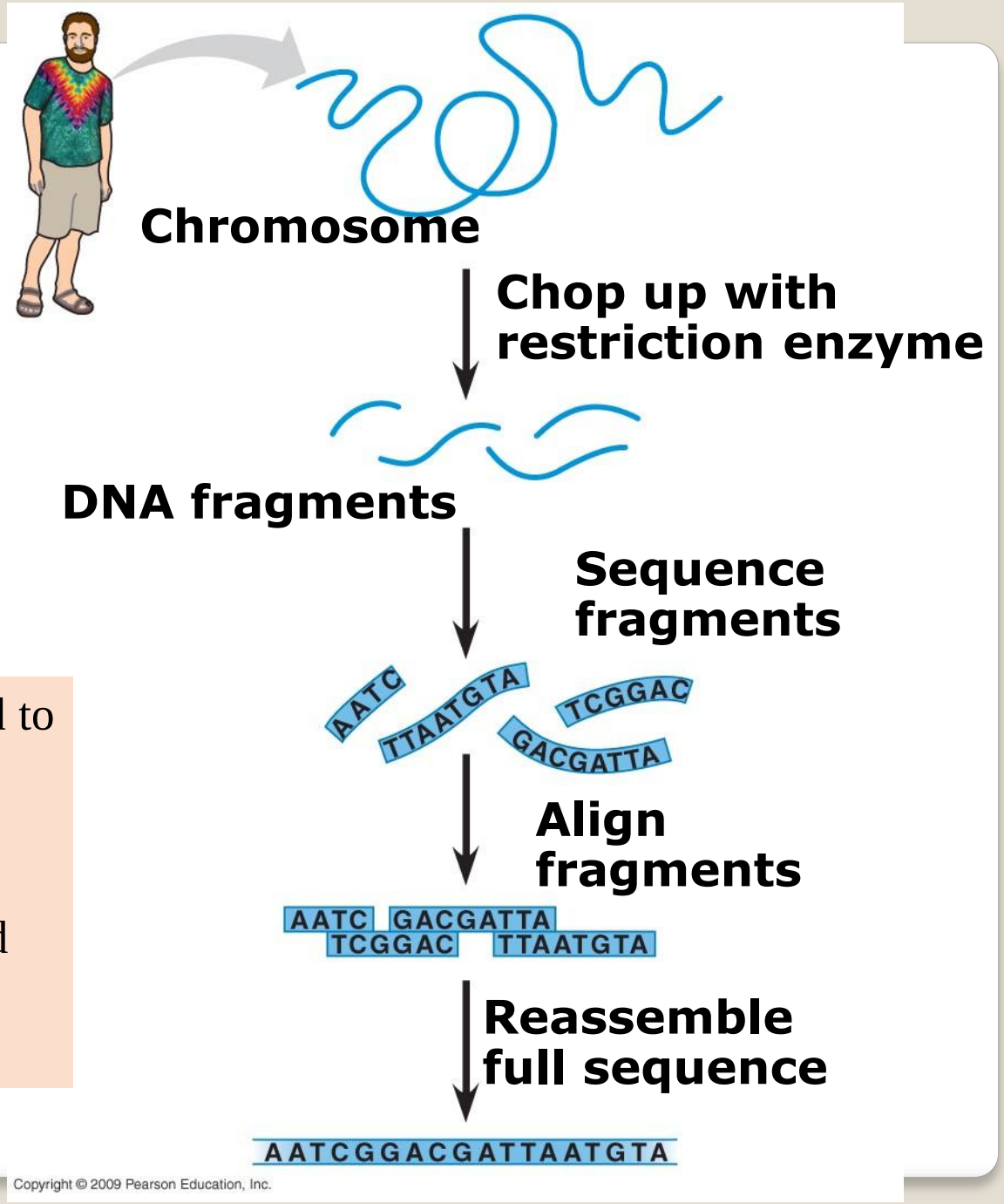


An example of a portion of a chromatogram from automated sequencing

Approaches to Genome Analysis

- Prior to the availability of entire genome sequences, the study of genomes required several approaches that complement each other.
- Of primary interest is the size of a genome, the number of genes it contains, and their distribution (gene density), function, and evolution.
- Two basic approaches to sequencing a genome can be distinguished: Clone-by-clone sequencing:
- individual DNA clones of known relation to each other are isolated, arranged in their proper alignment, and sequenced.
- The shotgun approach:
- breaks the genome into millions of fragments of unknown relation. The individual DNA clones for which prior knowledge of their precise origin is lacking are sequenced. Subsequently, the clones are aligned by high-capacity computers.
- The two approaches complement each other.

Whole-genome shotgun method for sequencing the human genome

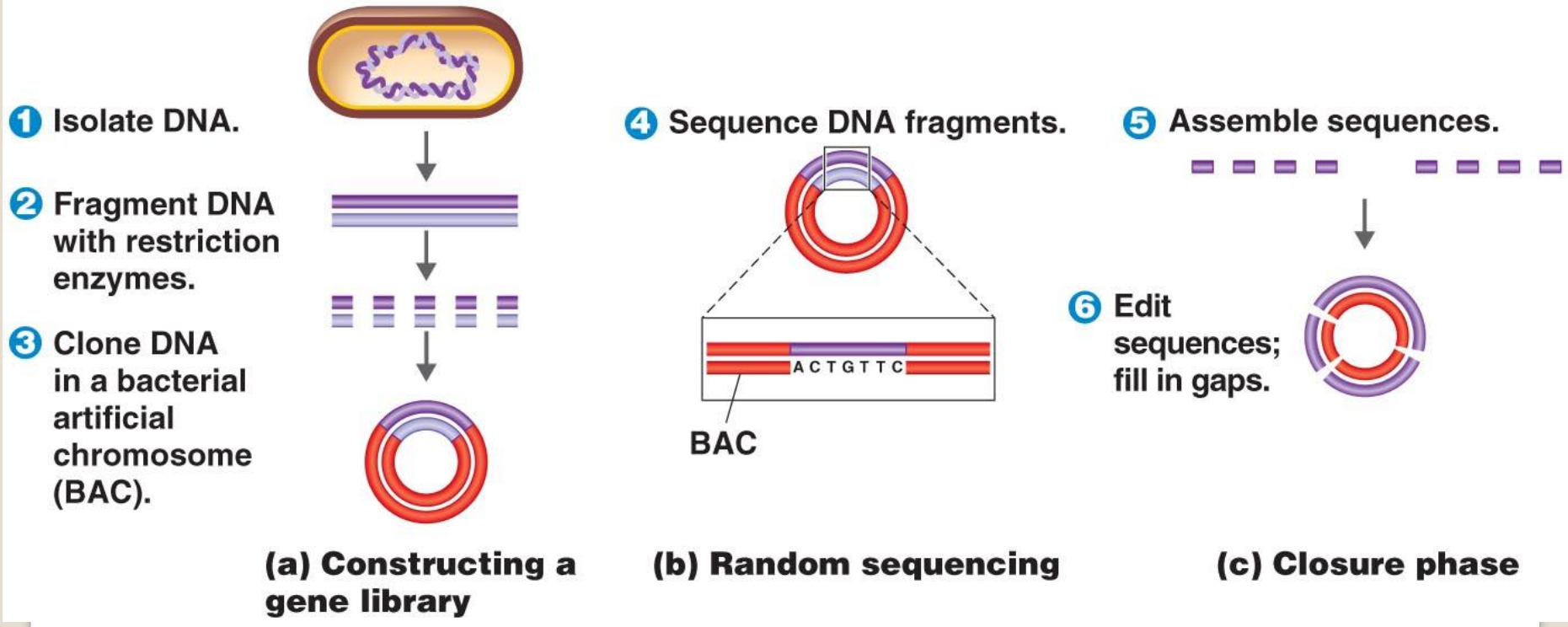


Restriction enzymes were used to produce fragments that were cloned and sequenced

- Computer analysis assembled the sequence by aligning overlapping regions

Shotgun sequencing

Shotgun sequencing is a method of DNA sequencing whereby a long stretch of DNA is physically broken into small (approximately 2,000 base-pair) fragments which are cloned, sequenced, and assembled using computer analysis.



Random Shotgun Sequencing

Thank you