334 MBIO
Biochemical Instrumentation Techniques

- Lab 5 -

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2025

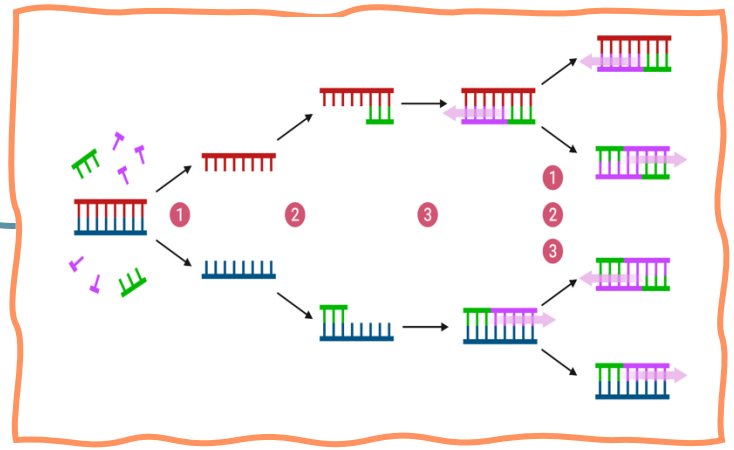
Investigations!

- Have you ever wondered how tiny DNA samples solve crimes?
- Have you ever wondered how viruses' infections are detected from DNA?
- Have you ever wondered how forensic experts identify suspects?

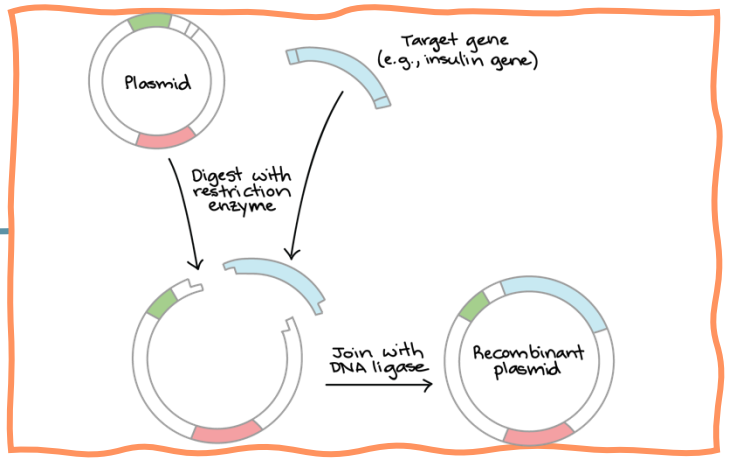
- The solution is to do **amplification of parts of DNA**.

Mainly there are two methods:

Polymerase chain reaction



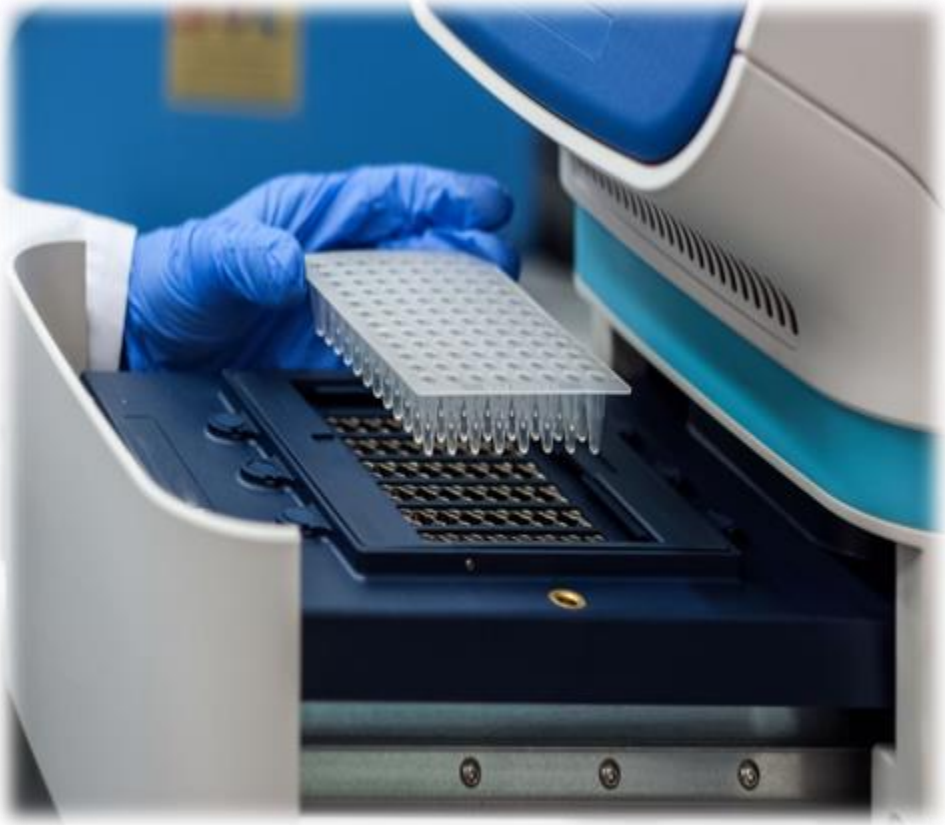
DNA Cloning



Polymerase Chain Reaction (PCR) for DNA Amplification

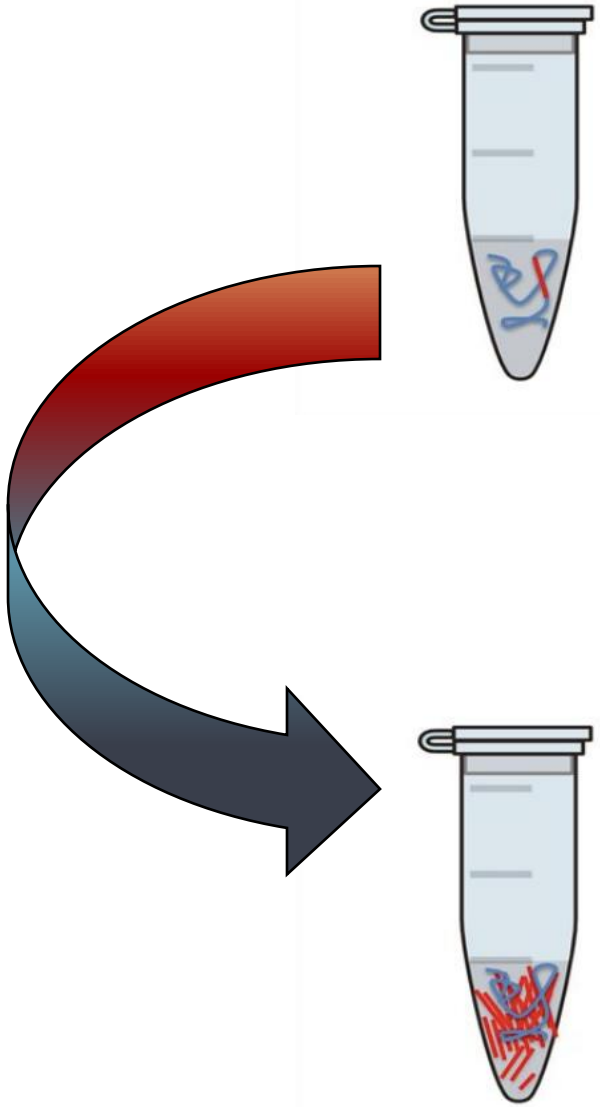
Polymerase Chain Reaction (PCR)

- A powerful and widely used technique in molecular biology that **amplifies specific DNA sequences** to generate millions of copies of a target region.
- PCR is essential for many applications, including **cloning, sequencing, and diagnostics**.



Polymerase Chain Reaction (PCR)

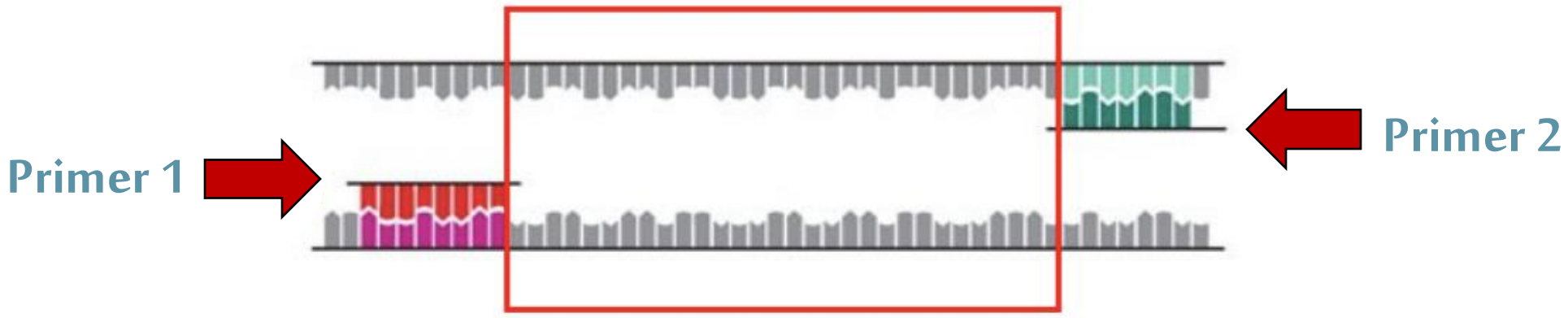
- It is a laboratory version of DNA Replication in cells.



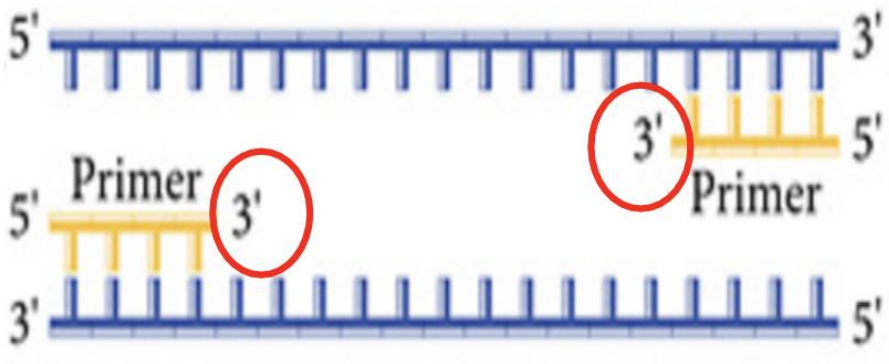
⟶ The laboratory version is commonly called "***in vitro***" since it occurs in a test tube while ***"in vivo"*** signifies occurring in a living cell.

Amplifications of specific target sequence

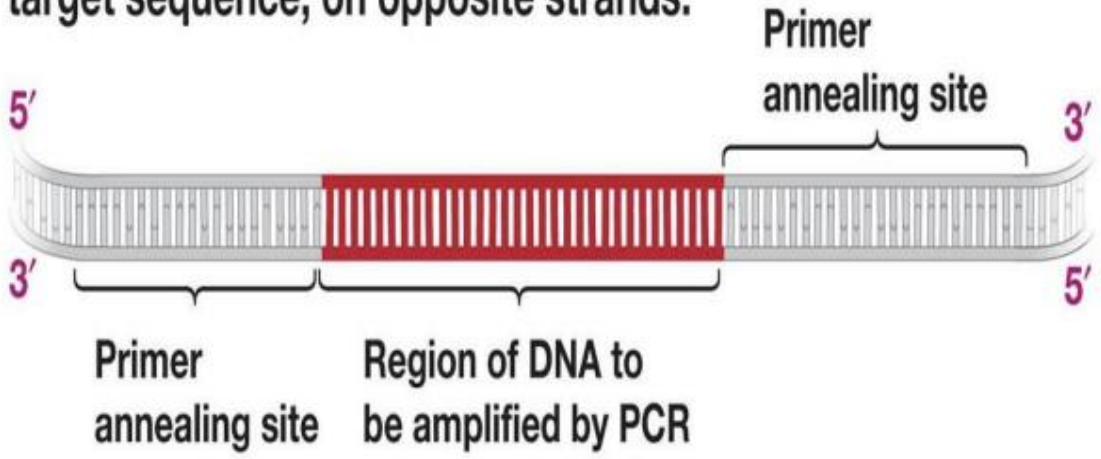
- **PCR** does not copy all the DNA in the sample. It copies only a **very specific sequence** of genetic code from a **template DNA**, targeted by **PCR primers**.
- It does require the knowledge of some DNA sequence information which flanks the fragment of DNA to be amplified (**target DNA**).



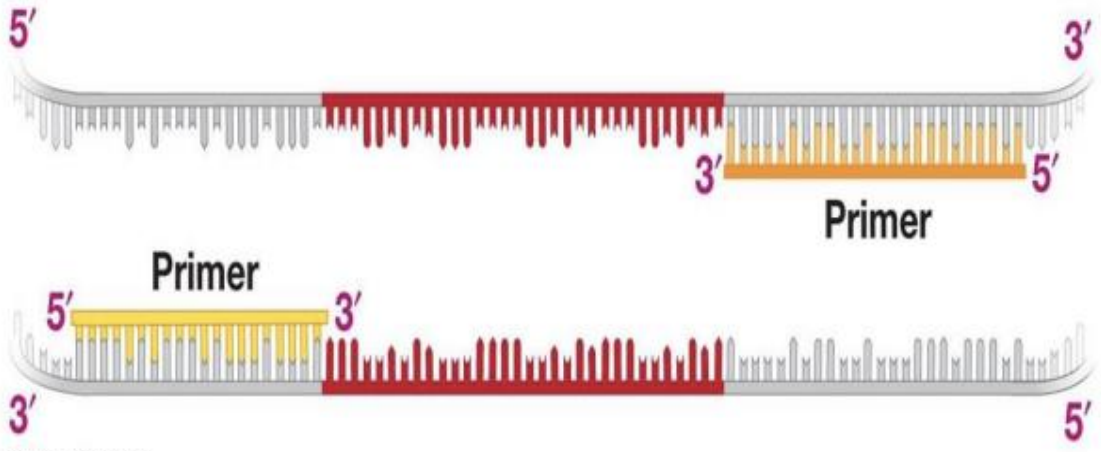
- **Two synthetic oligonucleotide primers** may be chemically synthesised each complementary to a stretch of DNA to **the 3′ side** of the target DNA.
- One oligonucleotide for each of the two DNA strands (DNA polymerase can add a nucleotide only onto **a preexisting 3'-OH group**)



(a) PCR primers must bind to sequences on either side of the target sequence, on opposite strands.



(b) When target DNA is single stranded, primers bind and allow DNA polymerase to work.

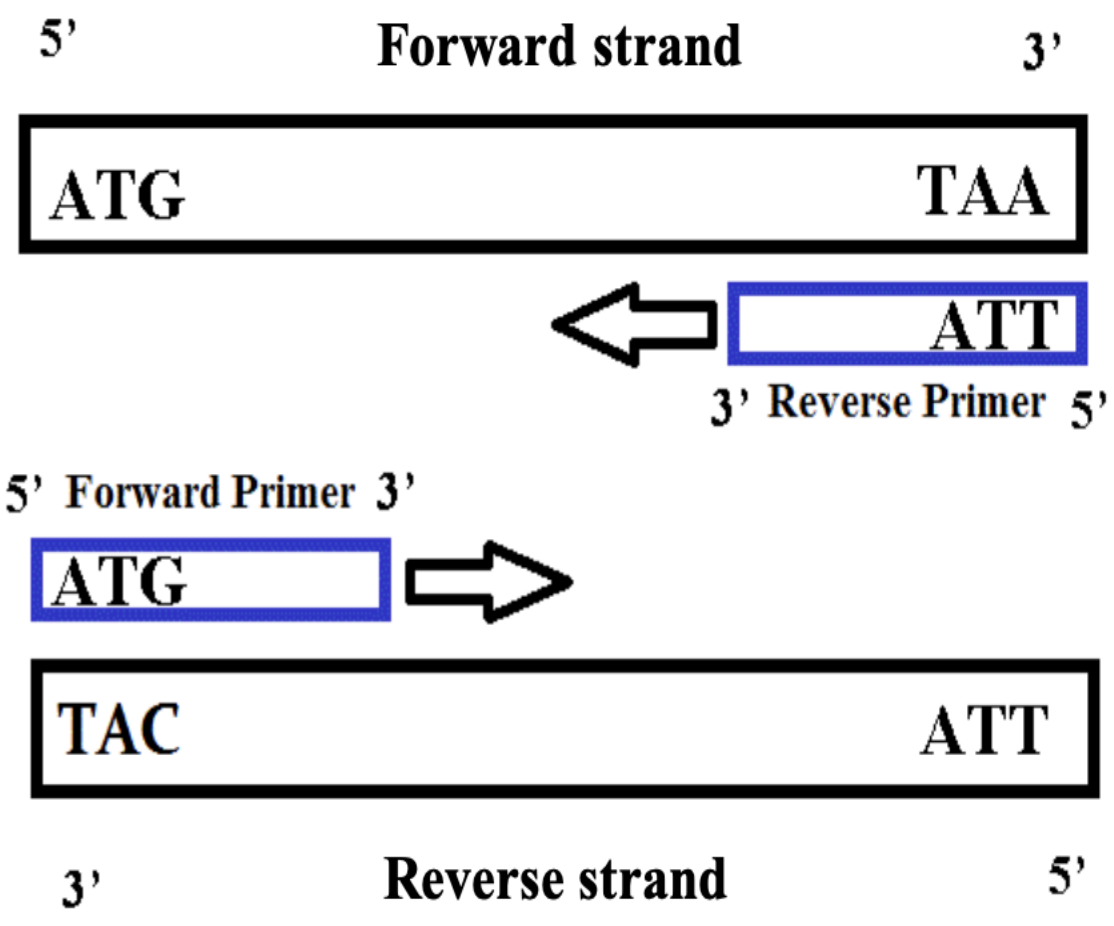


Why are Two Primers Needed?

- In a PCR reaction, two primers are needed to amplify the target sequence:

→ **One called:** Forward primer, which have the same sequence of forward DNA strand and bind to the complementary reverse strand.

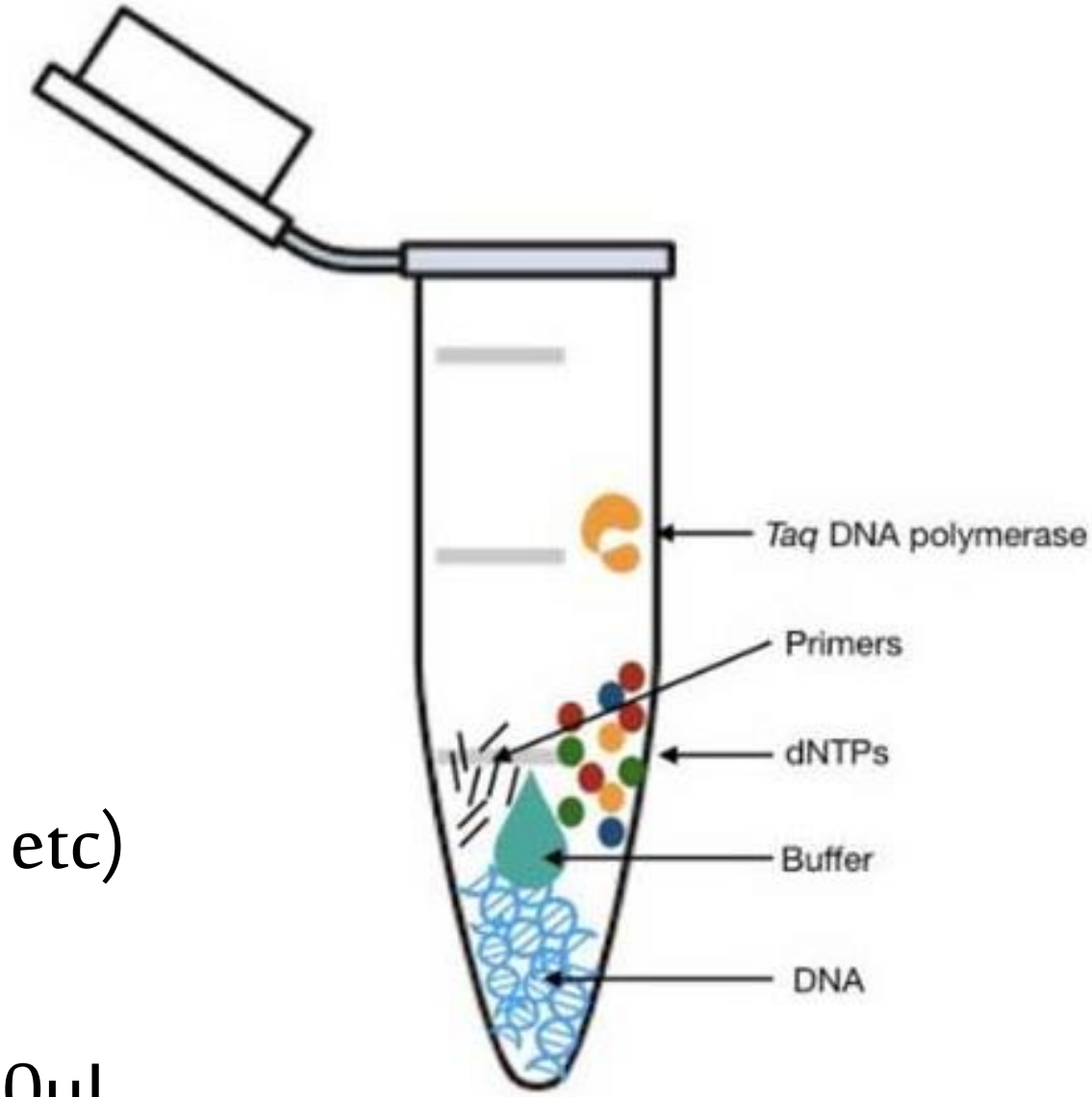
→ **The second called:** Reverse primer, which have the same sequence of reverse DNA strand and bind to the complementary forward strand.



Components of PCR Reaction

- **A single PCR reaction** consists of:

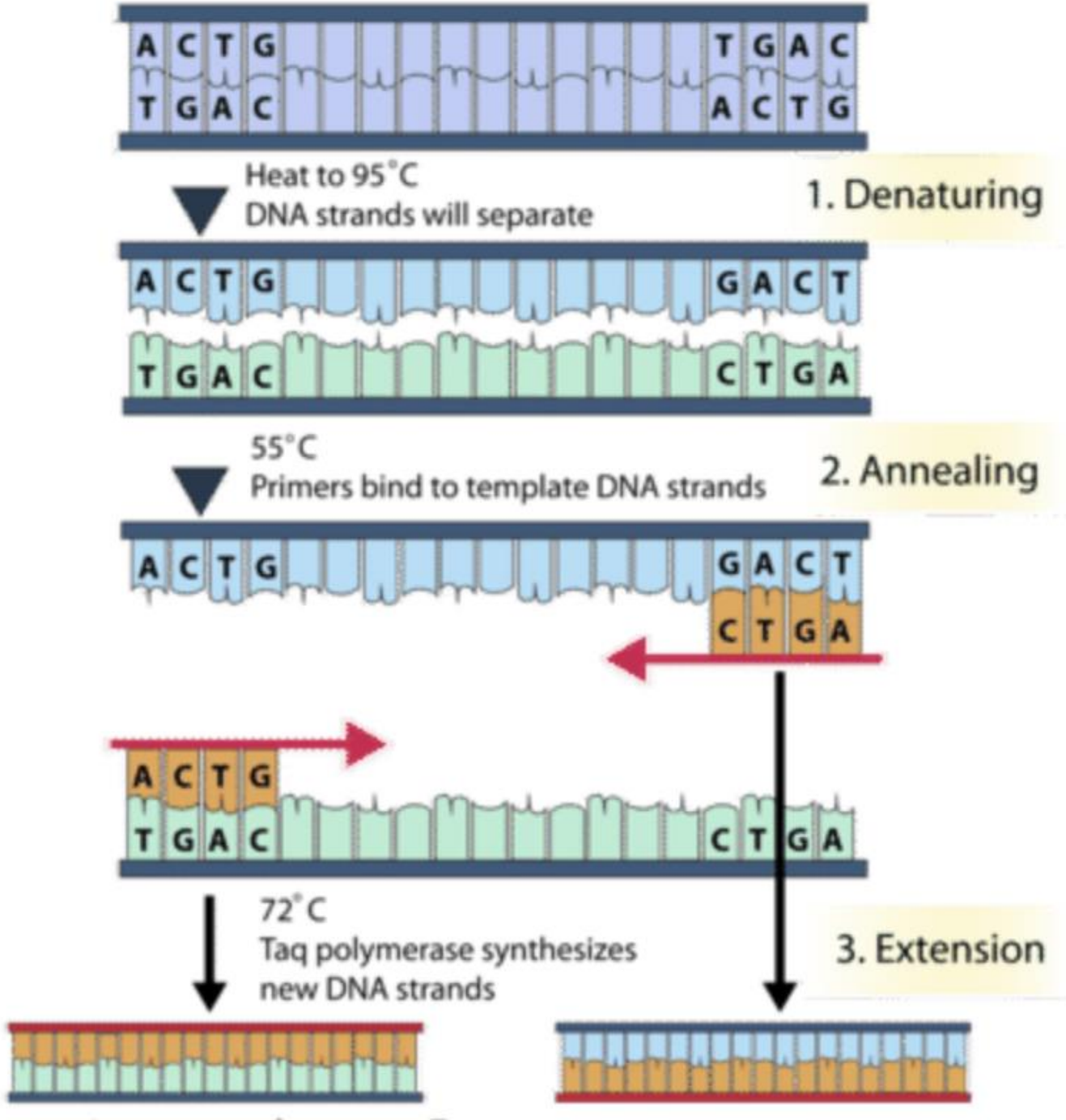
- DNA template
- Forward and reverse primers
- Nucleotides (dNTPs)
- Mixing buffer (e.g., Milli-Q H_2O)
- DNA polymerase (e.g., Taq, high fidelity, etc)



- Typical PCR volumes range around 20-50uL.

The PCR Cycle

- PCR proceeds in **THREE** distinct steps governed by **Temperature**:

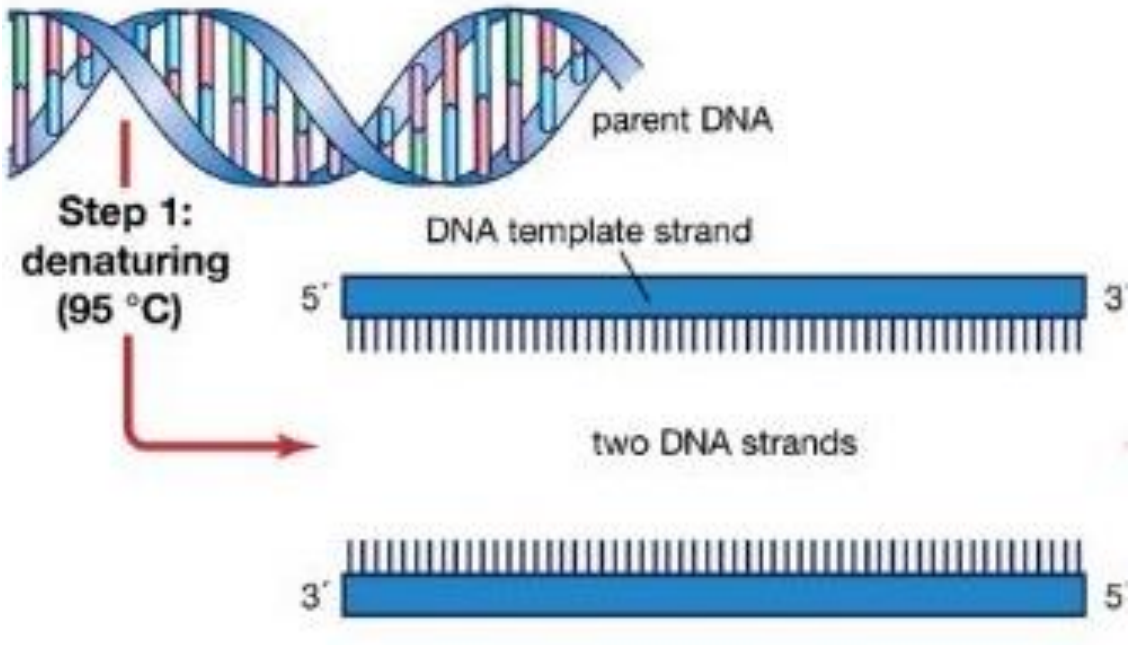


1. Denaturation

- The double-stranded template DNA is denatured by heating, to separate the double stranded DNA (**why?**).

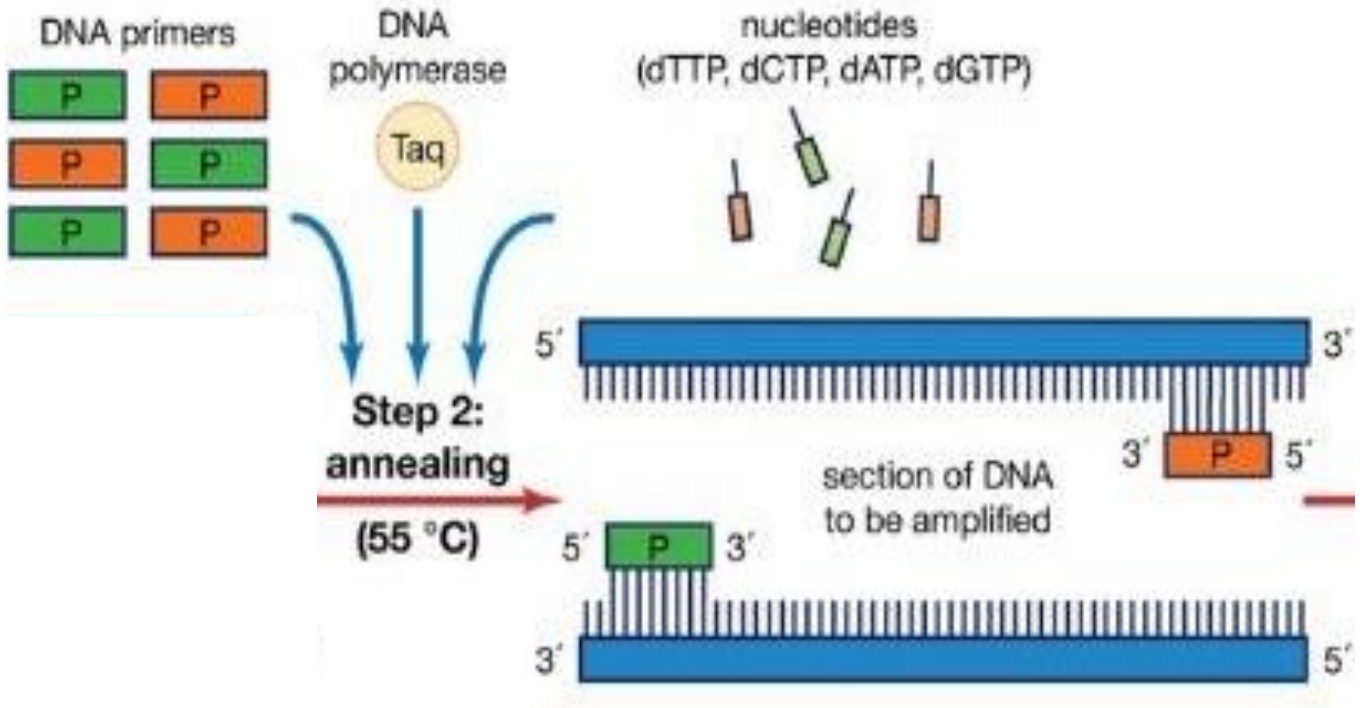
- Breaking the _____ bonds.

- Step 1 ⟶ **94 – 97 °C**



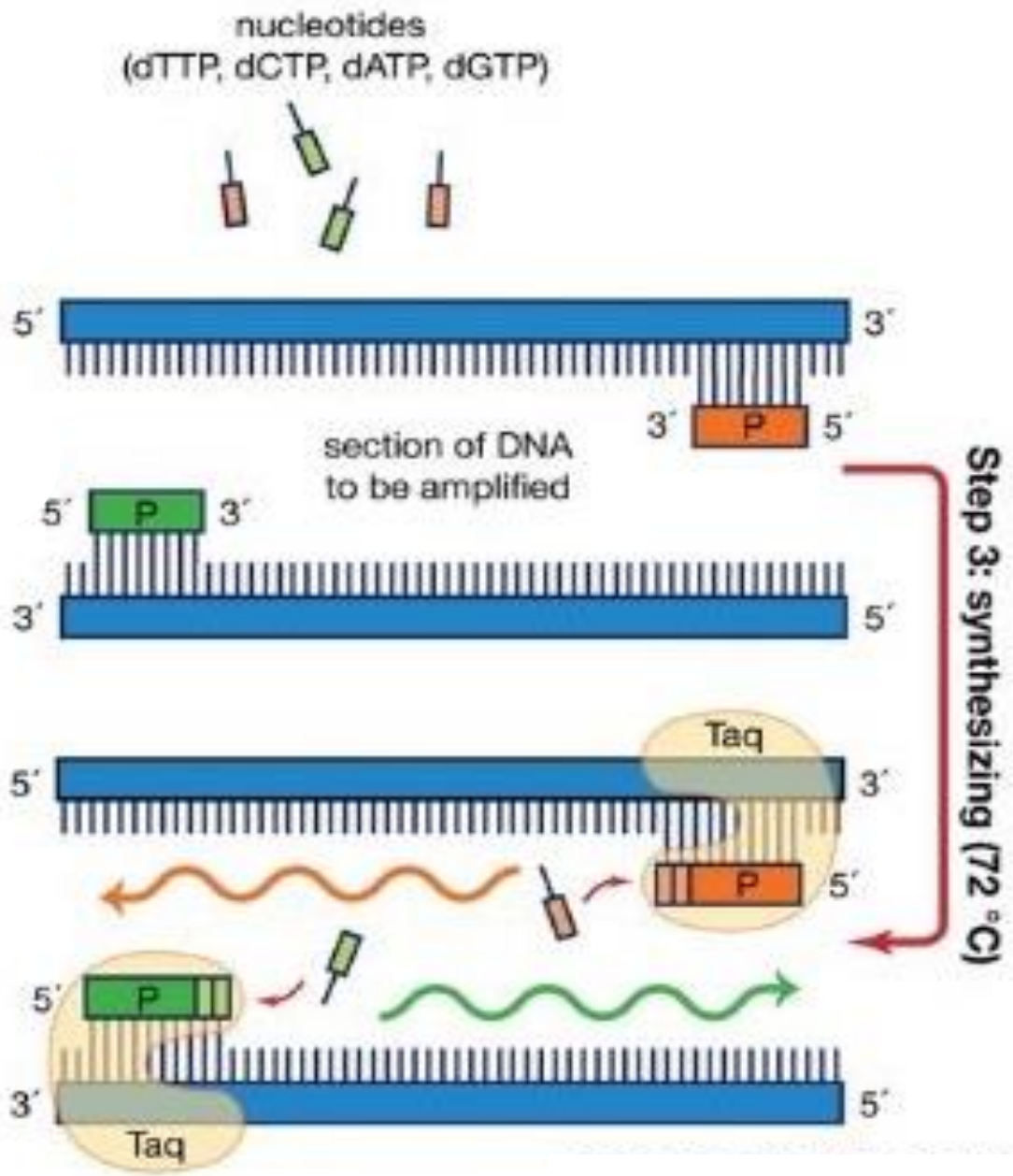
2. Annealing

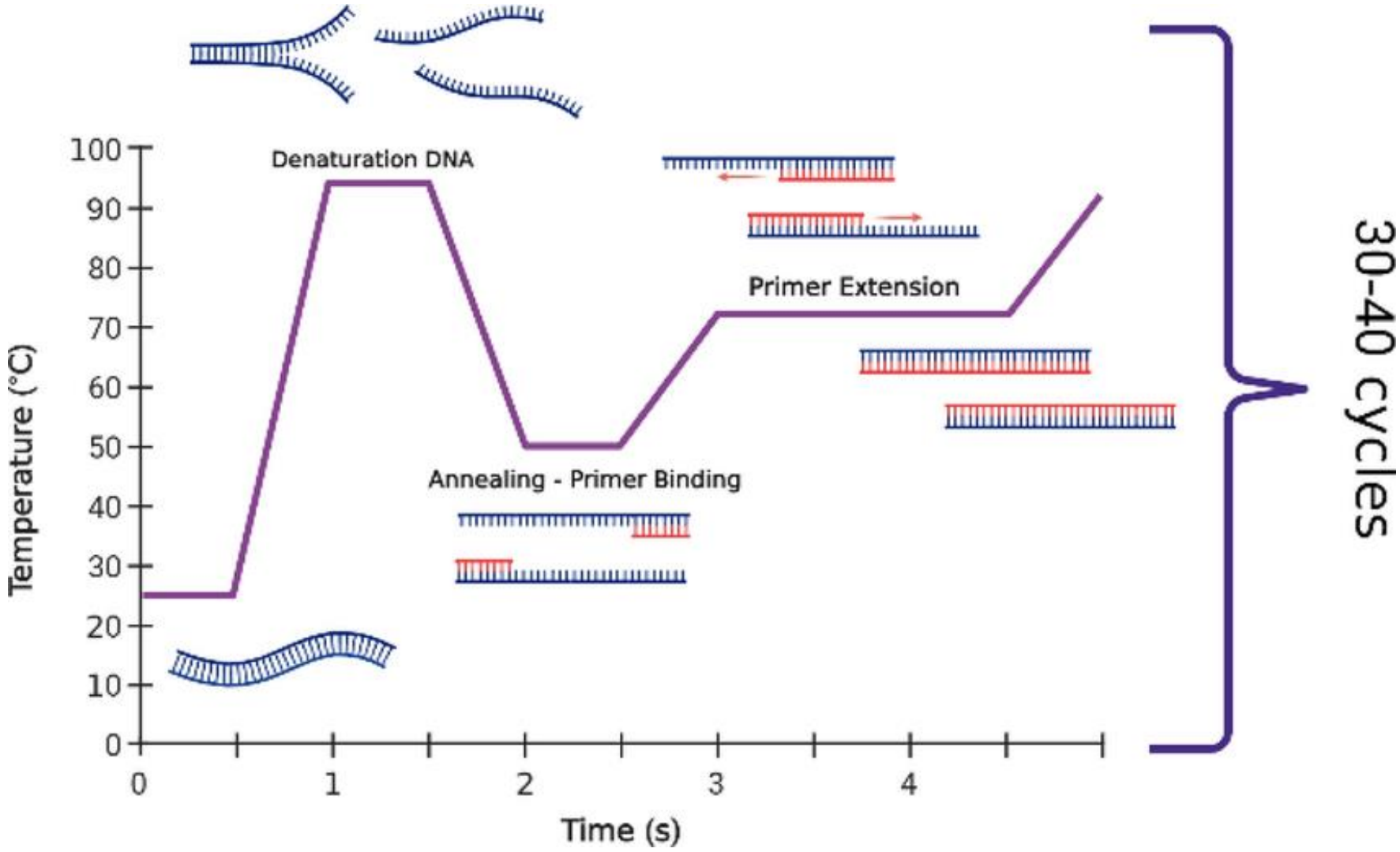
- The reaction is rapidly **cooled to the primer annealing temperature,** to allow the primers to hybridize to single stranded template.
- Primer will anneal only to sequences that are complementary to them (target sequence).
- **What is the type of the bond?**
- Step 2 ⟶ **50-65 °C**



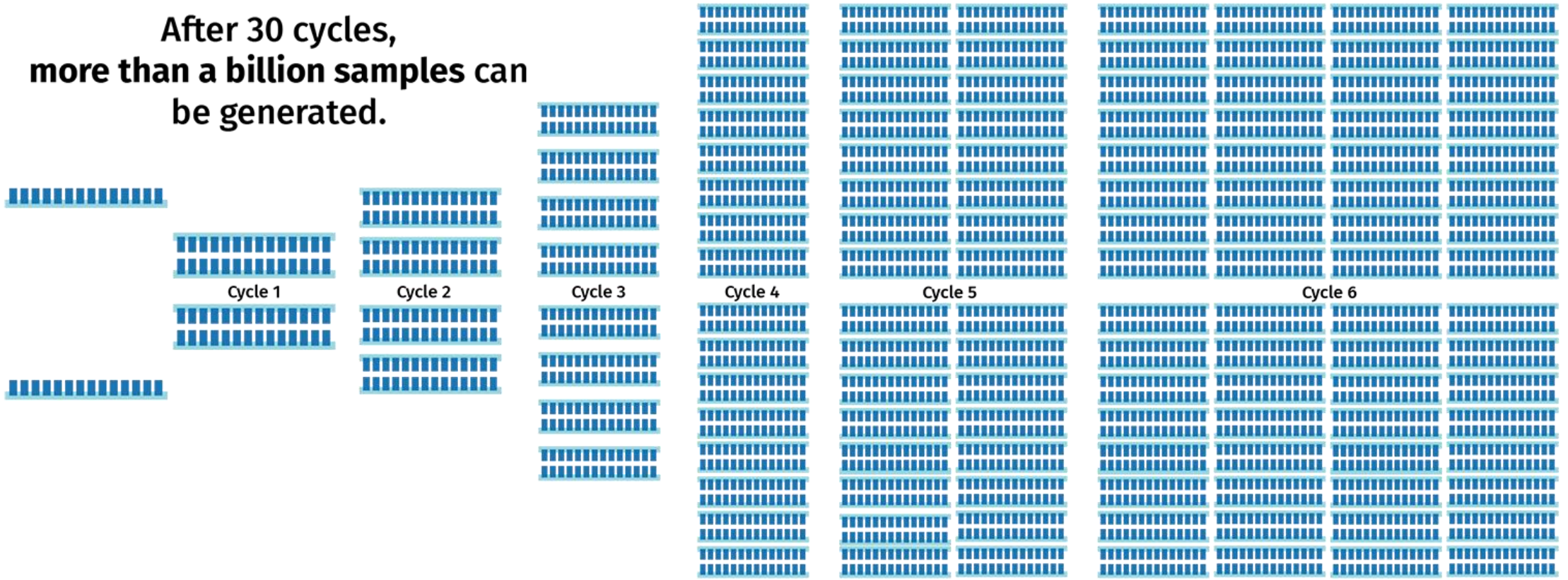
3. Extension

- The reaction is **heated** to a temperature depends on the **DNA polymerase** used.
- Commonly a temperature of **72°C** is used with this enzyme.
- At this step, the DNA polymerase **synthesizes a new DNA** strand complementary to the DNA template
- Step 3 ⟶ **72 °C**





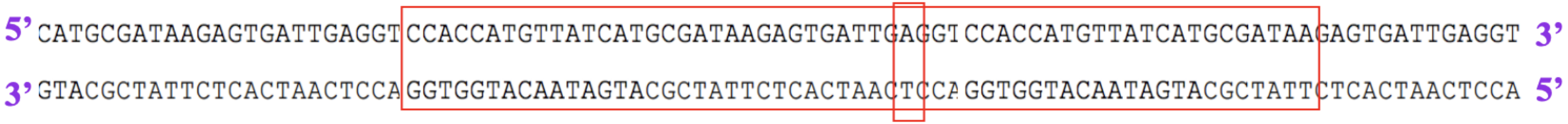
- At the end of the PCR reaction, the specific sequence will be accumulated in billions of copies (**amplicons**).
- In only 20 cycles, PCR can product about a million (2^{20}) copies of the target.



Example

- You want to study a **mutation in a DLG3 gene** and how it relate to memory:

The segment that you want to amplified is in the red square



Start the PCR

1. Denaturation:

95 °C 

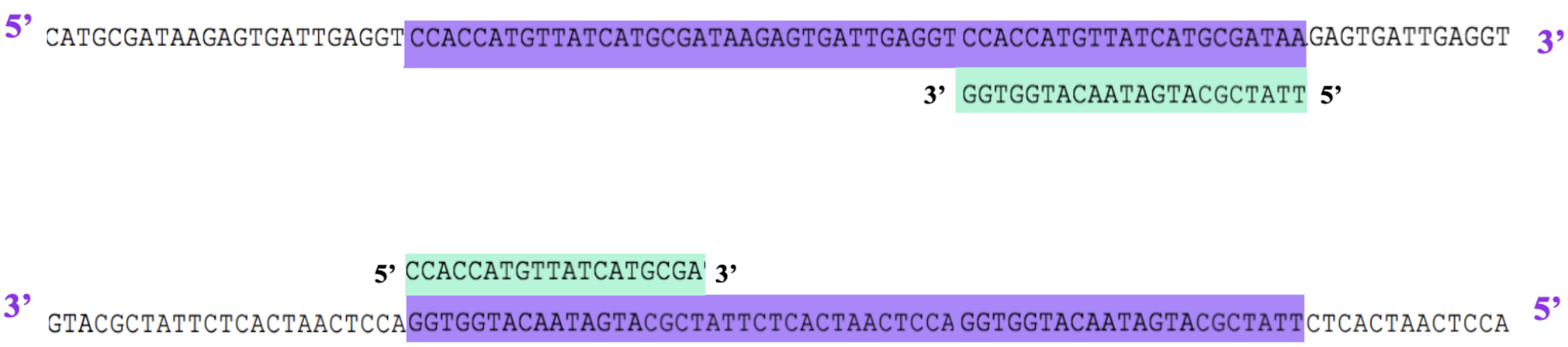
5’ CATGCGATAAGAGTGATTGAGGT CCACCATGTTATCATGCGATAAGAGTGATTGAGGT CCACCATGTTATCATGCGATAA GAGTGATTGAGGT 3’

3’ GTACGCTATTCTCACTAACTCCA GGTGGTACAATAGTACGCTATTCTCACTAACTCCA GGTGGTACAATAGTACGCTATT CTCACTAACTCCA 5’

Start the PCR

2. Annealing:

58 °C



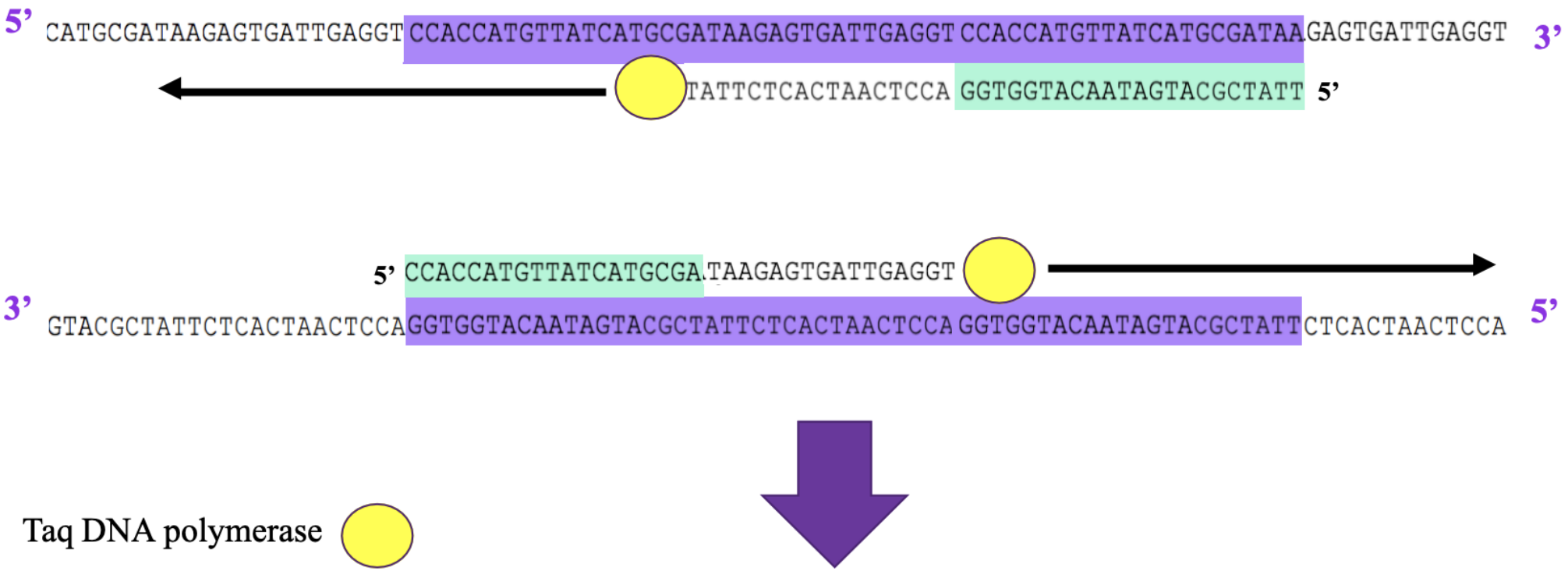
Forward primer: 5’ CCACCATGTTATCATGCGA 3’

Reverse primer: 3’ GGTGGTACAATAGTACGCTATT 5’

Start the PCR

3. Extension:

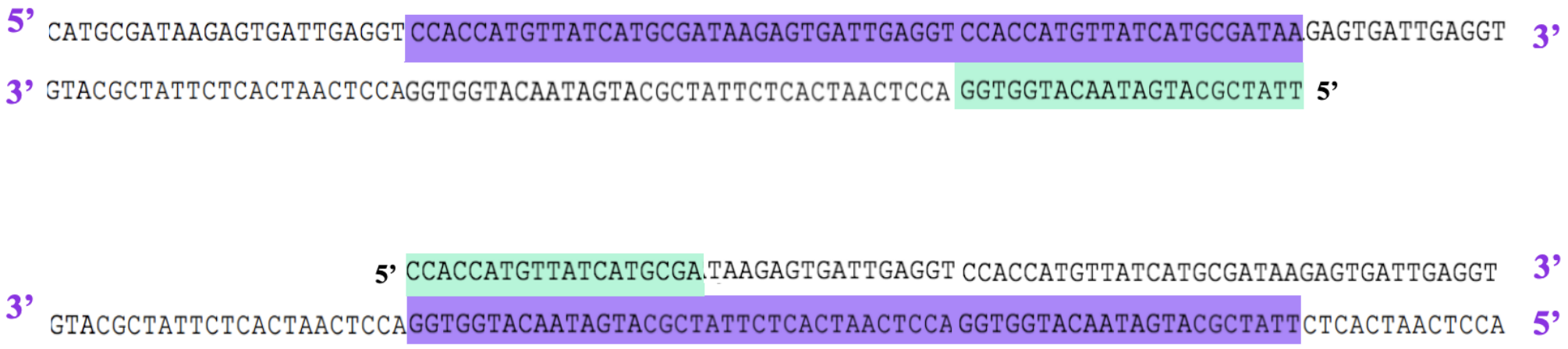
72 °C



Start the PCR

3. Extension:

72 °C

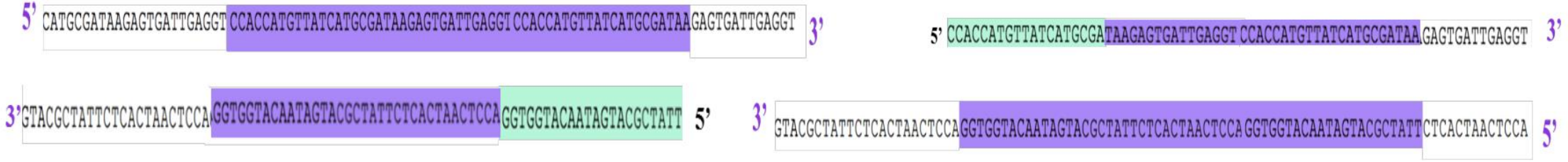


Cycle # 1:
1 DNA amplified to 2 DNA

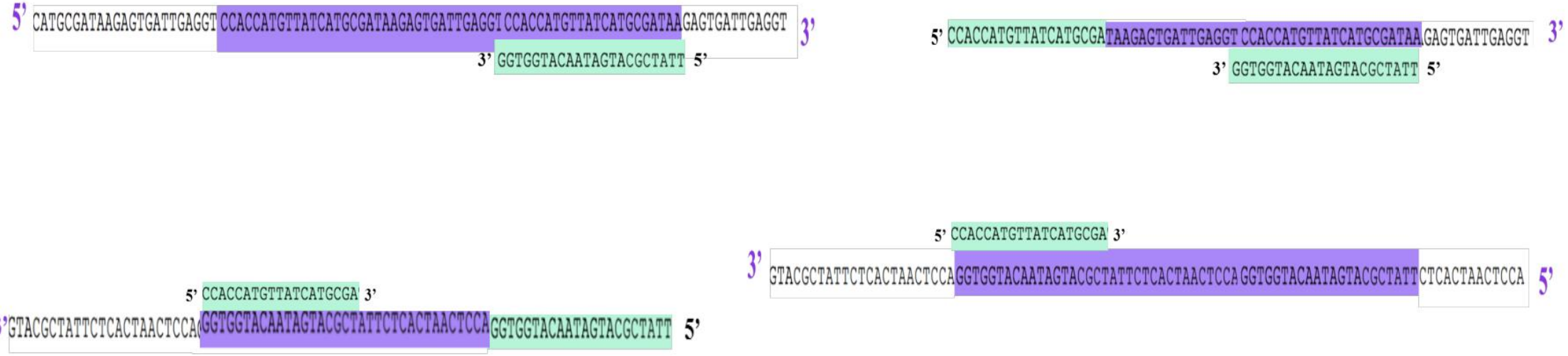
Start the PCR

Cycle 2

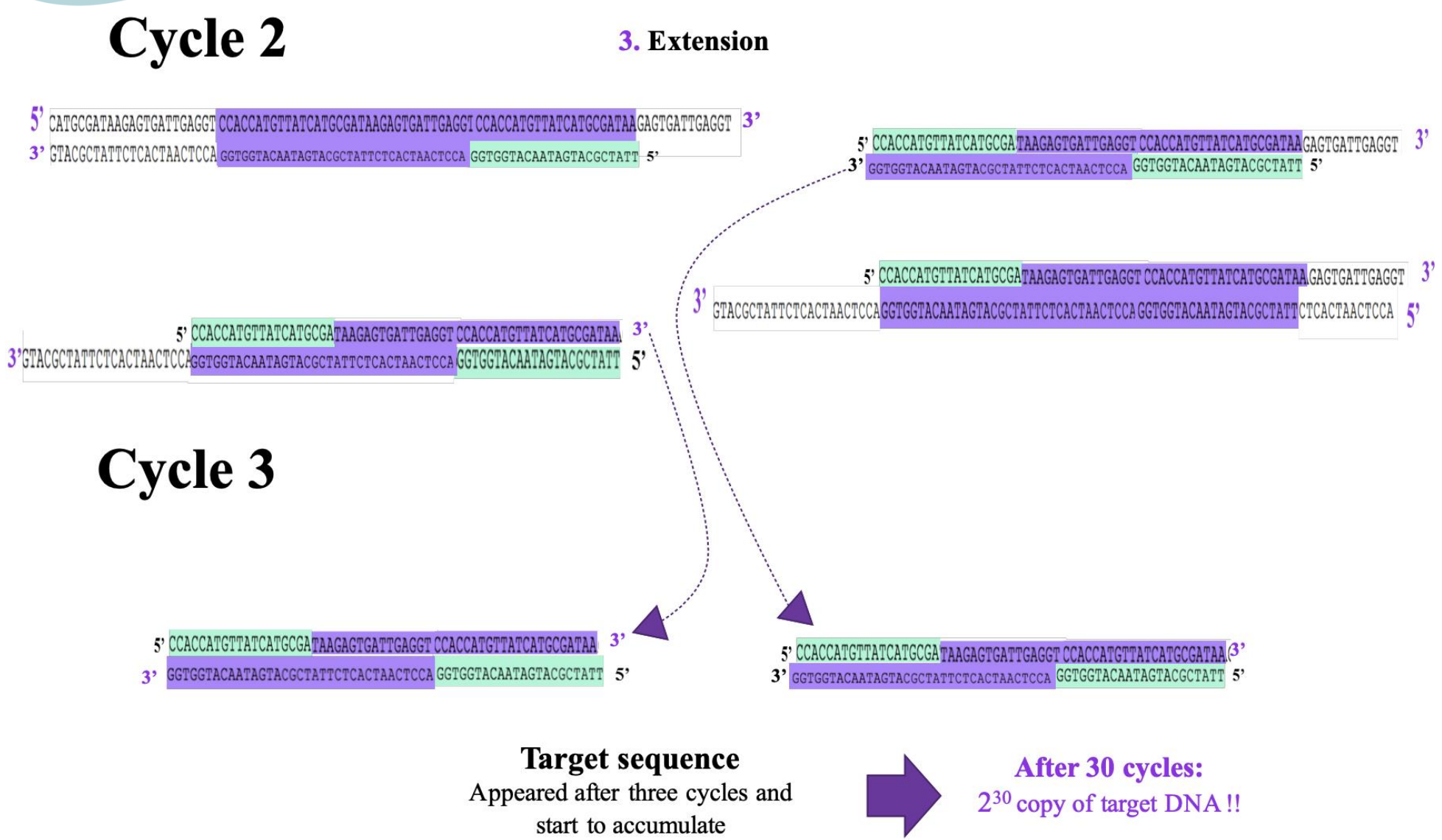
1. Denaturation



2. Annealing

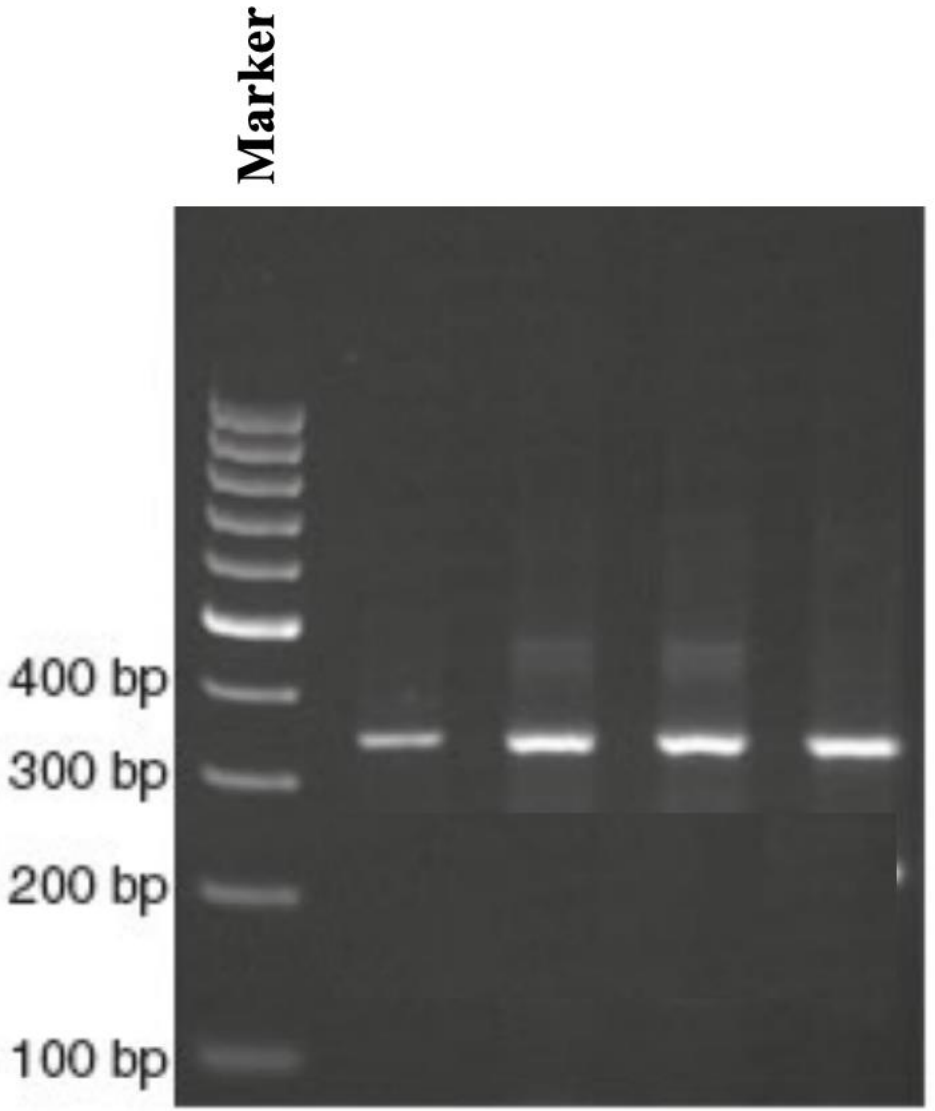


Start the PCR



How will you make sure that you target sequence is amplified?

- It is very important to know your product size.
- Only one band is formed.
- **Target sequence size of DLG3 gene is 350 bp.**



PCR Advantages

- **High Sensitivity** – Can amplify DNA from tiny amounts (even a single molecule).
- **Specificity** – Targets specific DNA sequences, minimizing non-specific amplification.
- **Rapid** – Generates millions of copies in just a few hours.
- **Automation** – Performed with minimal manual intervention using thermal cyclers.
- **Quantification** – Real-time PCR (qPCR) allows precise DNA measurement

PCR Applications

- **Genotyping** ⟶ Identifies genetic variations among individuals.
- **RT-PCR** ⟶ Detects and quantifies RNA expression levels.
- **Cloning** ⟶ Amplifies DNA for insertion into vectors.
- **Mutation** ⟶ Identifies/Generate genetic mutations in DNA.
- **Forensics** ⟶ Identifies individuals using DNA evidence.
- **Paternity testing** ⟶ Confirms biological relationships through DNA comparison.

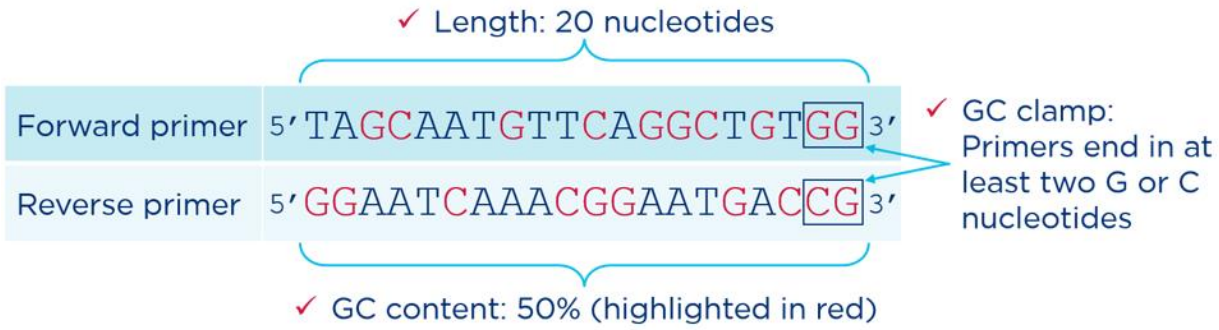
Primer Design Guidelines

- Primer Sequence
- Primer Length
- GC Content
- Melting Temperature (T_m)
- Annealing Temperature (T_a)

1. Primer Sequence:

Must be complementary to flanking sequences of target region. Avoiding each of:

- Complementary sequences between primers.
- Repeat (ex: ATATATAT) ⟶ **misprime**
- Cross Homology.



2. Primer Length:

- It is generally accepted that the optimal length of primers is **18-25 bp.**

3. GC Content:

- GC% = Number of guanine (G) and cytosine (C) in the primer as a percentage of the total bases.
- Should be **40-60%.**
- Presence of G or C bases within **the last five bases** from the 3' end of primers
- Not more than **2 G's or C's .**

5'-CAACATAATAGCGACAACACTAGA-3'

4. Melting Temperature (T_m):

- Melting temperatures in the range of **50-60 °C** generally produce the best results.
- **Maximum difference** between primer pairs is **5°C**.
- The T_m of the primer can be calculated by the following formula:

$$T_m = [(G + C) \times 4] + [(A + T) \times 2]$$

5. Annealing Temperature (T_a):

- The primer melting temperature is the estimate of the DNA-DNA hybrid stability and critical in **determining the annealing temperature**.
- Depends directly on length and GC composition of the primers.
  - **Too high T_a** ⟶ produce insufficient primer-template hybridization.
  - **Too low T_a** ⟶ lead to non-specific products caused by a high number of base pair mismatches.
- The Ta can be calculated by the following formula:

$$T_a = (0.3 \times T_m \text{primer}) + (0.7 \times T_m \text{product}) - 14.9$$

Exercise 1:
Primers Designing

- **You aim to study Neisseria Factor H binding protein (FHbp), which interacts with human Factor H and contributes to *Neisseria meningitidis* infection.**

The steps that you should follow is as following:

1. Find the sequence of the gene from scientific data (e.g., Ensebmle, PDB)
2. FHbp in PDB code is **GNA1870**
3. From Download files ⟶ download the **FASTA sequence**
4. Click on Display sequence in **FASTA form.**
5. Go to **EMBOSS Backtranseq** sequence back translation to get the DNA sequence
6. Click on new DNA file in SnapGene and paste the sequence
7. Design the primers using primer design tool (e.g., Primer3, SnapGene, etc), **FOLLOWING the GUIDLINES**

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