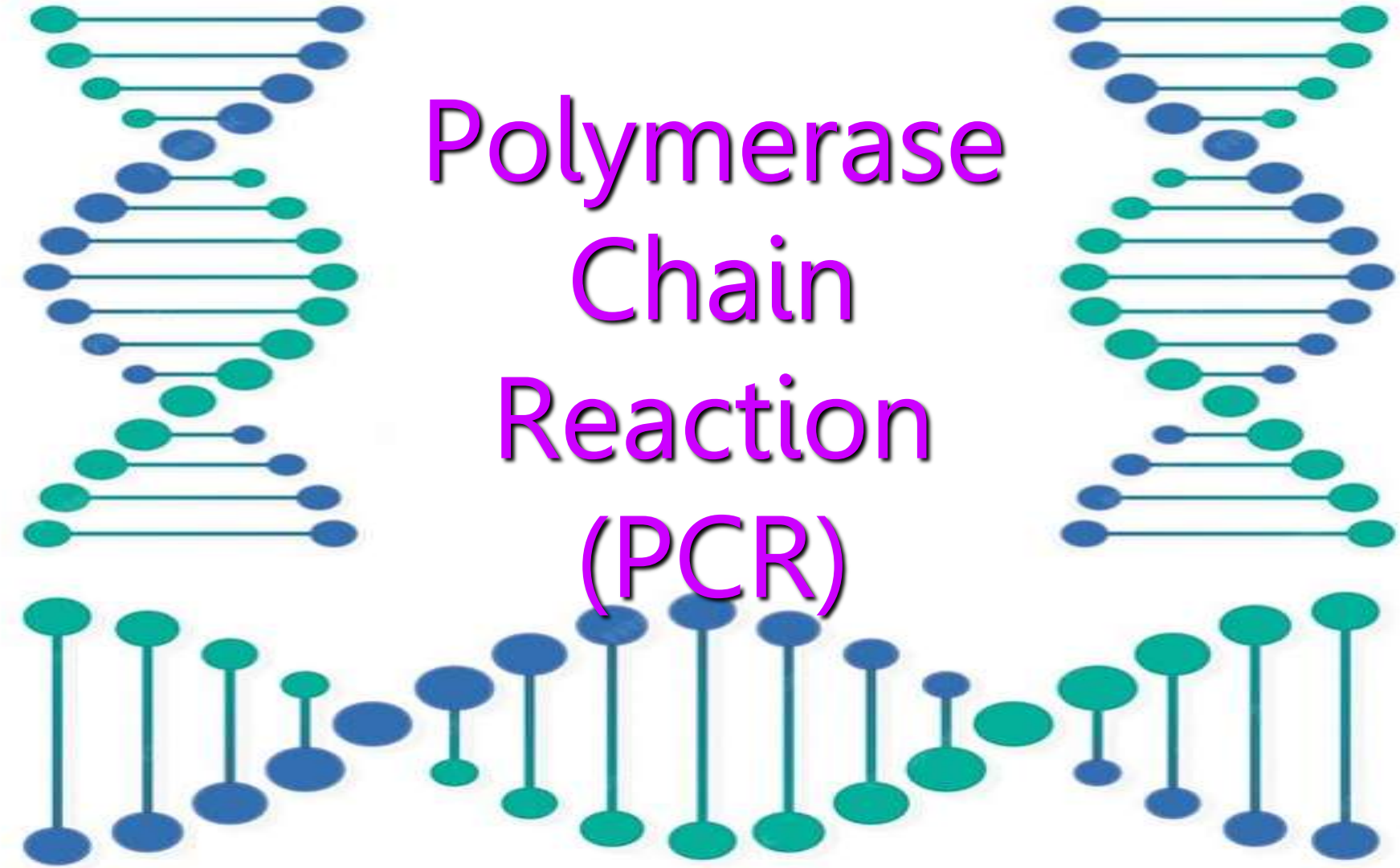
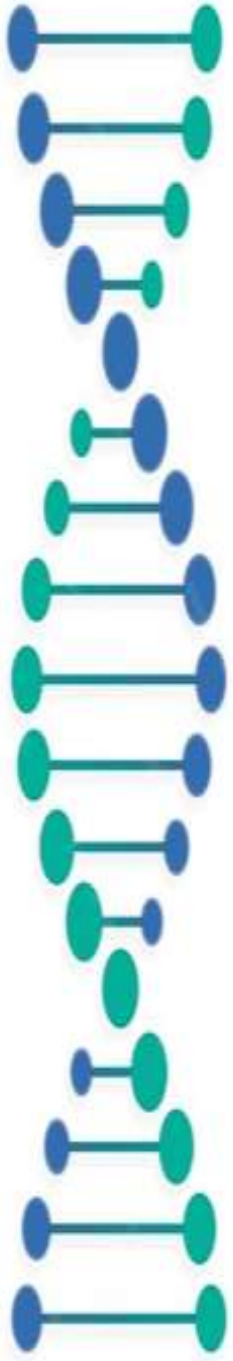
HOUSE-KEEPING GENES to read too!!



# Polymerase Chain Reaction (PCR)



- PCR
- History of PCR
- Thermal cycler
- Components of PCR
- Three basic steps
- PCR program in thermal cycler
- General guidelines for primer.
- Application of PCR
- Advantages and disadvantages of PCR

## What is PCR?

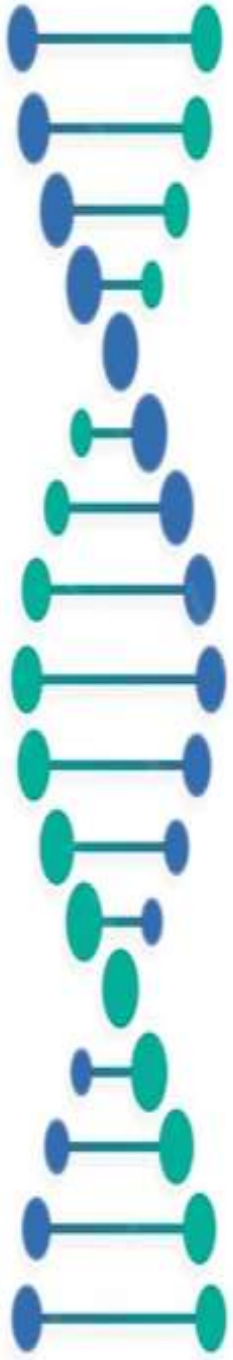
- PCR is an exponentially progressing synthesis of the defined target DNA sequences in vitro.

## Why “Polymerase”?

- It is called “polymerase” because the only enzyme used in this reaction is DNA polymerase.

## Why “Chain”?

- It is called “chain” because the products of the first reaction become substrates of the following one, and so on.



## The "Reaction" Components

1) Target DNA - contains the sequence to be amplified.

2) Pair of Primers - oligonucleotides that define the seq to be amplified. They are complementary to the 3' ends of each of the sense and anti-sense strand of the DNA target and needed to initiate DNA synthesis.

3) dNTPs - deoxynucleotidetriphosphates: DNA building

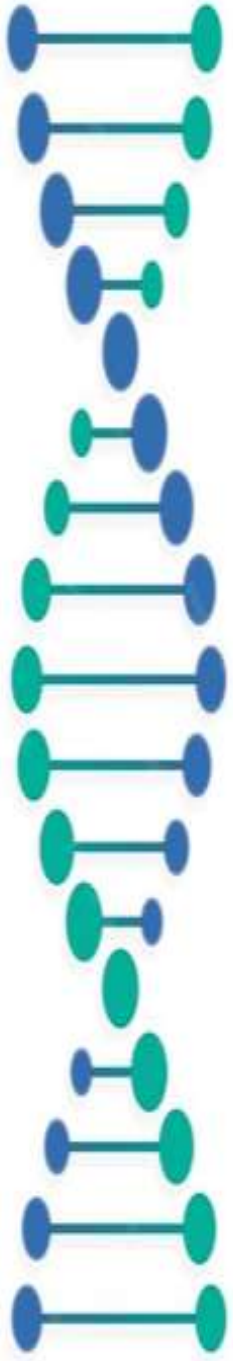
4) Thermostable DNA Polymerase - enzyme that catalyzes the reaction

5) Mg++ ions - cofactor of the enzyme

6) Buffer solution - maintains pH and ionic strength of the reaction solution suitable for the activity of the enzyme

PCR tube

THERMOCYCLER



## PCR (Polymerase chain reaction)

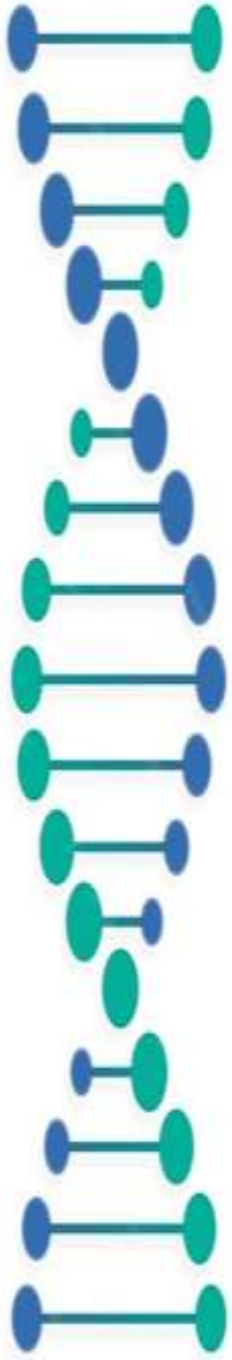
- A technique to make many copies of a specific DNA region in vitro.
- Primer mediated enzymatic amplification of specially cloned or genomic DNA sequences.
- Possible to generate thousands to millions of copies of a particular section of DNA from a very small amount of DNA.
- Common tool used in medical and biological research labs.

## Principle of PCR

- It is based on the enzymatic replication of DNA, were a short segment of DNA is amplified using primer mediated enzymes. DNA Polymerase synthesises new strands of DNA complementary to the template DNA. The DNA polymerase can add a nucleotide to the pre-existing 3'-OH group only. Therefore, a primer is required. Thus, more nucleotides are added to the 3' prime end of the DNA polymerase

## History of PCR

- Great mind behind this PCR : was an American biochemist Kary Banks Mullis
- Developed PCR in 1985 and was awarded the Nobel Prize in Chemistry in 1993 for his pioneering work.
- PCR machine otherwise called Thermocycler.
- -1983-Kary Mullis, a scientist working for the Cetus Corporation was driving along US Route 101 in northern California when he came up with the idea for the polymerase chain reaction.
- ln 1985 Cetus Corp. Scientists isolate Thermostable Taq Polymerase (from T. aquaticus), which revolutionized PCR & introduced to the scientific community at a conference in October .
- Cetus rewarded Kary Mullis with a $10,000 bonus for his invention.
- Later, during a corporate reorganization, Cetus sold the patent for the PCR process to a pharmaceutical company Hoffmann-LaRoche for $300 million.



## Three basic steps

### 1. Denaturation ds DNA template (96°C)

Heat the reaction strongly to separate, or denature, the DNA strands. This provides single-stranded template for the next step.

### 2. Annealing of primers (55-65°C)

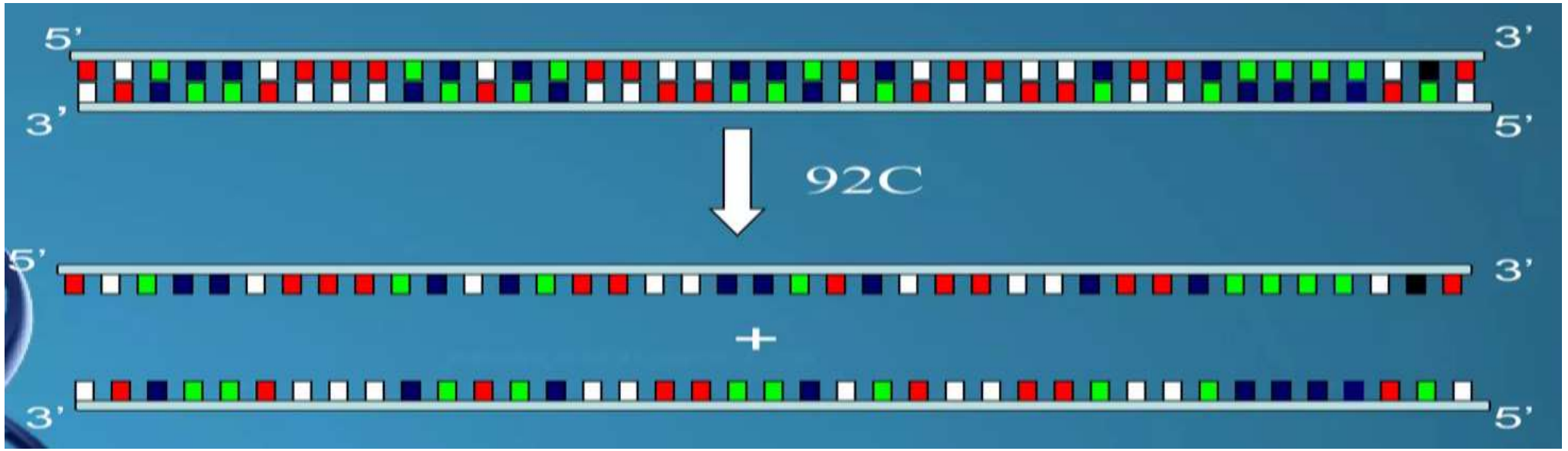
Cool the reaction so the primers can bind to their complementary sequences on the single-stranded template DNA.

### 3. Extension ds DNA molecules (72°C)

Raise the reaction temperatures so Taq polymerase extends the primers, synthesizing new strands of DNA.

## Denaturation

- The reaction mixture is heated to a temperature between 90-98º C so that the ds DNA is denatured into single strands by disrupting the hydrogen bonds between complementary bases.
- Duration of this step is 1-2 mins.
- Temperature: 92-94C.
- Double stranded DNA melts → single stranded DNA.

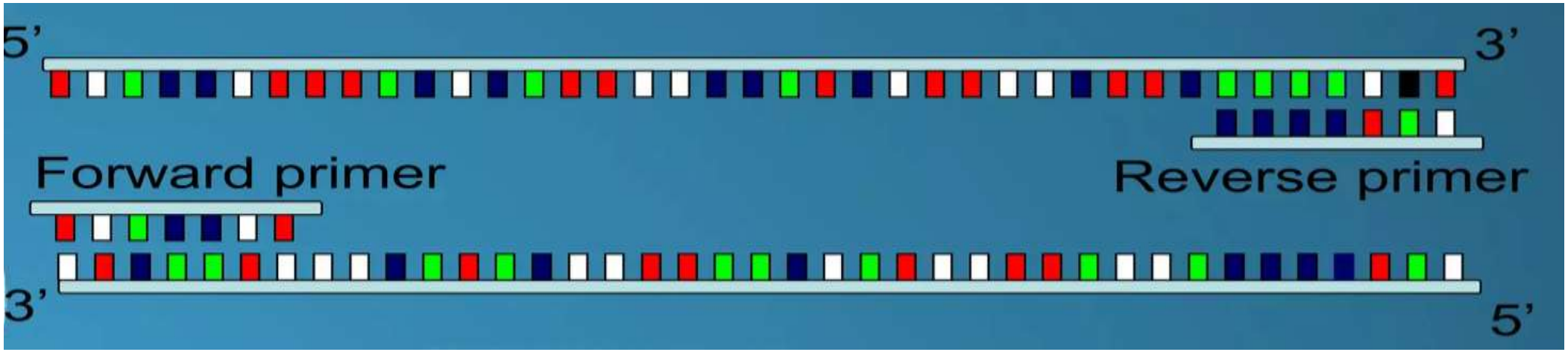


# Annealing

Temperature of reaction mixture is cooled to 45-60º C

- Primers are jiggling around caused by ???????
- Primers base pair with the complementary sequence in the DNA.
- Hydrogen bonds reform.
- Annealing fancy word for renaturing.

Temperature: ~45-70C (dependant on the melting temperature of the expected duplex).



# Extension

DNA polymerase binds to the annealed primers and extends DNA at the 3' end of the chain

The temperature is now shifted to 72º C which is ideal for polymerase.

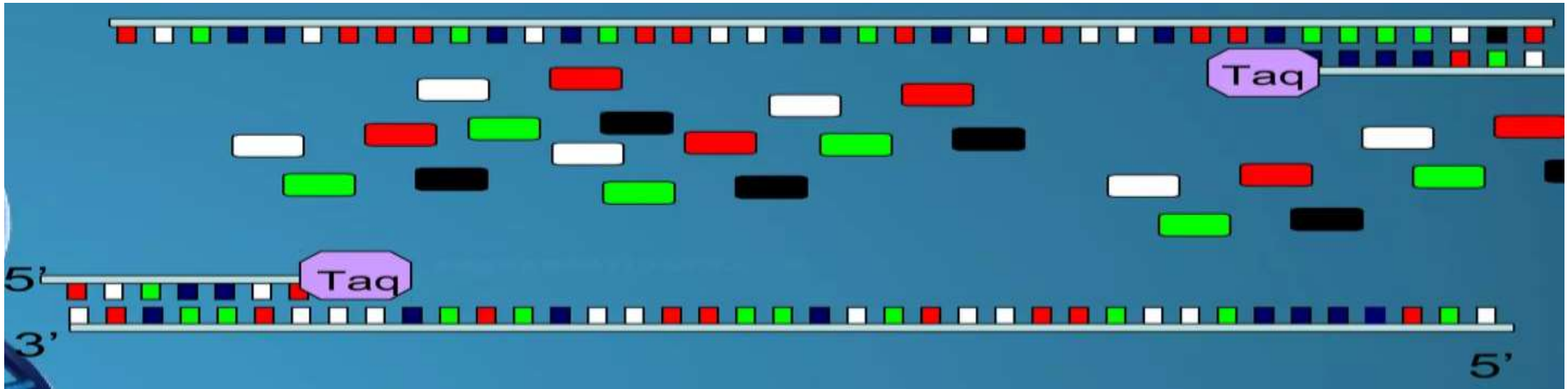
Primers are extended by joining the bases complementary to DNA strands.

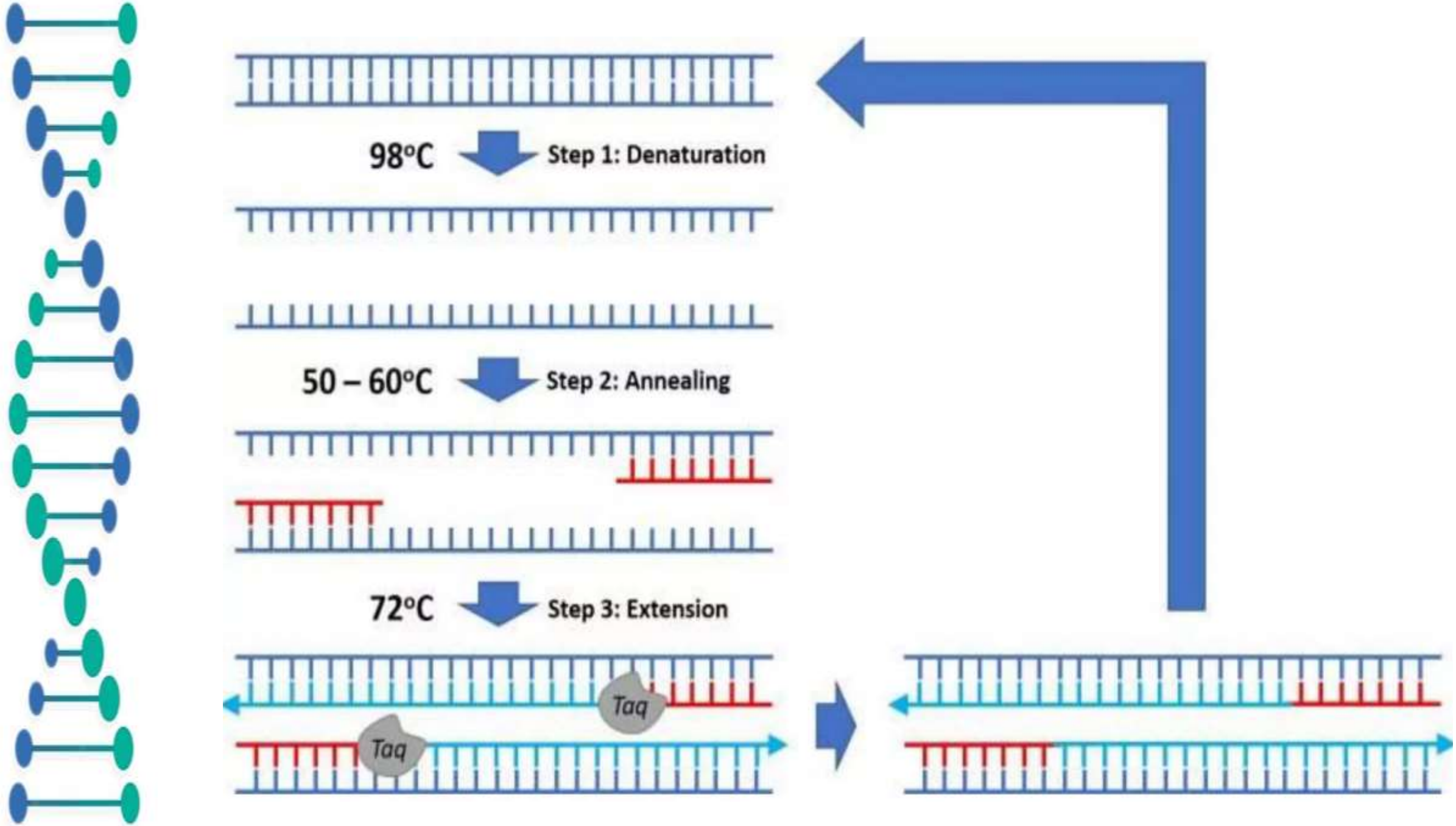
➤Elongation step continues where the polymerase adds dNTP's from 5' to 3', reading the template from 3' to 5' side, bases are added complementary to the template.

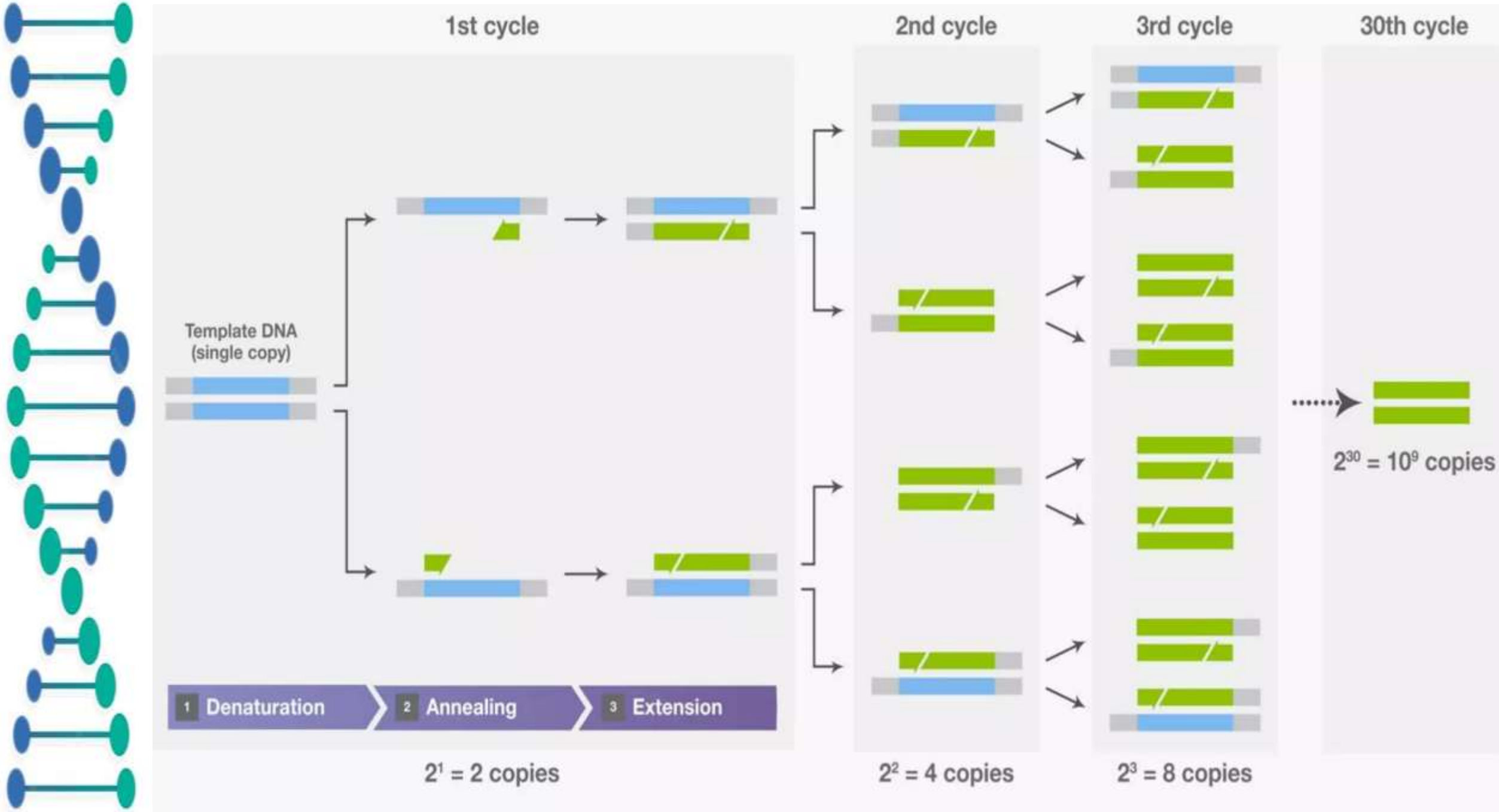
➤Now first cycle is over and next cycle is continued,as PCR machine is automated thermocycler the same cycle is repeated upto 30-40 times.

Temperature: ~72C

Time: 0.5-3min



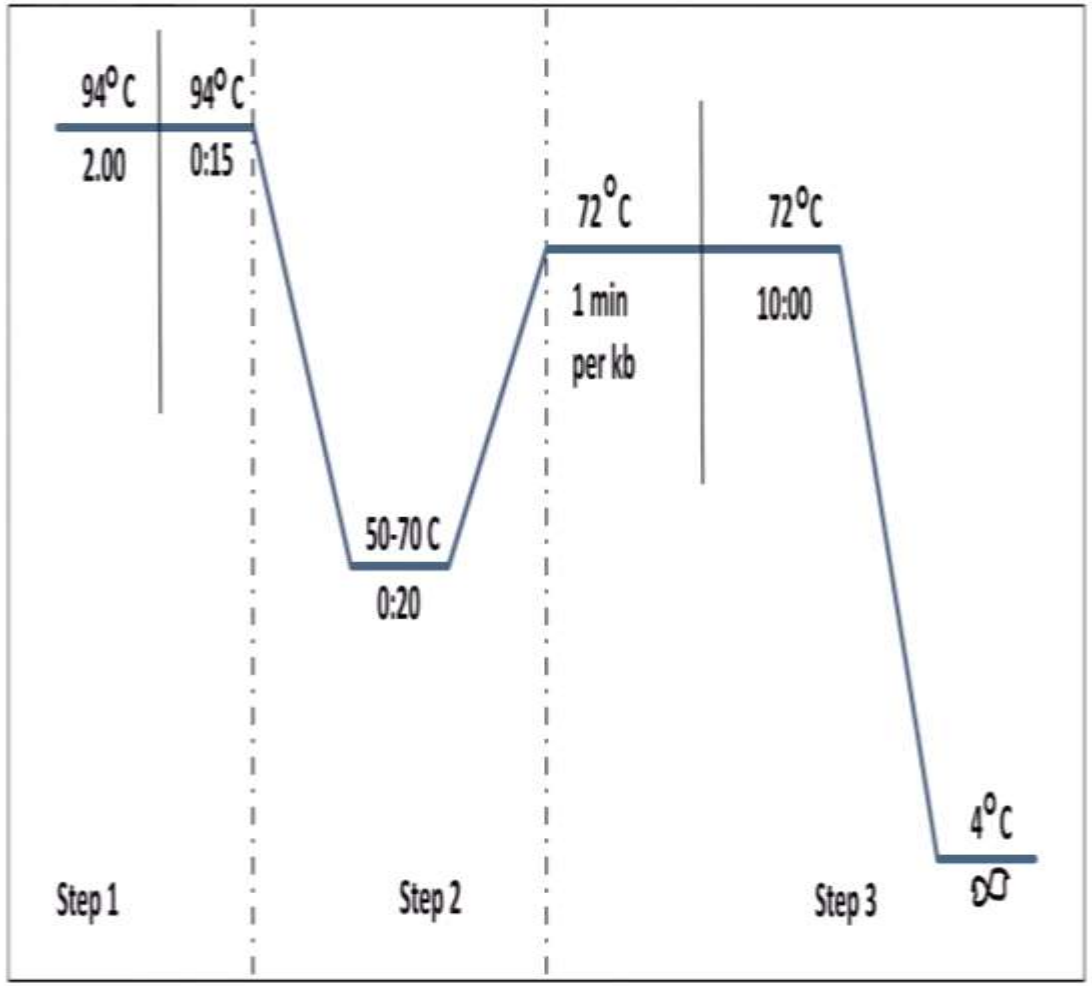

98°C
Step 1: Denaturation
50 – 60°C
Step 2: Annealing
72°C
Step 3: Extension
Taq
Taq


1st cycle
2nd cycle
3rd cycle
30th cycle
Template DNA
(single copy)
1 Denaturation
2 Annealing
3 Extension
$2^1$ = 2 copies
$2^2$ = 4 copies
$2^3$ = 8 copies
$2^{30} = 10^9$ copies

# PCR Program in a Thermal Cycler

| | | | |
|---|---|---|---|
| Denaturation | Step 1 | 94-96º C | 1-2 min/10-15 min |
| | Step 2 | 94-96º C | 30 sec to 1 min |
| Primer annealing | Step 3 | 50-70º C | 30 Sec to 1 min |
| Extension | Step 4 | 72º C (68º C) | 1 kb/min |
| | Step 5 | Go to step 2 34 more times | |
| Final extension | Step 6 | 72º C | 10 min |
| Hold | Step 7 | Hold at 16ºC/4°C | |

# Pcr reaction in graph



## General Guidelines for primers

### 1. Length:

- Shorter primers have a tendency to go and anneal to the non-target sequence of the DNA template.
- Short primer may offer sufficient for a simple template such as a small plasmid.
- But a long primer may be required when using eukaryotic genomic DNA as template. In practice, 20-30 nucleotides is generally satisfactory.

### 2. Mismatches:

- Do not need to match the template completely.
- Often beneficial to have C or G as the 3' terminal nucleotide which makes the binding of the 3' end of the primer to the template more stable.

## General Guidelines for primers

### 3. Melting Temperature Tm:

Melting temperature is the temperature at which one half of the DNA duplex will dissociated and become single stranded. Typically the annealing temperature is about 3-5 degrees Celsius bellow the Tm of the primers used. Primers with melting temperatures in the range of 52-58°C generally produce the best results. Primers with melting temperatures above 65°C have a tendency for secondary annealing.

Tm can be calculated from the following formula:

$$Tm = (4 X [G+C]) + 2 x [A+T])$$

## General Guidelines for primers

### 4. Internal Secondary Structure:

Should be avoided in order to prevent the primer to fold back on itself and not be available to bind to the template.

### 5. Primer-Primer Annealing:

Also important to avoid the two primers being able to anneal to each other. Extension by DNA polymerase of two self-annealed primers leads to formation of a primer dimer.

### 6. G/C content:

Ideally a primer should have a near random mix of nucleotides, a 50% G/C content.

# FACTORS FOR OPTIMAL PCR:

## ❑ PCR Primers

-correctly designed pair of primers is required

-primer dimer,hairpin formation should be prevented

-length of primer

## ❑ DNA Polymerase

-Thermus aquaticus-170° F

-Taq polymerase is heat resistant

-It lacks proof reading exonuclease activity

-Other polymerases can be used .eg:

Tma DNA Polymerase from Thermotoga maritama,

Pfu DNA Polymerase from Pyrococcus furiosus.

## ❑ Annealing Temperature

- Very important since the success and specificity of PCR depend on it because DNA-DNA hybridization is a temperature dependent process.
- If annealing temperature is too high,pairing between primer and template DNA will not take place then PCR will fail.
- Ideal Annealing temperature must be low enough to enable hybridization between primer and template but high enough to prevent amplification of nontarget sites.
- Should be usually 1-2° C or 5° C lower than melting temperature of the template-primer duplex

## ❑ Melting Temperature

- Temperature at which 2 strands of the duplex dissociate.

It can be determined experimentally or calculated from formula

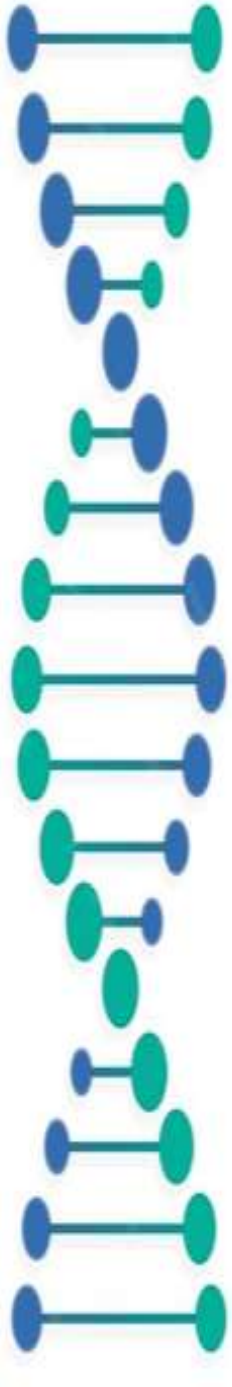
$$Tm = (4(G+C)) + (2(A+T))$$

## ❑G/C content

- ideally a primer should have a near random mix of nucleotides, a 50% GC content
- there should be no PolyG or PolyC stretches that can promote non-specific annealing

# Templates for PCR

- Body Fluids (Blood, CSF, Synovial, Sputum, Semen, Menstrual blood, Stool, Urine etc).
- Tissues
- Dried blood
- Semen stains
- Vaginal swabs
- Single hair
- Fingernail scrapings
- Insects in Amber
- Egyptian mummies
- Buccal Swab
- Toothbrushes
- Microorganisms (Bacteria, Fungi, Virus etc)



## Things to try if PCR does not work

### A) If no product (of correct size) produced:

1 Check DNA quality.

2 Reduce annealing temperature.

3 Increase magnesium concentration.

4 Add dimethylsulphoxide (DMSO) to assay (at around 10%).

5 Use different thermostable enzyme.

6 Throw out primers - make new stocks.

### B) If extra spurious product bands present:

1 Increase annealing temperature

2 Reduce magnesium concentration

3 Reduce number of cycles

4 Try different enzyme

# Variations of the PCR

Colony PCR

Nested PCR

Multiplex PCR

AFLP PCR (Amplified fragment length polymorphism)

Hot Start PCR

In Situ PCR

Inverse PCR

Asymmetric PCR

Reverse Transcriptase PCR

Allele specific PCR

Real time/Qunatitative PCR

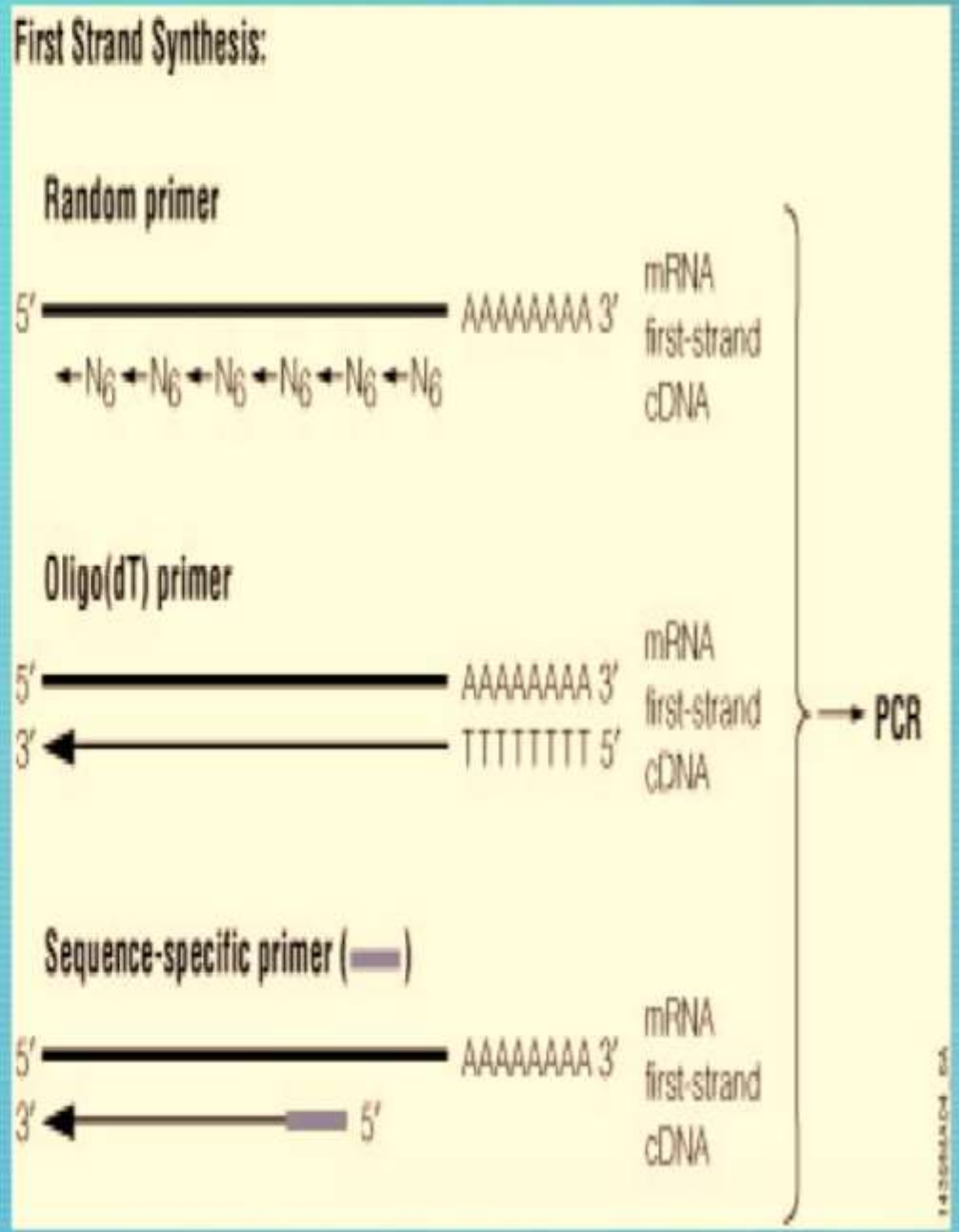
ARMS PCR (Amplification Refractory Mutation System)

Methyl Specific PCR

# ❑ Reverse transcriptase PCR

-It is employed for amplification of RNA molecules .

-RT-PCR is widely used in expression profiling, to determine the expression of a gene or to identify the sequence of an RNA transcript.



## ❑ Quantitative real time PCR (Q-RT PCR)

It is used to amplify and also for quantification and detection of DNA sample.

- ➢ Real time PCR using DNA dyes
- ➢ Fluorescent reporter probe method

-Detection and quantitation of fluorescent reporter the signal of which increases in direct proportion to the amount of PCR product in a reaction

-Does not measure the amount of end product but its production in real time

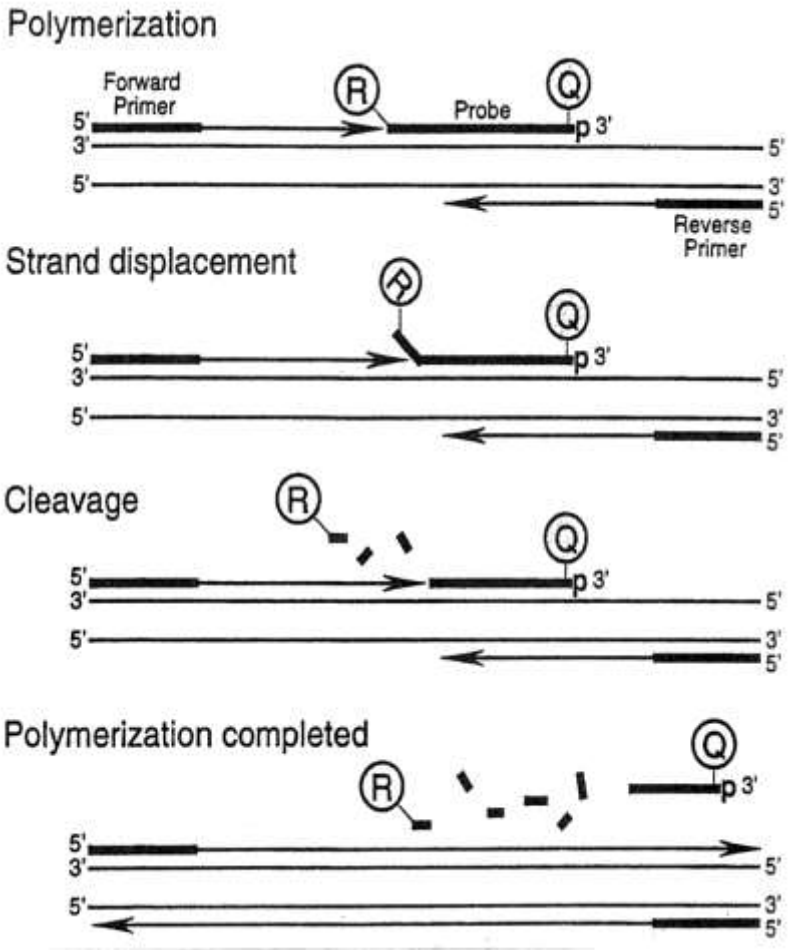
## *TaqMan™ Sequence Detection System*

- Several types of chemistries have been developed for this direct detection of PCR-copied sequences. One of the most popular is the TaqMan™ system illustrated here.

- In the TaqMan™ system, as each new copy of the target sequence is made – a hybridization probe which binds to the sequence is simultaneously hydrolyzed by the polymerase enzyme

- This causes two fluorescent dyes at either end of the probe to become separated and eliminates the Q dye quenching effect on the fluorescence of the reporter or R dye.

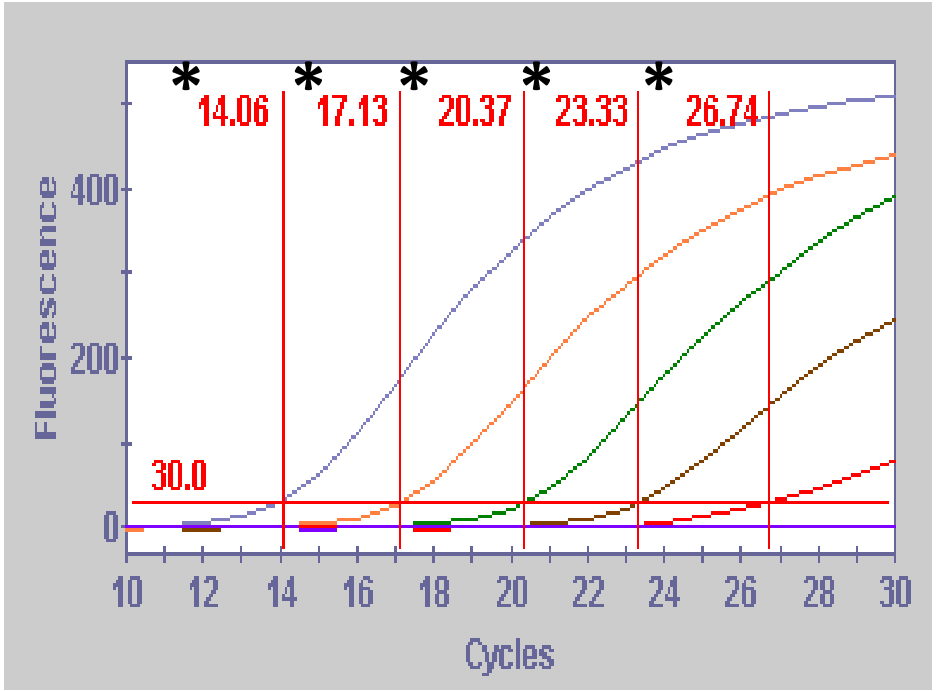
- For each new copy of the sequence that is made, the fluorescence of one reporter dye molecule becomes detectable by the instrument.



# QPCR Growth Curves

**- The quantitative capability of this system stems from the direct correlation that has been shown between the starting number of target sequence copies in the sample and the number of amplification cycles required for the instrument to first detect an increase in reporter dye fluorescence associated with the generation of new copies.**

**- The cycle numbers where the reporter dye fluorescence curves cross a threshold value (red line near the bottom of the figure) that is significantly above the background fluorescence (purple line at the very bottom) are automatically reported by real time PCR instruments.**




**100 pg DNA**

**10 pg DNA**

**10 ng DNA**

**1 pg DNA**

**1 ng DNA**

**no DNA**

*Cycle Threshold: Cycle # at which growth curve = 30 fluorescence units (significantly above background)

## Calculation of target organism cells in test samples from TaqMan assay cycle threshold results using the comparative cycle threshold method

| Target cells in sample | Sample type | $C_T$ | $\Delta C_T$ ($C_{T,test}$-$C_{T,calib}$) | Measured cells in test sample ($2^{-\Delta CT}$ x cells in calibrator) |
|---|---|---|---|---|
| 20000 | Calibrator | 19.8 | ---- | ---- |
| Unknown | Test | 22.9 | 3.1 | 0.11 x 20000 = 2200 |
| Unknown | Test | 26.2 | 6.4 | 0.012 x 20000 = 240 |

assuming amplification efficiency = 2

- Information from the standard curve and results from a single calibrator sample containing known target cell numbers - that is extracted and run with the test samples - can also be used to determine target cell numbers in the test samples using a simple calculation called the comparative cycle threshold method as illustrated here.

# Applications of PCR

**Molecular Identification**
- Molecular Archaeology
- Molecular Epidemiology
- Molecular Ecology
- DNA fingerprinting
- Classification of organisms
- Genotyping
- Pre-natal diagnosis
- Mutation screening
- Drug discovery
- Genetic matching
- Detection of pathogens

**Sequencing**
- Bioinformatics
- Genomic Cloning
- Human Genome Project

**Genetic Engineering**
- Site-directed mutagenesis
- Gene Expression Studies

# Application of PCR

## Medical Application

- Genetic testing for presence of genetic disease mutations.
- Detection of disease causing genes in suspected parents who act as carrier.
- Study of alteration to oncogenes may help in customization of therapy.
- Can also be used as part of a sensitive test for tissue typing, vital to organ transplantation genotyping of embryo.
- Helps to monitor the gene therapy.

## Infectious Disease Application

- Analyzing clinical specimens for the presence of infectious agents, including HIV, hepatitis, malaria, tuberculosis etc.
- Detection of new virulent subtypes of organism that is responsible for epidemics

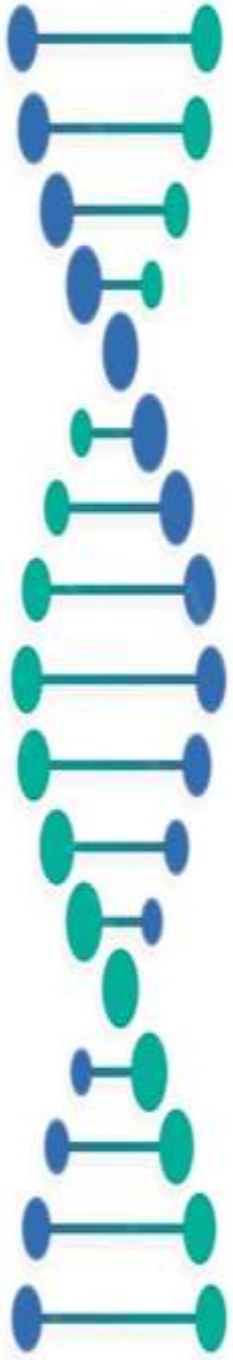
# Application of PCR

## Forensic Application

- Can be used as a tool in genetic fingerprinting.
- This technology can identify any one person from millions of others in case of crime scene, paternity testing etc.

## Research and Molecular Genetics

- Helps to compare the genomes of two organisms and identify the difference between them.
- In phylogenetic analysis, minute quantities of DNA from any source such a fossilized material, hair, bones, mummified tissues.
- In Human genome project for aim to complete mapping and understanding of all genes of human beings.



# Advantages of PCR

Automated, fast, reliable (reproducible) results.

Contained (less chances of contamination).

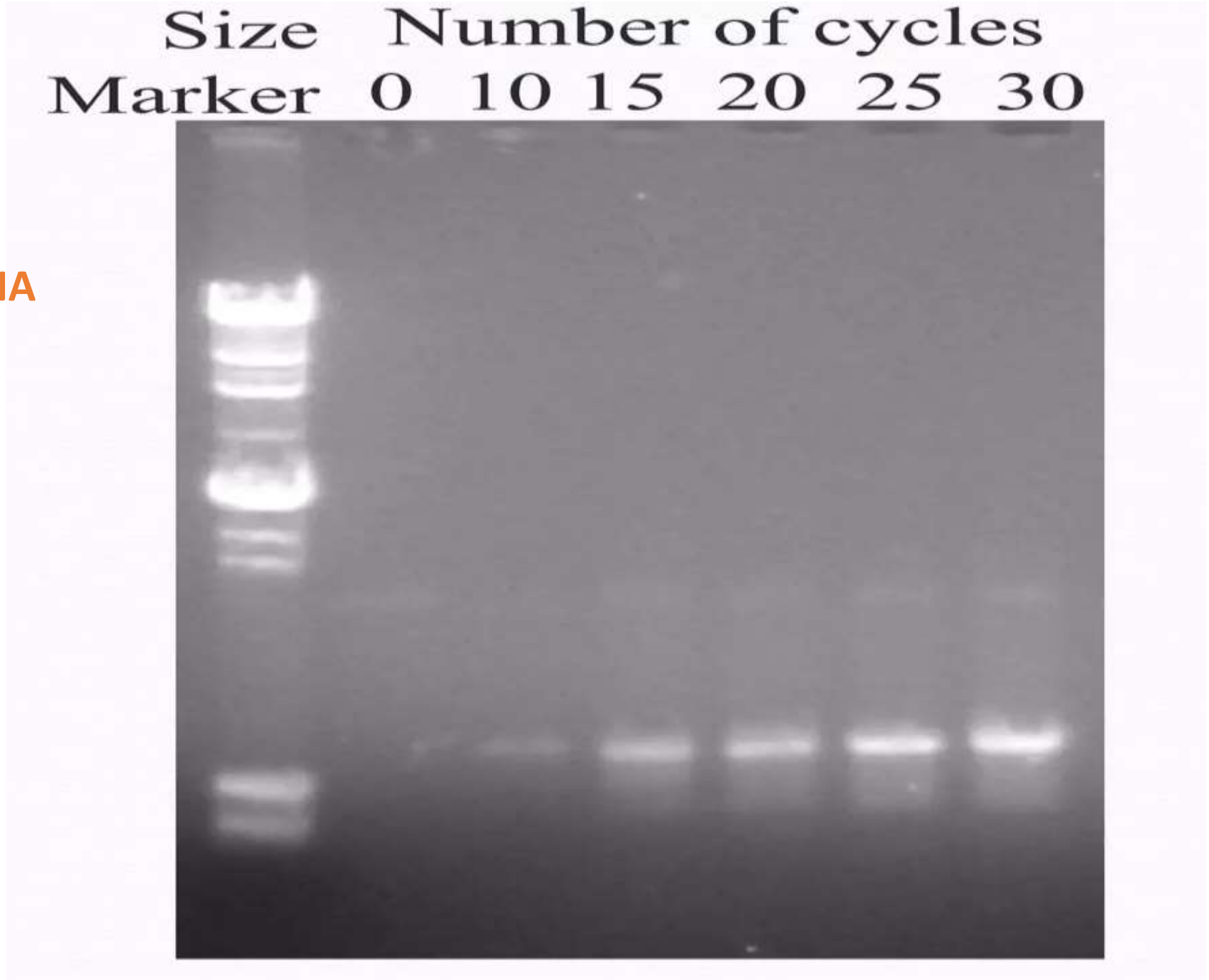
• High output.

Sensitive.

Broad uses.

Defined, easy to follow protocols.

More Cycles = More DNA

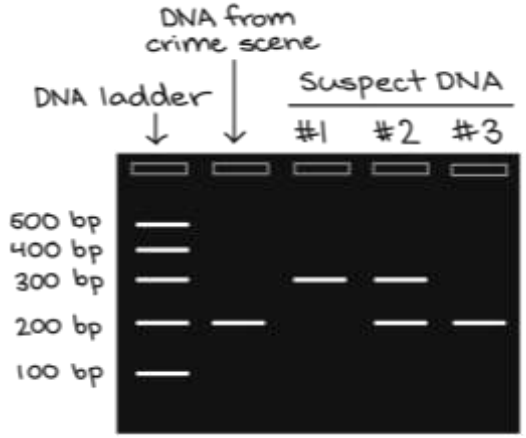


**Sample problem: PCR in forensics**

Suppose that you are working in a forensics lab. You have just received a DNA sample from a hair left at a crime scene, along with DNA samples from three possible suspects. Your job is to examine a particular genetic marker and see whether any of the three suspects matches the hair DNA for this marker.

The marker comes in two alleles, or versions. One contains a single repeat (brown region below), while the other contains two copies of the repeat. In a PCR reaction with primers that flank the repeat region, the first allele produces a 200 bp DNA fragment, while the second produces a 300 bp DNA fragment:


You perform PCR on the four DNA samples and visualize the results by gel electrophoresis, as shown below:




**Which suspect's DNA matches the DNA from the crime scene at this marker?**

**Suspect 3**

- Humans are *diploid*, meaning that they have two copies of most of their DNA. Thus, there will be two copies of the marker we are examining in each of the DNA samples.
- If a person has two different alleles of the marker (is *heterozygous*), two different-sized bands ( 200 bp and 300 bp) will be amplified during PCR. These will appear as bands of DNA in the gel at the 200 bp and 300 bp locations.
- If a person has two copies of the same allele (is *homozygous*), only one band will be amplified during PCR. If the person is homozygous for the 200 bp allele, only a 200 bp band will be visible on the gel. Similarly, if the person is homozygous for the 300 bp allele, only a 300 bp band will be visible on the gel.
- The marker genotypes of the DNA samples are:
- Crime scene DNA: homozygous 200 bp allele
- Suspect 1: homozygous 300 bp allele
- Suspect 2: heterozygous
- Suspect 3: homozygous 200 bp allele
- Both the DNA sample from the crime scene and the DNA sample from suspect are homozygous for the 200 bp version of the marker. That is, the two samples match for this marker.

## 1980s

TC1 DNA Thermal Cycler

TC 480 Thermal Cycler

## 1990s

GeneAmp PCR System 9600

GeneAmp PCR System 9700

GeneAmp PCR System 2400

## 2000s

GeneAmp PCR System 2700

GeneAmp PCR System 9700 Dual 96 Well

Veriti Thermal Cycler

GeneAmp PCR System 9700 Autolid 384W

GeneAmp PCR System 9800

2720 Thermal Cycler

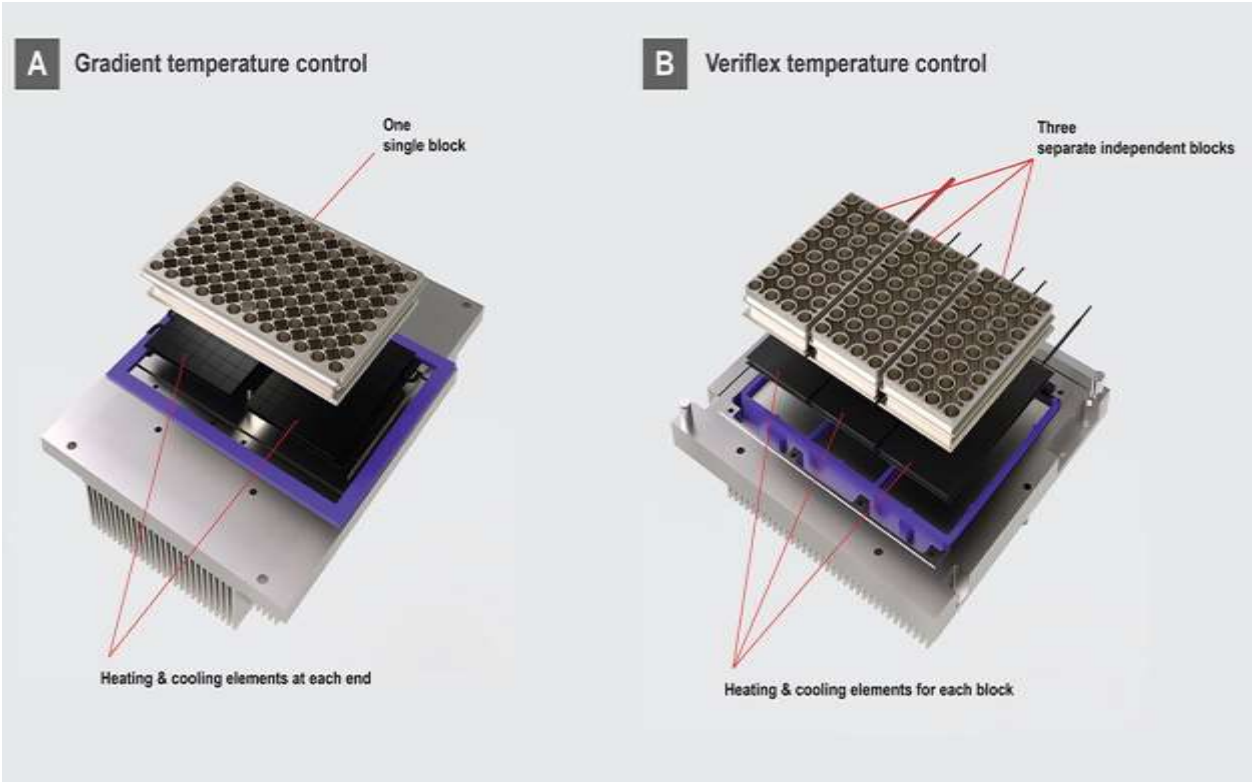
## 2010s

ProFlex PCR System

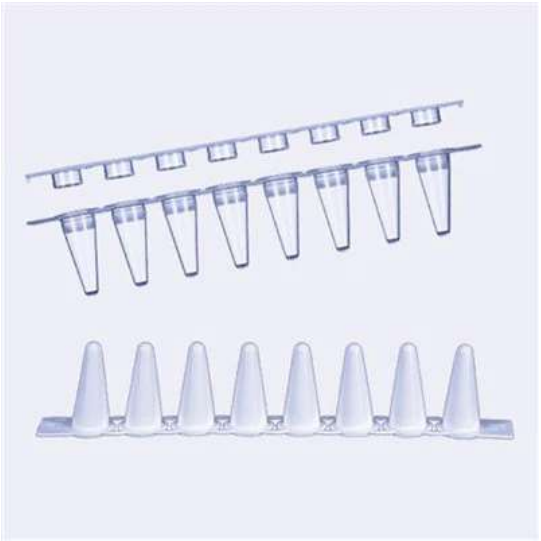
Automated Thermal Cycler

SimpliAmp Thermal Cycler

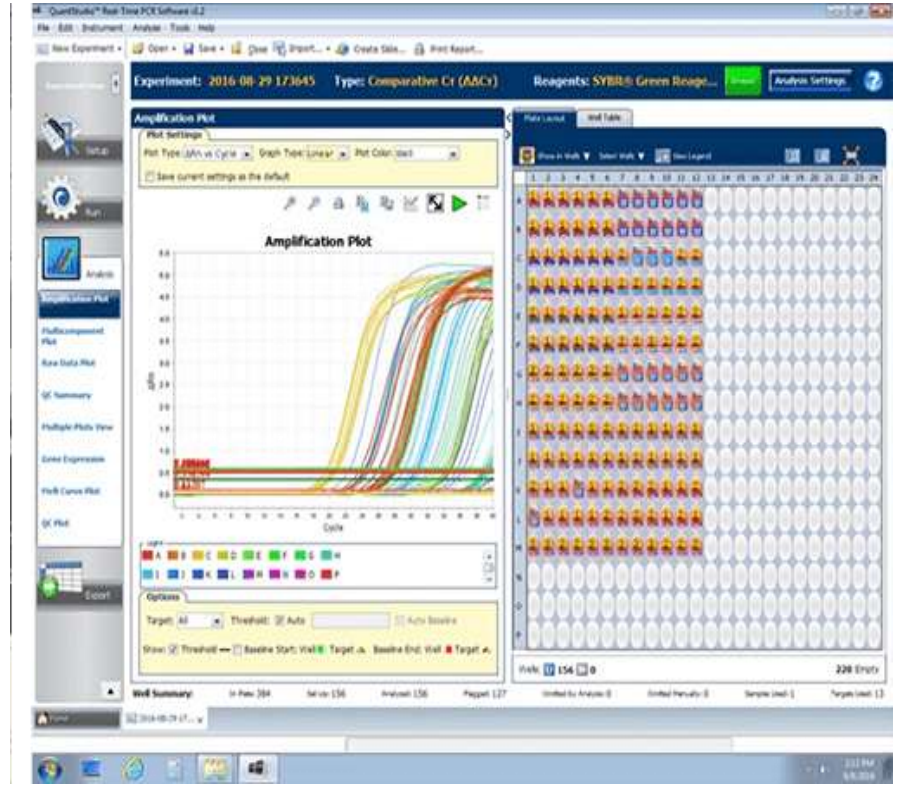
MiniAmp Thermal Cycler




(A) Gradient temperature control of one single block with heating and cooling elements at each end. (B) [Applied Biosystems VeriFlex Block] with “better-than-gradient” temperature control, featuring three separate independent blocks and individual heating and cooling elements for each block.



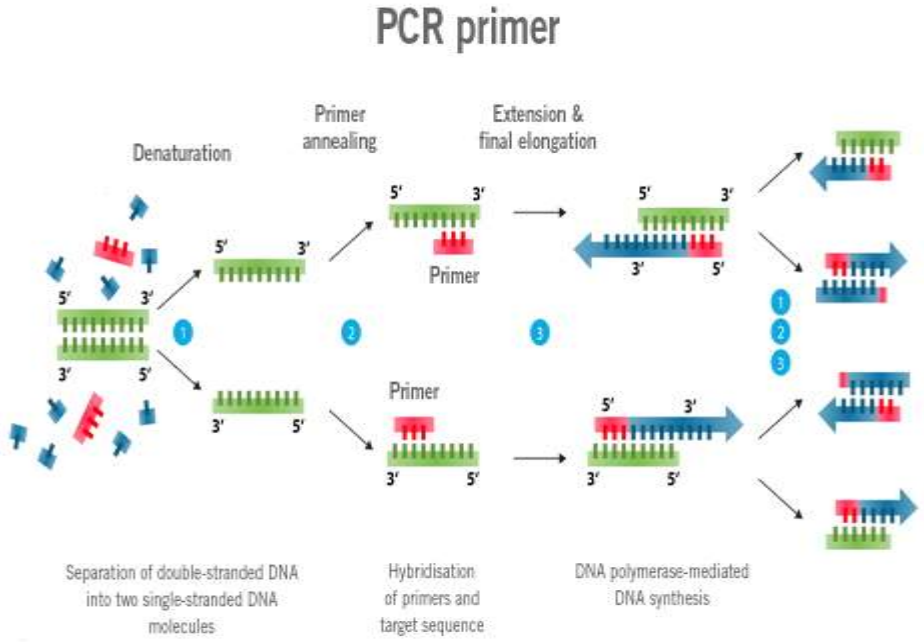

QuantStudio 7 Pro

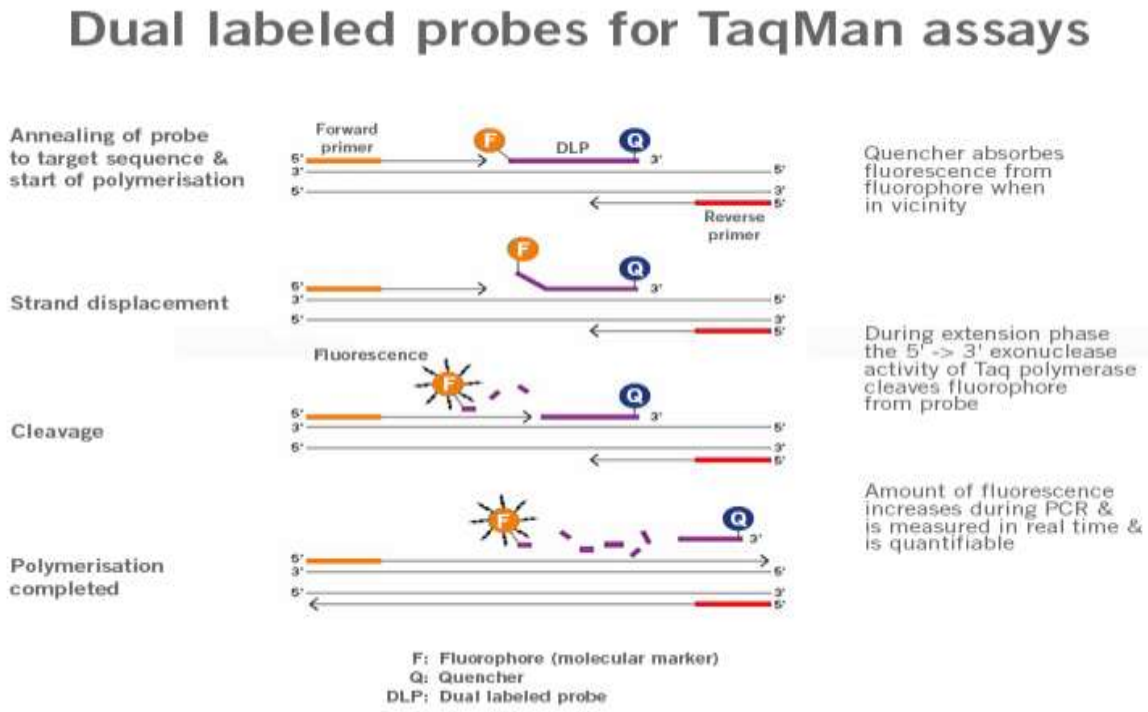

Experiment: 2016-08-29 173645
Type: Comparative Ct (ΔΔCt)
Reagents: SYBR® Green Reage...
Amplification Plot
Amplification Plot
Cycle

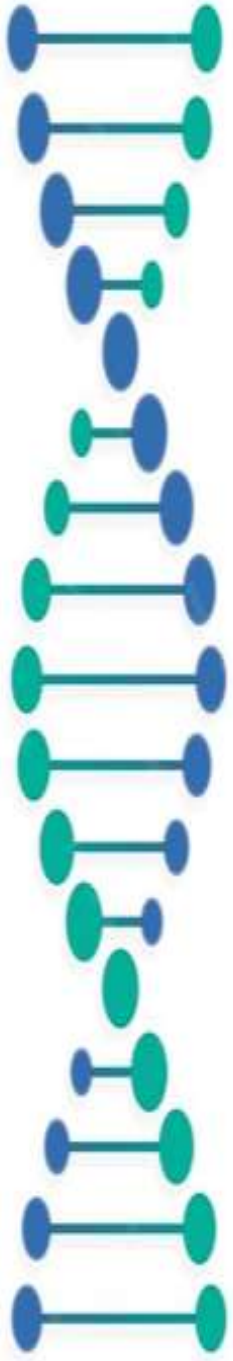
## Difference between primers and probes

Primers are the starting point of the polymerase chain reaction (PCR) with single-stranded DNA/RNA. Primers are usually designed to bind to a specific DNA/RNA sequence. In the cell, RNA primers are the starting point of DNA replication.

qPCR probes describe DNA sequences similar to primers that are typically labelled with a fluorophore as signalling molecules (molecular marker). The use of these probes allows for the quantification of specific DNA sequences present in a sample (image 1).





## Make sure it’s the right length

## The specificity of a primer depends on its length.

- Primers, such as PCR primers, should be designed with a length of **18 to 24 nucleotides** for ideal amplification.
- Long primers have a slower hybridisation rate and a lower chance of annealing to the intended target sequence.
- Slower hybridisation produces **inadequate specificity and inadequate binding to the target sequence**, whereas faster hybridisation rates result in high target concentrations and maximum binding to the target sequence.

The risk of a slower hybridisation increases when a primer is **longer than 30 base pairs**.

Even though long primers have a higher level of specificity than short primers, they are less efficient during the annealing phase and produce less amplicon yield. Due to the build-up of by-products and the loss of components necessary for DNA synthesis during a PCR, more cycles can result in less efficient outcomes. Short primers, however, anneal to their target sequence more effectively and need fewer PCR cycles for amplicon generation compared to long primers (Wu et al., 2010).

Unlike primers, the optimal length of probes is highly target-specific. The length generally selected by experts is between 15 and 30 nucleotides. However, when longer probes are used instead of shorter ones, fewer probes per gene may be required.

## Choose the optimal melting temperature (Tm) range

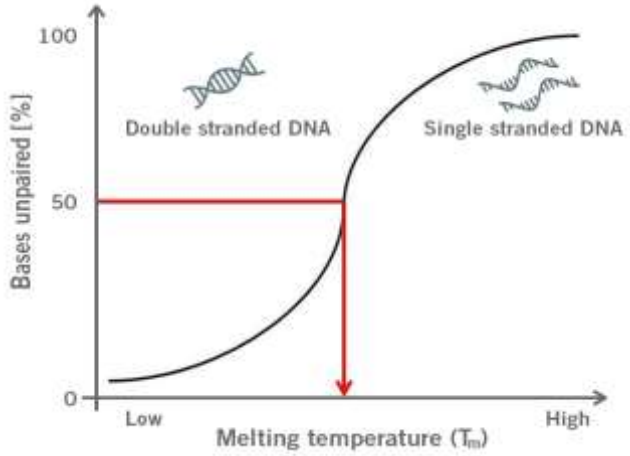
Melting temperature (Tm) plays an integral part in both PCR and qPCR experiments. This is the temperature at which the DNA duplex, commonly known as double-stranded DNA, splits into two single-stranded DNA parts.

Moreover, Tm also determines the annealing temperature Ta, where primer binding occurs at the highest efficiency and specificity. Ta affects the final amount of PCR and qPCR product yield.

The optimal melting temperature for maintenance of primer specificity is 54°C or higher (54°C to 65°C). However, Ta of a primer is often above its Tm, usually in a range of 2-5°C.

When a primer is designed, its Tm should not be above 65°C, as it increases the risk of secondary annealing.

During a PCR, Tm describes the temperature at which primers have annealed to 50% of the target sequences and the other 50 % of target sequences are free. Both states are in equilibrium (image).



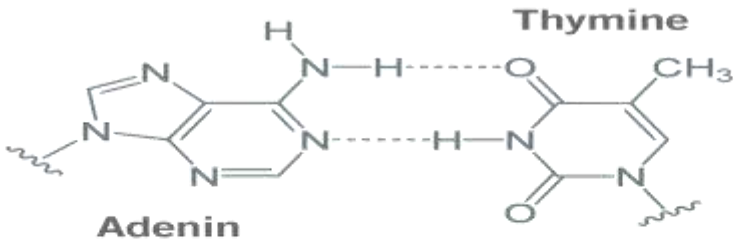

The Tm of a PCR can vary based on buffer composition, metal ion concentration, pH, and additives such as DMSO.

## Choose the appropriate percentage of GC content

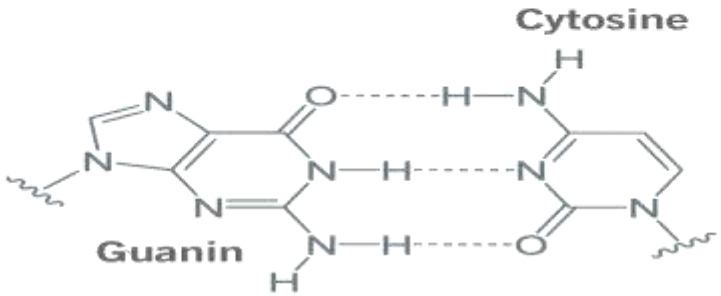
- The GC content of primers and probes is the percentage of guanine (G) and cytosine (C) in the primer. It is recommended to keep the GC content between 40% to 60% when designing a primer. A primer with a length of 20 nucleotides should contain 8 to 12 Gs or Cs.

- The reason for this GC content range is simple. When primers and probes anneal to their target sequence, GC base pairs form three hydrogen bonds and adenine (A) and thymine (T) form two hydrogen bonds (image).

- As three hydrogens bonds are stronger than two, the separation of G and C requires more energy (in the form of heat) than for A and C.




- A higher GC content in the primer will lead to stronger binding of the single DNA strands (ssDNA), resulting in a higher Tm. This could be an issue during PCR and qPCR, as higher GC content can cause mismatches and the formation of primer-dimers, i.e. the hybridisation of two primers with each other .




- If the GC content of the primers is less than 40%, their lengths may need to be increased in order to maintain the optimal Tm.

## GC clamp

- The term “GC clamp” refers to the presence of Gs or Cs in the last five nucleotides at the 3′ end of primers.
- The benefit of a GC clamp is that it promotes complete primer binding.
- However, the presence of more than 3 G’s or C’s at the 3′ end of a primer can lead to non-specific binding and false-positive results.
- For probes, the ideal GC content is between 35% and 60% to promote probe specificity and avoid false-positive results.
- Probes should not contain a G at the 5′ end, as this could interfere with the fluorescence from the reporter molecule (fluorophore) that is attached to this end.

5’-CTATGTACGGTCACGA**C**TA**C**-3’

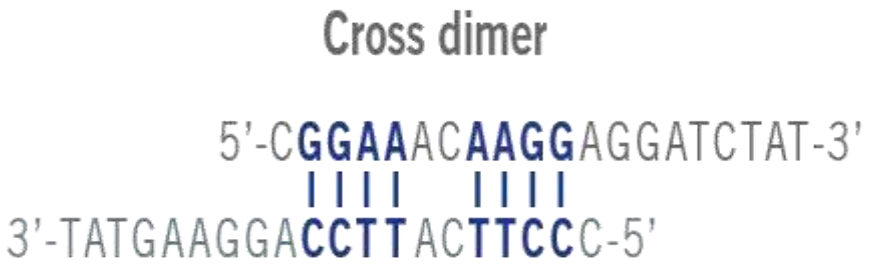
5’-CGCTACCACTATCGATT**G**AT-3’

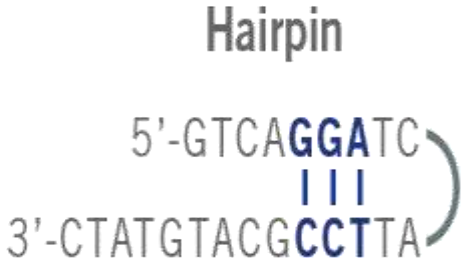
5’-GCATCTGGATGCATG**C**TAT**G**-3’

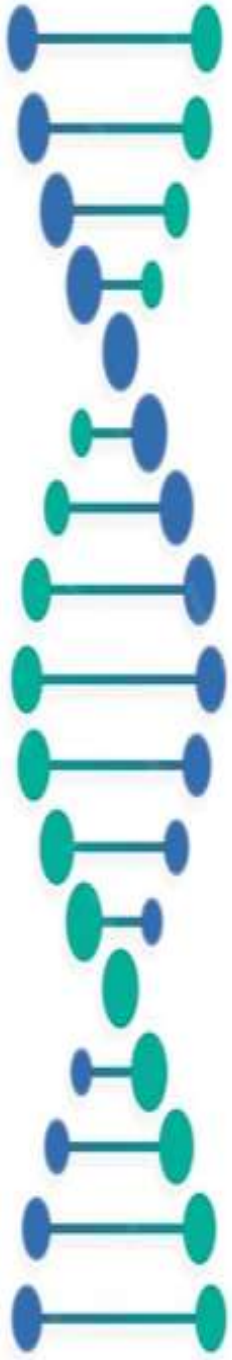
## Avoid secondary structures and primer-dimers

- Primer-dimers and hairpin loops are two types of secondary structures that can form during PCRs and qPCRs.
- Primer-dimers are formed due to the presence of **complementary sequences within a utilised primer or complementary sequences shared by two primers.**
- This is represented by the parameter "self-complementarity" in primer design tools. The two types of primer dimers are:
- **Self-dimer** refers to, for example, the hybridisation of two forward primers to each other due to intra-primer homology.
- **Cross-dimer** refers to the hybridisation of the forward and reverse primer due to inter-primer homology.
- Primer-dimers prevent primers from annealing to the target sequence. Hence, most of the final PCR product is just the amplification of the primers themselves rather than the amplicon.

- Hairpins are formed due to the **intramolecular interaction of the primer.**
- Here, two regions of three or more nucleotides within the primer are complementary to each other. When they anneal, a hairpin is formed.
- The probability of forming a hairpin is represented by the parameter "**self 3′ complementarity**".
- Hairpins can impact the amplification step and lead to non-specific amplicons or even no amplicon yield.
- Significantly, hairpins can also form when the annealing temperature is too low.





- For both parameters (self-complementarity and self 3′-complementarity), the lower the number, the better.
- However, secondary structures can be avoided by adjusting the annealing temperature (in most cases, an increase in temperature), avoiding cross homology, and changing the primers or DNA concentration. The DNA concentration should be balanced with the number of cycles required in the reaction to enable the best possible results.
- Runs of three or more Cs or Gs at the 3'-ends of primers may promote mispriming at G or C-rich sequences (because of stability of annealing), and should be avoided;
- 3'-ends of primer should not be complementary.