Principles of DNA Sequencing

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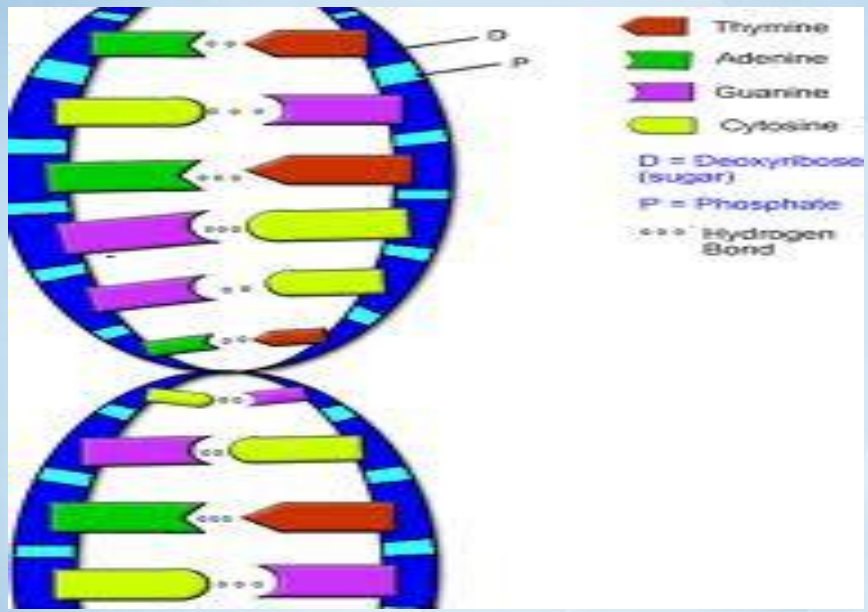
What is DNA Sequencing?

- DNA Sequencing is finding the order of nucleotides in a fragment of DNA



- It is involving various biochemical, biophysical and computational techniques to determine the order of the nucleotide bases- adenine, guanine, thymine & cytosine in a molecule of DNA.

Methods of DNA Sequencing

Sanger Method

DNA sequencing by enzymatic synthesis

Nobel Prize 1958, seq. of insulin

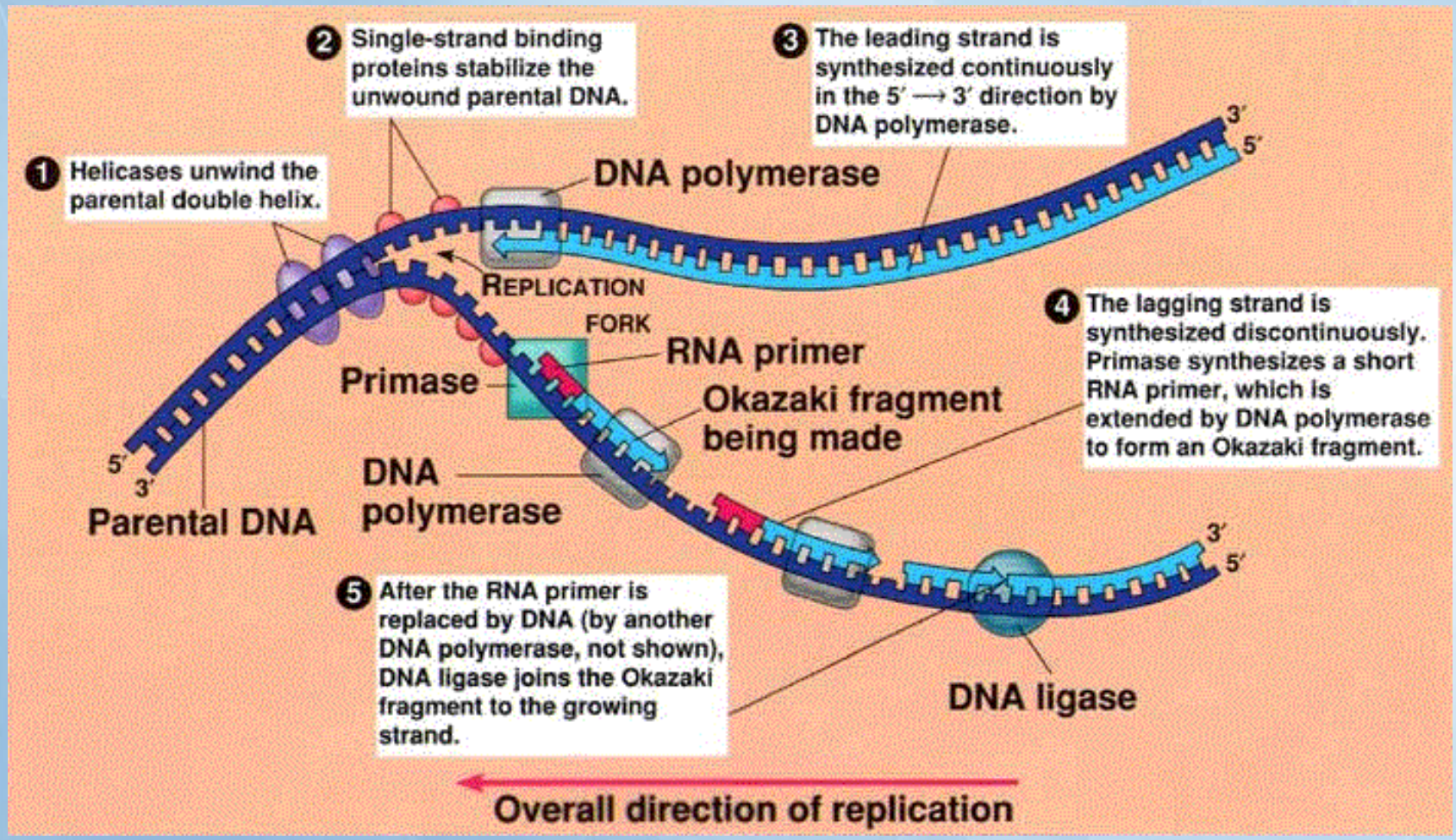
Nobel Prize 1980, DNA seq.

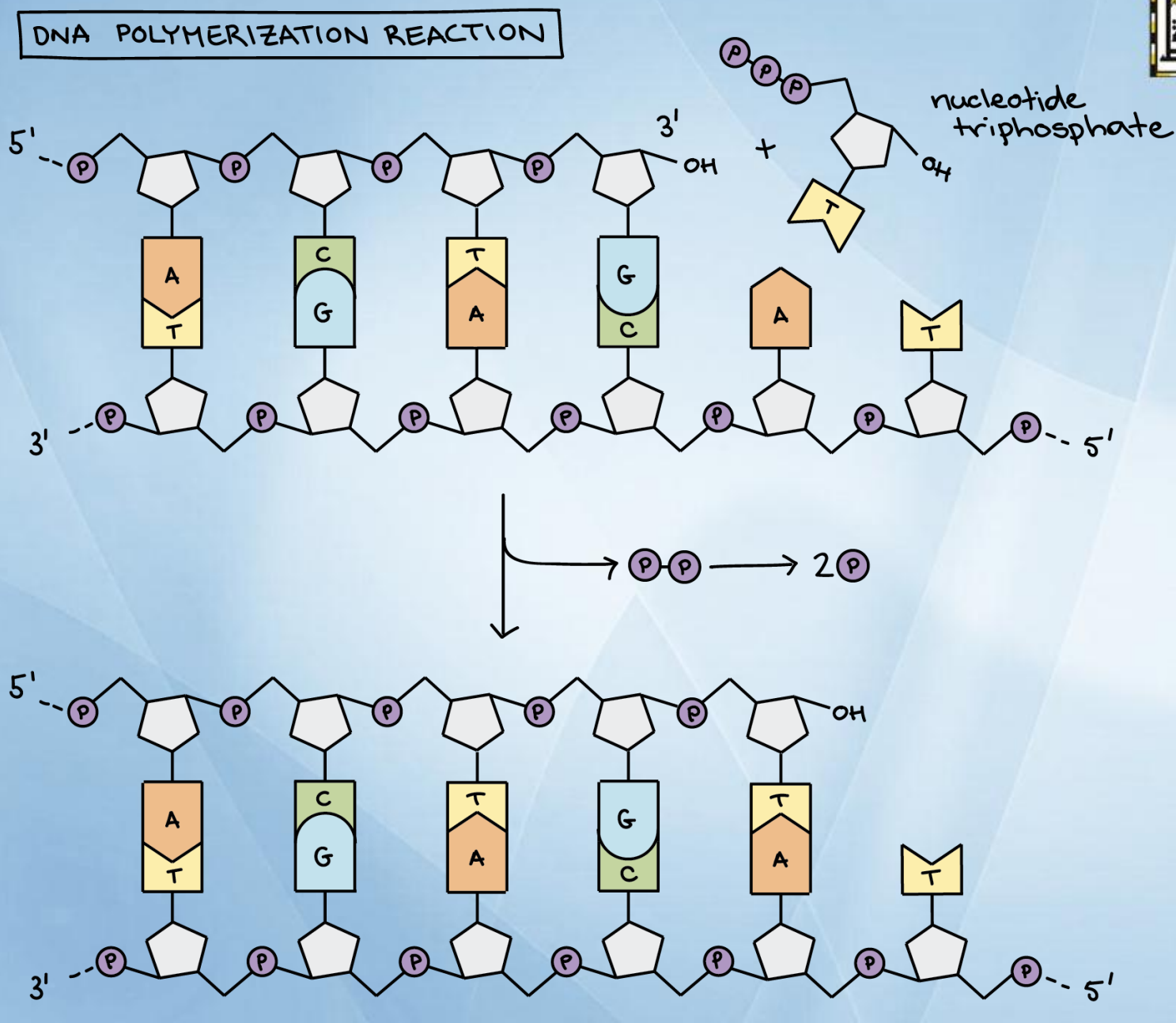
Maxam–Gilbert Method

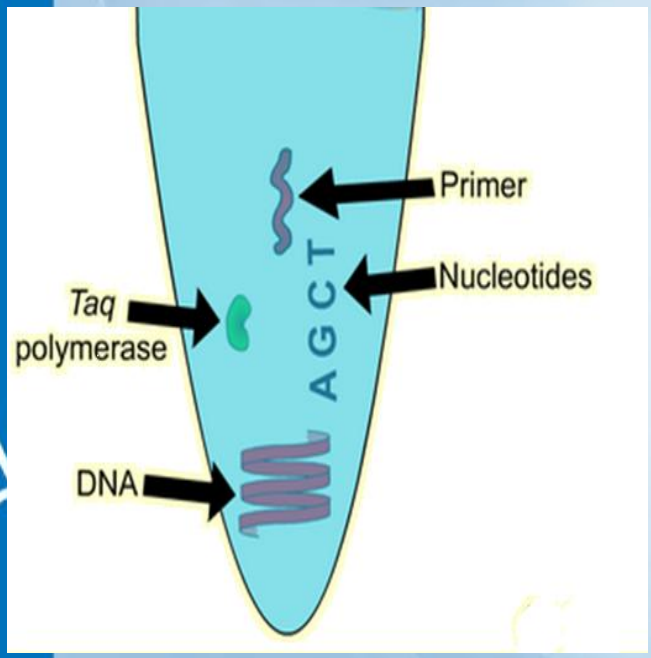
DNA sequencing by chemical degradation

Nobel Prize 1980, DNA sequence

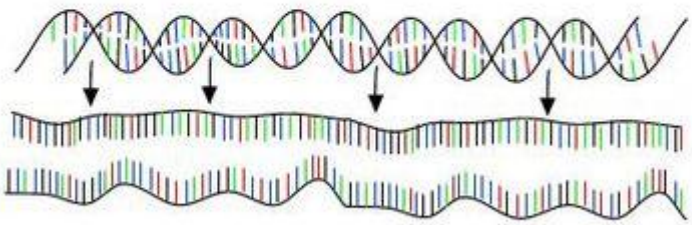
Modern sequencing equipment uses the principles of the Sanger technique


2 Single-strand binding proteins stabilize the unwound parental DNA.
3 The leading strand is synthesized continuously in the 5′ ⟶ 3′ direction by DNA polymerase.
1 Helicases unwind the parental double helix.
DNA polymerase
3′
5′
REPLICATION FORK
RNA primer
Primase
Okazaki fragment being made
4 The lagging strand is synthesized discontinuously. Primase synthesizes a short RNA primer, which is extended by DNA polymerase to form an Okazaki fragment.
5′
3′
Parental DNA
DNA polymerase
3′
5′
5 After the RNA primer is replaced by DNA (by another DNA polymerase, not shown), DNA ligase joins the Okazaki fragment to the growing strand.
DNA ligase
Overall direction of replication


DNA POLYMERIZATION REACTION
nucleotide triphosphate
5'
3'
OH
A
T
C
G
T
A
G
C
A
T
P
2P

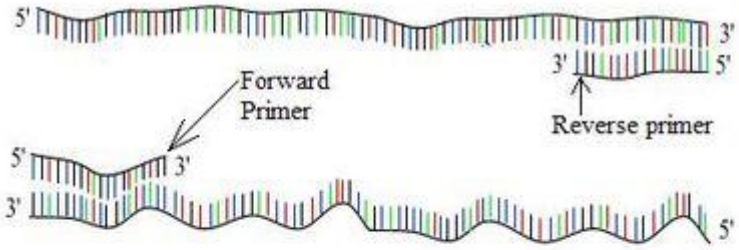


PCR : Polymerase Chain Reaction



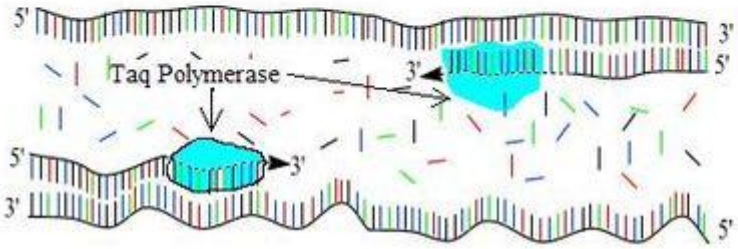
Step 1 : denaturation

94 °C



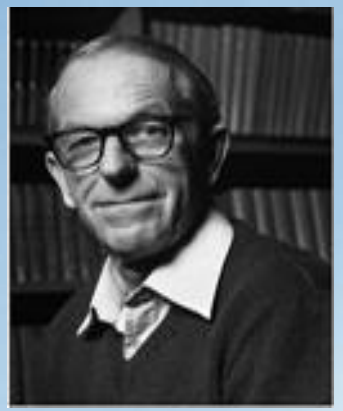
Step 2 : annealing

54 °C



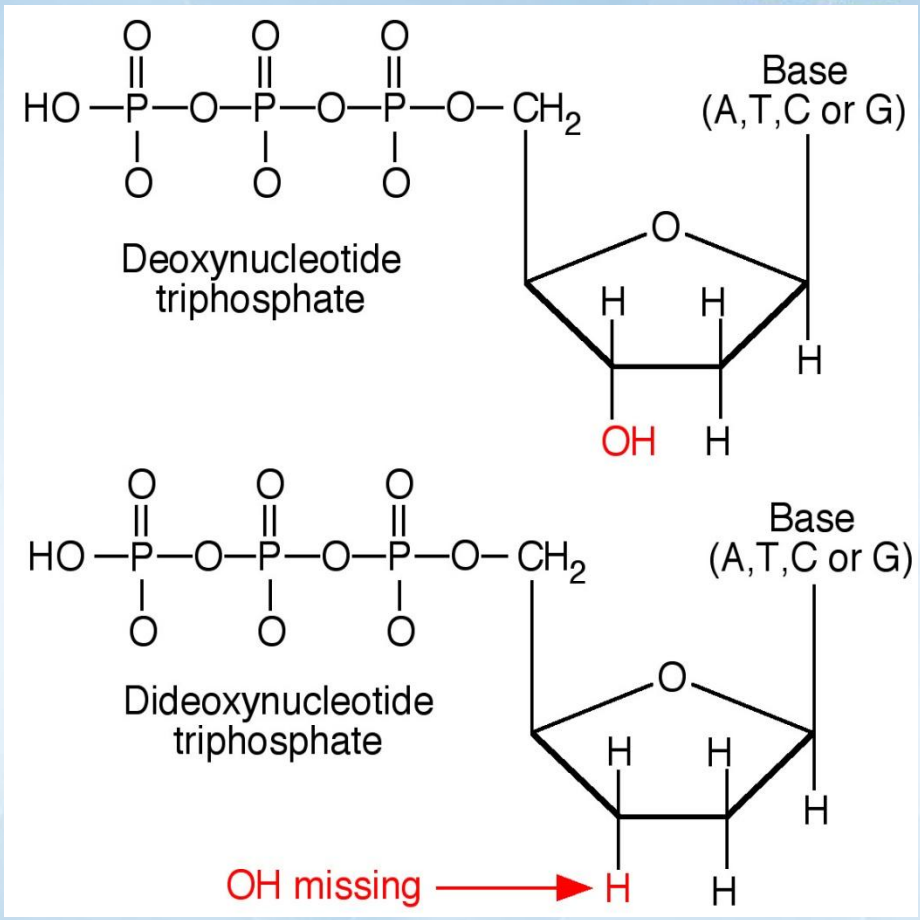
Step 3 : extension

72 °C



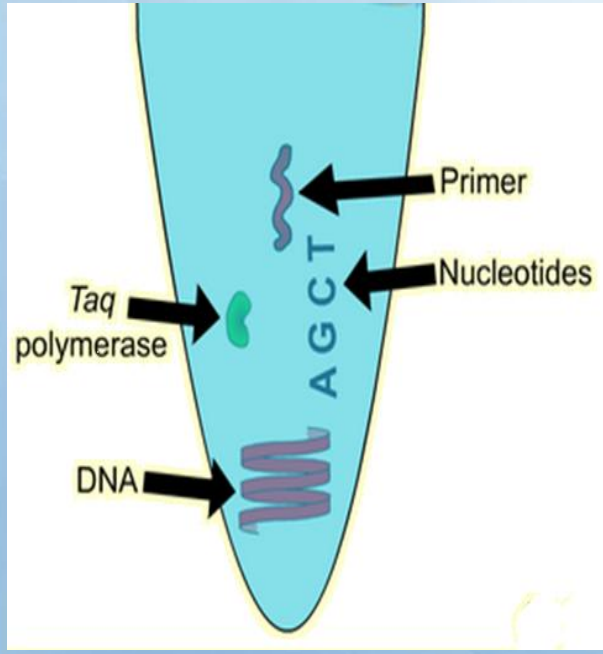
The Sanger method

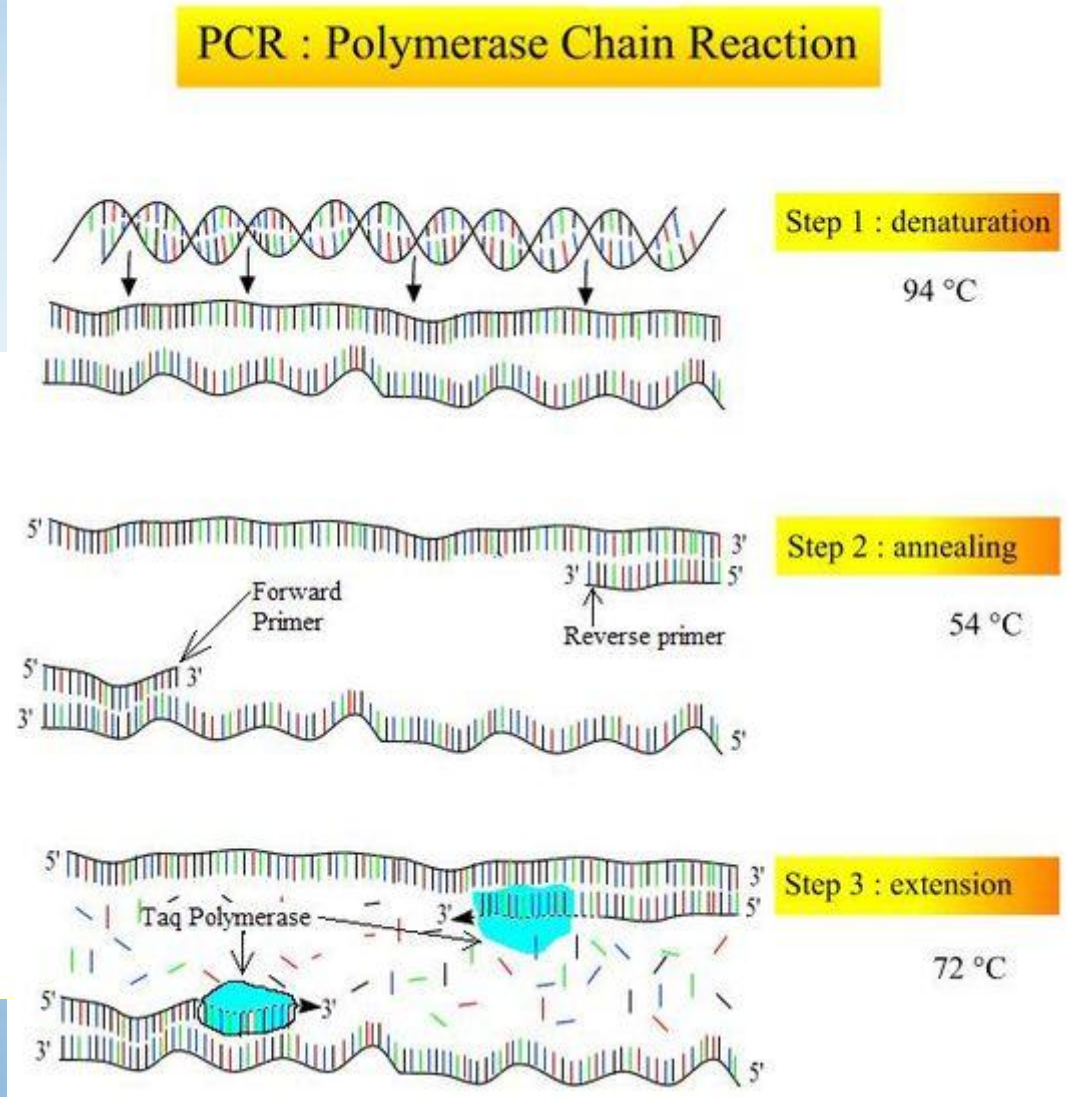
Uses dideoxy nucleotides to terminate DNA synthesis.

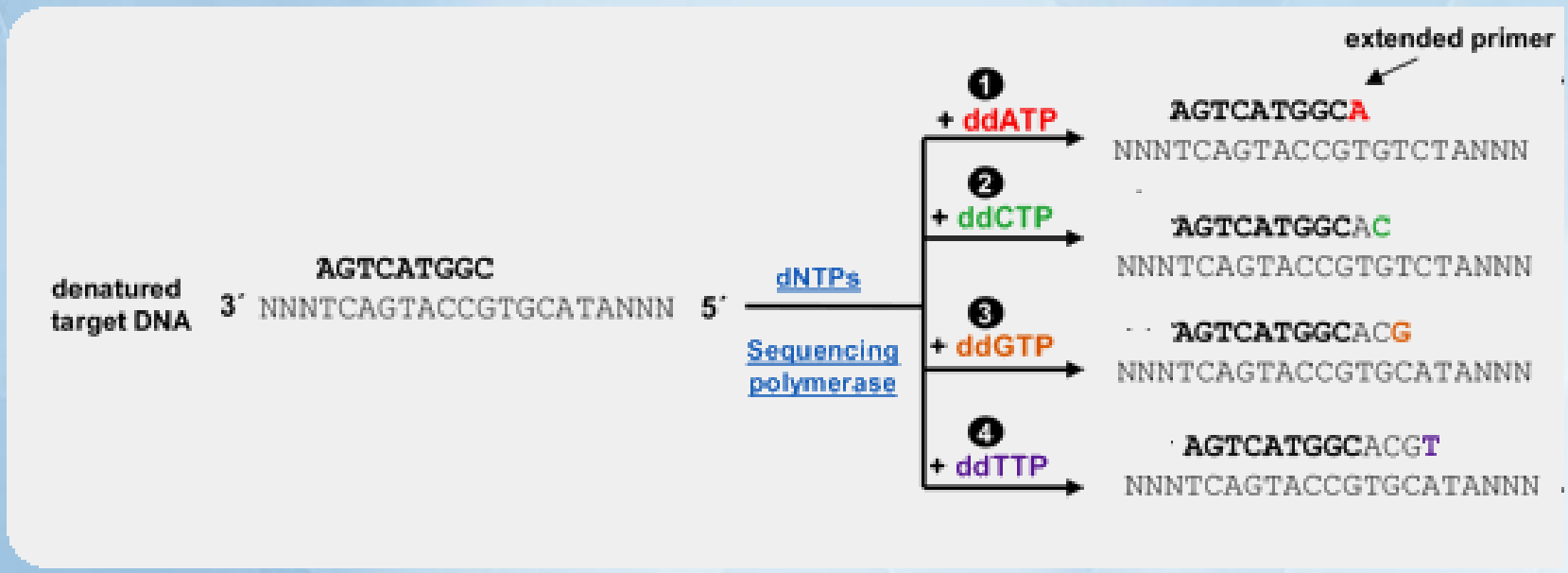


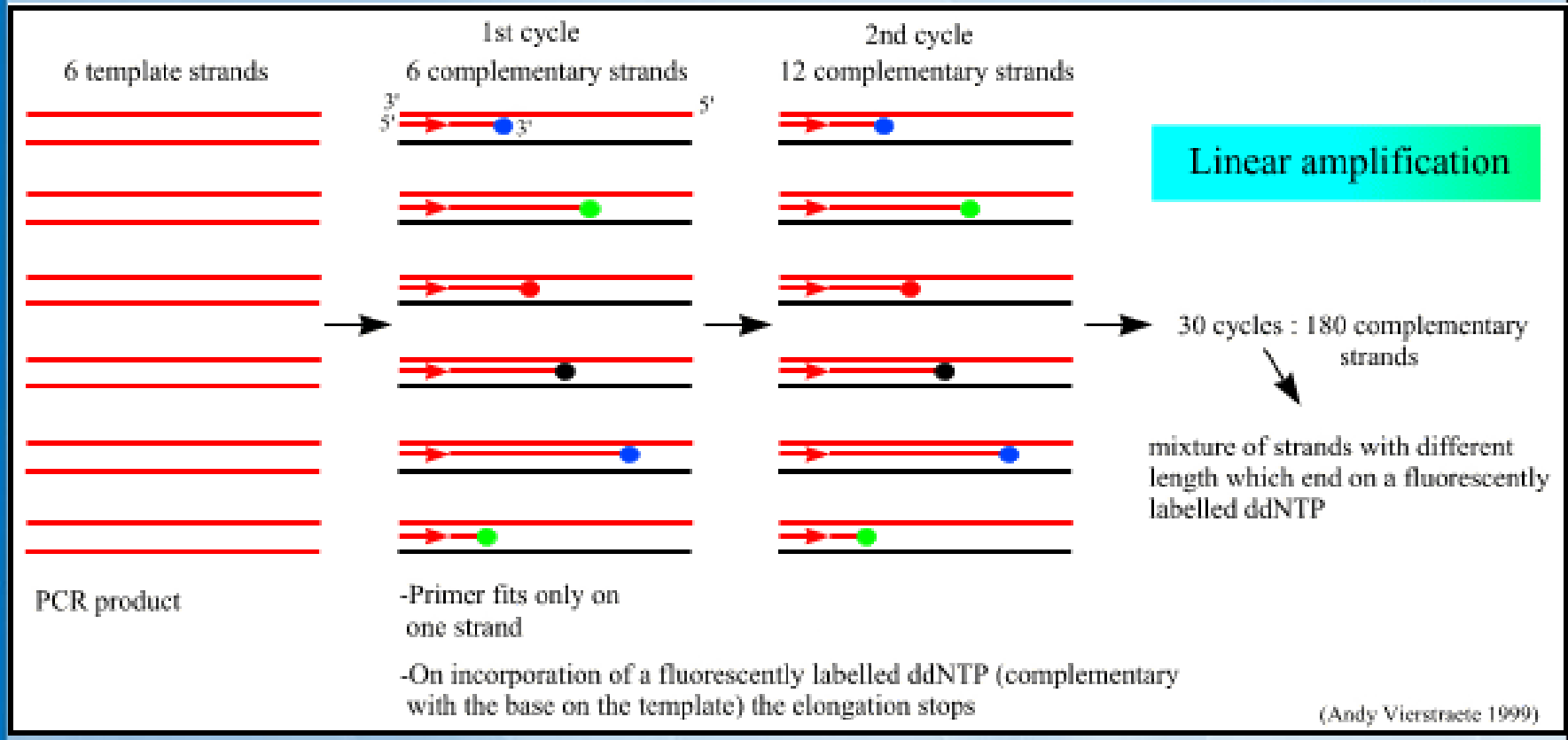
Because they lack the –OH, replication stops

What will happen if ddATP, ddGTP, ddCTP, ddTTP are added ?



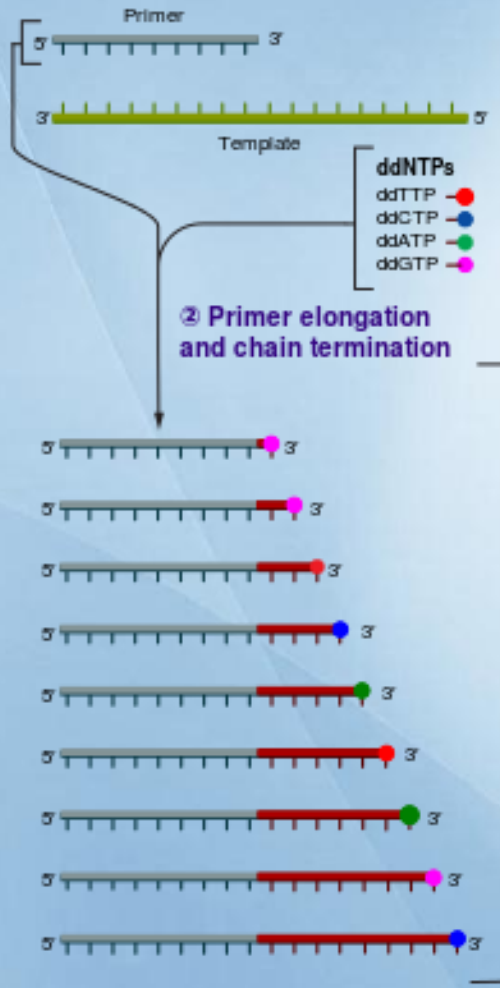



extended primer
denatured target DNA
3´ AGTCATGGC NNNTCAGTACCGTGCATANNN 5´
dNTPs
Sequencing polymerase
1 + ddATP
AGTCATGGCA
NNNTCAGTACCGTGTCTANNN
2 + ddCTP
AGTCATGGCAC
NNNTCAGTACCGTGTCTANNN
3 + ddGTP
AGTCATGGCACG
NNNTCAGTACCGTGCATANNN
4 + ddTTP
AGTCATGGCACGT
NNNTCAGTACCGTGCATANNN

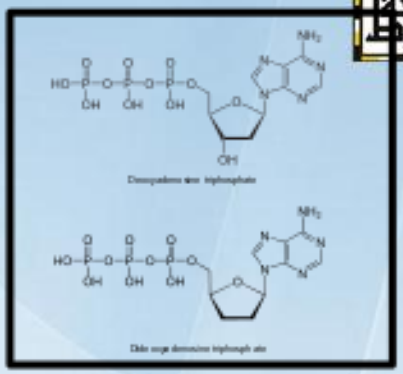

6 template strands
1st cycle
6 complementary strands
2nd cycle
12 complementary strands
3'
5'
5'
3'
Linear amplification
30 cycles : 180 complementary strands
mixture of strands with different length which end on a fluorescently labelled ddNTP
PCR product
-Primer fits only on one strand
-On incorporation of a fluorescently labelled ddNTP (complementary with the base on the template) the elongation stops
(Andy Vierstraete 1999)

① Reaction mixture

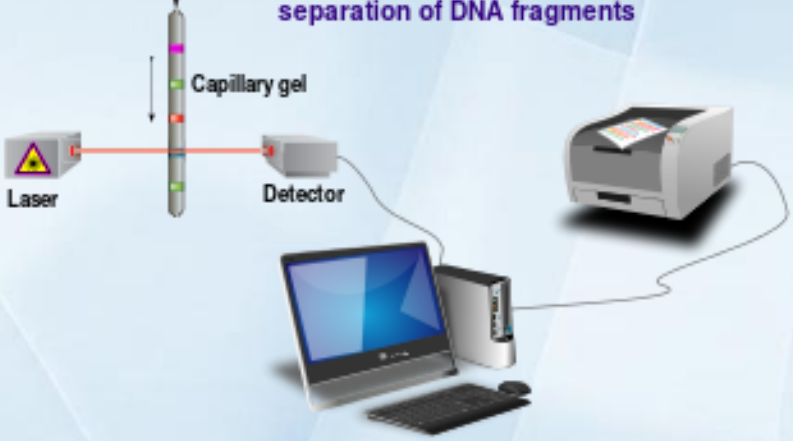
- Primer and DNA template
- DNA polymerase
- ddNTPs with flourochromes
- dNTPs (dATP, dCTP, dGTP, and dTTP)

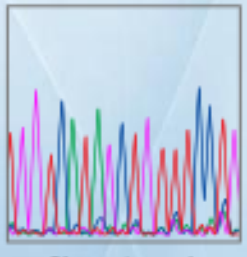


② Primer elongation and chain termination

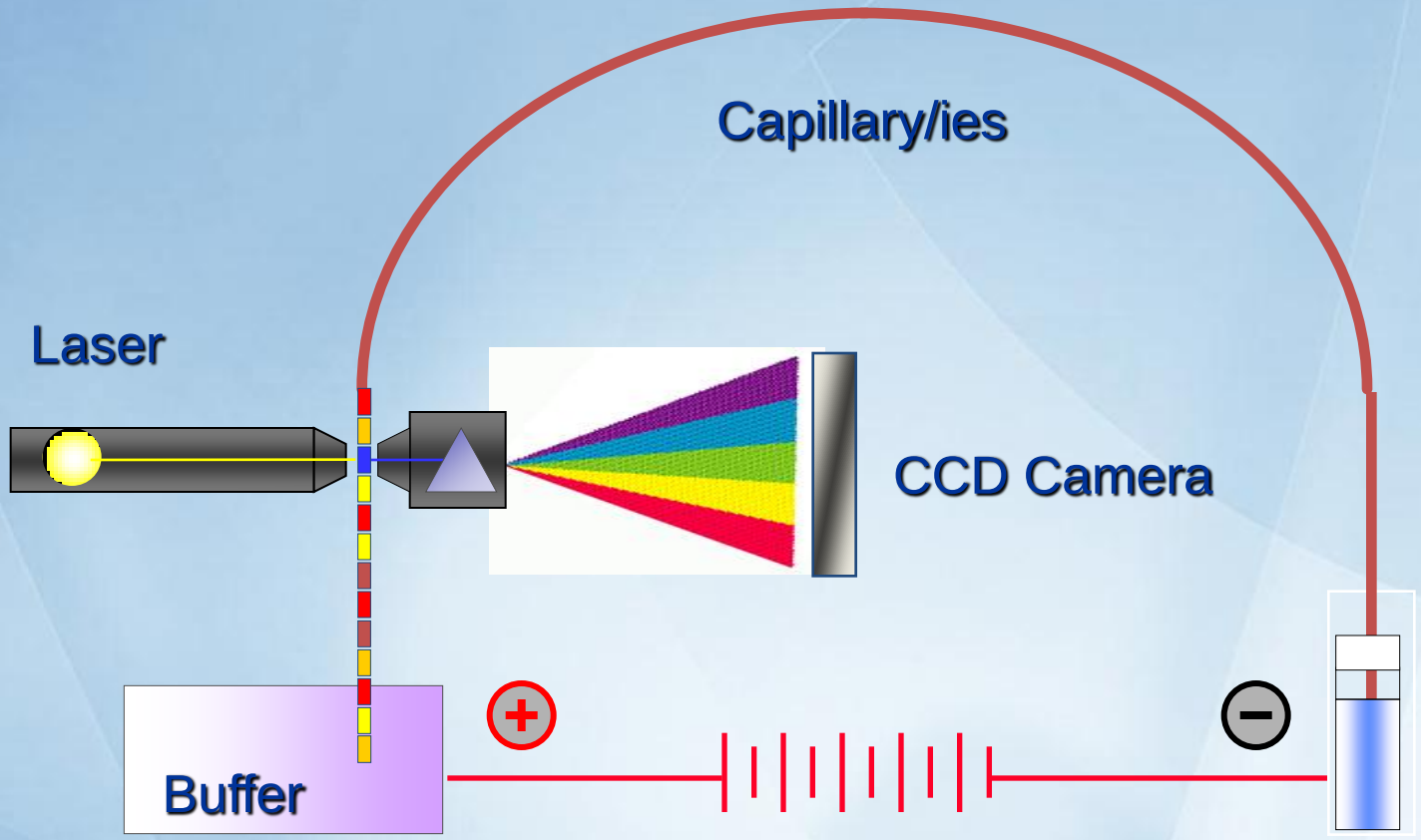


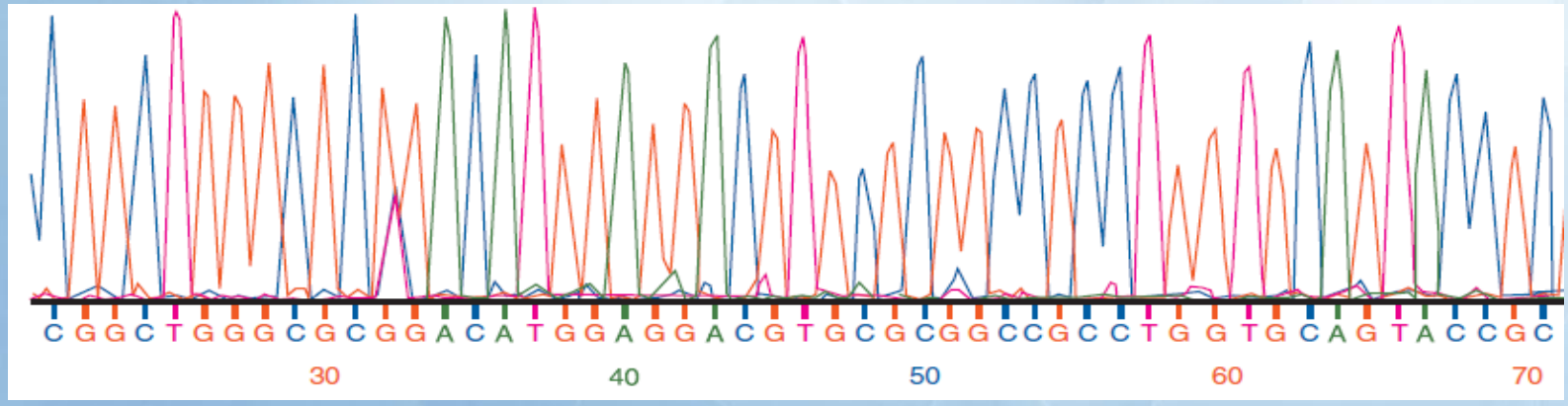
③ Capillary gel electrophoresis separation of DNA fragments





④ Laser detection of flourochromes and computational sequence analysis


Capillary/ies
Laser
CCD Camera
Buffer
+
−


C G G C T G G G C G C G G A C A T G G A G G A C G T G C G C G G C C G C C T G G T G C A G T A C C G C
30
40
50
60
70

When voltage applied, strands separate by size in capillary, smallest go through first

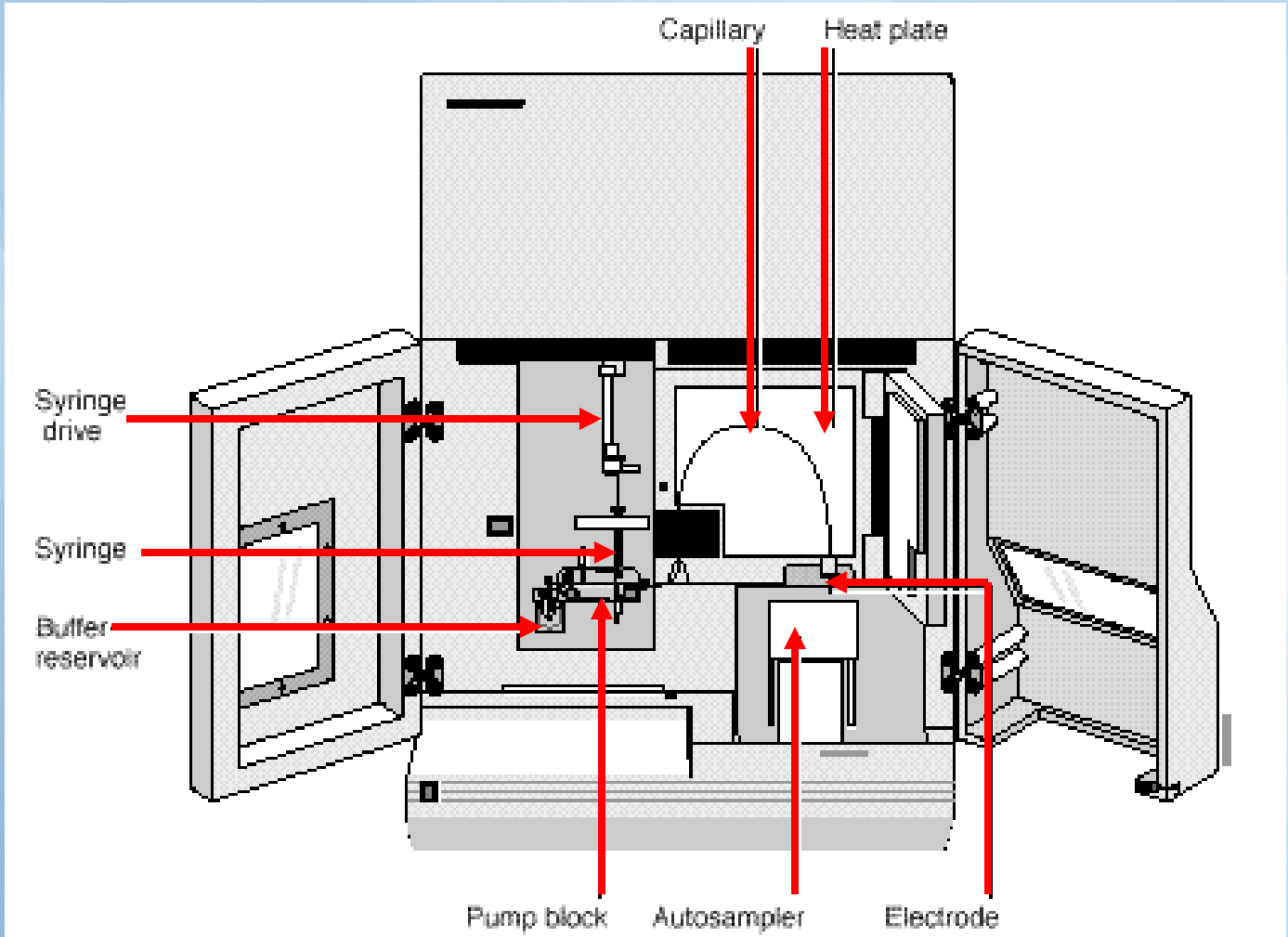
Movie

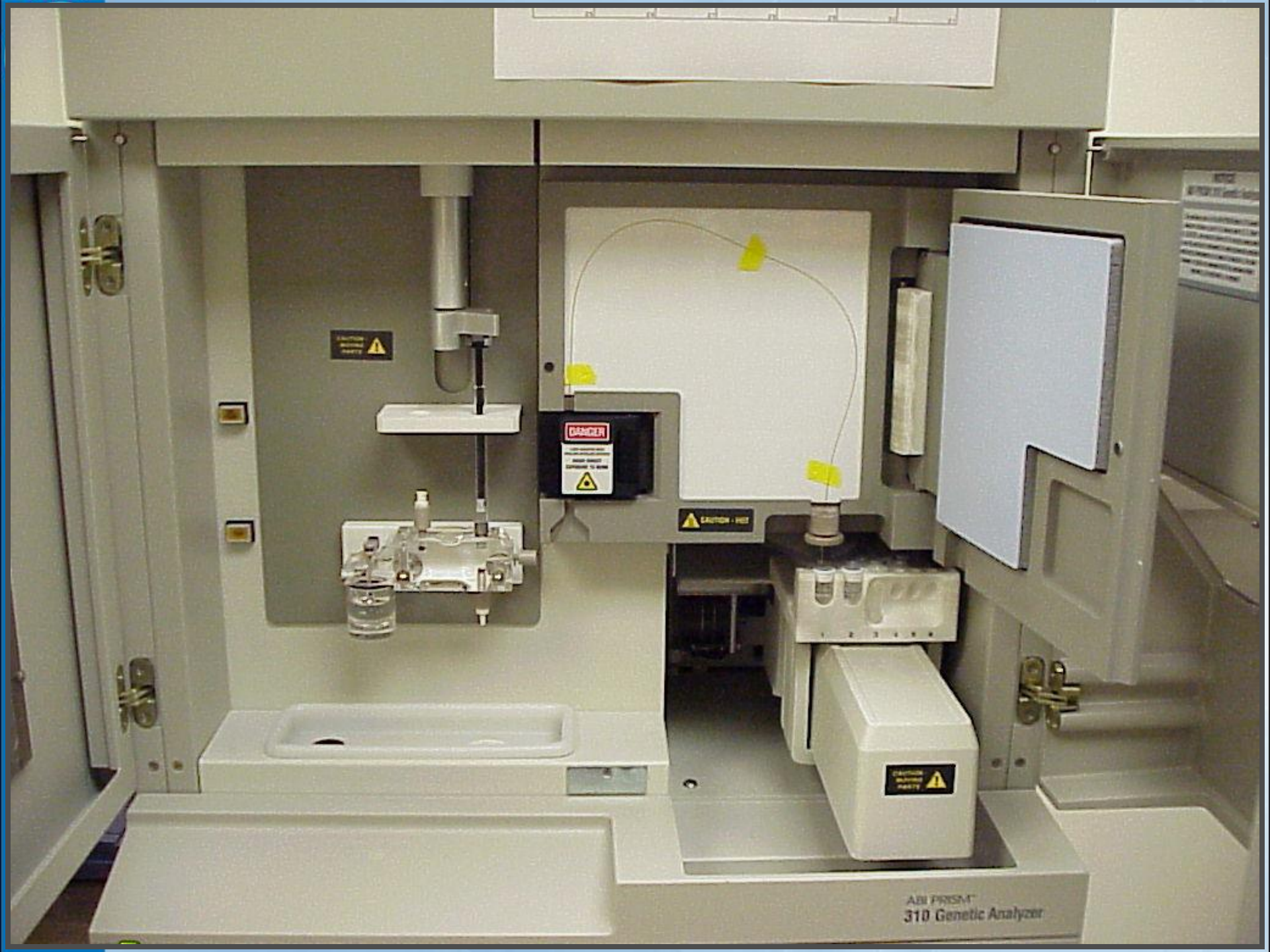
ABI prism 310

Capillary electrophoresis

ABI prism 310

Capillary electrophoresis

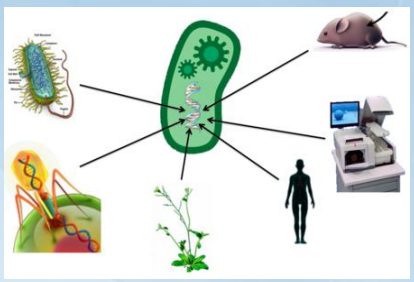


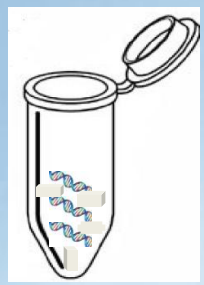

DANGER
CAUTION - HOT
ABI PRISM
310 Genetic Analyzer

Setting up sequencing reaction

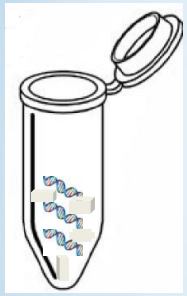
Sequencing workflow

1. DNA isolation

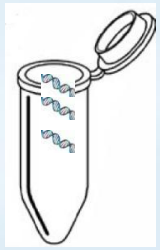




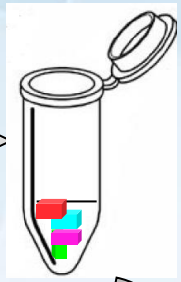
2. PCR amplification of the target gene



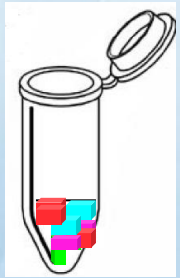
3. Purify PCR product



4. Set up and Perform sequencing reaction

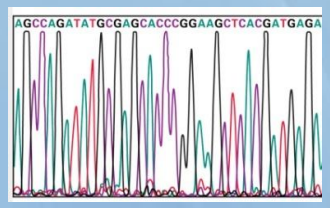


5. Purification PCR product



6. Resolve sequence fragments

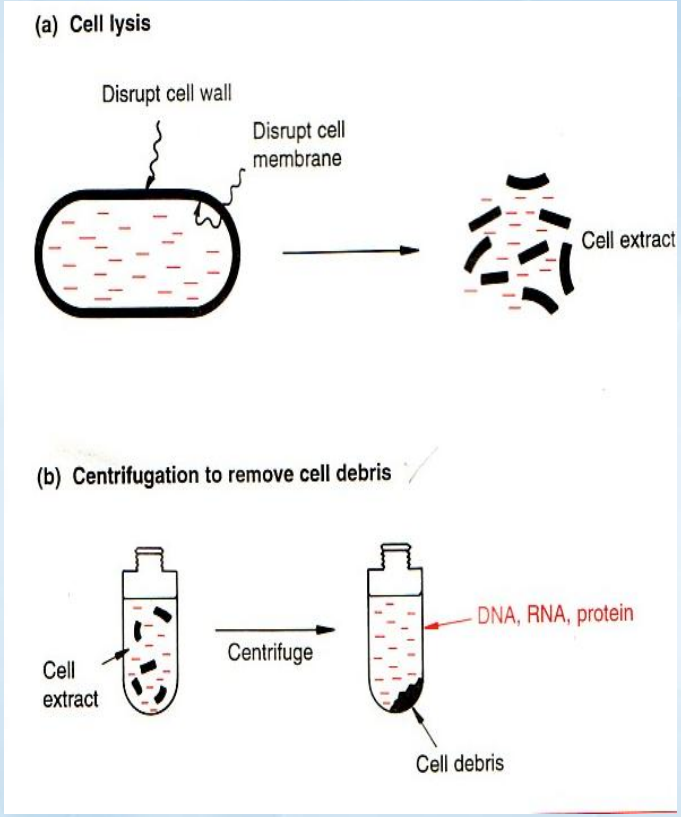
7. Read order of terminators (DNA sequence)



1.DNA isolation

Most DNA extraction protocols consist of 3 parts

1. **Cell lysis**
2. **Removing contaminating proteins, RNA, or macromolecules**
3. **Recovery of the DNA**

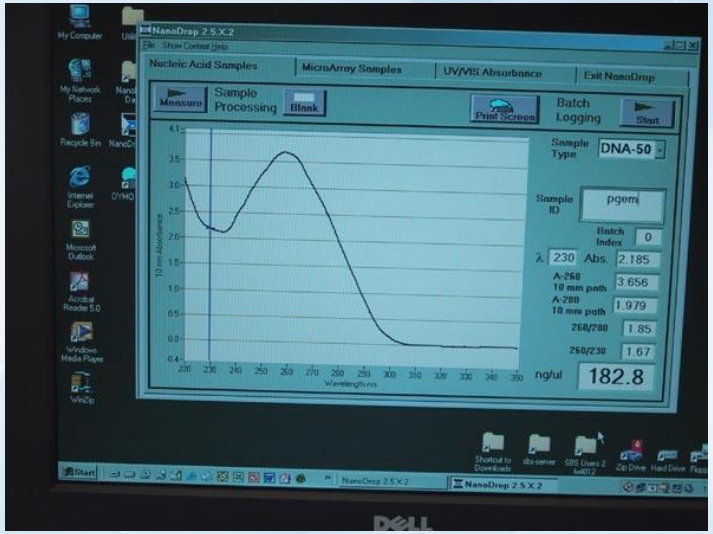


NanoDrop spectrophotometer

Check samples on a Nanodrop first to measure:

1. Purity
2. Concentration

Can accurately measure tiny volumes of sample as low as 1 µl.



dsDNA	50 – 100 fmol
ssDNA	25 – 50 fmol
Purified PCR product	10 – 50 fmol

2.Amplify the target Gene Using (PCR)

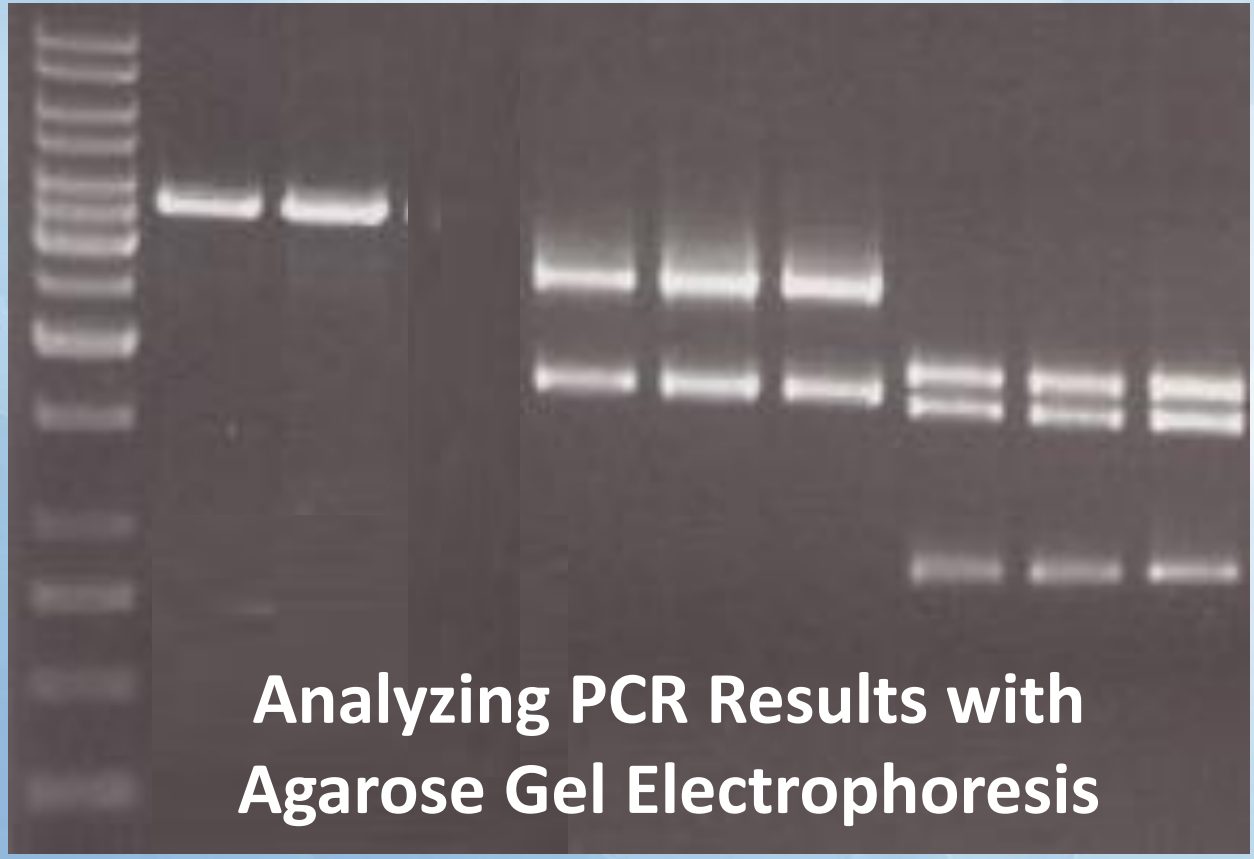
PCR Ingredients

1. DNA “template”
2. *Taq* Polymerase
3. Deoxynucleotides (dNTPs)
4. Primers (Fw + Rv)
5. Buffer and water

PCR Program

Step 1: 5 min at 95˚C
Step 2: 40 cycles of:
30 sec at 95˚C
30 sec at 50-60˚C
1 min at 72˚C
Step 3: 7 min at 72˚C
Step 4: hold at 4˚C

DID YOUR PCR WORK?



Factors Influencing PCR Success

- Contamination
- DNA
- Cycle parameters
- Primers

Avoiding Contamination

- Sample preparation, reaction mixture assemblage should be performed in separate areas.
- A Laminar Flow Cabinet with a UV lamp is recommended for preparing the reaction mixture.
- New gloves should be used for DNA/RNA purification.
- The use of tips with filters for both sample and reaction mixture preparation
- Autoclaving of all buffers is recommended.

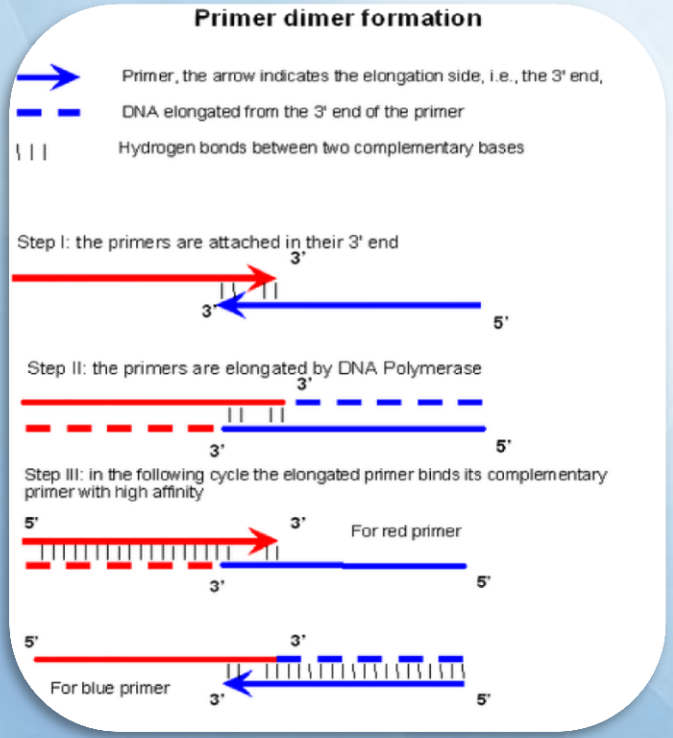
DNA Influencing PCR Success

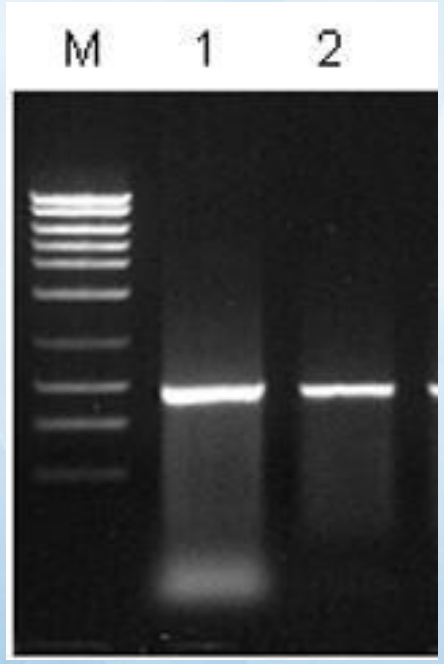
- Tissue type used for DNA extraction
- Quantity of the Sample
- Length of the DNA fragment to be amplified
- PCR Inhibitors
  - Detergent
  - Phenol
  - Heparin
  - Heme
  - Dyes (bromphenol blue)
  - Urine
  - High concentration of DNA

Primers

- **The primer should not be self-complementary or complementary to any other primer in the reaction mixture, to prevent primer-dimers.**

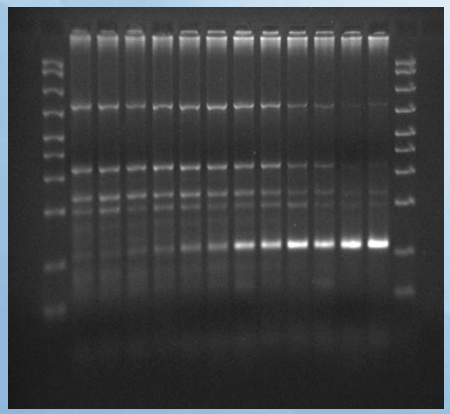
- Primer-dimers interferes with Sequencing



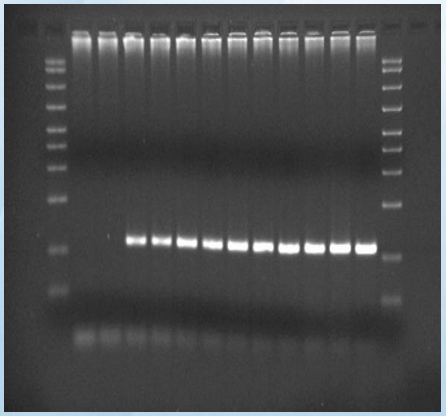


Cycle parameters

- **Annealing temperature**
  - starting approximately 5°C below calculated T_m
- **Extension time**
  - every 1kb of amplicon: 1 minute (Is it always true?)
- **Number of cycles**
  - 25-40 cycles

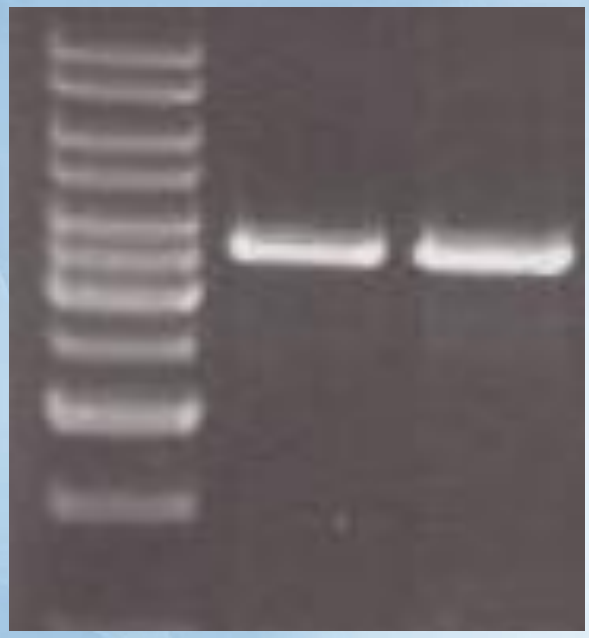


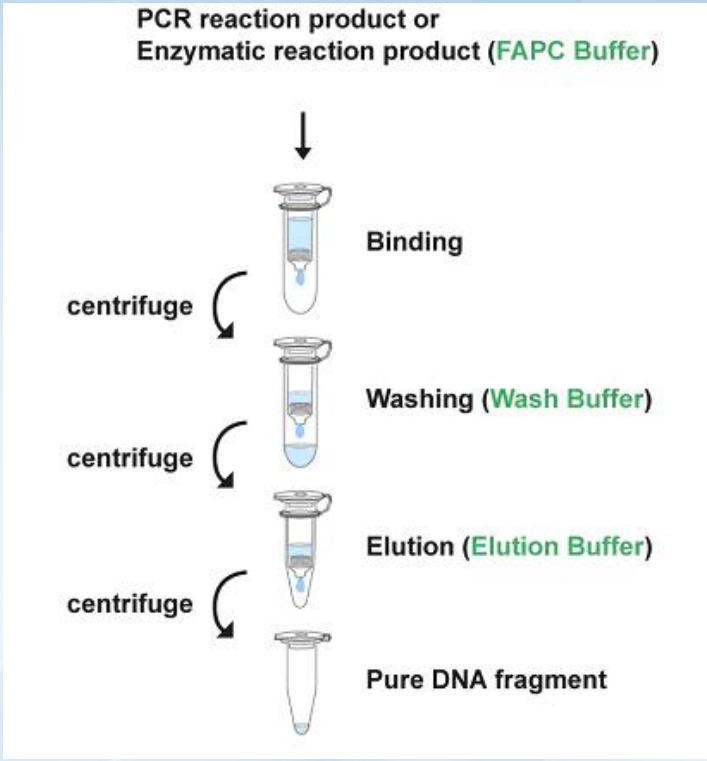
Not optimized



Well optimized

PCR purification





Prepare templates properly:

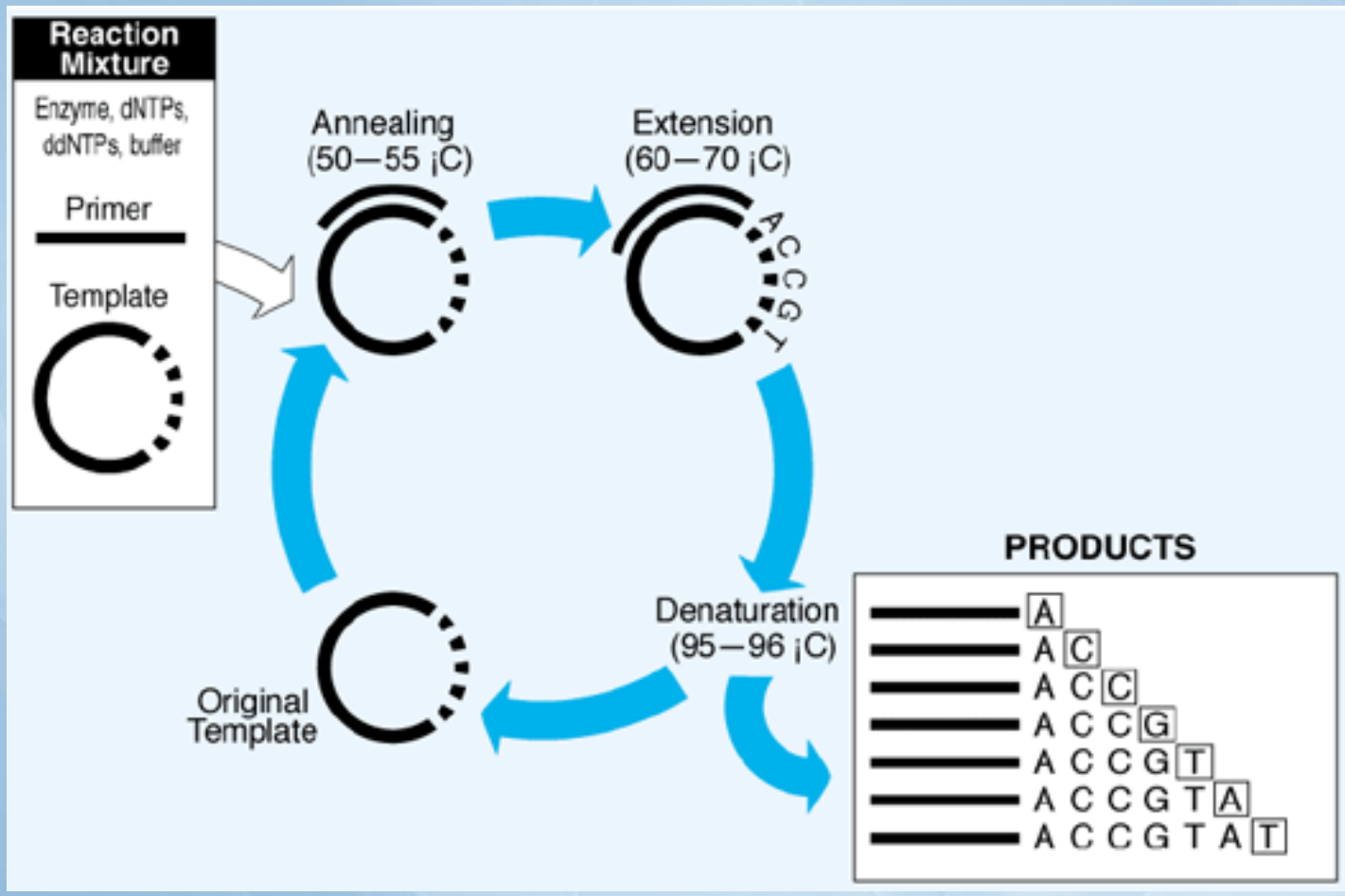
Resuspend in water.

Do not resuspend in TE or EDTA reagents.

Best to use a commercial kit for purification!

*****REMEMBER: HIGH QUALITY TEMPLATE WILL LEAD TO HIGH QUALITY DATA!!!!**

Preparation of Sequencing Reaction

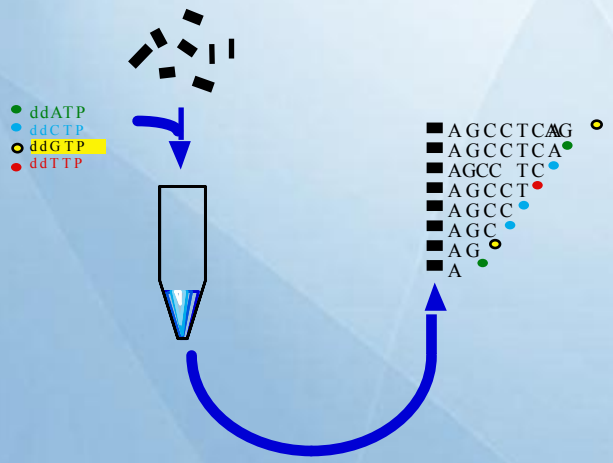


- Cycle sequencing is linear amplification
- Only one primer per reaction

Sequencing Reaction

PCR Ingredients

1. DNA “PCR prouduct”
2. Primers (Fw **OR** Rv)
3. Bigdye terminator
4. Seqeuncing buffer



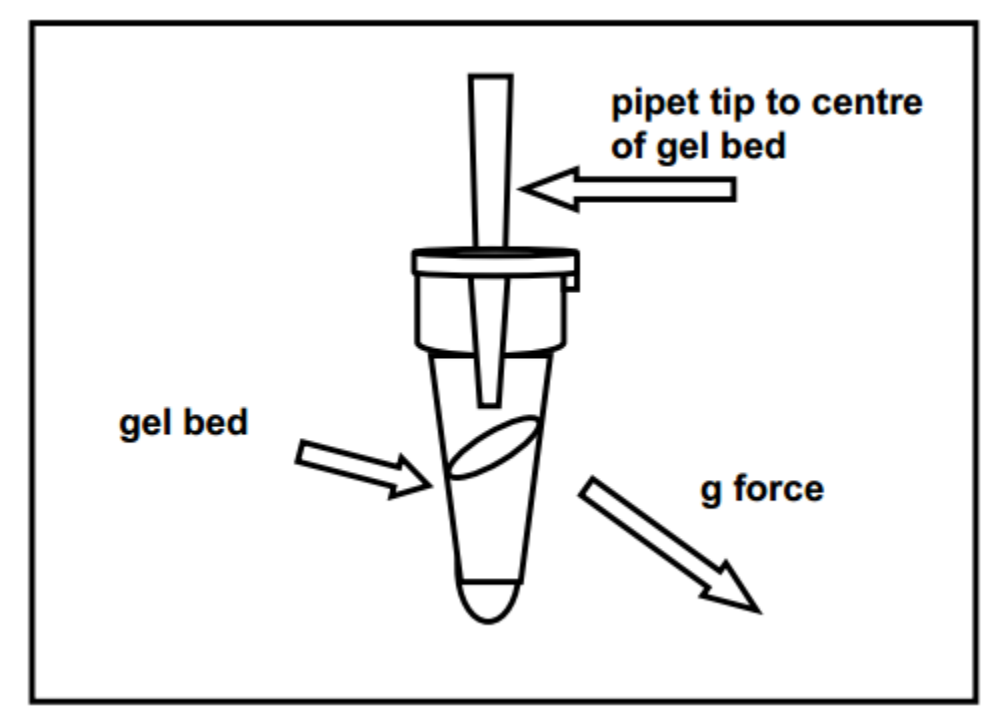
PCR Program

Step 1: 1 min at 96˚C
Step 2: 25 cycles of:
10 sec at 96˚C
5 sec at 50˚C
4 min at 60˚C
Step 3: hold at 4˚C

Sequencing Reaction Purification

Centri - Sep Protocol

These are useful for removing excess, nucleotides, small primers, radioactive and fluorescent precursors salts and buffers

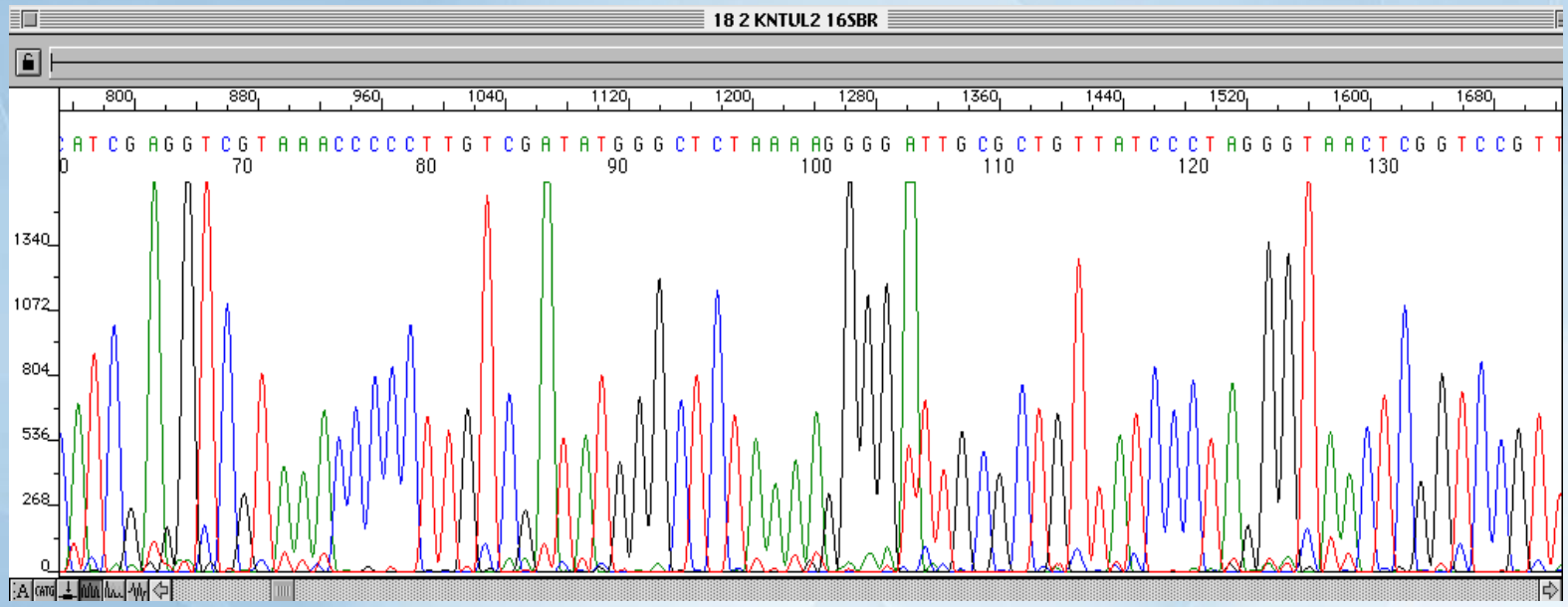


Loading the Reaction on the 310 ABI sequencer

Accuracy is approx 99%

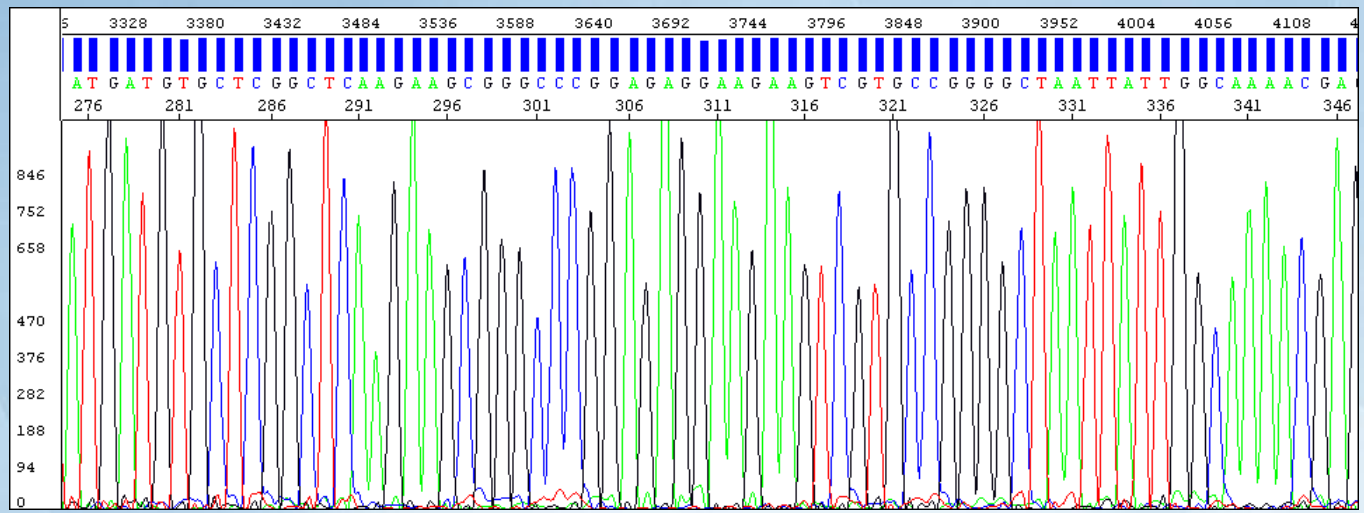
Data output

Data in electropherogram format shows peaks .abi file
Free software sequence scanner v1.0 (Life Tech).



Data in sequence file format shows text .seq file

Recommendations for successful sequencing



Good quality sequence data, with sharp peaks, no N's and high quality value scores

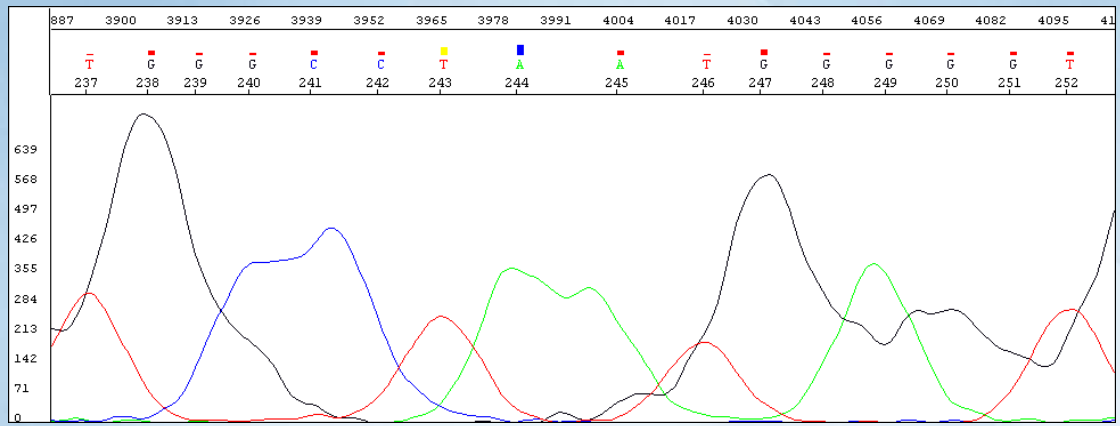
* Check sample using a Nanodrop. Check for contaminants.
* Check the primer melting temperature..
* Submit samples at the correct concentrations. Use water not EDTA or TRIS.
* For Ethanol precipitation always use fresh stocks and ensure it is completely removed.

Troubleshooting

Contaminants such as excess salt, RNA or protein in your sample:

These cause the bands to be distorted and wide and the quality scores are low.

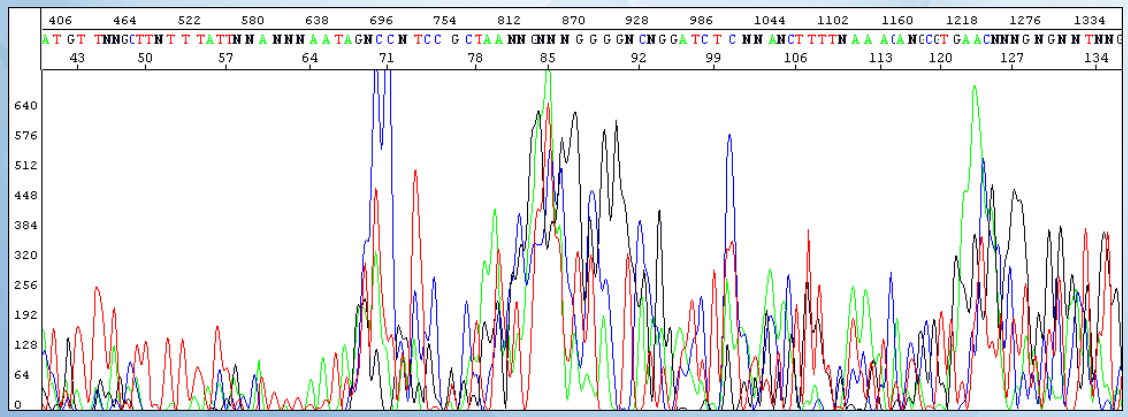
The software has problems calling the right bases.



A failed reaction:

There are many Ns called as the reaction has failed due to template and/or primer problems with high background noise observed.

The software cannot call the correct bases.



Submitting your template

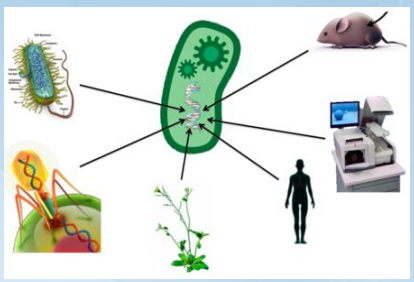
Sample Requirements

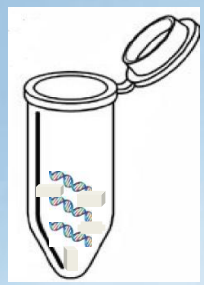
Please supply your templates as detailed below:

Template type	Plasmid	M13	PCR product
Template concentration	200 ng/µL	100 ng/µL	5-20 ng/µL
Template volume	5 µL	5 µL	5 µL
Primer concentration	5 pmol/µL	1 pmol/µL	5 pmol/µL
Primer volume	5 µL	5 µL	5 µL

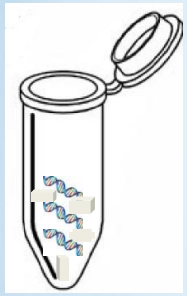
Sequencing workflow

1. DNA isolation

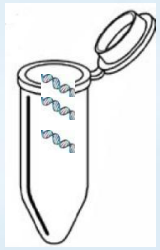




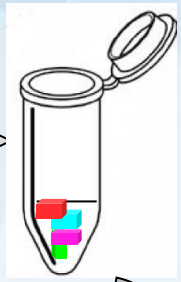
2. PCR amplification of the target gene



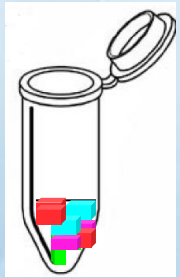
3. Purify PCR product



4. Set up and Perform sequencing reaction



5. Purification PCR product



6. Resolve sequence fragments

7. Read order of terminators (DNA sequence)