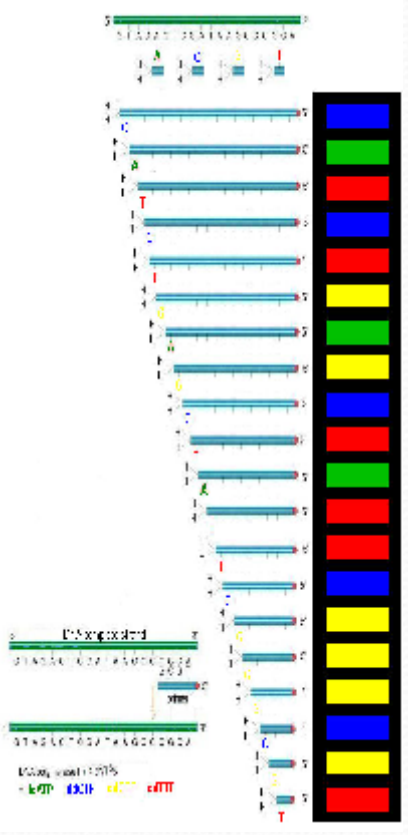
Principles of DNA Sequencing



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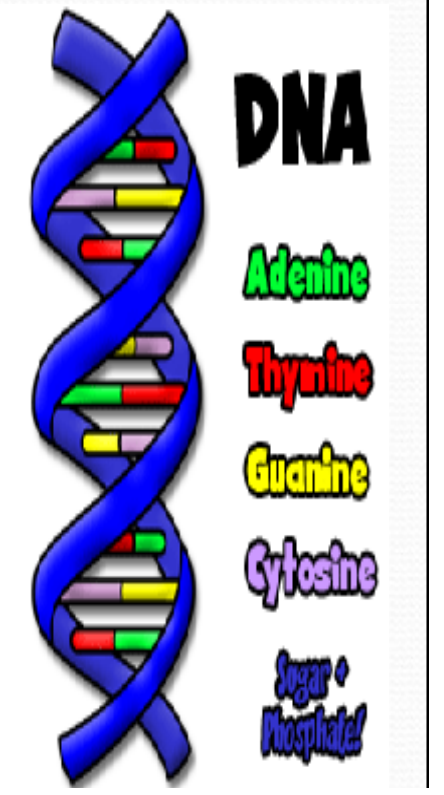
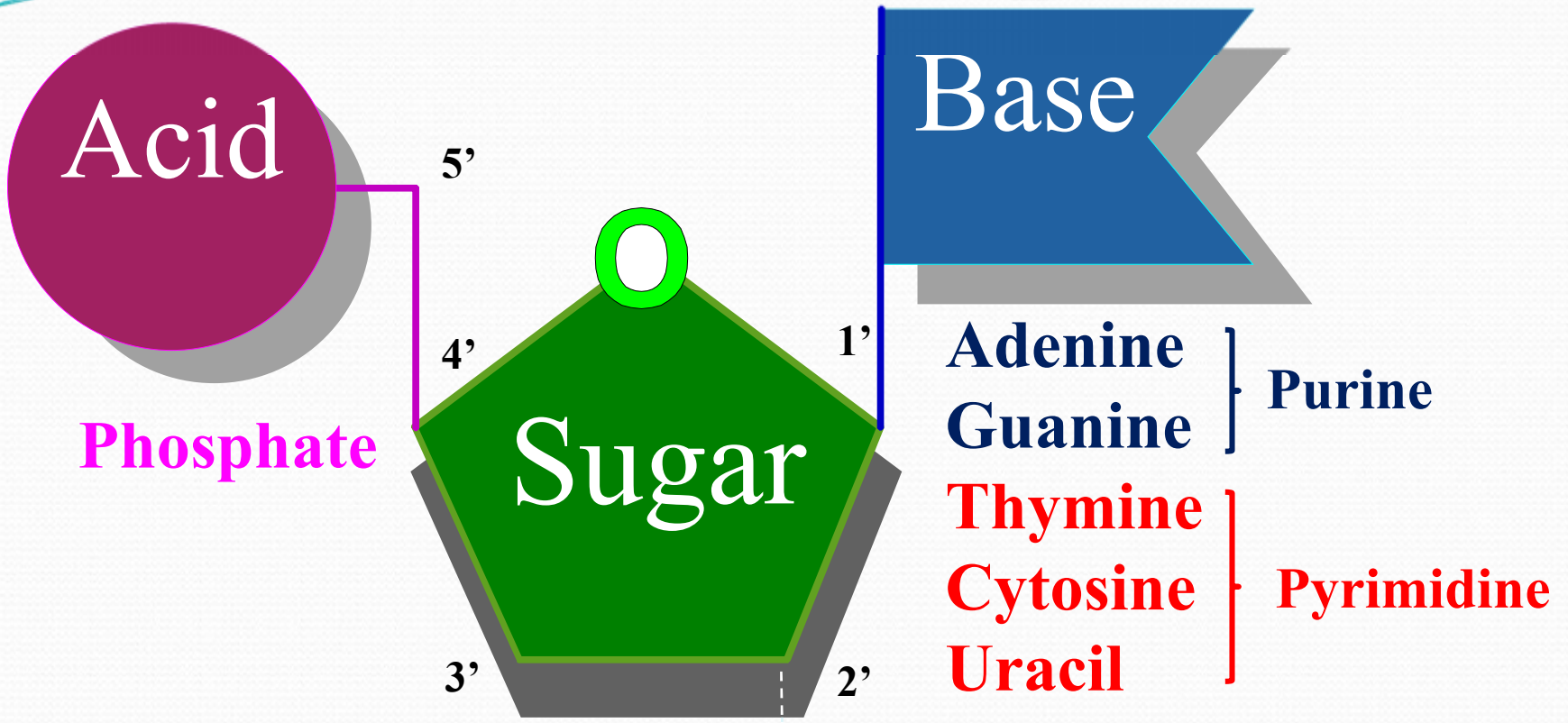
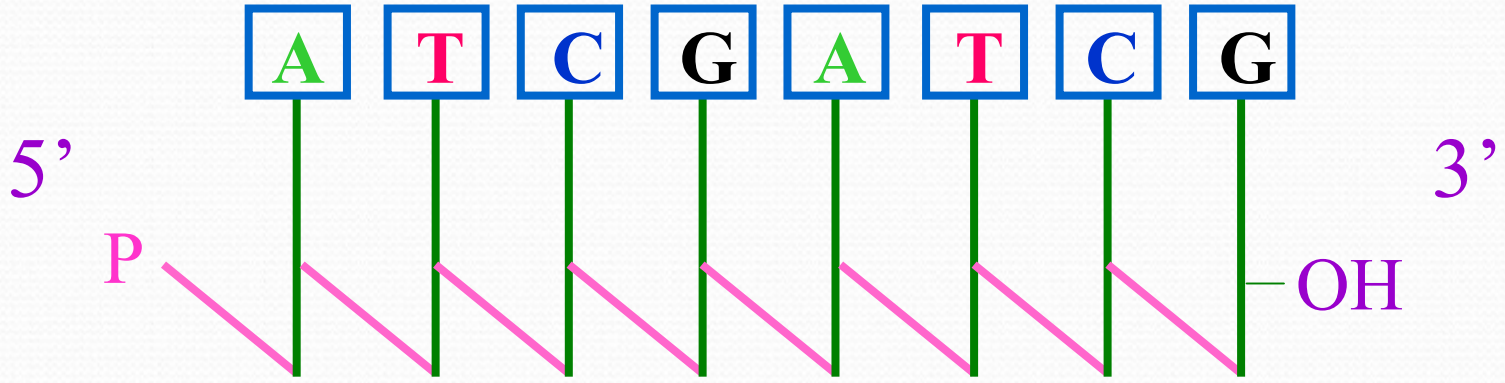
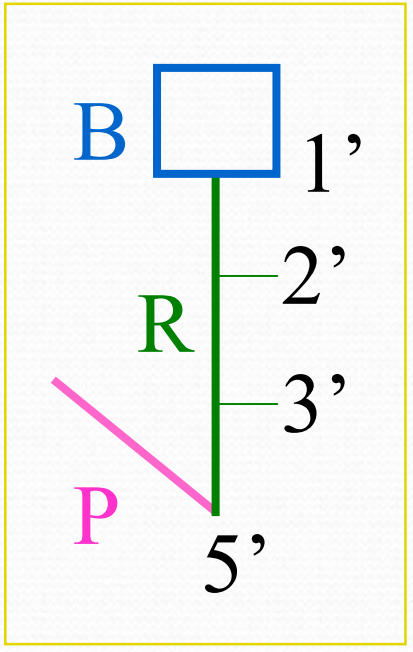


Basic structure of nucleic acid



Nucleotides linked by phosphodiester





5’ pApTpCpGpApTpCpG-OH 3’

5’ pATCGATCG-OH 3’

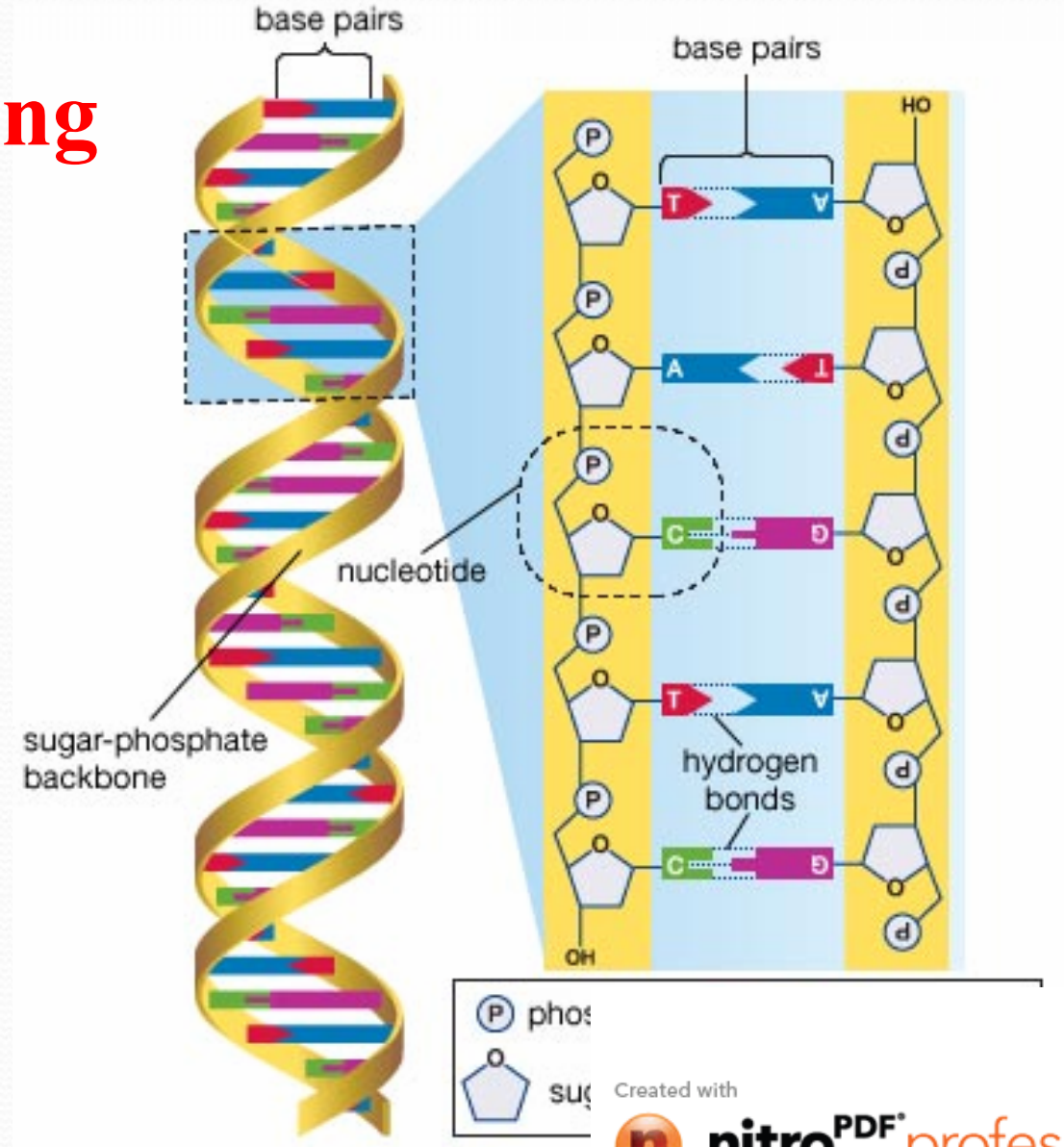
ATCGATCC

Nucleic acid pairing

hydrogen bonds

G ≡ C

A = T

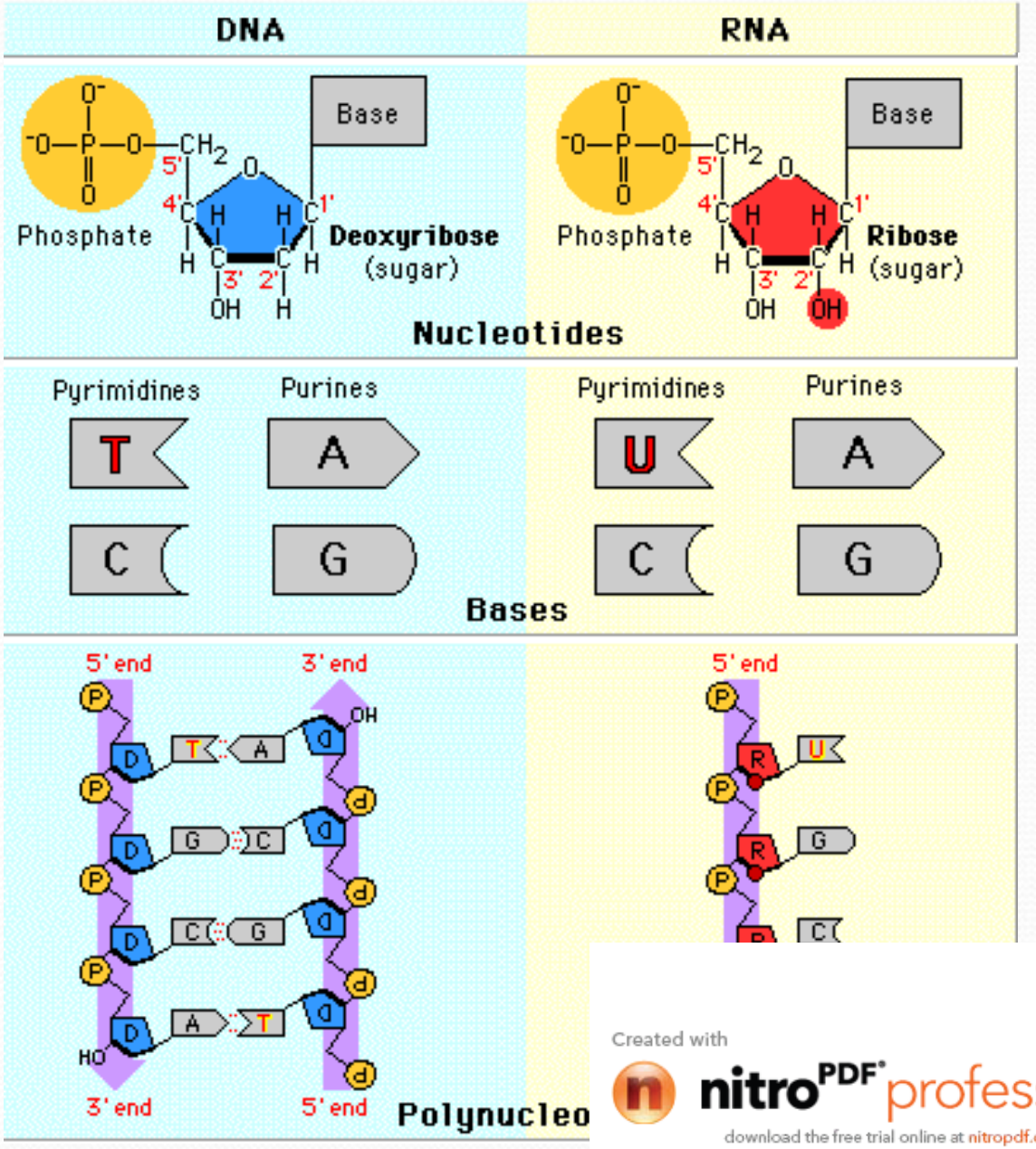


Two strands are anti-parallel

5’ pApTpCpGpApTpCpG-OH 3’

3’ HO-TdApGdCpTdApGdCd 5’

DNA vs RNA



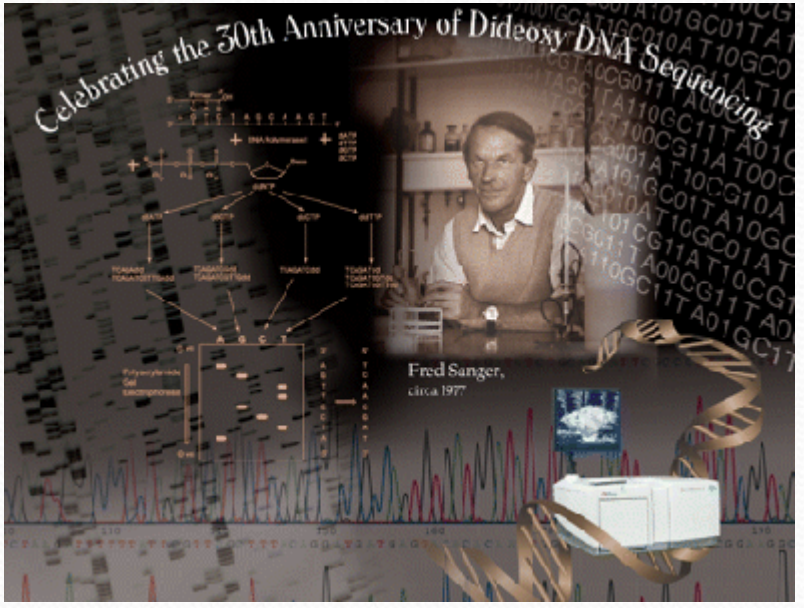
What is DNA sequencing?

-Determining the precise order of nucleotides in a piece of DNA

-DNA sequence is useful in studying fundamental biological processes and in applied fields such as diagnostic or forensic research

-DNA sequencing methods have been around for 40 years, and since the mid-1970s

Founders of sequencing technology



Sanger

Gilbert

The Nobel Prize in Chemistry 1980

Shared with Walter Gilbert and Paul Berg

Sequencing methods

Two basic methods for DNA sequencing :-

A- Chemical cleavage method (Maxam and Gilbert, 1977)

- Base-specific cleavage of DNA by certain chemicals
- Four different chemicals, one for each base
- A set of DNA fragments of different sizes
- DNA fragments contain up to 500 nucleotides

B- Enzymatic method (Sanger, 1981)

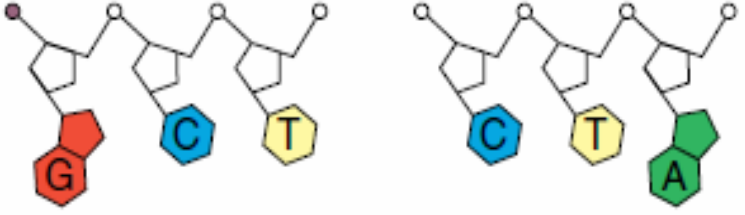
The chain cleavage reaction

DNA labeled at one end with ^{32}p

Base modification

Release or displacement of reacted bases

Strand scission



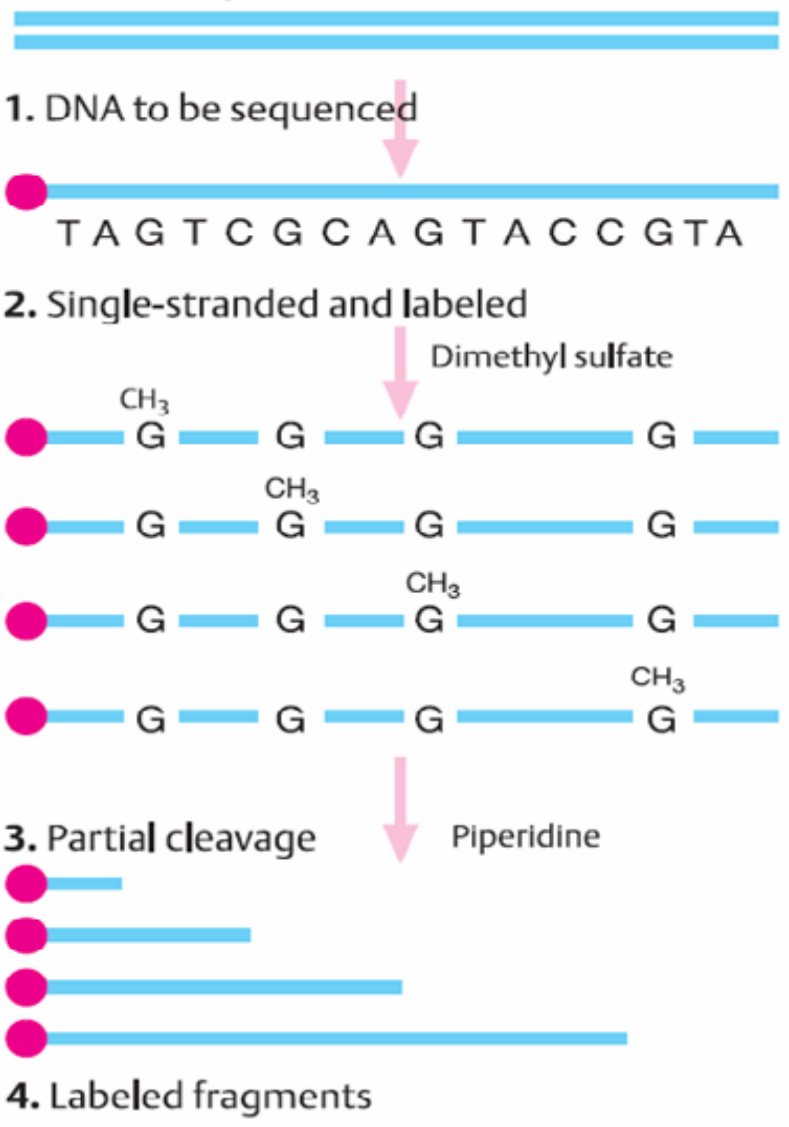
- Dimethyl sulfate (DMS) methylates G -Acid (A)

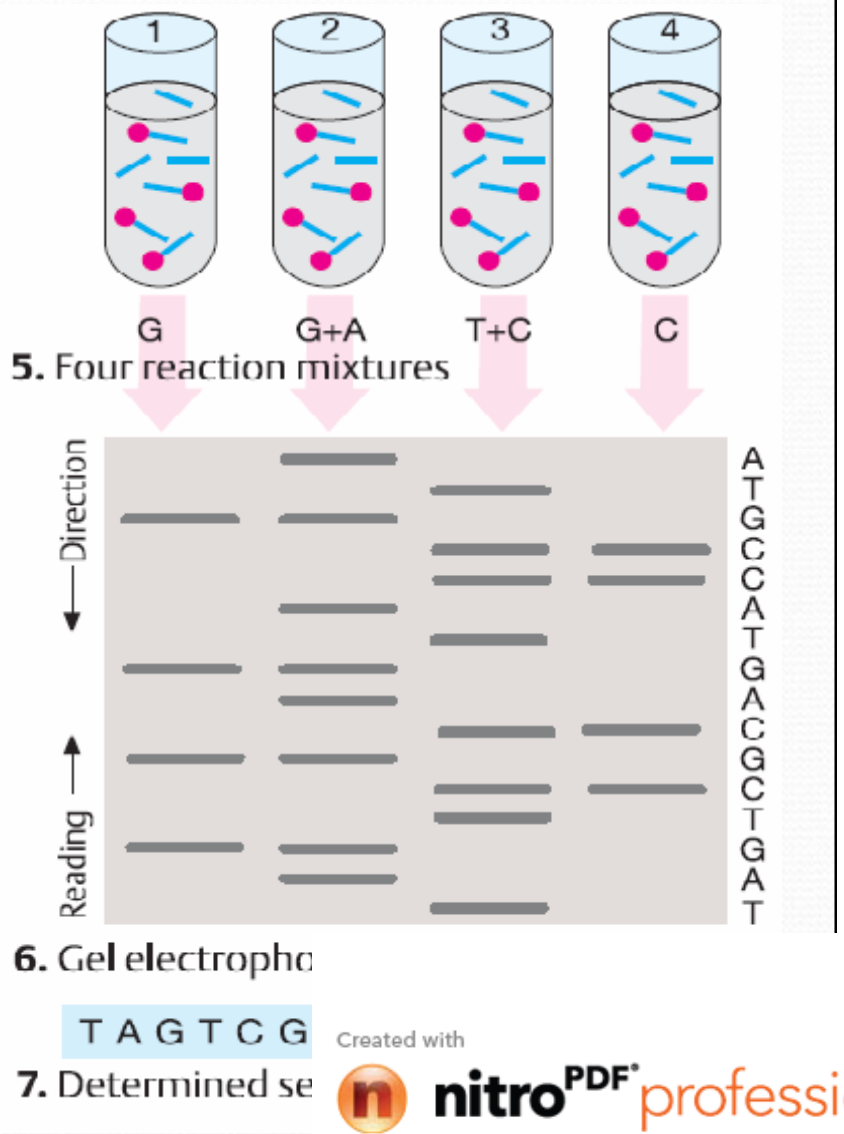
-Hydrazine (C)
- Hydrazine & NaCl (T)
- Piperidine

The fragments created by chain cleavage at guanines

^{32}pGpCpTpGpCpTpApGpGpTpGpCpCpGpApGpC
G G G G G G
^{32}p
^{32}pGpCpTp
^{32}pGpCpTpGpCpTpAp
^{32}pGpCpTpGpCpTpApGp
^{32}pGpCpTpGpCpTpApGpGpTp
^{32}pGpCpTpGpCpTpApGpGpTpGpCpCp
^{32}pGpCpTpGpCpTpApGpGpTpGpCpCpGpAp
^{32}pGpCpTpGpCpTpApGpGpTpGpCpCpC

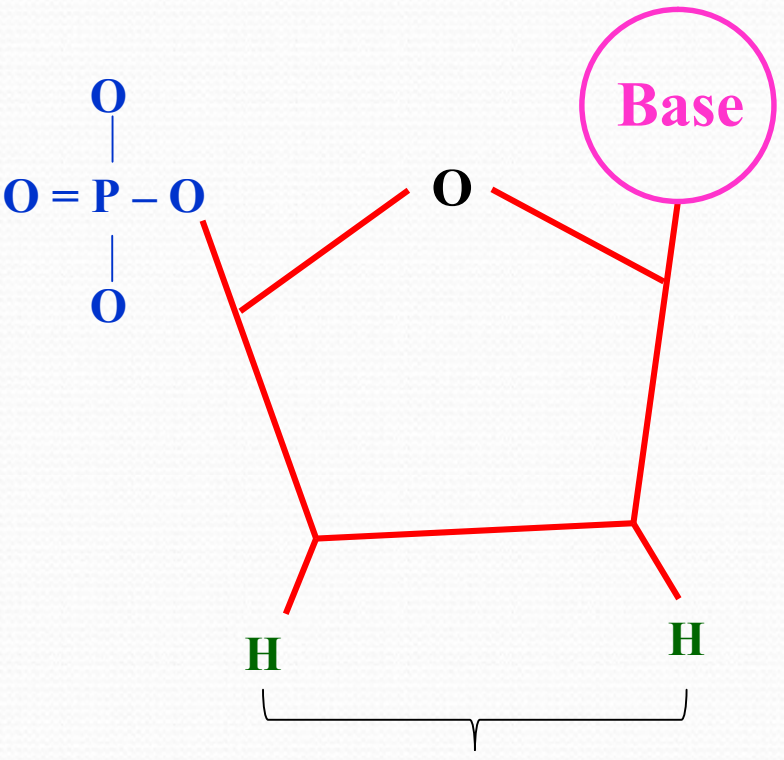
Chemical degradation method (Maxam–Gilbert method)





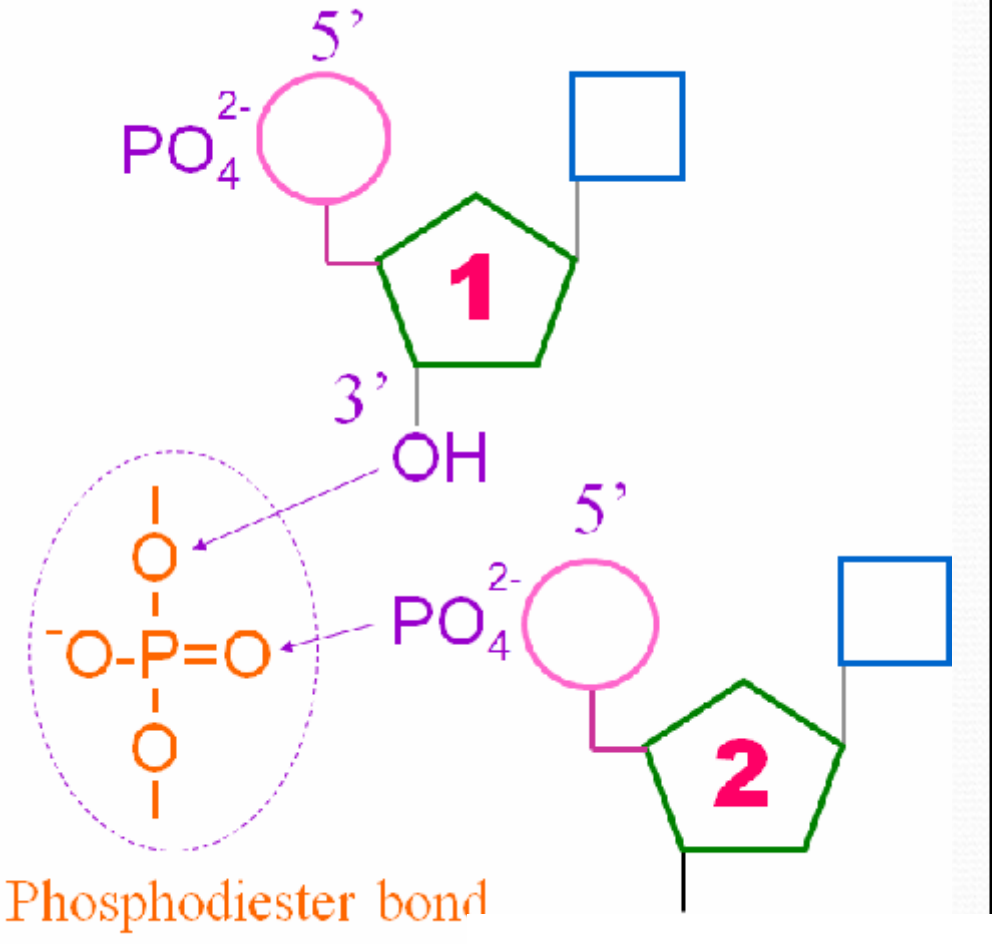
The Sanger DNA sequencing method

- Uses dideoxy nucleotides to terminate DNA synthesis.
- DNA synthesis reactions in four separate tubes
- Radioactive dATP is also included in all the tubes so the DNA products will be radioactive.
- Yielding a series of DNA fragments whose sizes can be measured by electrophoresis.
- The last base in each of these fragments is known.

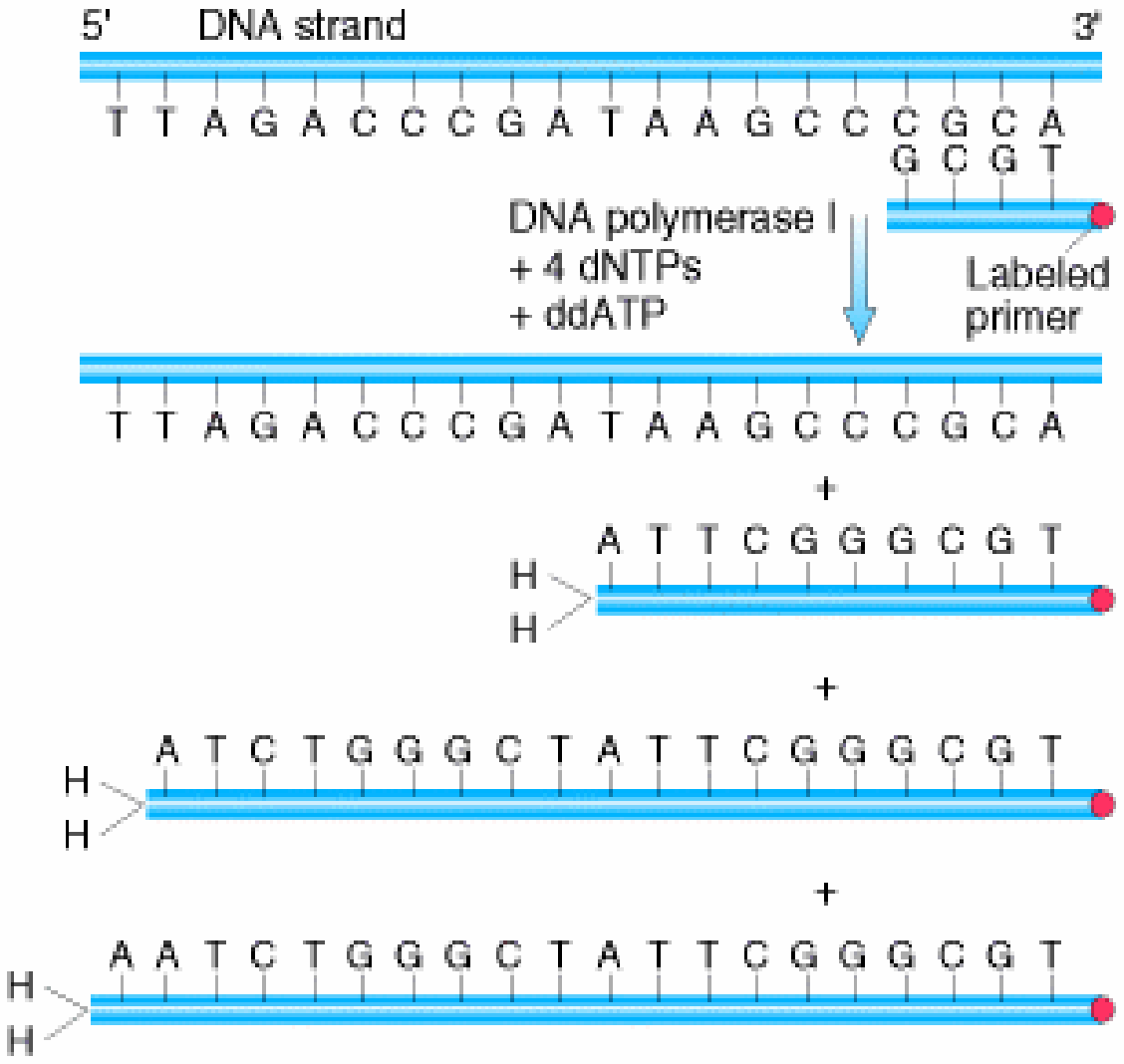


2’, 3’ dideoxy nucleotide

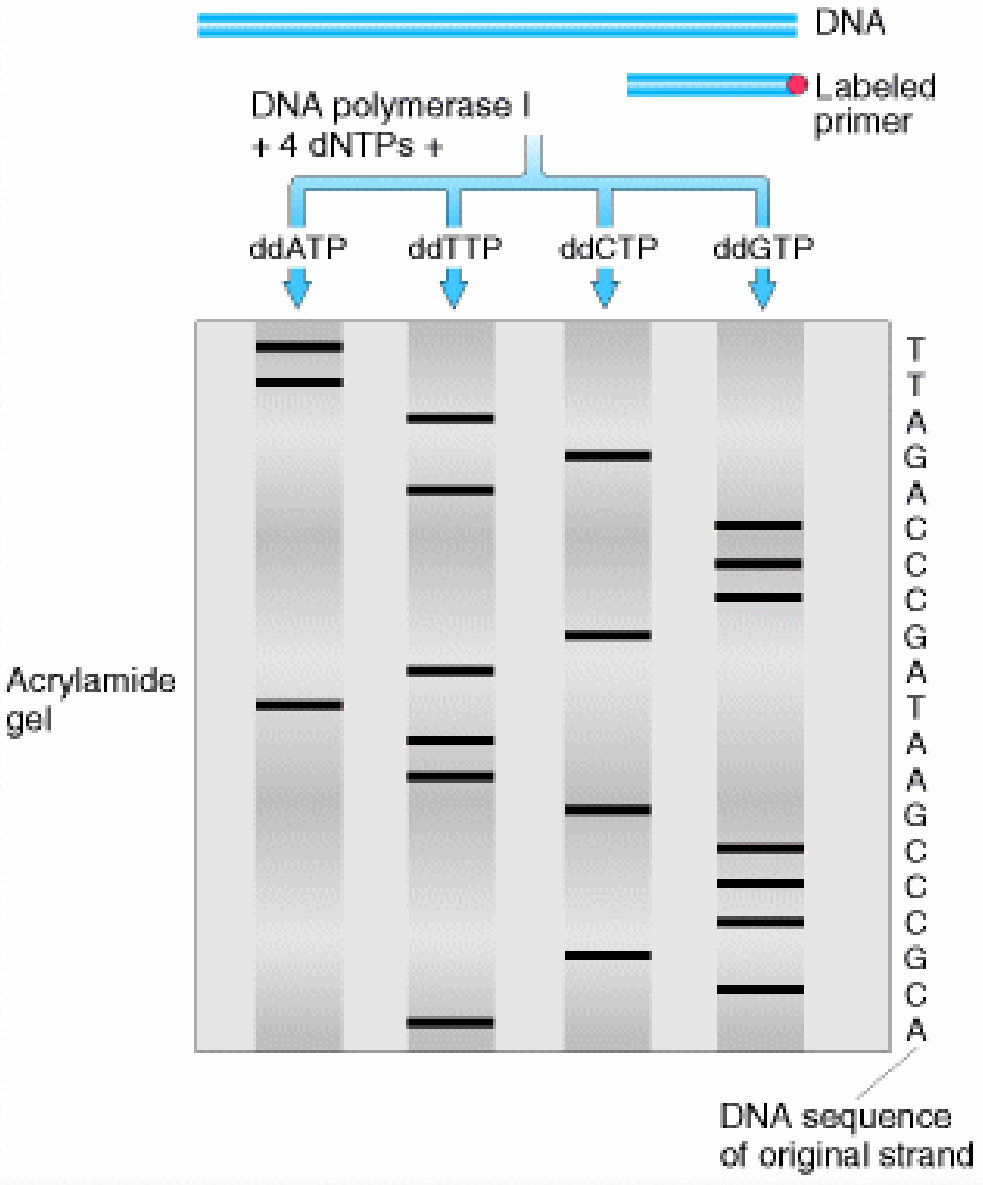
Can not form phosphodiester bound with next coming dNTP



The dideoxy sequencing method (Sanger method)



A labeled primer is used to initiate DNA synthesis. The addition of four different dideoxy nucleotides randomly arrests synthesis.



The resulting fragments are separated elect
and subjected t

Automated DNA sequencing

-The primer extension reactions are run in the same way as in the manual method

-Reaction carried out in one tube and all possible products are actually produced

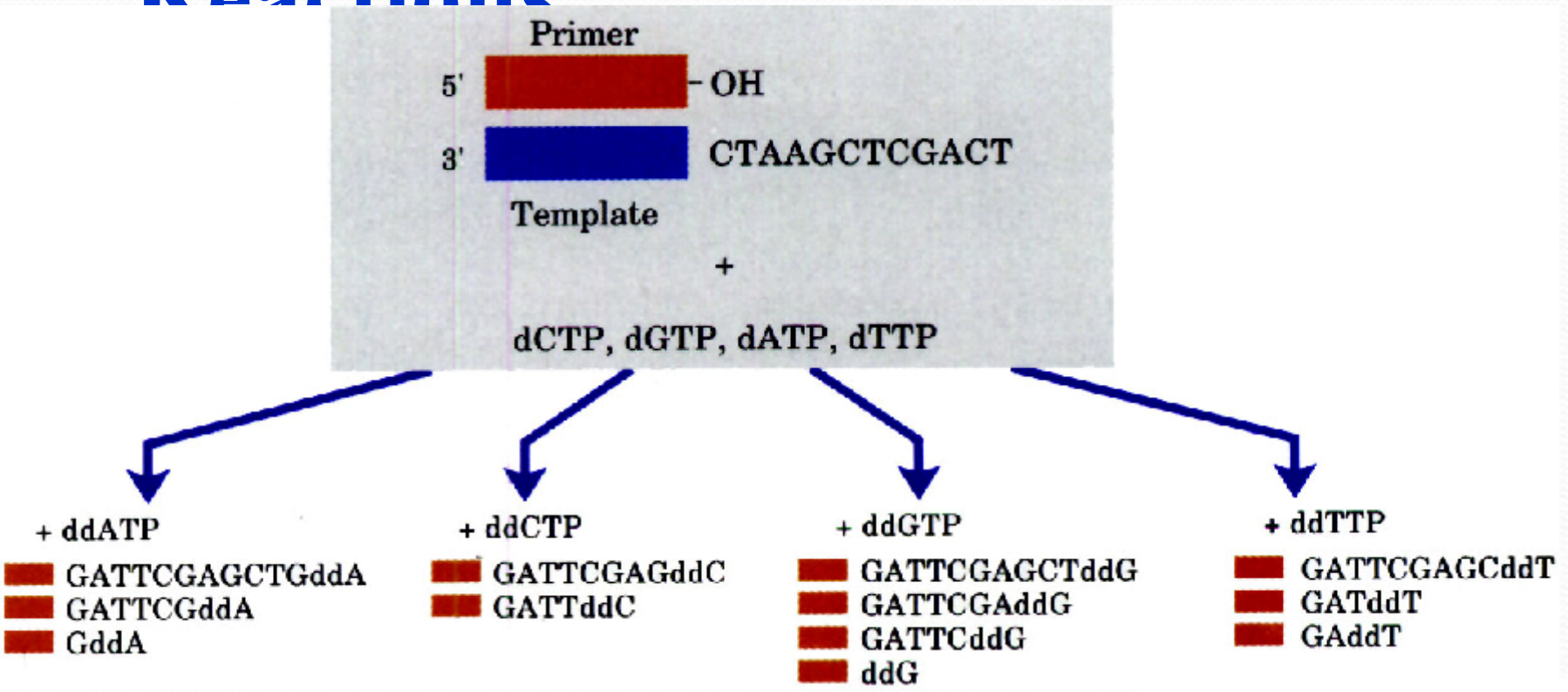
- The various reaction products separate according to size on gel electrophoresis

-The bands are color-coded according to the termination reaction that produced them

-A laser scanner excites the fluorescent tag on each band as it passes by, and a detector analyzes the color of the resulting emitted light

- Each colored peak is a plot of the fluorescence intensity of a band as it passes through the laser beam

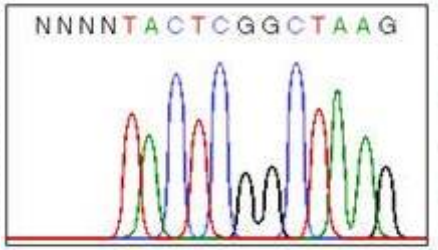
DNA Sequencing Reactions

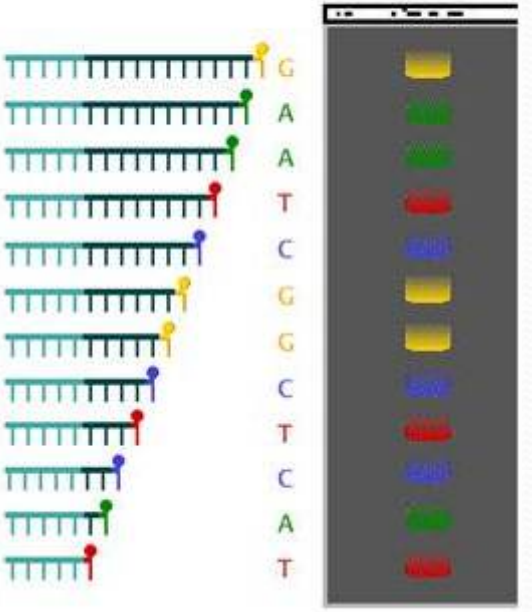


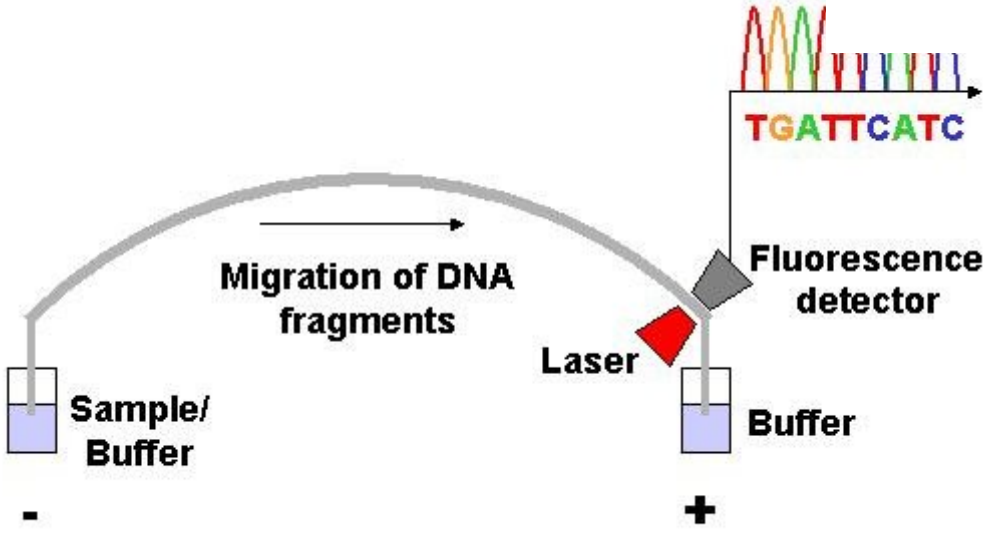
Gel electrophoretic Fractionation

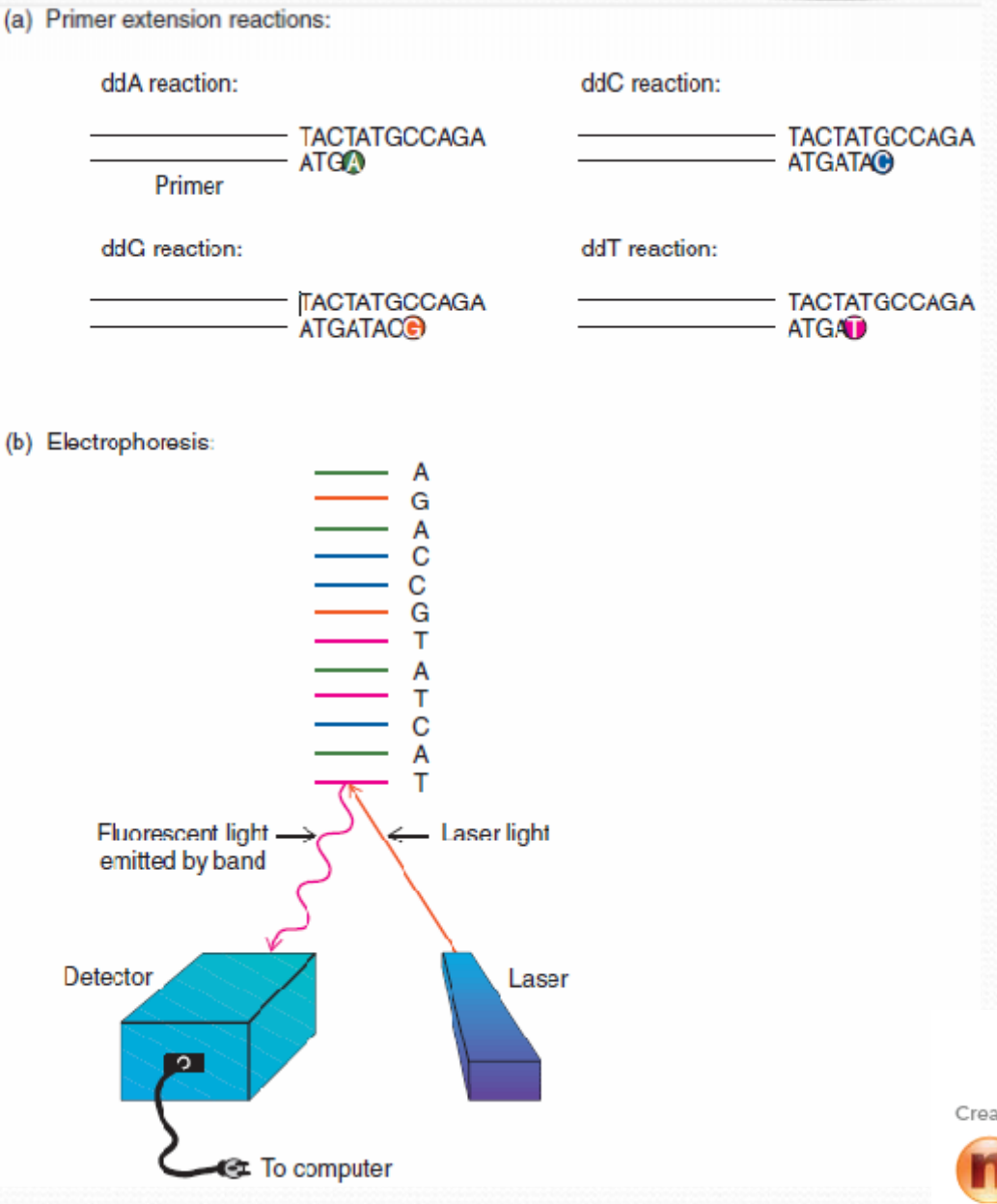
Cycle Sequencing

The simulated gel image is read from bottom to top, starting with the smallest fragment.

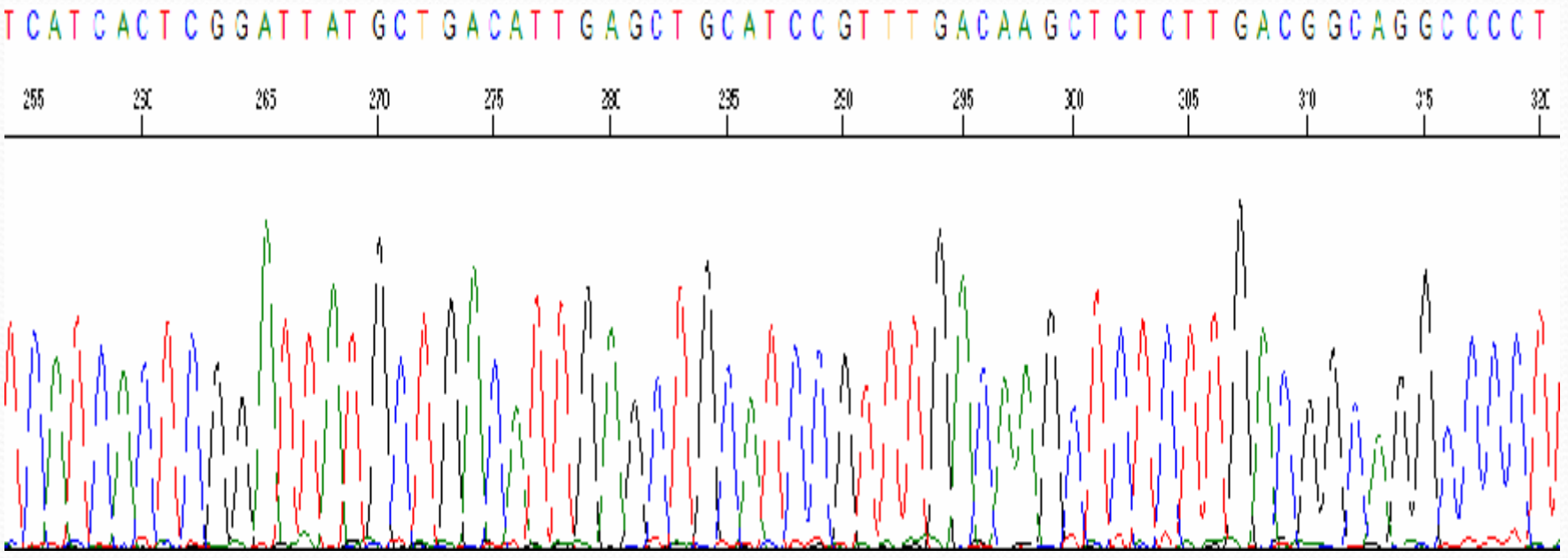




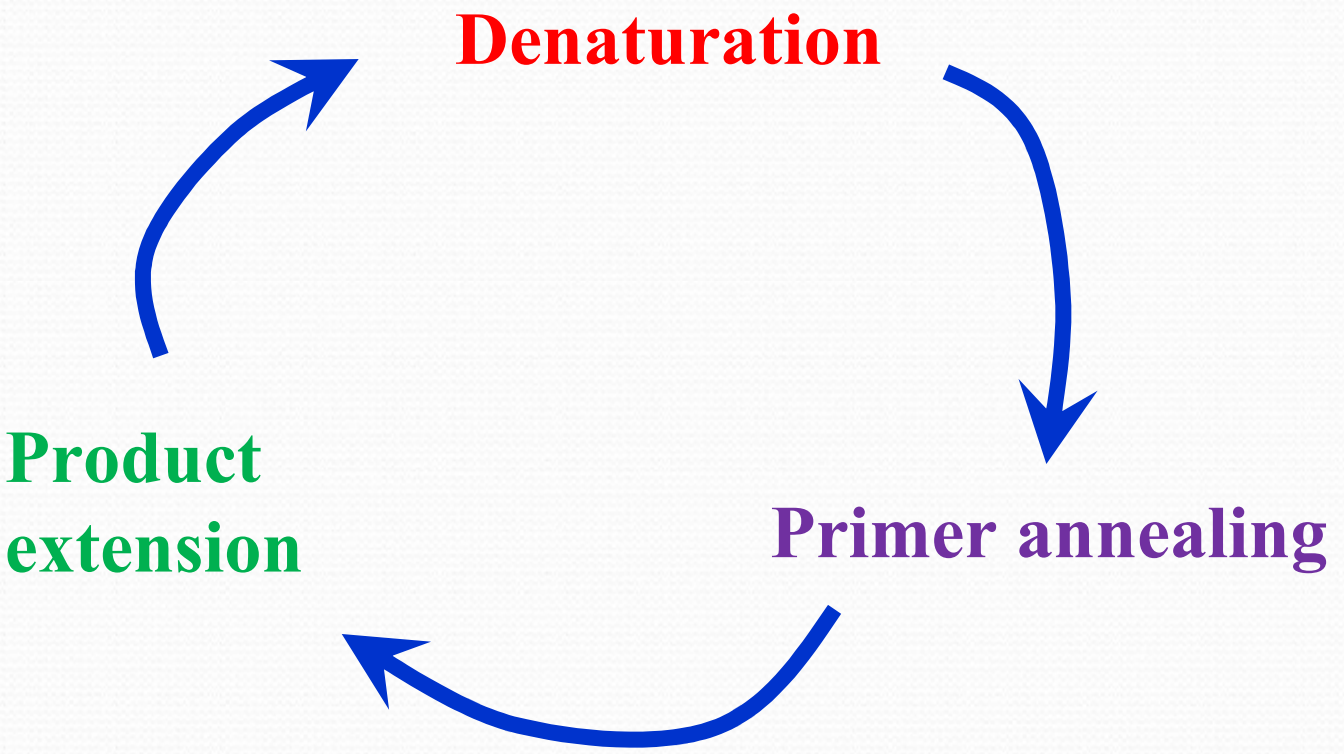




Printout of an automated DNA sequencing



Set up cycle sequencing



Performing DNA sequencing reaction

1- DNA template preparation

a. DNA quality

1- Agarose gel electrophoresis

2- Spectrophotometry

A_{260}/A_{280} (1.7 – 1.9)

(Smaller ratio indicates contamination by protein and organic chemicals)

- Non specific PCR products

There are several methods for purifying PCR products

- Column
- Ethanol
- Gel

b. Quantity of template DNA

(critical for successful sequencing reaction)

PCR products

100 – 200 bp	1 – 3 ng
200 – 500 bp	3 – 10 ng
500 – 100 bp	5 – 20 ng
1000 – 2000 bp	10-40 ng

2- Primer

-Tm > 45°C better results

-Longer than 18 bases decrease secondary hybridization

- Conc. 1pmol/ul

3- Reagents handling and storage

The freshest reagents are likely to perform the best

-Divided reagents (Thawing freezing)

-Shield from light

4- Preparing Cycle Sequencing reaction

Terminator Ready reaction mix

Sequencing buffer

DNA template

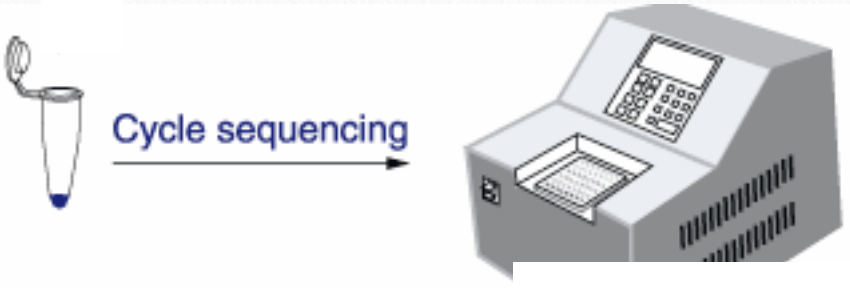
Primer

Deionized water

5-Cycle sequencing conditions

96 ◦ C / 10 sec
50 ◦ C / 5 sec
60 ◦ C / 4min

Repeated for 25 cycles



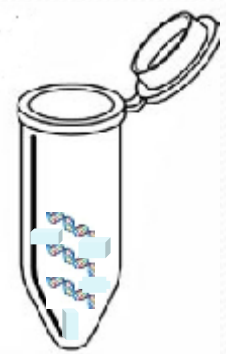
6- Preparing products for electrophoresis

Excess dye terminator obscure data at the beginning of the sequencing

- Spin column (Centri – Sep)
- Ethanol ppt

7-Loadig samples for electrophoresis

Pathway of sequencing reaction



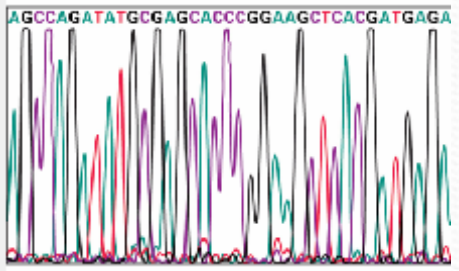
1. Purify PCR product

2. Set up sequencing reaction

3. Perform cycle sequencing

4. Resolve seque fragments

5. Read order of terminators (DNA sequence)



ABI 310 Sequencer