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# Polymerase Chain Reaction (PCR)



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# INTRODUCTION

- Polymerase chain reaction (PCR) is a technique used in molecular biology to amplify a single copy or a few copies of a segment of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.



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# History

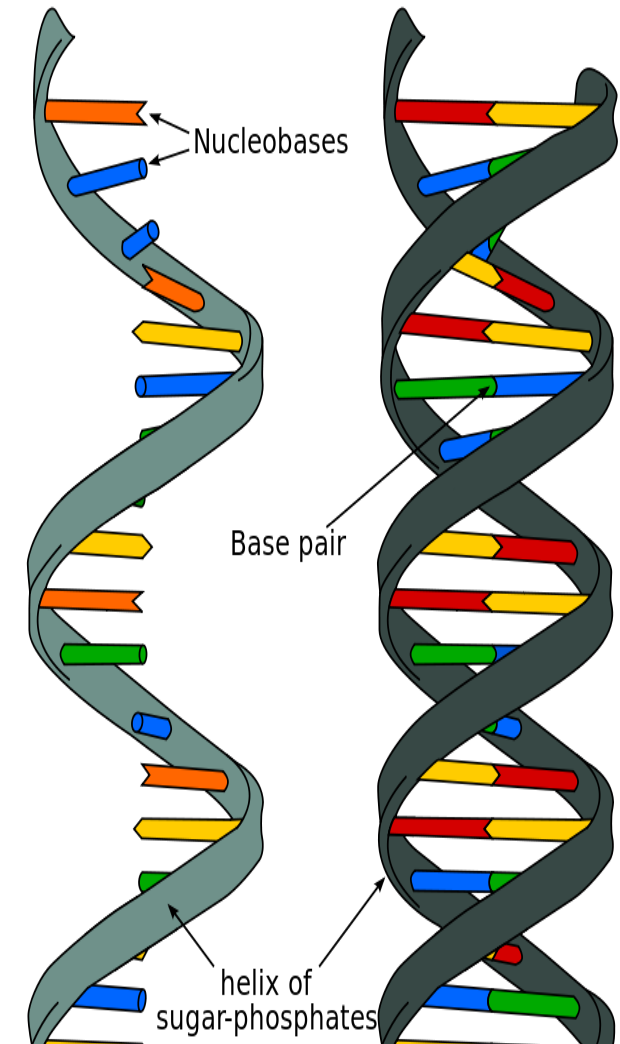
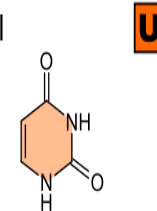
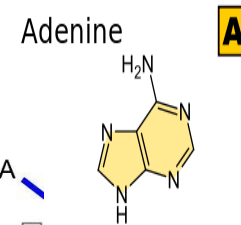
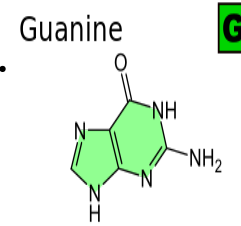
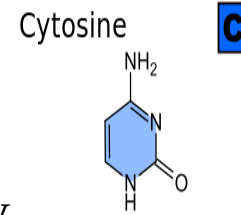
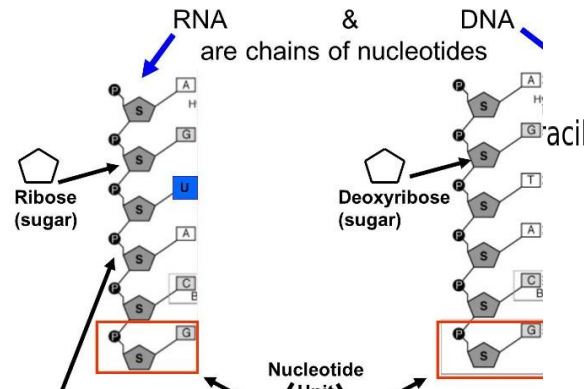
- Developed in 1983 by Kary Mullis, PCR is now a common technique used in clinical and research laboratories for a broad variety of applications.
- In 1993, Mullis was awarded the Nobel Prize in Chemistry for his work on PCR.



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# DNA

- DNA is a nucleic acid that is composed of two complementary nucleotide building block chains.
- The nucleotides are made up of a phosphate group, a five carbon sugar, and a nitrogen base.





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# DNA

- DNA has four nitrogen bases.
- **Two are purines** ( 2 ringed base )
  - Adenine ( **A** ), Guanine ( **G** )
- **Two are pyrimidines** ( 1 ringed base )
  - Cytosine ( **C** ), Thymine ( **T** )



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# DNA

- These four bases are linked in a repeated pattern by hydrogen bonding between the nitrogen bases.
- The linking of the two complementary strands is called **hybridization**.



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# DNA

- Example of bonding pattern.

Primary strand

**CCGAATGGGATGC**

**GGCTTACCCTACG**

Complementary strand



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# DNA

- A purine always links with a pyrimidine base to maintain the structure of DNA.
- Adenine ( A ) binds to Thymine ( T ), with two hydrogen bonds between them.
- Guanine ( G ) binds to Cytosine ( C ), with three hydrogen bonds between them.





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# What does PCR need?

1. Template (the DNA you are exploring)
2. Sequence-specific primers flanking the target sequence, Forward & Reverse.
3. Polymerases
4. Nucleotides (dATP, dCTP, dGTP, dTTP) }
  - Deoxyadenosine triphosphate
  - Deoxycytidine triphosphate
  - Deoxyguanosine triphosphate
  - Thymidine triphosphate
5. Magnesium chloride (enzyme cofactor)
6. Buffer
7. Water, mineral oil



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# Components for PCR

## 1. DNA template:

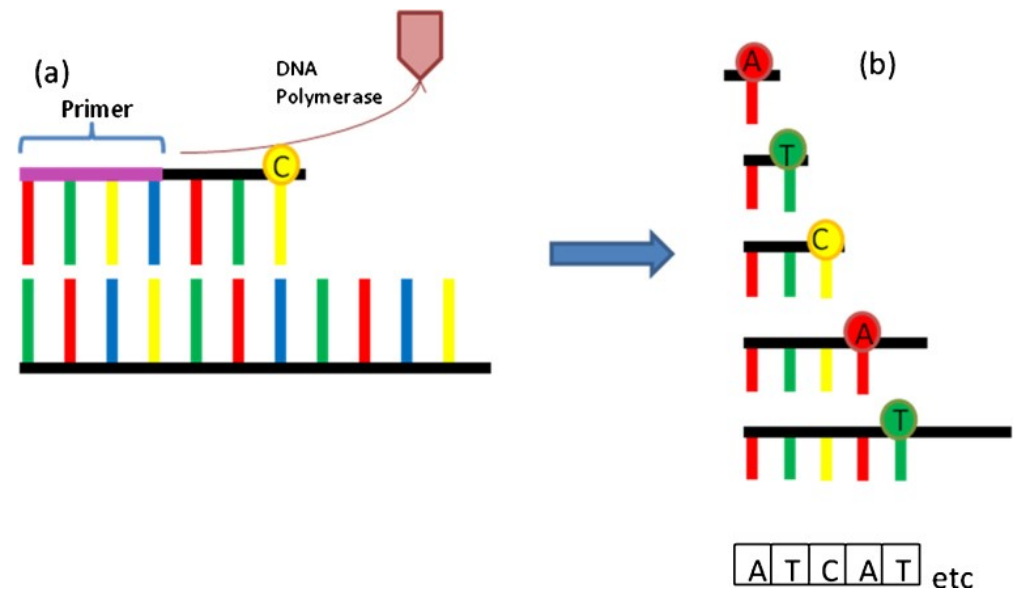
- DNA template is DNA target sequence.
- DNA template is the DNA molecule that contains the DNA region (segment) to be amplified, the segment we are concerned which is the target sequence.



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## 2. Primers:

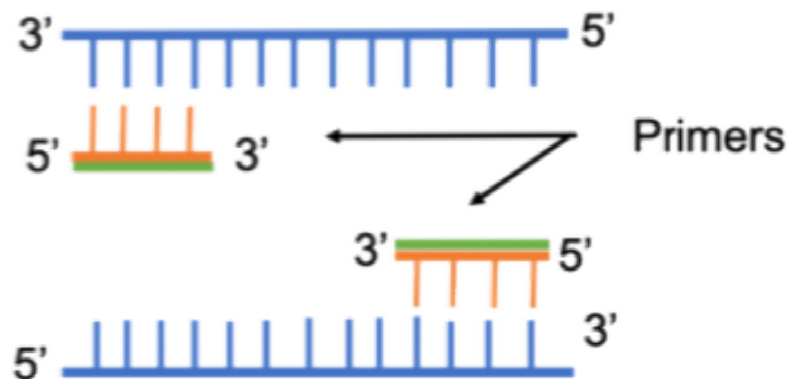
- Primers are synthetic DNA strands of about 18 to 25 nucleotides complementary to 3' end of the template strand.
- DNA polymerase starts synthesizing new DNA from the 3' end of the primer .





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- Two primers must be designed for PCR; the forward primer and the reverse primer. The forward primer is complementary to the 3' end of antisense strand (3'-5') and the reverse primer is complementary to the 3' end of sense strand (5'-3').
- If we consider the sense strand (5'-3') of a gene, for designing primers, then forward primer is the beginning of the gene and the reverse primer is the reverse-complement of the 3' end of the gene.





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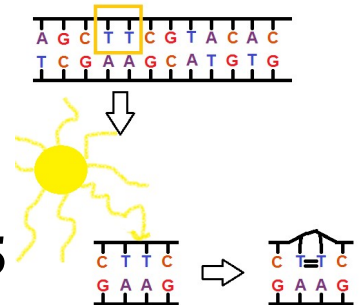
## Primer Problems

- Primers should flank the sequence of interest
- Primers that match multiple sequences will give multiple products
- Repeated sequences can be amplified -but only if unique flanking regions can be found where primers can bind
- A primer may form a dimer with itself or with the other primer.

• 5'-ACCGGTAGCCACGAATTCGT-3'

|||||

3'-TGCTTAAGCACCGATGGCCA-5





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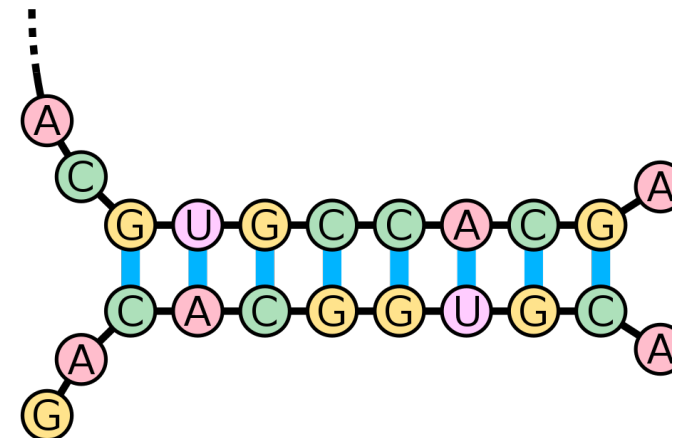
## Primers That Form Hairpins

- Primers can have self-annealing regions within each primer (i.e. hairpin and fold back loops)
- A primer may be self-complementary and be able to fold into a hairpin:

5'-GTTGACTTGATA

|||||T

3'-GAACTCT



- The 3' end of the primer is base-paired, preventing it annealing to the target DNA.



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## **Designing PCR Primers:**

- Primer sequences should be unique
- Primers should be ~20 bases long.
- The G/C content should be 45–55%.
- The annealing temperatures should be within 1°C of one another.
- The 3'-most base should be a G or C.
- The primers must not base pair with each other or with themselves or form hairpins.
- Primers must avoid repetitive DNA regions.



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### 3. DNA polymerase:

- DNA polymerase sequentially adds nucleotides complimentary to template strand at 3'-OH of the bound primers and synthesizes new strands of DNA complementary to the target sequence.
- The most commonly used DNA polymerase is *Taq* DNA polymerase (from *Thermus aquaticus*, a thermophilic bacterium) found in 176°F hot springs in Yellow Stone National Forest.
- The optimum temperature for *Taq* Polymerase is 72°C





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- Another enzyme *Pfu* DNA polymerase (from *Pyrococcus furiosus*) is also used widely because of its higher fidelity (accuracy of adding complimentary nucleotide).
- $Mg^{2+}$  ions in the buffer act as co-factor for DNA polymerase enzyme and hence are required for the reaction.



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## Disadvantages of *Taq* Pol

- *Taq* Polymerase lacks 3' to 5' exonuclease proof reading activity, commonly present in other polymerases.
- *Taq* mis-incorporates 1 base in  $10^4$ .
- A 400 base pair target will contain an error in 33% of molecules after 20 cycles.
- Error distribution will be random.



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#### **4. Nucleotides (dNTPs or deoxynucleotide triphosphates):**

- All types of nucleotides are "building blocks" for new DNA strands and essential for reaction. It includes

**Adenine(A)**

**Guanine(G)**

**Cytosine(C)**

**Thymine(T)**

or

**Uracil(U).**



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## 5. Magnesium:

- Magnesium affects primer annealing and template denaturation, as well as enzyme activity. An excess of magnesium gives non-specific amplification products, while low magnesium yields lesser amount of desired product.



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## 6. Buffer:

• **PCR** is carried out in a **buffer** that provides a suitable chemical environment for activity of DNA polymerase. The **buffer** pH is usually between 8.0 and 9.5 and is often stabilized by Tris-HCl. For *Taq* DNA polymerase, a common component in the **buffer** is potassium ion ( $K^+$ ) from KCl, which promotes primer annealing.



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## 7. Water and Mineral oil:

- **Water** has been **used** to make up the final volume of the sample in polymerase chain reaction.
- The template DNA and the DNA polymerase should be added just before the start of the **PCR** reaction. When a **PCR** machine is **used** without a heated lid, 30 µl of **mineral oil** should be added to the mixture to prevent evaporation.



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# PCR Requirements

- Magnesium chloride: .5-2.5mM
- Buffer: pH 8.3-8.8
- dNTPs: 20-200 $\mu$ M
- Primers: 0.1-0.5 $\mu$ M
- DNA Polymerase: 1-2.5 units
- Target DNA: 1  $\mu$ g



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# Steps in PCR

- Denaturation 93 to 95°C 1min
- Annealing 50 to 55°C 45sec
- Elongation 70 to 75°C 1-2min





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# How does PCR work?

- Heat ( $94^{\circ}\text{C}$ ) to denature DNA strands
- Cool ( $54^{\circ}\text{C}$ ) to anneal primers to template
- Warm ( $72^{\circ}\text{C}$ ) to activate *Taq* Polymerase, which extends primers and replicates DNA
- Repeat multiple cycles



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## **1. Denaturation:**

- Denaturation is the first step in PCR, in which the DNA strands are separated by heating to 95°C.
- The Hydrogen bonds between the two strands breaks down and the two strands separates.



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## **2. Annealing:**

- Annealing is the process of allowing two sequences of DNA to form hydrogen bonds.
- The annealing of the target sequences and primers is done by cooling the DNA to 55°C.
- Time taken to anneal is 45 seconds.



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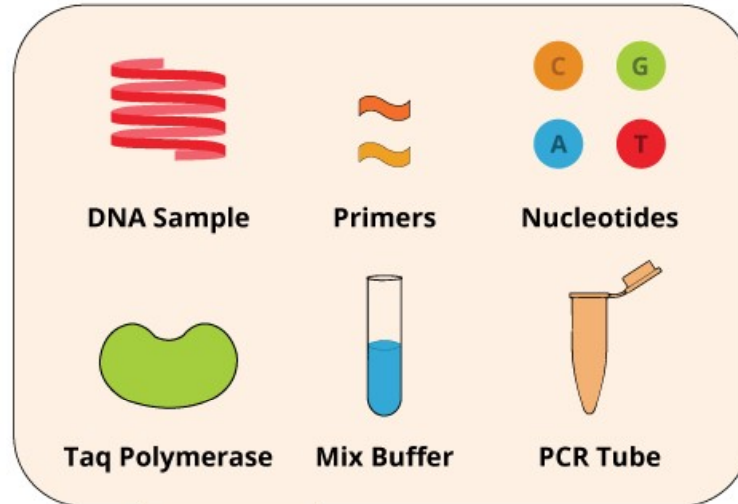
### **3. Elongation:**

- *Taq* polymerase binds to the template DNA and starts adding nucleotides that are complementary to the first strand.
- This happens at 72°C as it is the optimum temperature for *Taq* Polymerase.



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## PCR Components

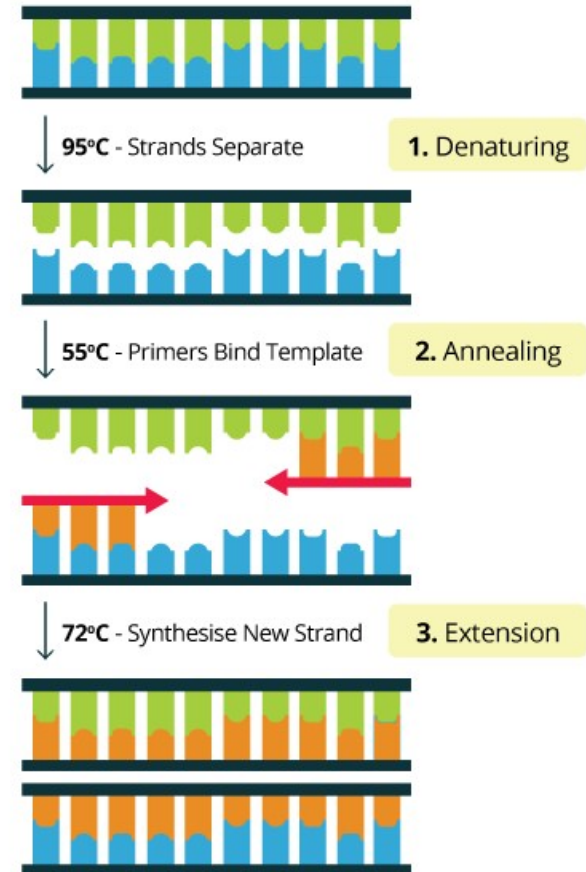


Thermal Cycler



PCR Cycle

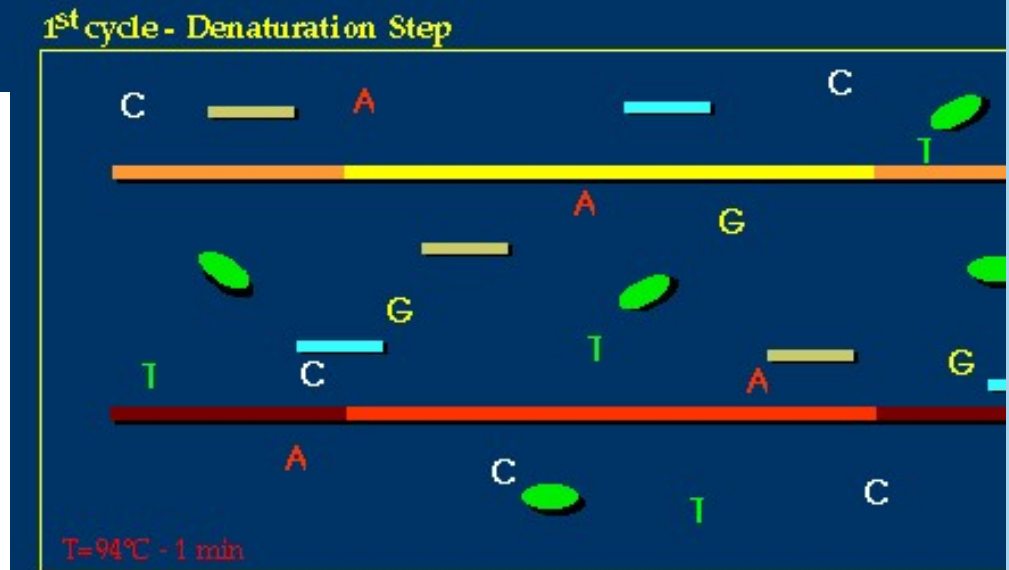
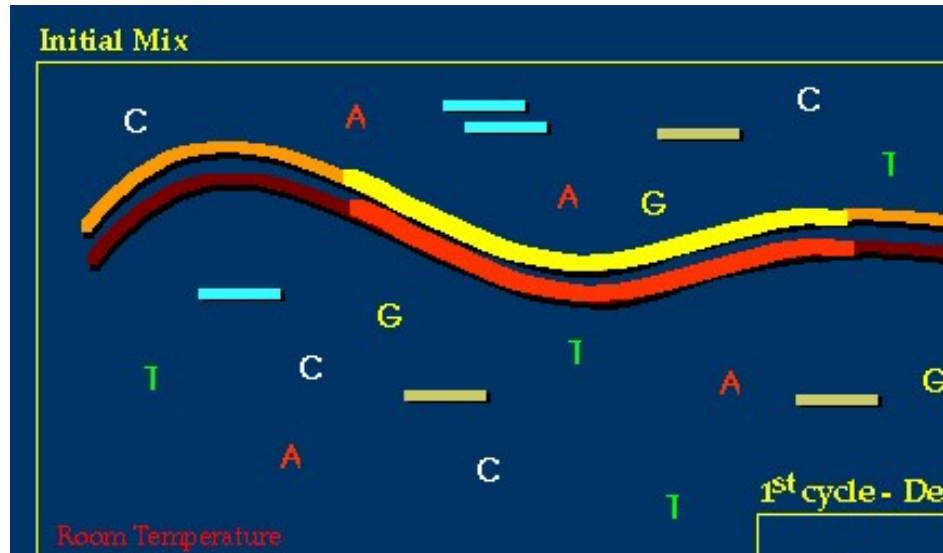
## PCR Process (One Cycle)





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# PCR Cycles

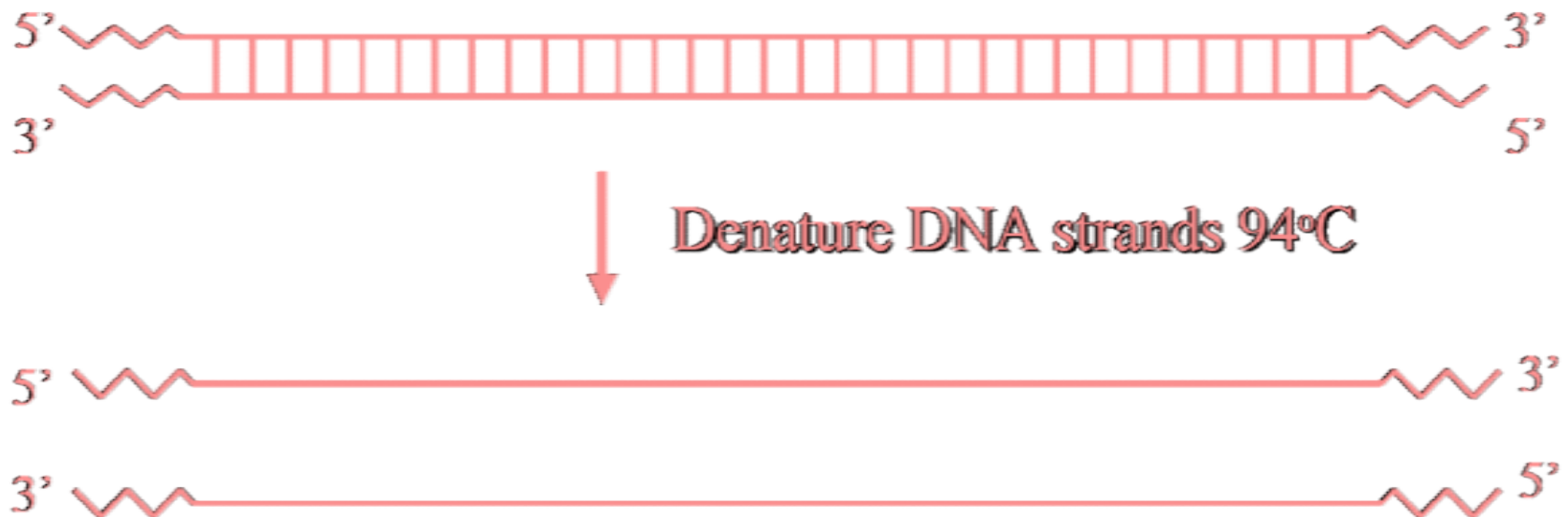
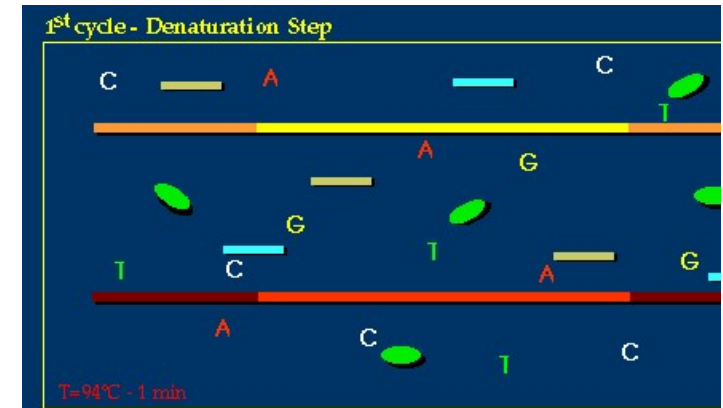




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# Denaturing Template

- Heat causes DNA strands to separate

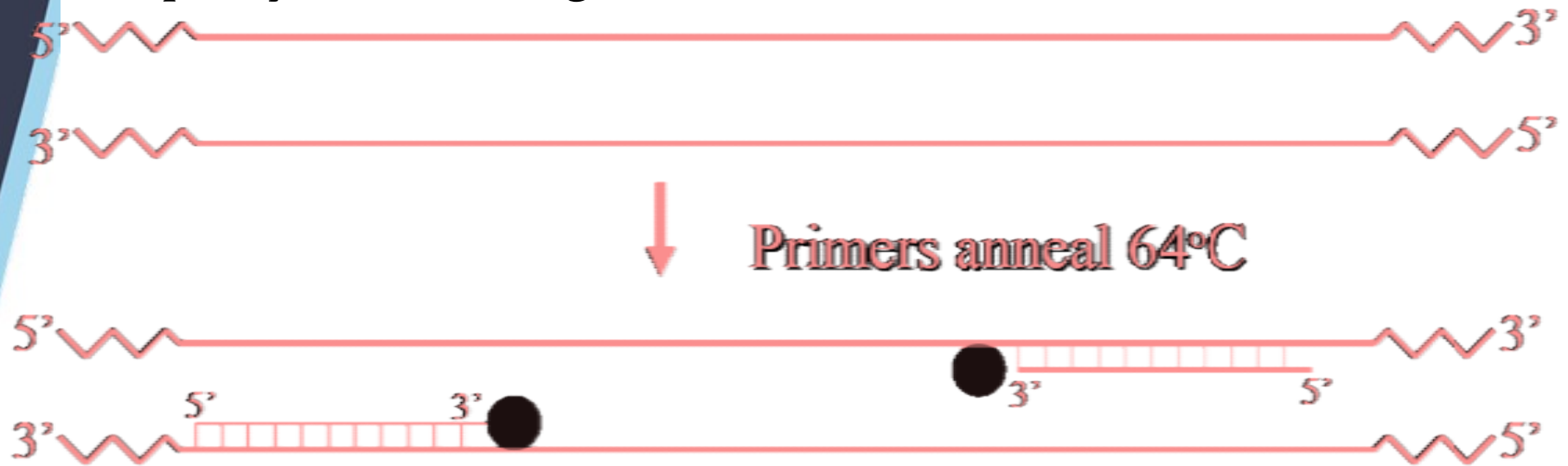
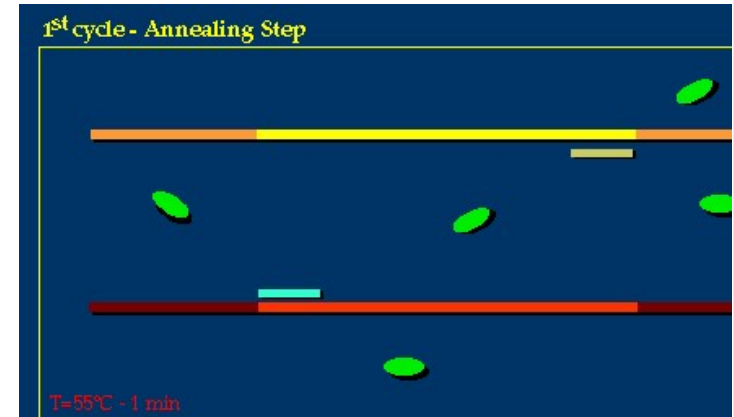




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# Annealing Primers

- Primers bind to the template sequence
- *Taq* Polymerase recognizes double-stranded substrate



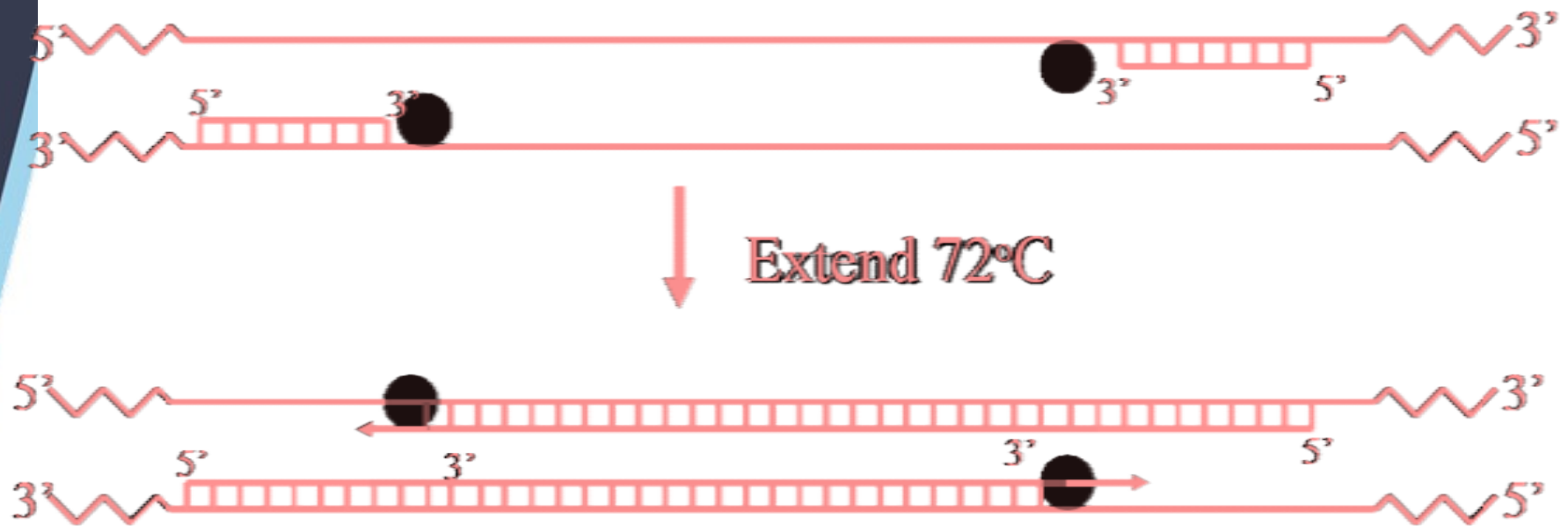
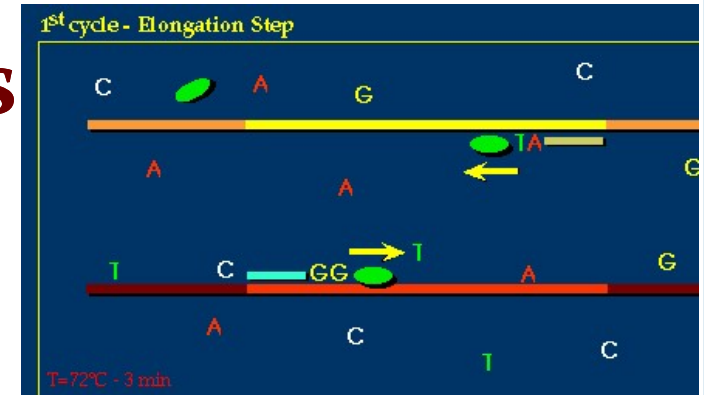




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# Taq Polymerase Extends

- Taq Polymerase extends primer
- DNA is replicated



Repeat denaturing, annealing, and extending 30 cycles



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# The target product is made in the third cycle

Cycle 1



Cycle 2



Cycle 3





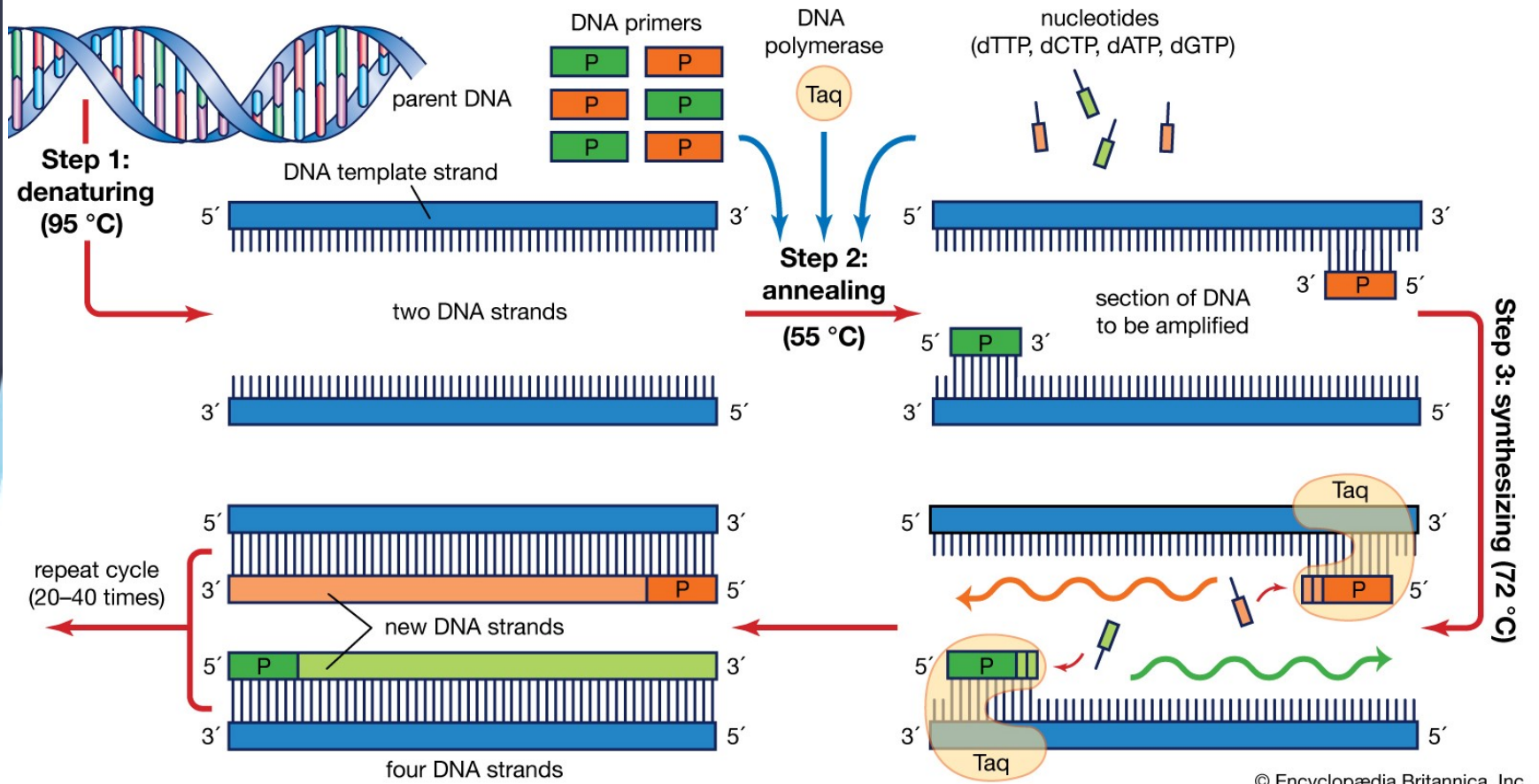
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# PCR Cycles Review

- **Denaturation:** 94°- 95°C
- **Primer Annealing:** 55°- 65°C
- **Elongation of DNA:** 72°
- **Number of Cycles:** 25-40
- No target products are made until the third cycle.
- At 30 cycles there are 1,073,741,764 target copies ( $\sim 1 \times 10^9$ ).



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# Limitations of PCR

- **Need for target DNA sequence information**

Primer Designing for unexplored ones.

Boundary regions of DNA to be amplified must be known.

- **Infidelity of DNA replication.**
- *Taq* Pol – no Proof reading match – Error 40% after 20 cycles
- **Short size and limiting amounts of PCR product**

Up to 5kb can be easily amplified .

Up to 40kb can be amplified with some modifications.

Cannot amplify gene >100kb

Cannot be used in genome sequencing projects.



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# How to overcome Difficulties?

- *Pfu* DNA Polymerase from *Pyrococcus furiosus* possesses 3' to 5' exonuclease proofreading activity.
- The error rate is only 3.5% after 20 cycles
- More amount of primer is added to avoid primer dimering.
- For unexplored genes, primers used in closely related species are used.



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# Advantages of PCR

- **Speed**
- **Ease of use**
- **Sensitivity**
- **Robustness**



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# Applications of PCR

- Screening human DNA samples for mutations associated with genetic diseases such as thalassemia and cystic fibrosis.
- Can detect the presence of viral DNA before it turns in to a killer.
- PCR enables rapid amplification of template DNA for screening of uncharacterized mutations
- Can obtain sequences from hair, blood stain, bones, other forensic specimens, other remains preserved at archaeological sites.





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# THANK YOU



**Happy to Answer if you have any question.....**