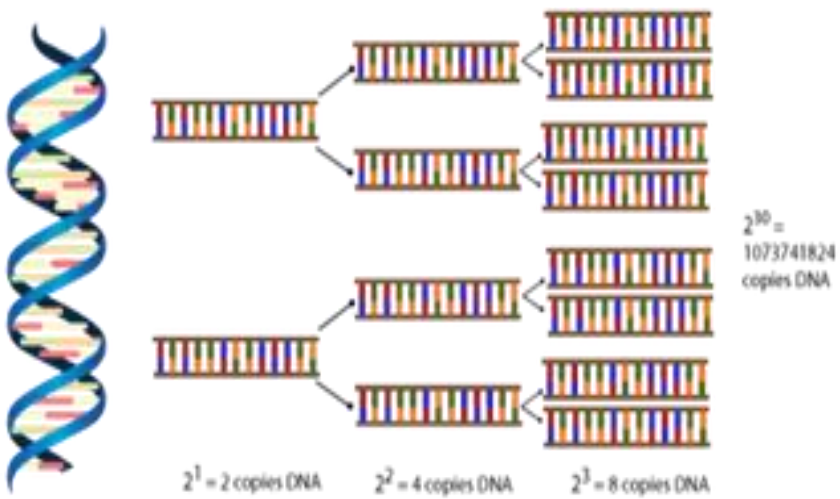
Polymerase chain reaction (PCR)

BCH361- Practical



Polymerase Chain Reaction (PCR)=DNA Photocopier

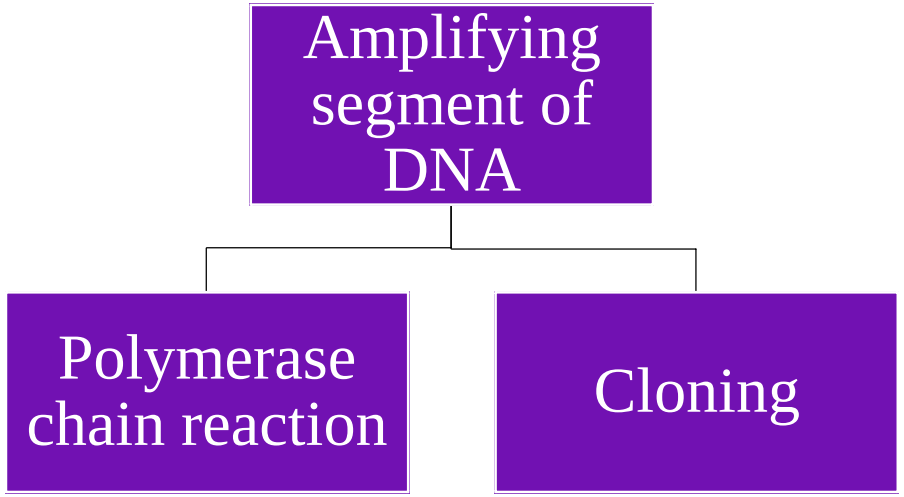
DNA Amplification:

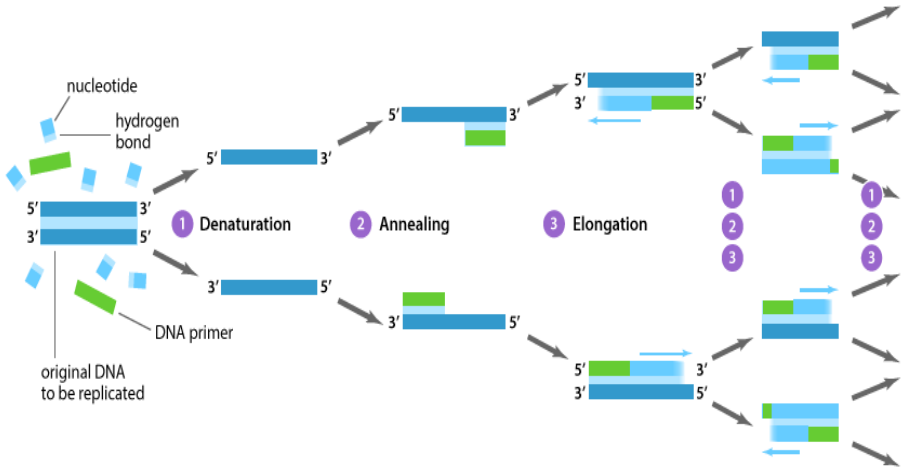
- In a crime scene, a sample of DNA was found, however **amount of DNA was not enough to** be analyzed.

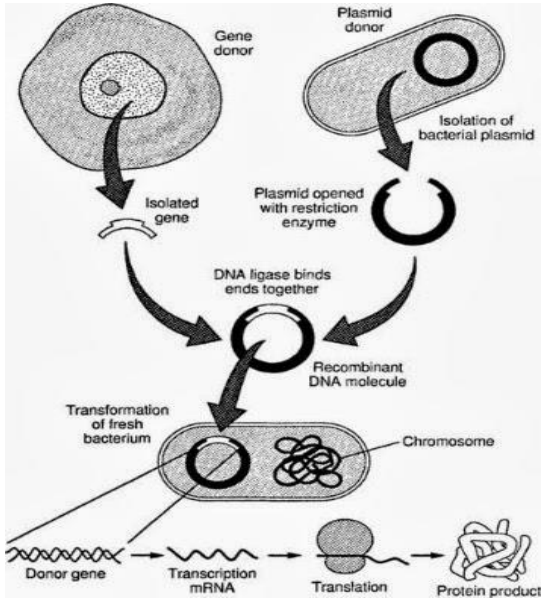
- After DNA extraction, the scientist want to study a specific **part of a gene** to do sequencing.

- How scientist solve these problem ?

- The solution is to do **amplification of parts of DNA**!!
- Mainly there are two methods:







PCR-Polymerase Chain Reaction:

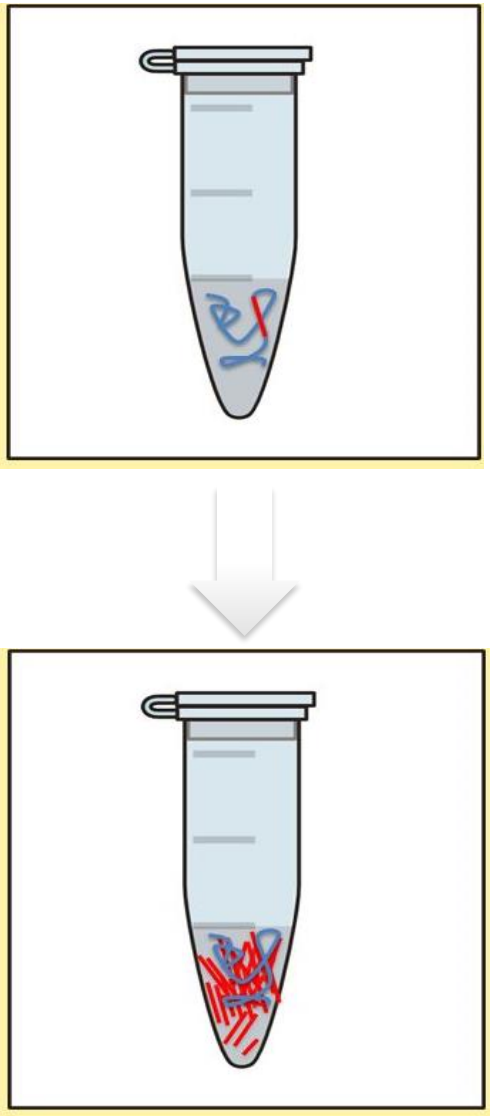
- PCR is a means to amplify a particular piece of DNA .

  ➔ **Amplify=** making numerous copies of a segment of DNA.

- PCR can make billions of copies of a **target sequence of DNA** in short time.

- 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.



- So…

➔ How the amplification will be done?

➔ How you will determine your target sequence?

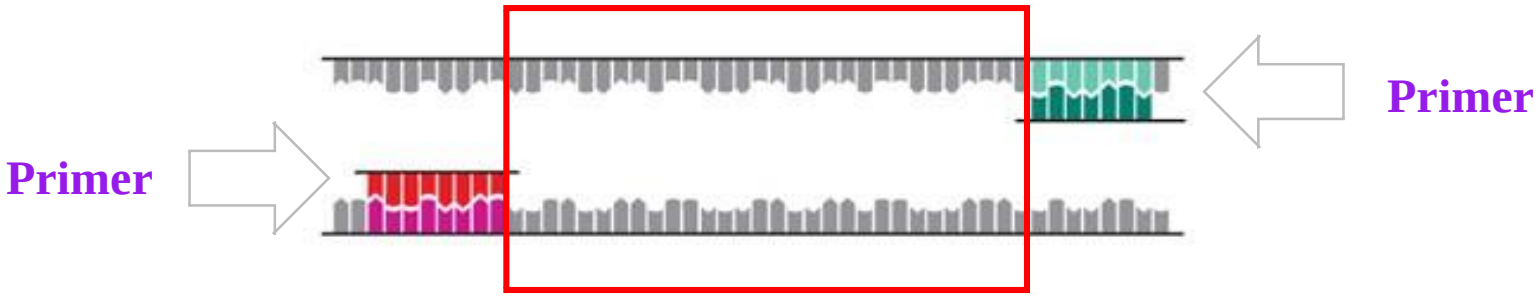
➔ How the amplification will be specific for certain segment?

You must to understand these questions

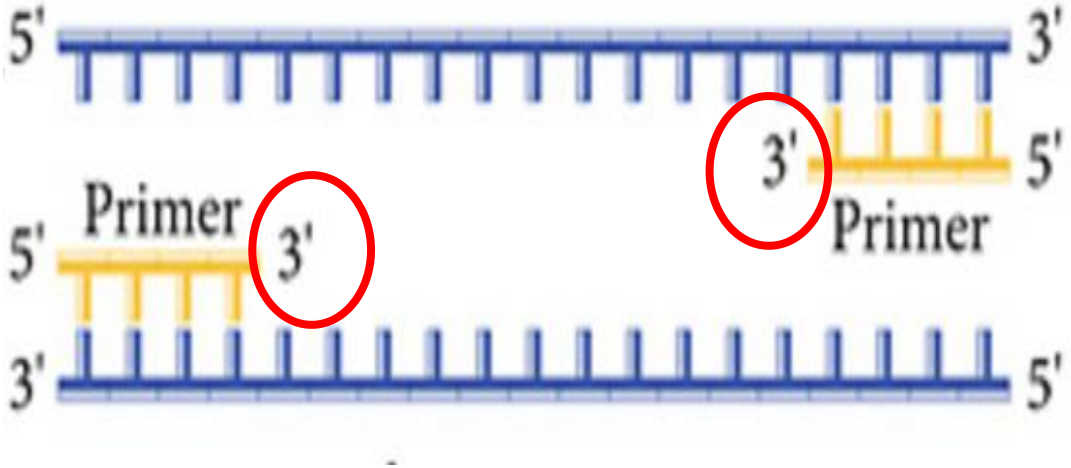
Amplification of a Specific Target Sequence:

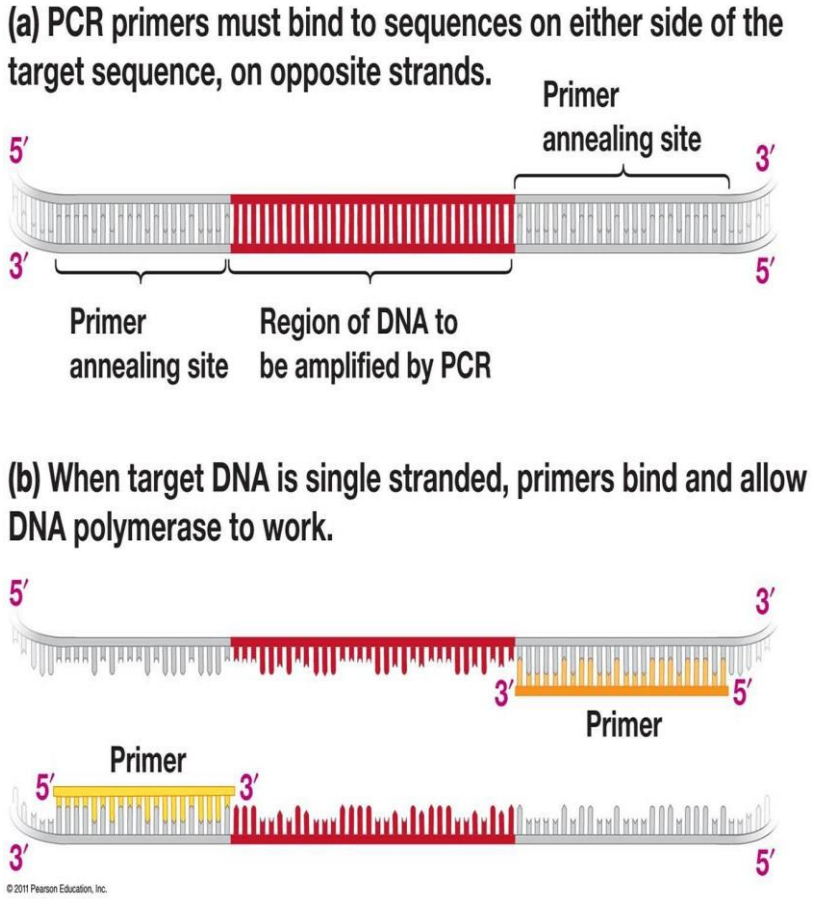
- PCR **does not copy all of the DNA** in the sample. It copies only a very specific sequence of genetic code from a template DNA, targeted by **PCR primers**.

- It does require the knowledge of some DNA sequence information which flanks the fragment of DNA to be amplified (**target DNA**).

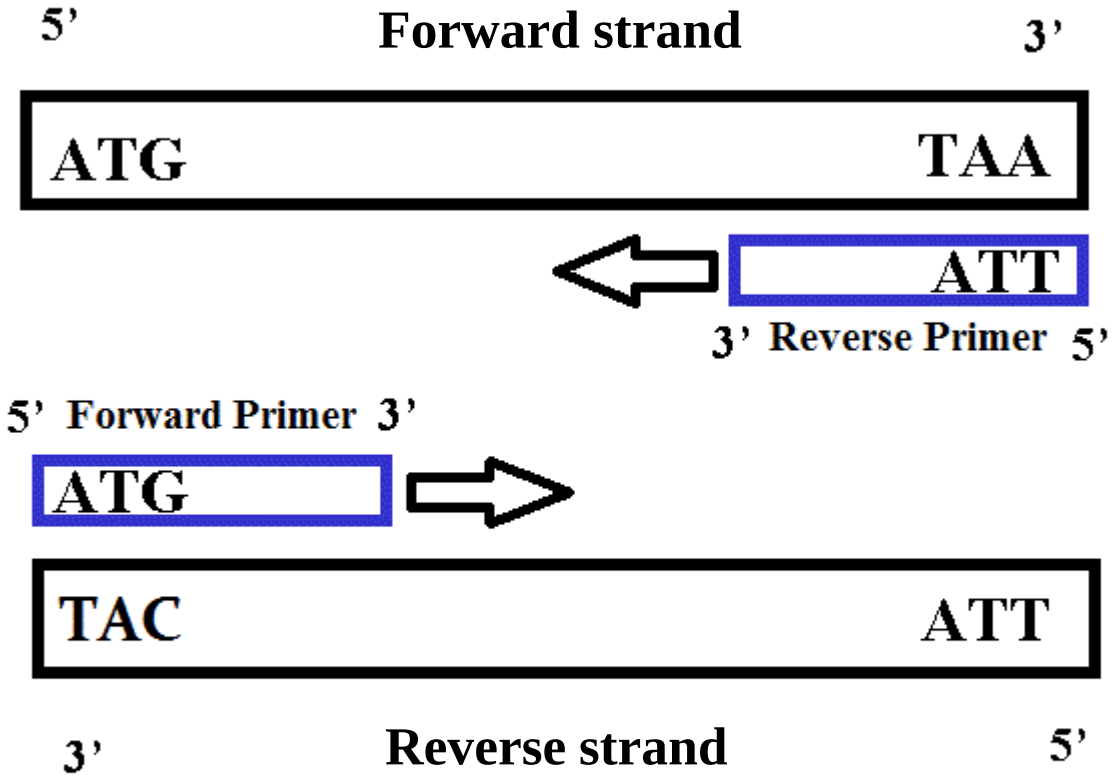


- From this information two synthetic oligonucleotide primers may be chemically synthesised each complementary to a stretch of DNA to the 3' side of the target DNA, one oligonucleotide for each of the two DNA strands (DNA polymerase can add a nucleotide only onto a **preexisting 3'-OH group**).





Why we need two primers ?



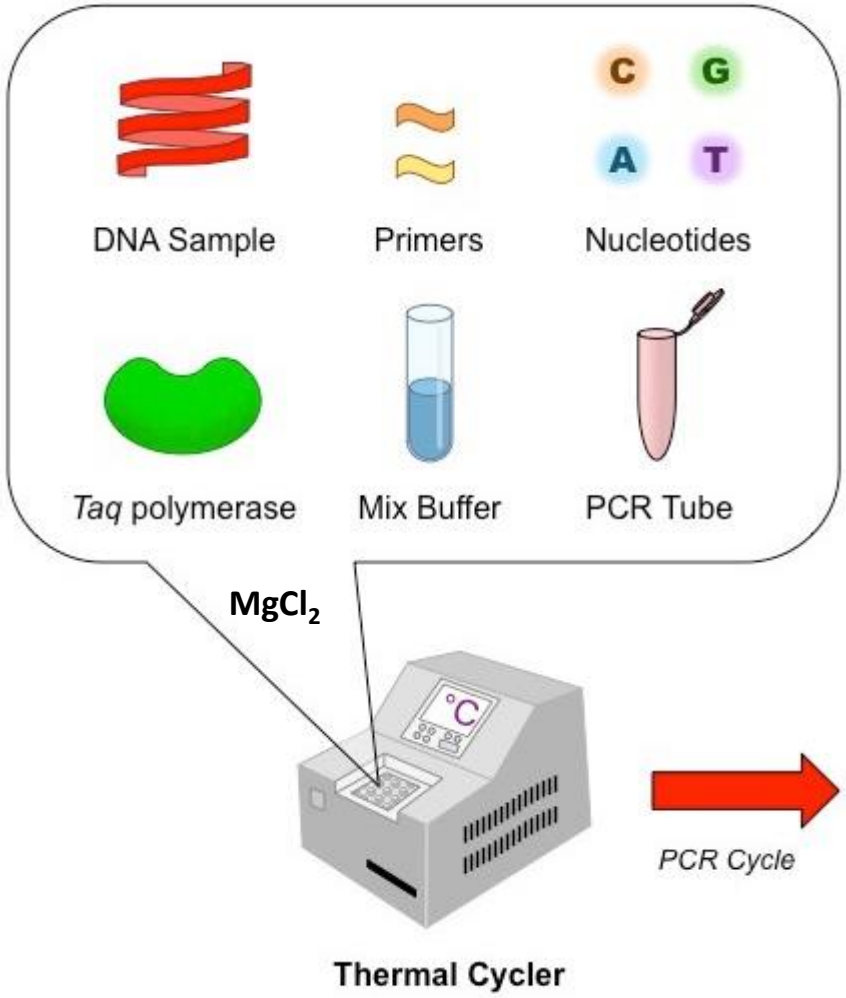
- In a PCR reaction you need **two primers** to amplify the target sequence:

➔**One called:** Forward primer, which have the same sequence of forward DNA strand and

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

*If there is only one primer, only one strand of the double stranded DNA will be amplified in the PCR reaction.

Components of PCR



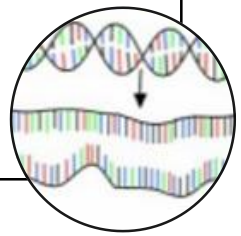
Additional reagents may included

The PCR Cycle :

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

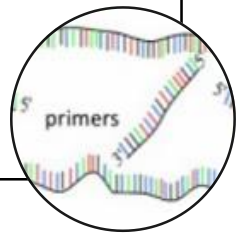
- The double-stranded template DNA is denatured by heating, typically to 95°C, to separate the double stranded DNA.

Denaturation:
(95⁰C)



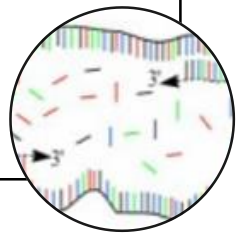
- The reaction is rapidly cooled to an annealing temperature to allow the oligonucleotide primers to hybridize to the template.

****Annealing**:
(50-65⁰C)



- The reaction is heated to a temperature, typically 72°C for efficient DNA synthesis by the thermostable DNA polymerase.

Extension:
(72⁰C)



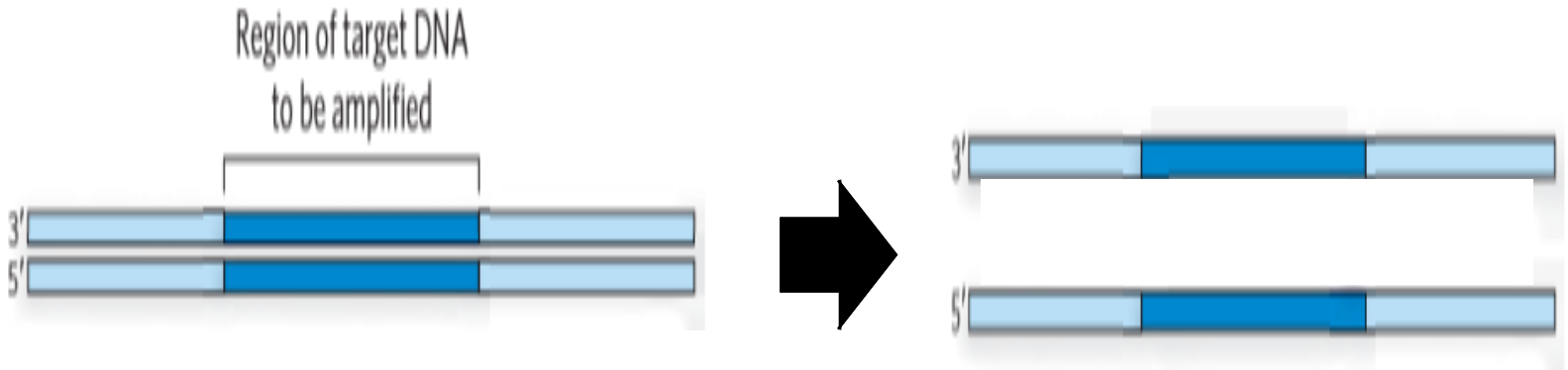
1. Denaturation:

- The double-stranded template DNA is denatured by heating, typically to **95°C**, to separate the double stranded DNA (why?).

- Breaking the _____ bonds.

Step 1

(94–97 °C)

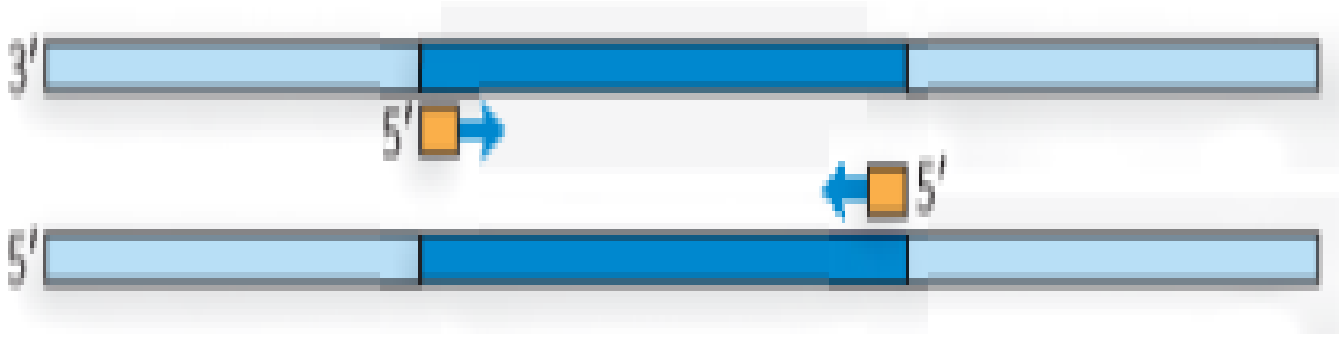


2. Annealing:

- The reaction is rapidly cooled to the primer annealing temperature (50-65 °C) to allow the oligonucleotide 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 **)**

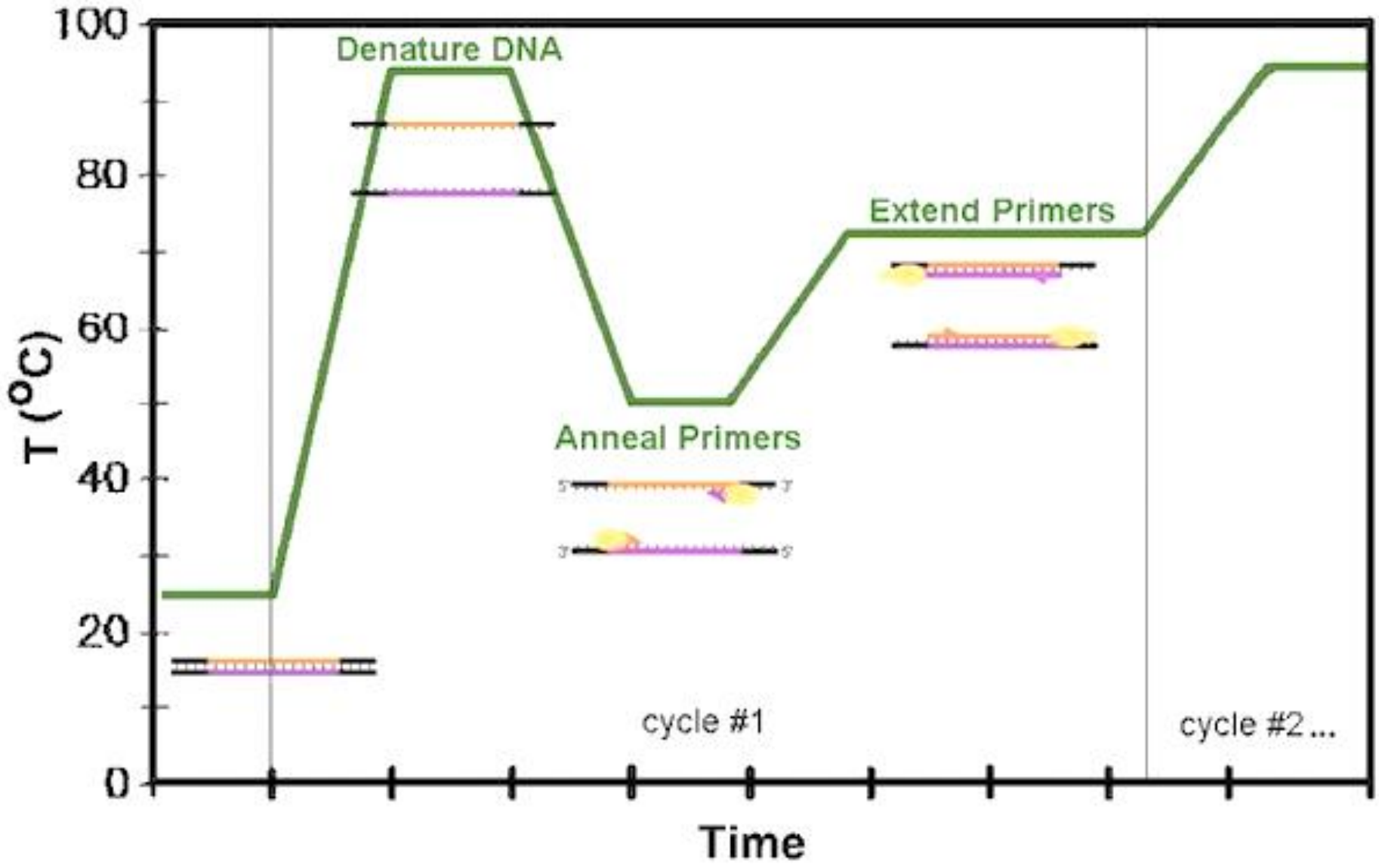


3. Extension:

- The reaction is heated to a temperature depends on the DNA polymerase used.
- **Commonly** a temperature of 72°C is used with this enzyme.
- This means that 72°C is the optimum …… of DNA polymerase.
- At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template

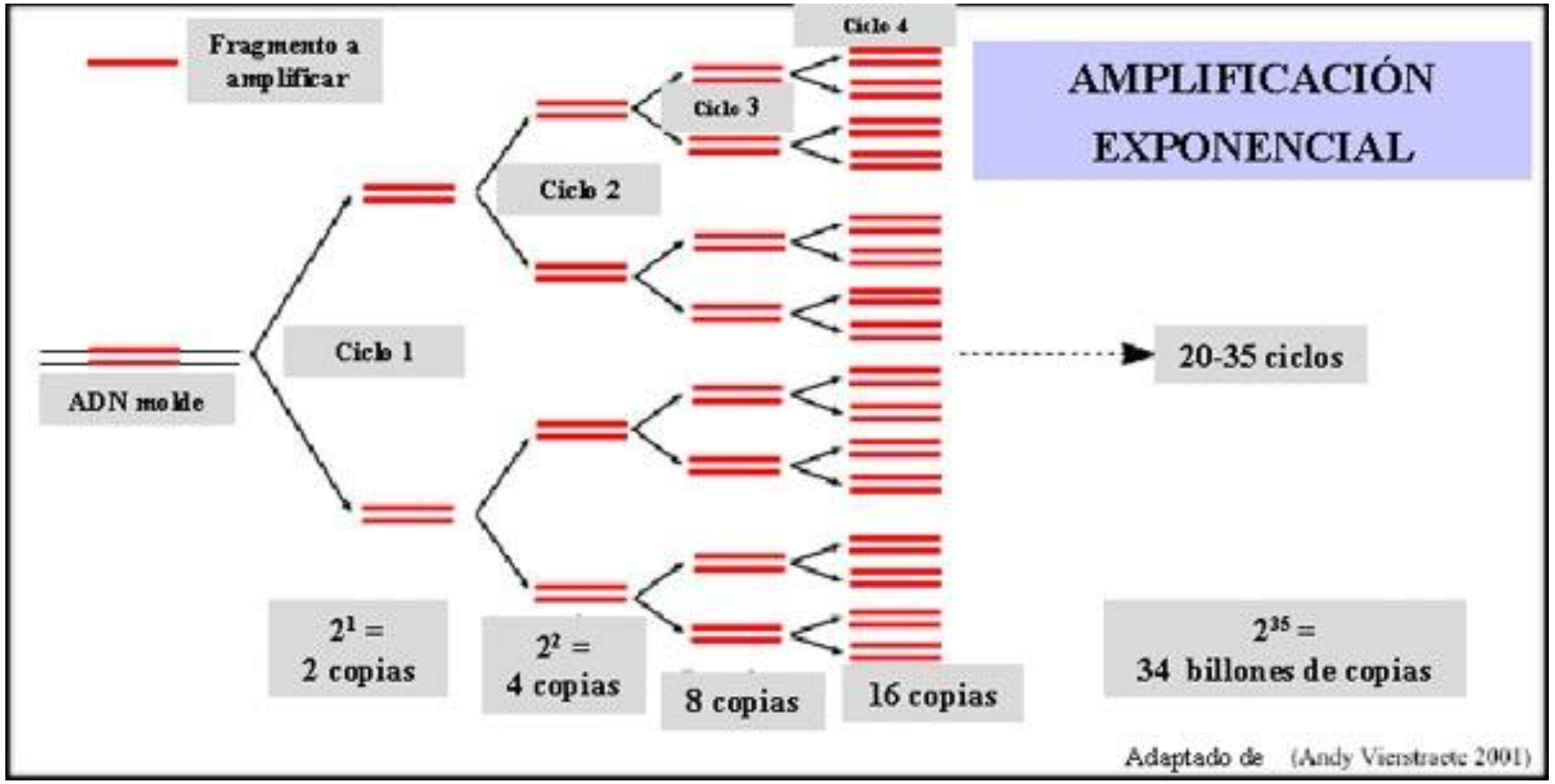
Step 3

(72 °C)

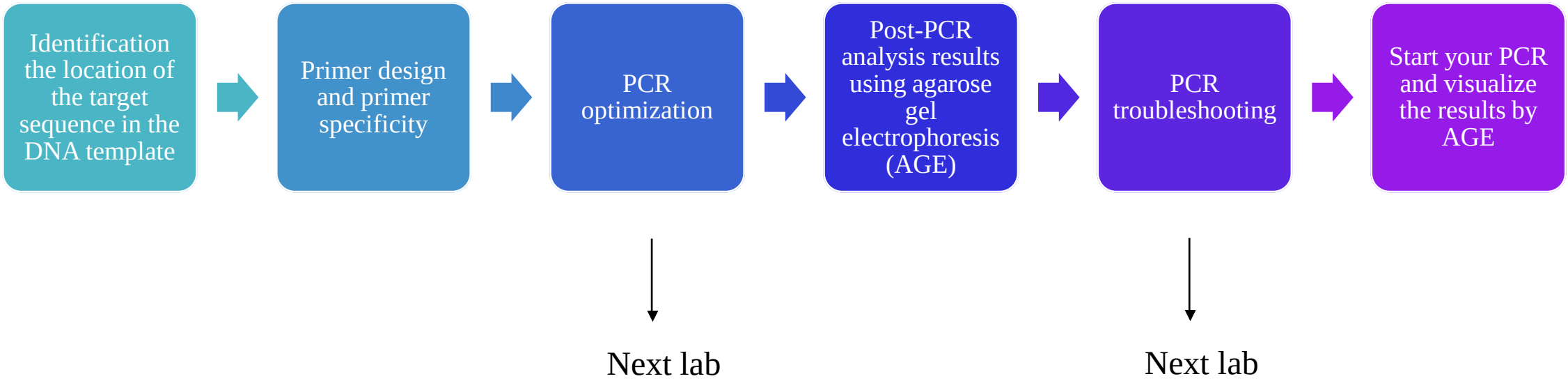


One cycle X 25

- 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.



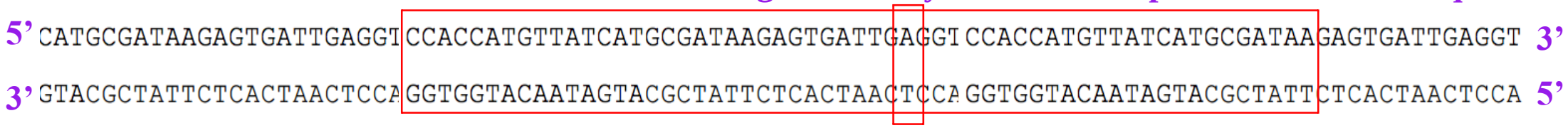
Performing PCR steps :



Example:

- You want to study a mutation in a DLG3 gene and how it relate to memory:
  1. Find the sequence of the gene from any website, eg.Ensebmle.
  2. Determine your **target region**.

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

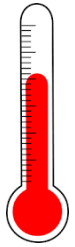


  3. Design the primers using primer design tool, eg.Primer3, then send them to any company who will synthesize them.
  4. Make sure that the area that you want to study is **between the primers (**the region to be studied should be between the forward and reverse primer**)**.
  5. Check primer specificity by BLAST.
  6. Optimize your PCR and trouble shooting.
  7. Start PCR.

Start your PCR !

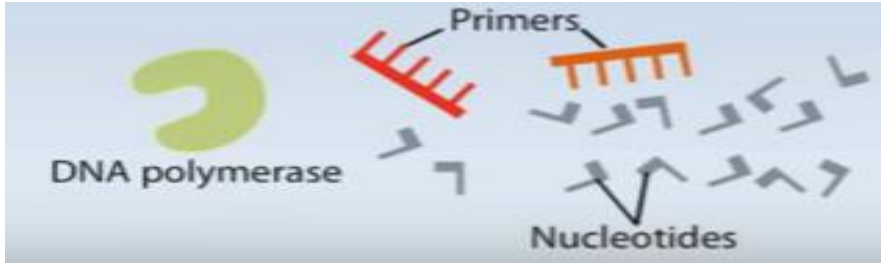
1. Denaturation:

95 °C



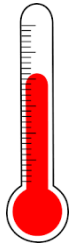
5’ CATGCGATAAGAGTGATTGAGGT CCACCATGTTATCATGCGATAAGAGTGATTGAGGT CCACCATGTTATCATGCGATAAGAGTGATTGAGGT 3’

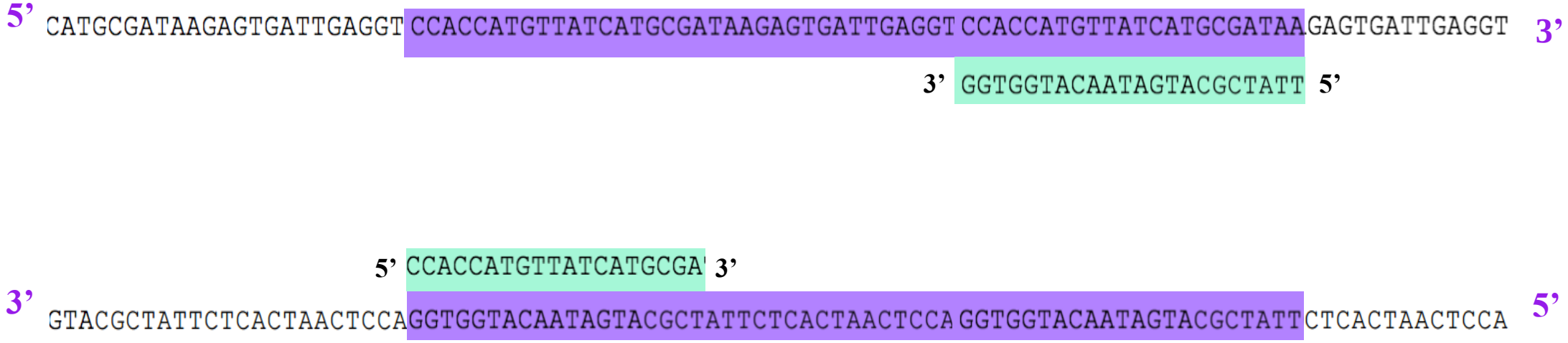
3’ GTACGCTATTCTCACTAACTCCAGGTGGTACAATAGTACGCTATTCTCACTAACTCCAGGTGGTACAATAGTACGCTATTCTCACTAACTCCA 5’



2. Annealing:

58 °C



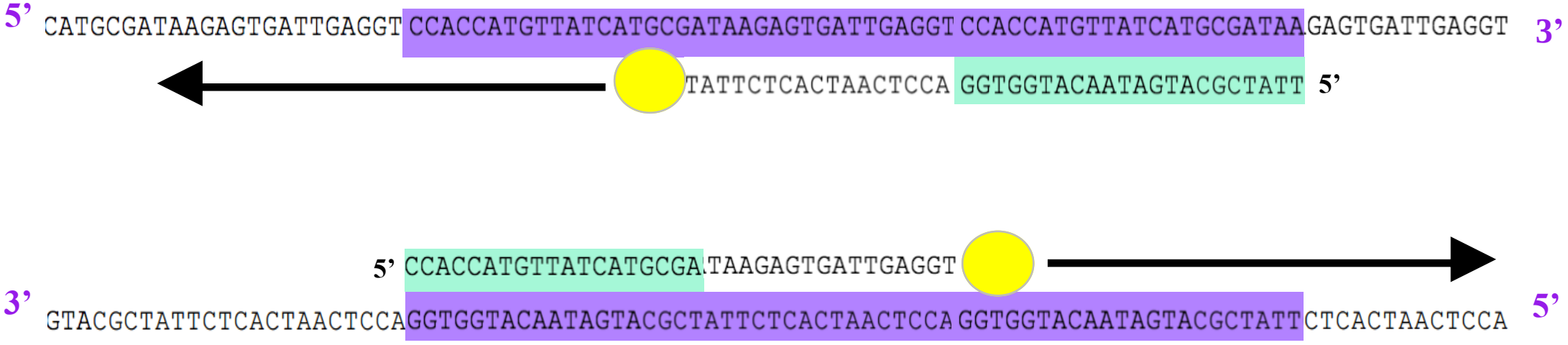


Forward primer: 5’ CCACCATGTTATCATGCGA 3’

Reverse primer: 3’ GGTGGTACAATAGTACGCTATT 5’

3. Extension:

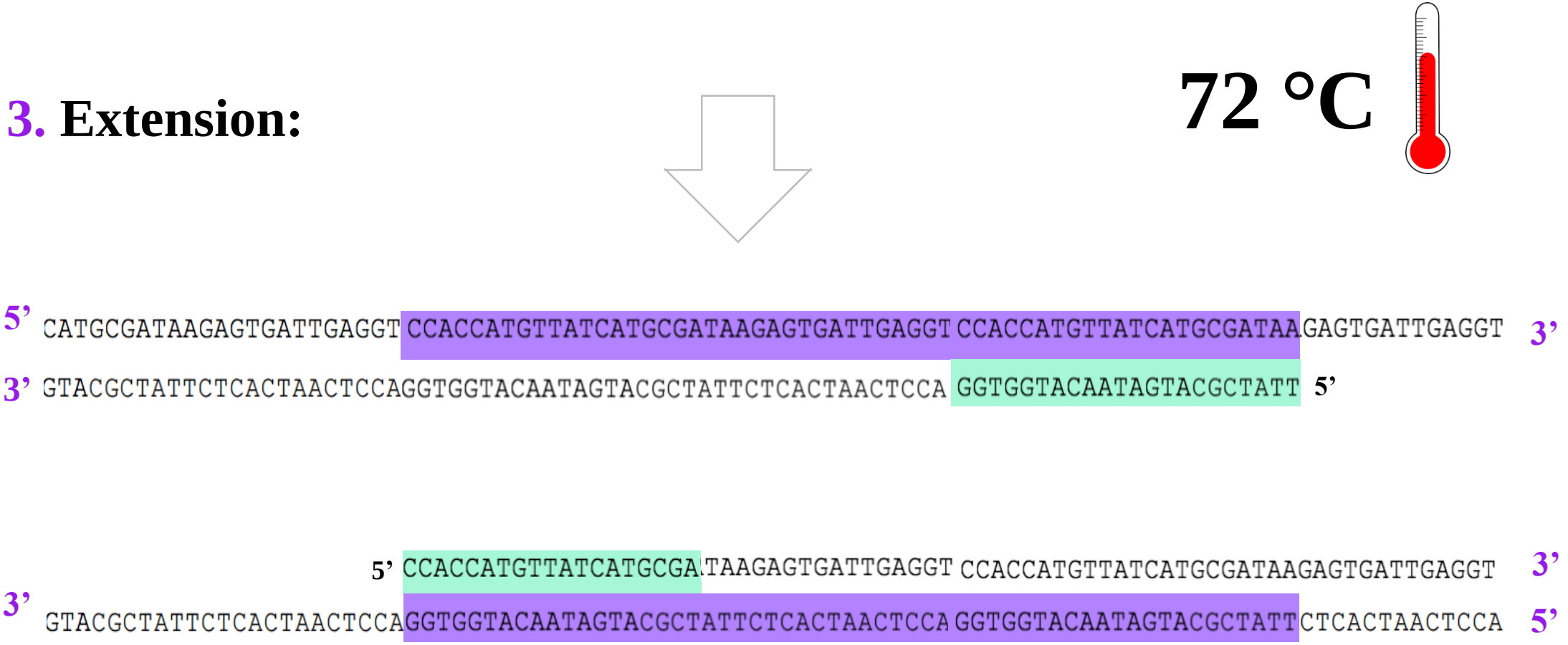
72 °C



Taq DNA polymerase

3. Extension:

72 °C

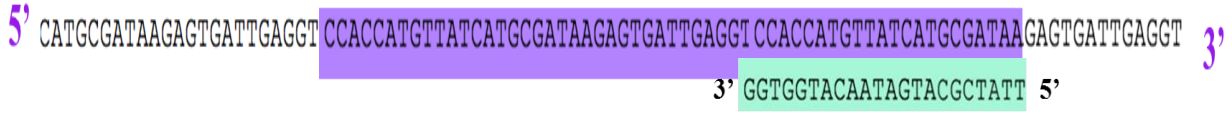


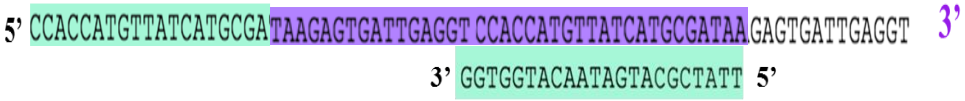
Cycle # 1:
1 DNA amplified to 2 DNA

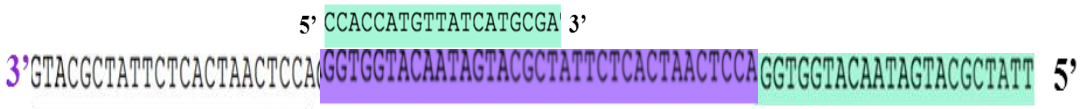
Cycle 2

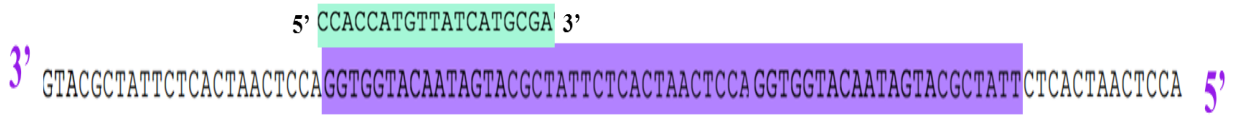
1. Denaturation

2. Annealing



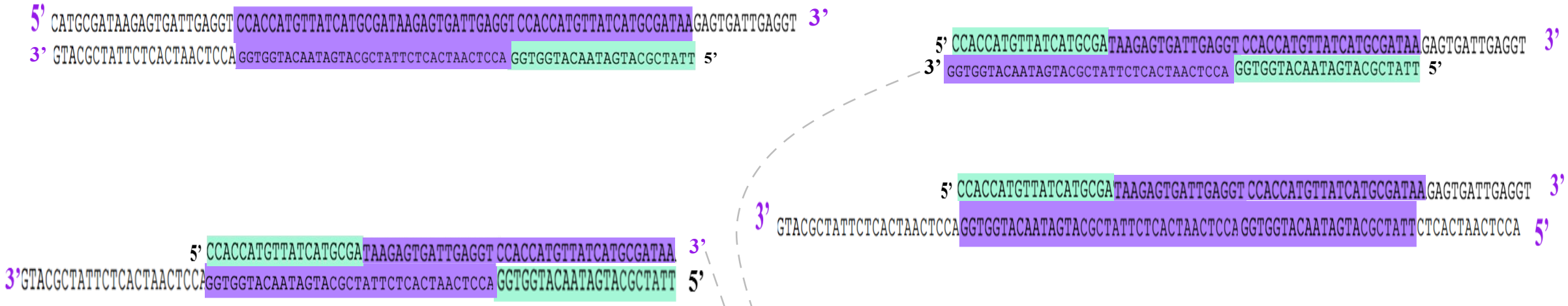




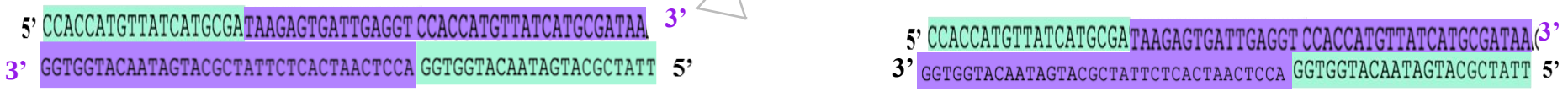


Cycle 2

3. Extension



Cycle 3



Target sequence

Appeared after three cycles and start to accumulate

After 30 cycles:

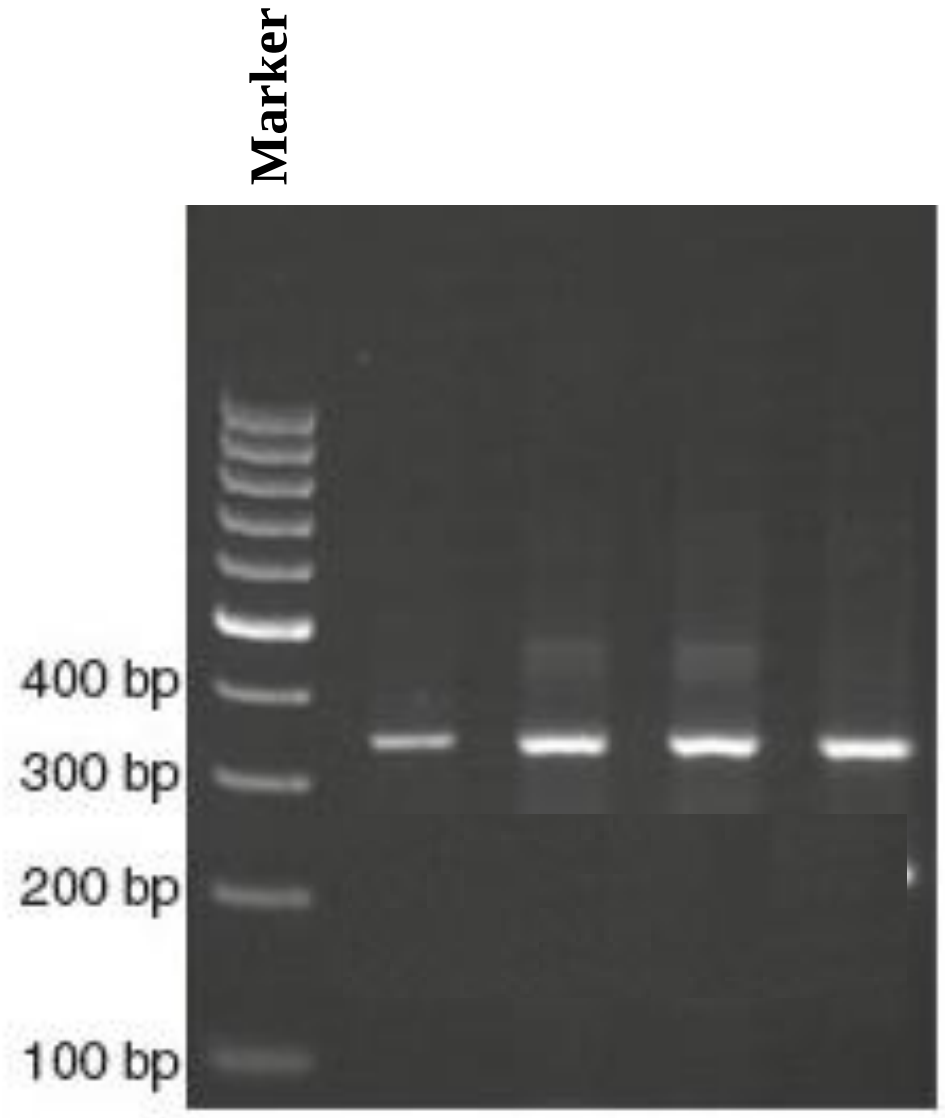
2^{30} copy of target DNA !!

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

It is very important to know your product size, why?

➔ **Our target sequence size is 350 bp**

AGE results



Target sequence

PCR advantages:

- Simplicity, easier methodology, sensitive, extensively validated standard operating procedure and availability of reagents and equipment

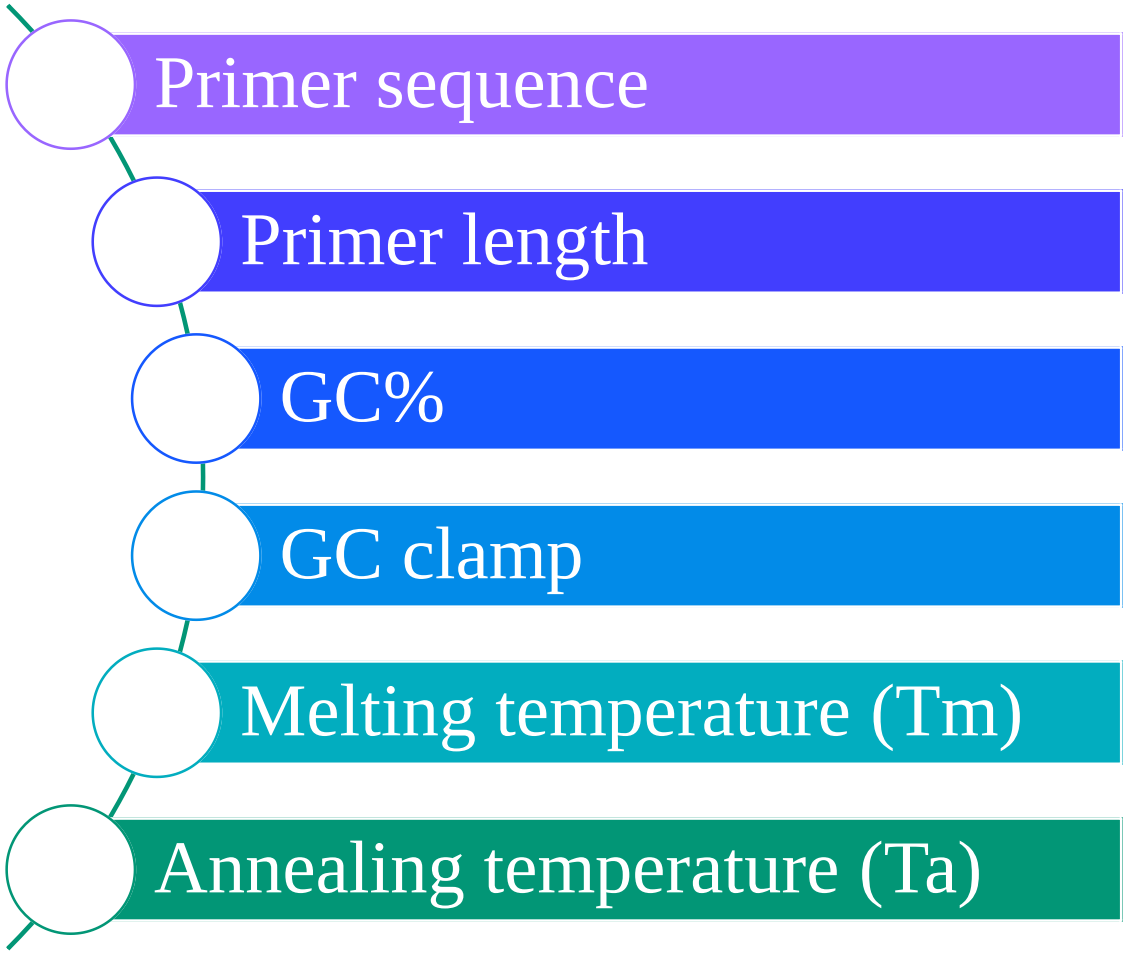
PCR application:

- Genotyping.
- RT-PCR.
- Cloning.
- Mutation detection.
- Sequencing.
- Microarrays.
- Forensics.
- Paternity testing.

PCR Optimization:

- There is no single set of conditions that is optimal for all PCR reactions.
- Next Lab.

Primer Design Guidelines:



Primer Design Guidelines:

1. Primer sequence:

- Must be complementary to flanking sequences of target region.
- Avoid:

➔Complementary sequences between primers.

➔Repeat (ex: ATATATAT) ➔ misprime.

➔Runs (ex: AGCGGGGGAT) ➔ misprime.

➔Mismatch at 3’ end.

➔Cross Homology.

2. Primer length:

- It is generally accepted that the optimal length of primers is **18-25 bp.**
- Not too long nor too short. (why?)

Primer Design Guidelines:

3. GC content:

- **GC% =** Number of G's and C's in the primer as a percentage of the total bases.
- Should be 40-60% (why?).

4. GC clamp:

- 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'

Primer Design Guidelines:

5. Melting temperature (Tm):

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

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

6. Annealing Temperature (Ta):

- 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 Ta ➔ produce insufficient primer-template hybridization.
- Too low Ta ➔ lead to non-specific products caused by a high number of base pair mismatches.

Now… you should be able to answer the following questions:

- How the amplification will be done?
- How you will determine your target sequence?
- How the amplification will be specific for certain segment?
- What are the requirements to carry PCR?

Home Work:

- Suppose you perform a PCR that begins with one double-strand of the following DNA template:

```
→5′-CTACCTGCGGGTTGACTGCTACCTTCCCGGGATGCCCAAAATTCTCGAG-3→
      ::::::::::::::::::::::::::::::::::::::::::::::::
←3′-GATGGACGCCCAACTGACGATGGAAGGGCCCTACGGGTTTTAAGAGCTC-5′←
```

A. Draw one cycle of PCR reaction below the following diagram.

B. Label the template DNA, the primers, and what is happening at each step.