POLYMERASE CHAIN REACTION

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Brief Outlines

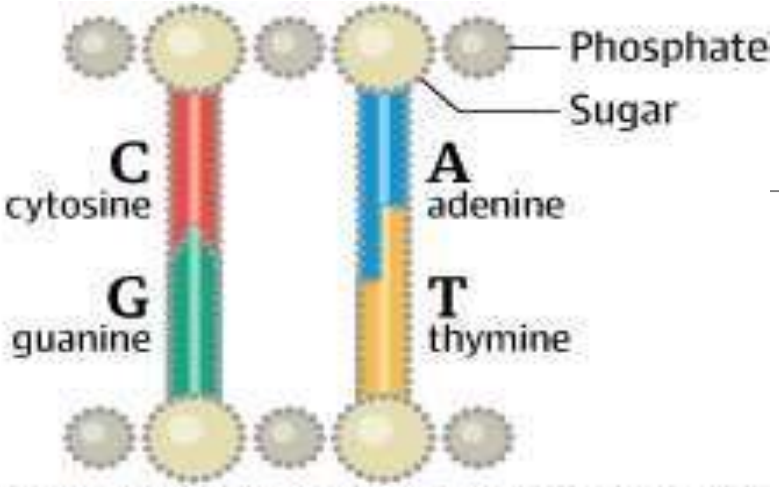
- Introduction
- History
- Components of PCR
- Basic requirements
- Instrumentation
- PCR programme
- Prevention of contamination in PCR
- Variants of PCR
- Advantages of PCR
- Applications of PCR

How PCR WORKS ?

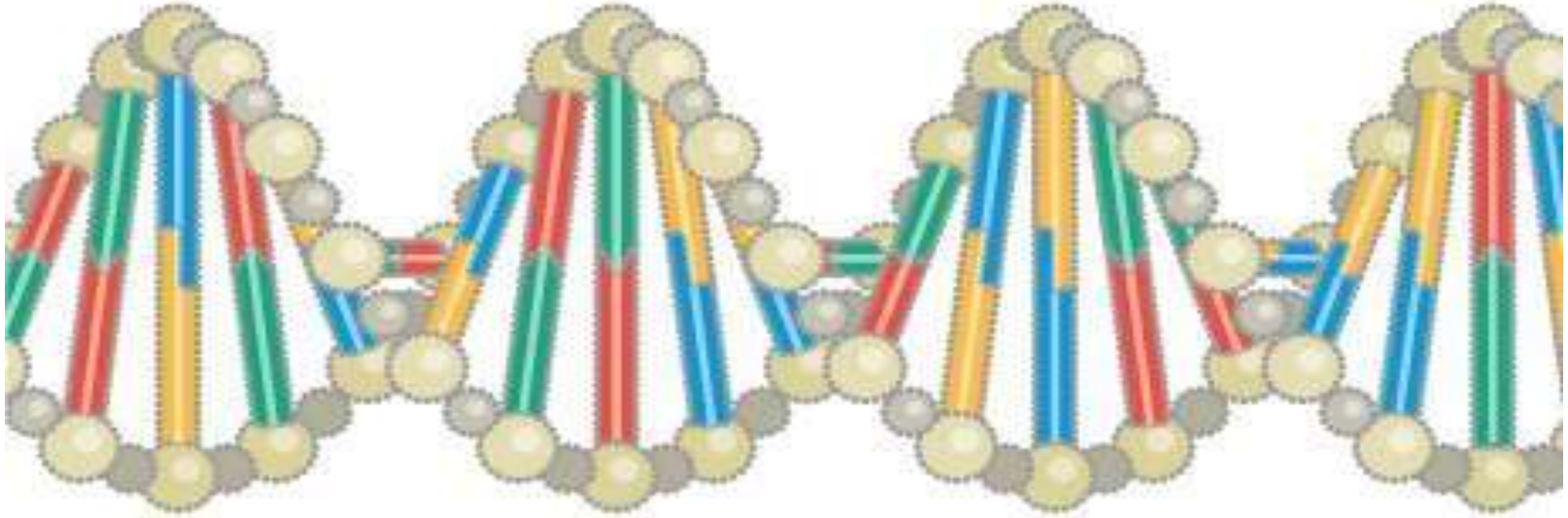
https://www.youtube.com/watch?v=a5jmdh9AnS4

Structure of DNA

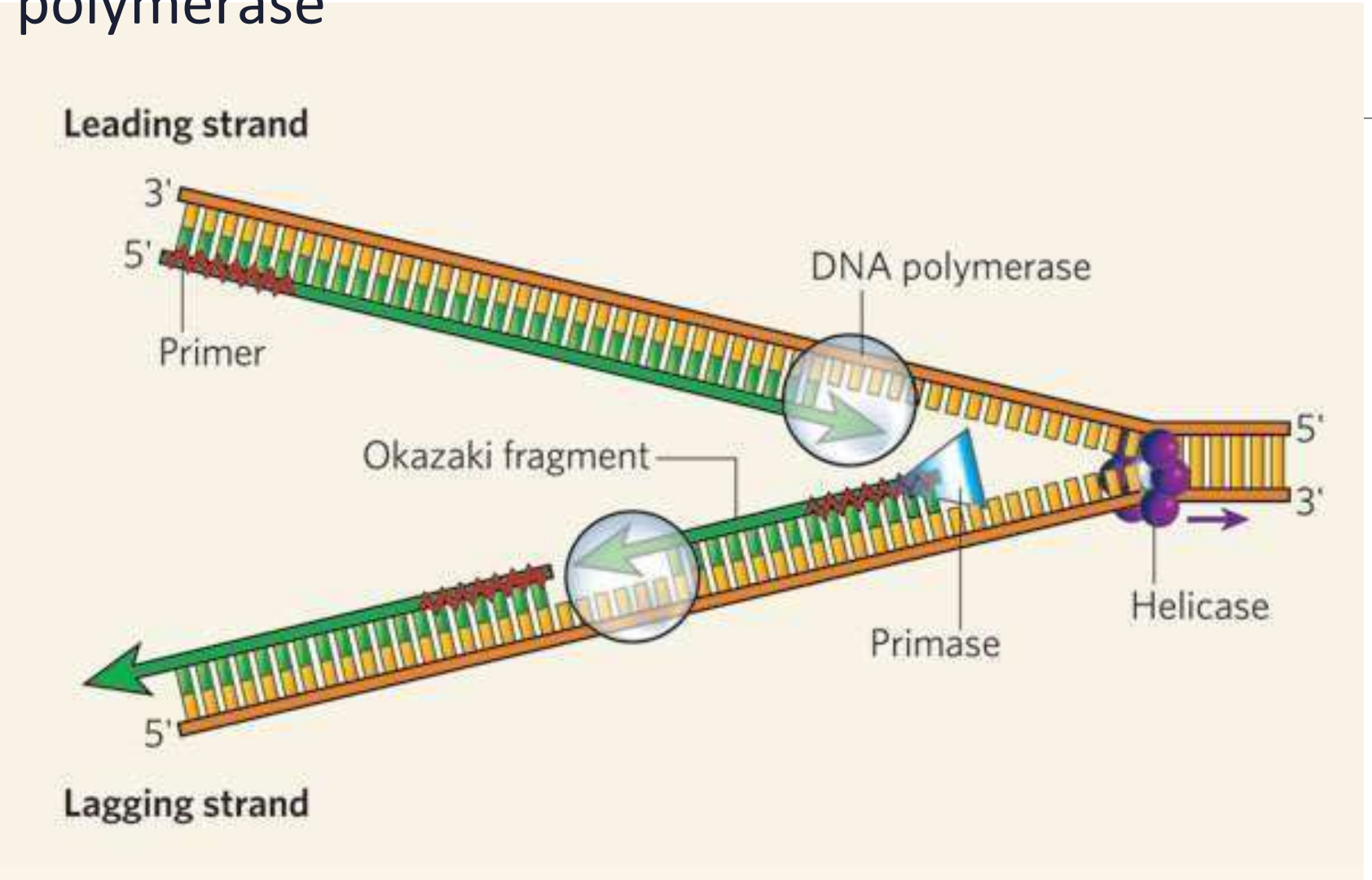
The famous double helix is two intertwined sugar and phosphate chains, linked by four kinds of chemical 'bases' – a, c, g and t – which make up the genetic code



Base pairs 'C' always pairs with 'G', and 'A' with 'T'



DNA replication and DNA polymerase



DNA Replication vs PCR

PCR 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

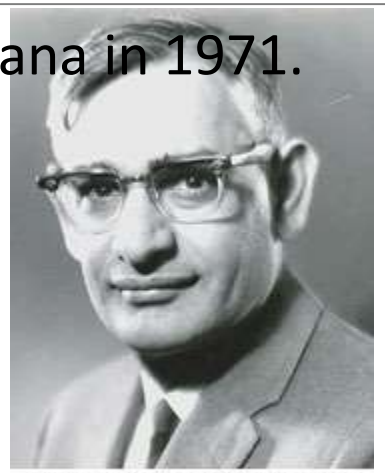
Key enzymes involved in DNA Replication

- DNA Polymerase
- DNA Ligase
- Primase
- Helicase- untwists the two parallel DNA strands
- Topoisomerase- relieves the stress of this twisting
- Single strand binding protein- binds to and stabilizes the unpaired DNA strands

- DNA replication is the copying of DNA
- It typically takes a cell just a few hours to copy all of its DNA
- DNA replication is semi-conservative (i.e. one strand of the DNA is used as the template for the growth of a new DNA strand)
- This process occurs with very few errors (on average there is one error per 1 billion nucleotides copied)
- More than a dozen enzymes and proteins participate in DNA replication

History

- Principle of replicating a piece of DNA using two primers had been described by Har Gobind Khorana in 1971.



Courtesy of the University of Wisconsin.
Noncommercial, educational use only.

- Progress was limited by primer synthesis and polymerase purification issues. How to proceed.?

?

Histor
y

- Kary Banks Mullis, who was driving his vehicle his girlfriend, who was also a chemist, when he had the idea to use a pair of primers to bracket the desired DNA sequence and to copy it using DNA polymerase
- Accordingly Mullis succeeded in demonstrating PCR December 16, 1983.
- In 1985: First publication of PCR by cetus corporation appear in Science.
- 1988: Perkin Elmer introduced the automayed thermal cycler

Histor
y

- 1986: Purified Taq polymerase is first used in PCR.
- 1989: Science declares Taq polymerase "molecule of the year"
- 1990: "real time" or "kinetic "PCR
- 1991: RT PCR (reverse transcriptase PCR) using single thermostable polymerase, Tth
- 1993: Kary Mullis was Awarded Nobel Prize for Chemistry for conceiving PCR technology.

Principle

PCR amplifies a specific region of a DNA strand (the DNA target). Most PCR methods amplify DNA fragments of between 0.1 and 10 kilo base pairs (kbp), although some techniques allow for amplification of fragments up to 40 kbp in size. The amount of amplified product is determined by the available substrates in the reaction, which become limiting as the reaction progresses.

A basic PCR set-up requires several components and reagents, including:

I. **DNA template** that contains the DNA region (target) to be amplified.

II. Two **primers** that are complementary to the 3’ ends of each of the sense and anti-

III. sense strand of the DNA target.

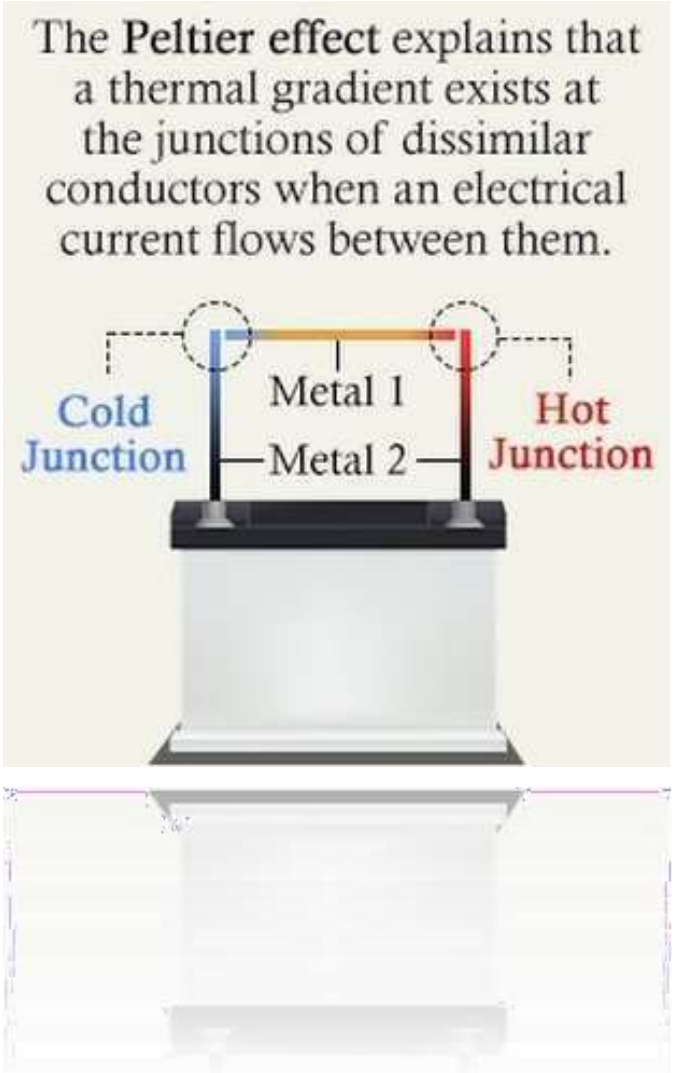
Taq polymerase or another Thermo-stable DNA polymerase with a temperature optimum at around 70 °C.

IV. **Deoxynucleoside triphosphates**(dNTPs)

V. **Buffer solutions**, (Tris, ammonium ions and/or potassium ions, magnesium ions)

Principl
e

- The reaction is commonly carried out in a volume of 10–200 µl in small reaction tubes (0.2–0.5 ml volumes) in a thermal cycler. The thermal cycler heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction .
- Many modern thermal cyclers make use of the *Peltier effect*, which permits both heating and cooling of the block holding the PCR tubes simply by reversing the electric current.
- Thin-walled reaction tubes permit favorable thermal conductivity to allow for rapid thermal equilibration. Most thermal cyclers have heated lids to prevent condensation at the top of the reaction tube



Principl
e

Procedur
e

- Typically, PCR consists of a series of 20–40 repeated temperature changes, called cycles, with each cycle commonly consisting of two or three discrete temperature steps.
- The cycling is often preceded by a single temperature step at a very high temperature (>90 °C (194 °F)), and followed by one hold at the end for final product extension or brief storage.
- The temperatures used and the length of time they are applied in each cycle depend on a variety of parameters, including

- the enzyme used for DNA synthesis,
- the concentration of bivalent ions and
- dNTPs in the reaction, and
- the melting temperature (*Tm*) of the primers.

Procedur
e

The individual steps common to most PCR methods are as follows:

- **Initialization**:

This step is only required for DNA polymerases that require heat activation by hot- start PCR.

It consists of heating the reaction chamber to a temperature of 94–96 °C (201–205 °F), or 98 °C (208 °F) if extremely thermostable polymerases are used, which is then held for 1–10 minutes.

Procedur
e

- There are three major steps in a PCR, which are repeated for 30 or 40 cycles.

1. Denaturation

1. Annealing.

1. Extension or elongation.

1) Denaturation at 94-98°C

:

Consists of heating the reaction to 94–98 °C for 30–90 seconds.

It causes separation of DNA template by disrupting the hydrogen bonds between
complementary bases of the DNA strands, yielding single strands of DNA.

2) Annealing at 54°C

:

- The reaction temperature is lowered to 50–65 °C for 30–120 seconds allowing annealing of the primers to the single-stranded DNA template.
- The primers are jiggling around, caused by the Brownian motion .
- Once the primers fits exactly the sequence in the single stranded template, it forms a stable bond.

On the little piece of double stranded DNA (template and primer), the polymerase can attach and starts copying the template

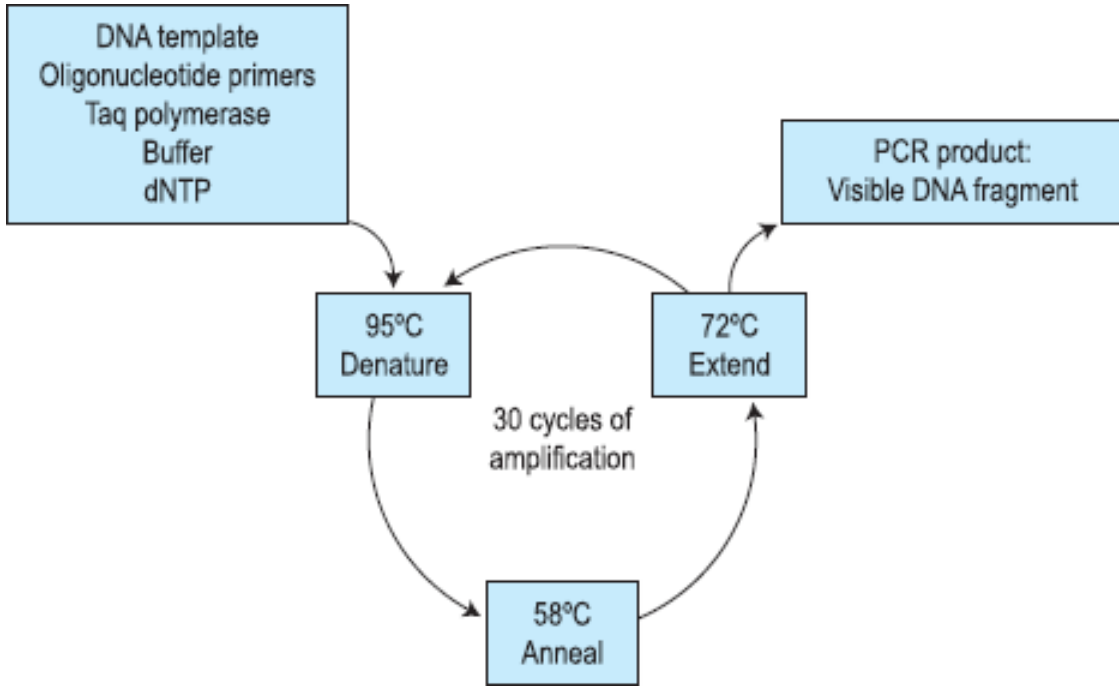
3) Extention or elongation at 72°C

:

- The DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, reading the template from 3' to 5' side.
- Results in the generation of a second target molecule, which then acts as a template for a subsequent cycle.

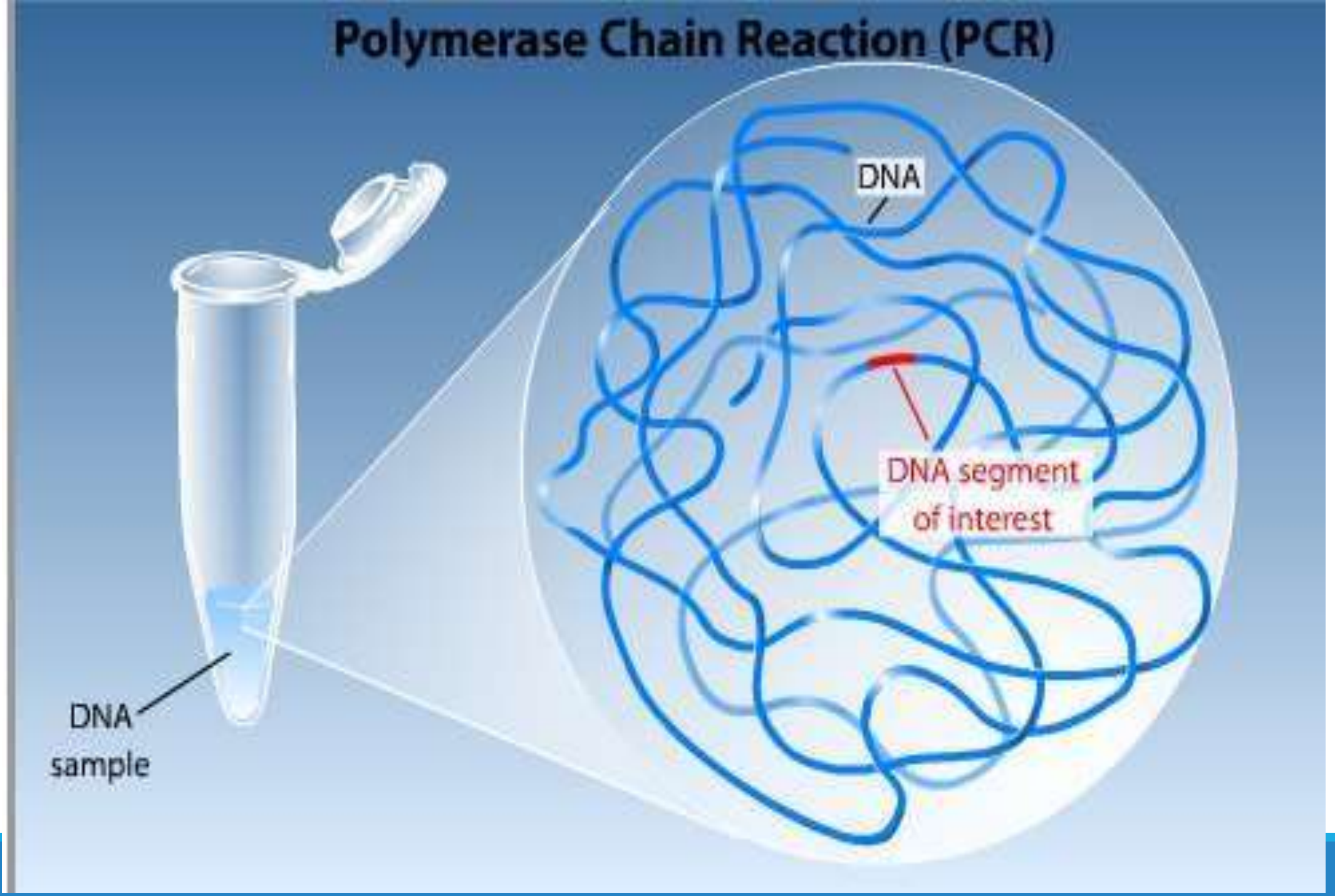
4) Final elongation:

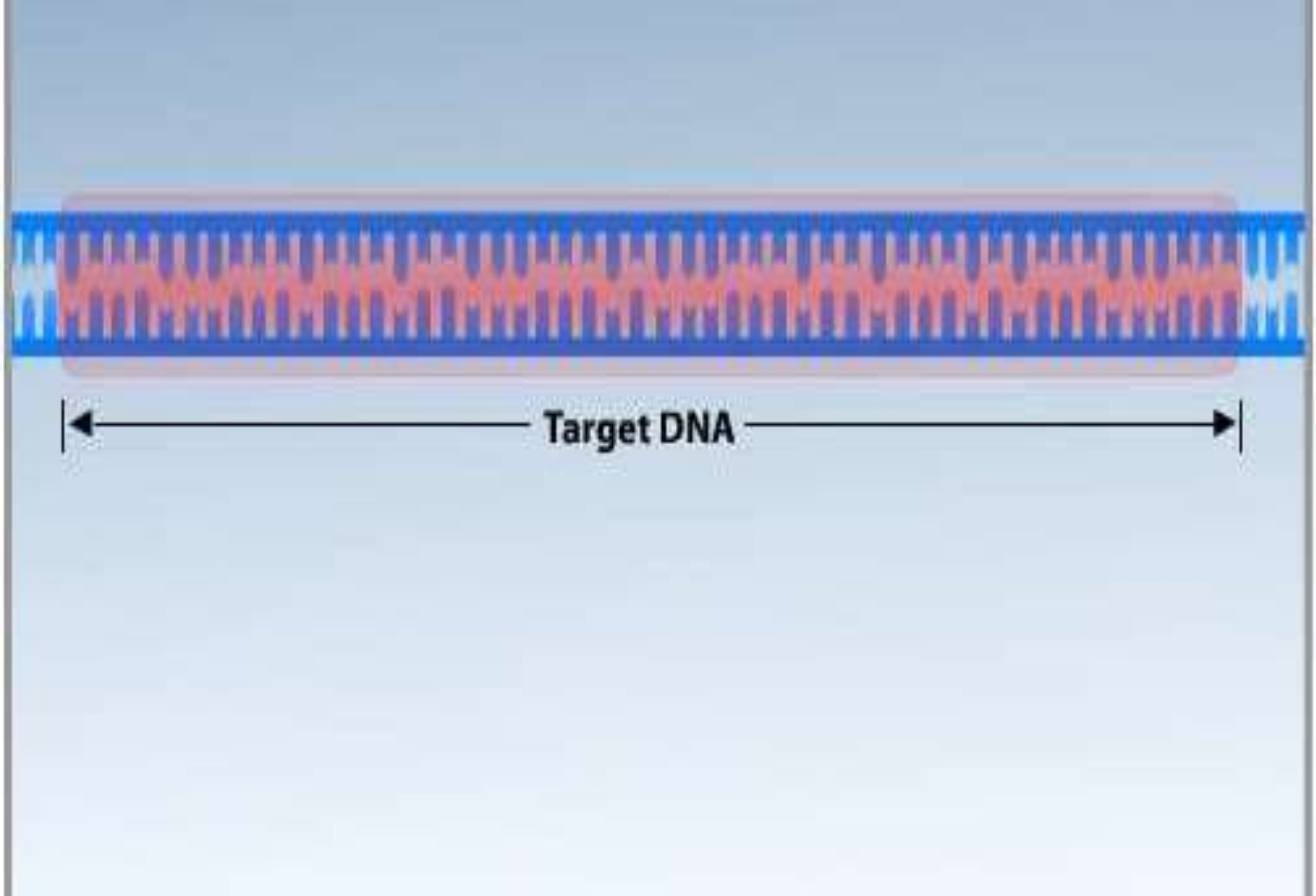
- This single step is *optional*, but is performed at a temperature of 70–74 °C (158–165 °F) (the temperature range required for optimal activity of most polymerases used in PCR) for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully elongated.

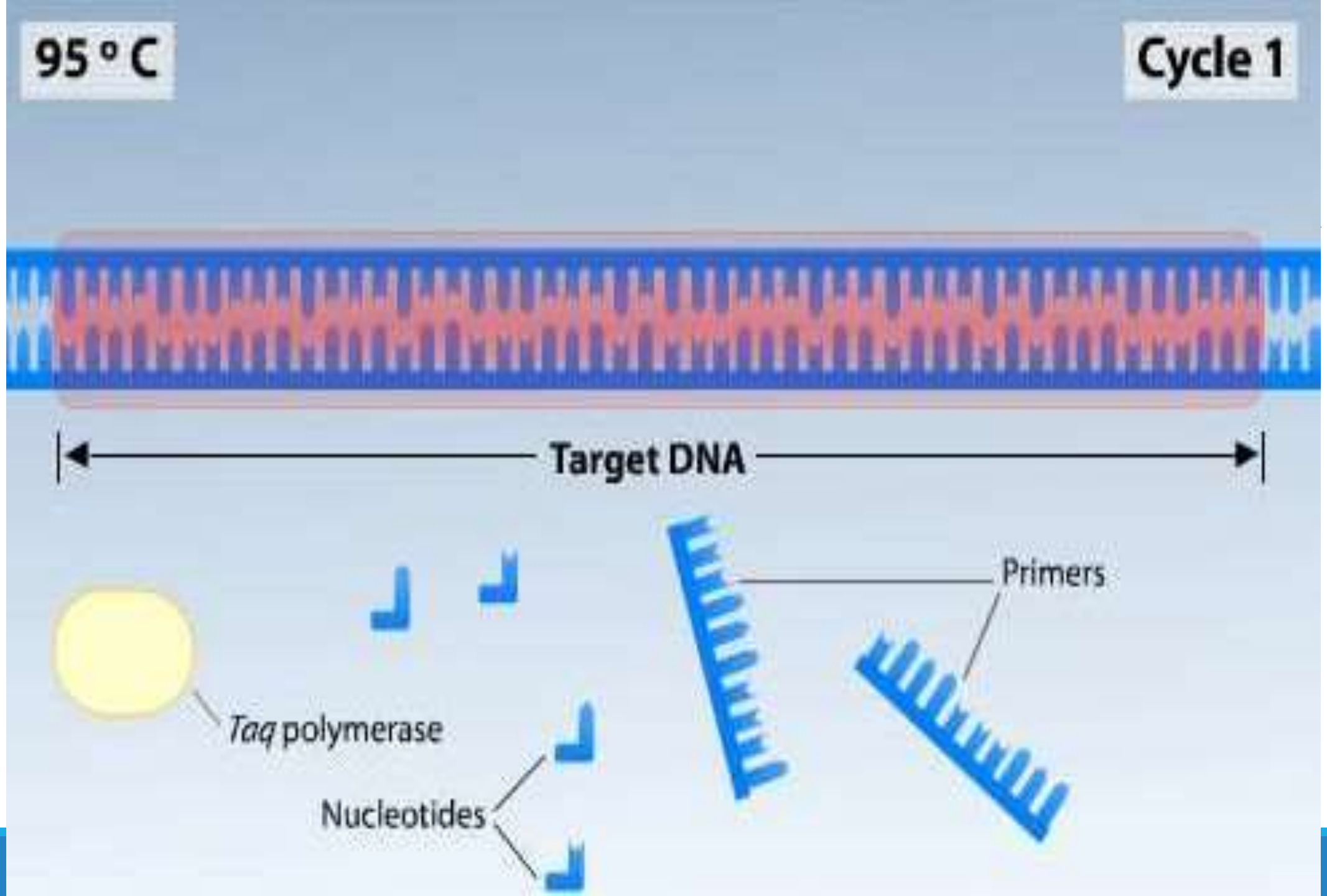


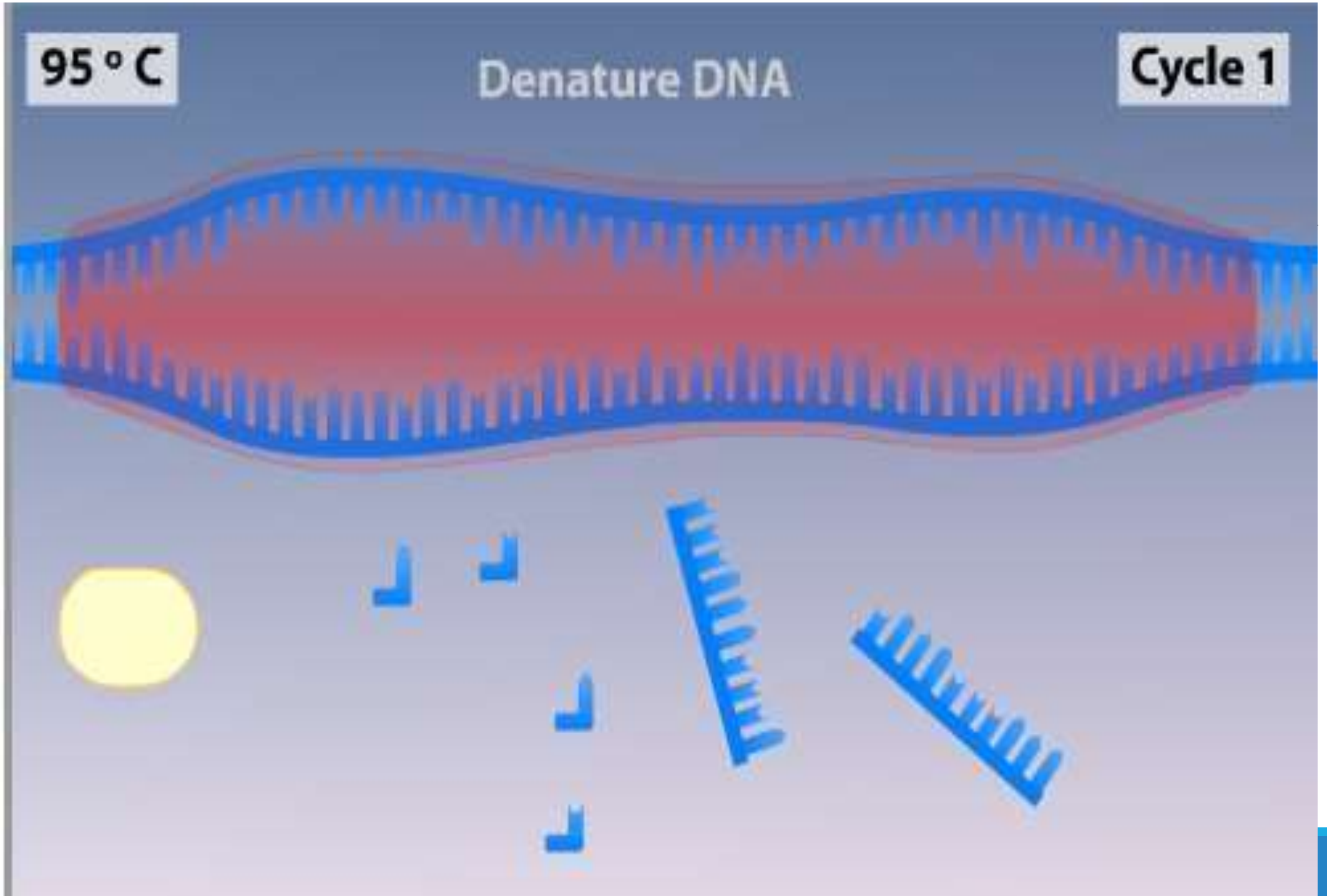
5) Final hold:

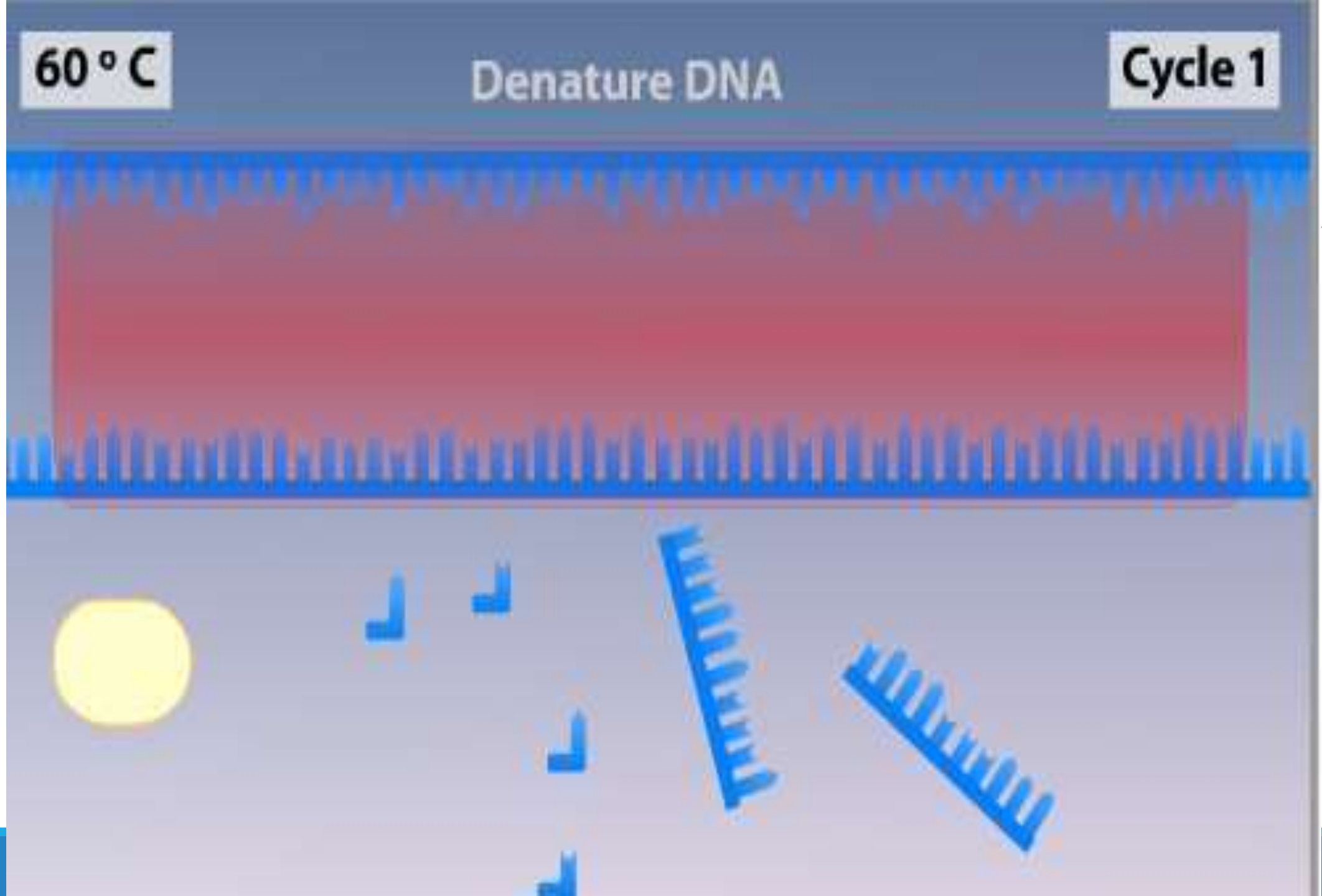
- The final step cools the reaction chamber to 4–15 °C (39–59 °F) for an indefinite time, and may be employed for short-term storage of the PCR products.

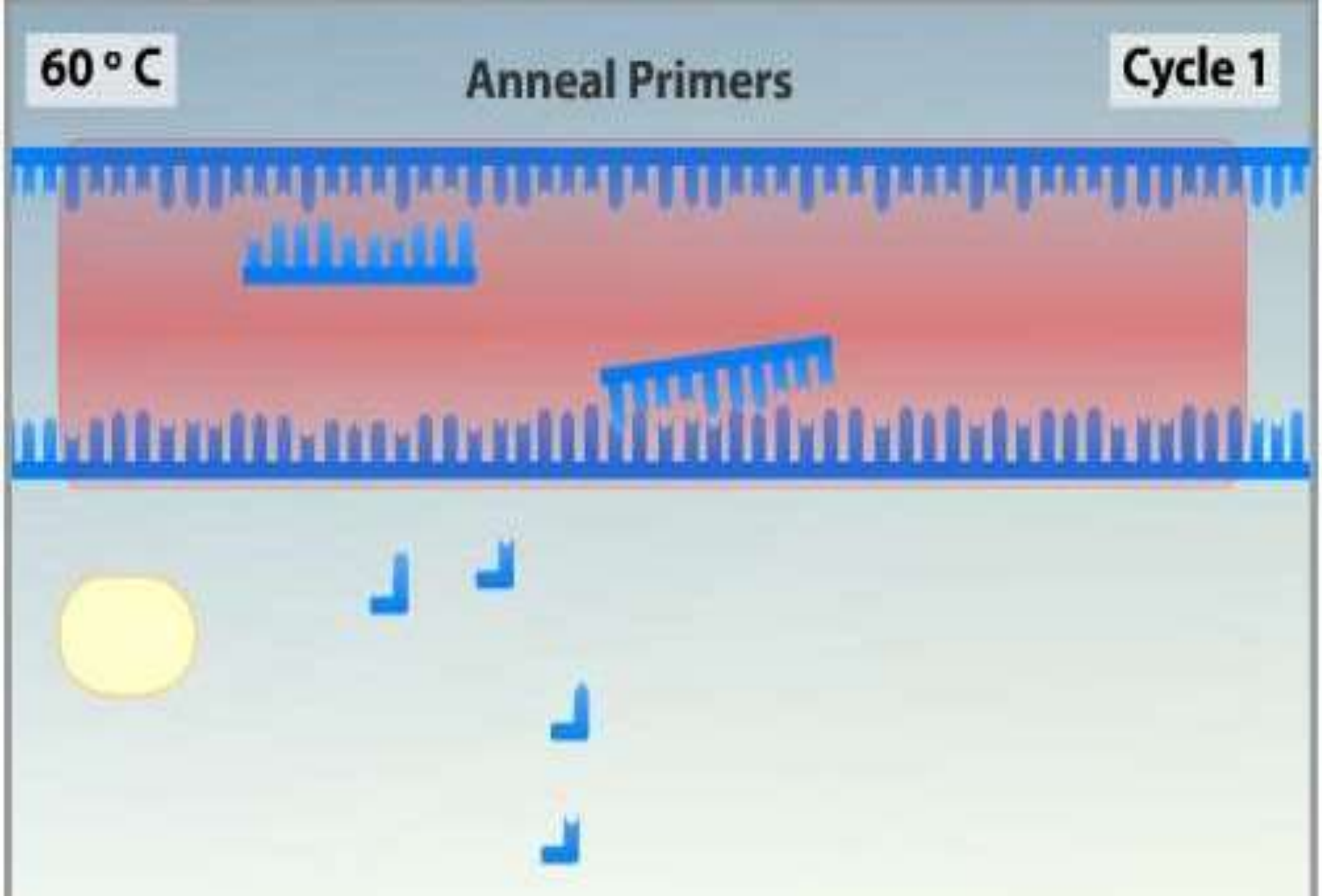

Polymerase Chain Reaction (PCR)
DNA
DNA segment of interest
DNA sample

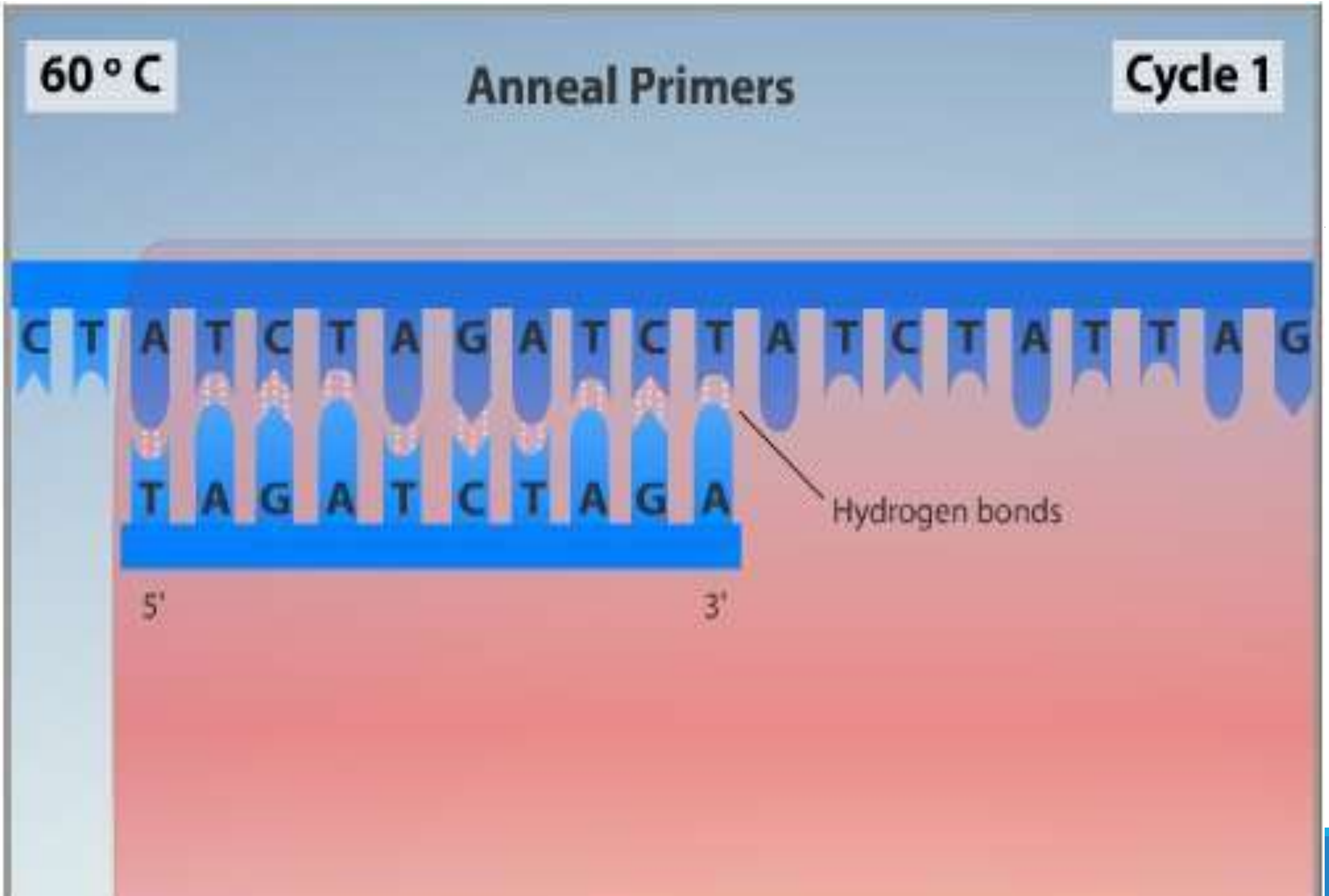

Target DNA

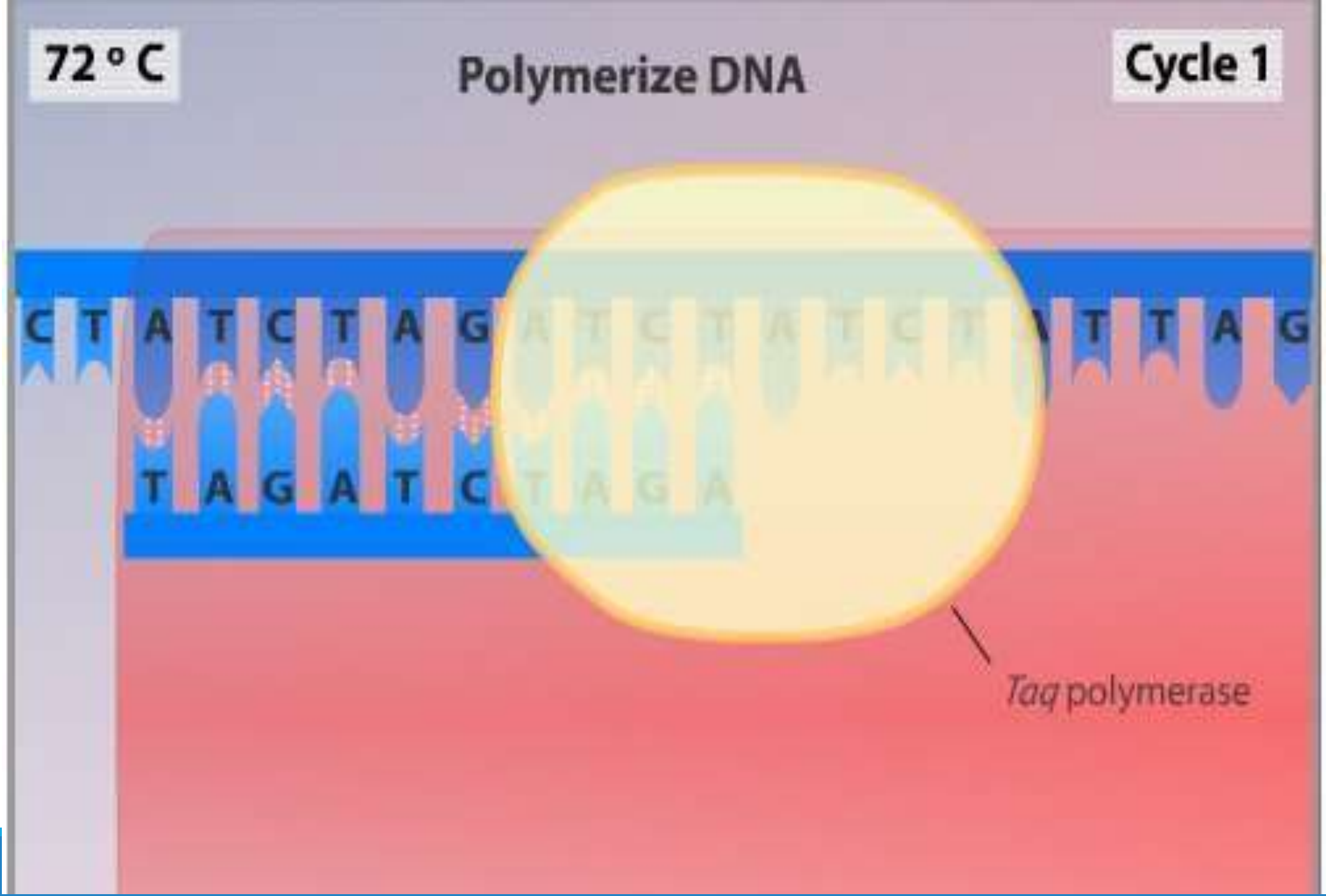

95 °C
Cycle 1
Target DNA
Primers
Taq polymerase
Nucleotides

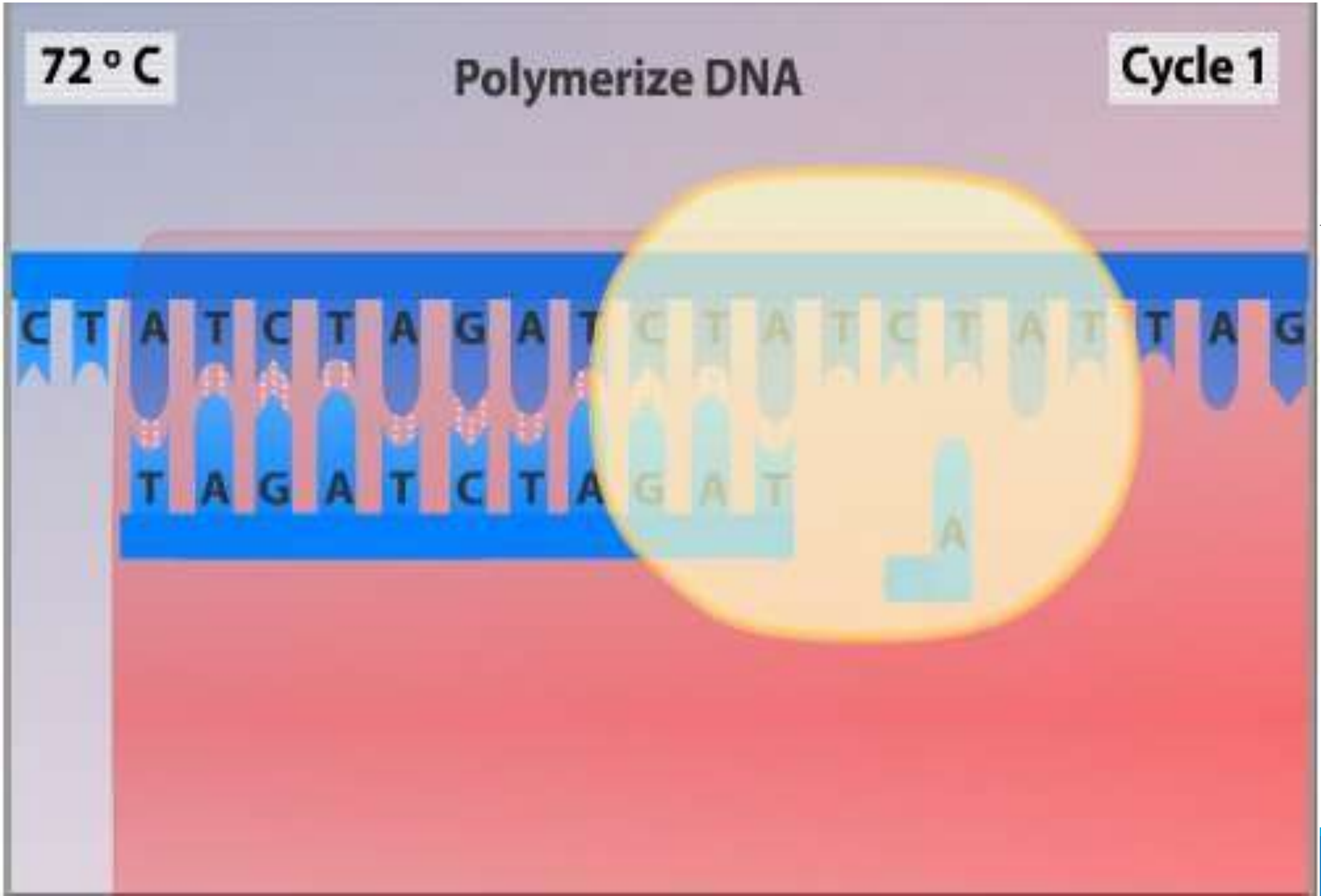

95 ° C
Denature DNA
Cycle 1

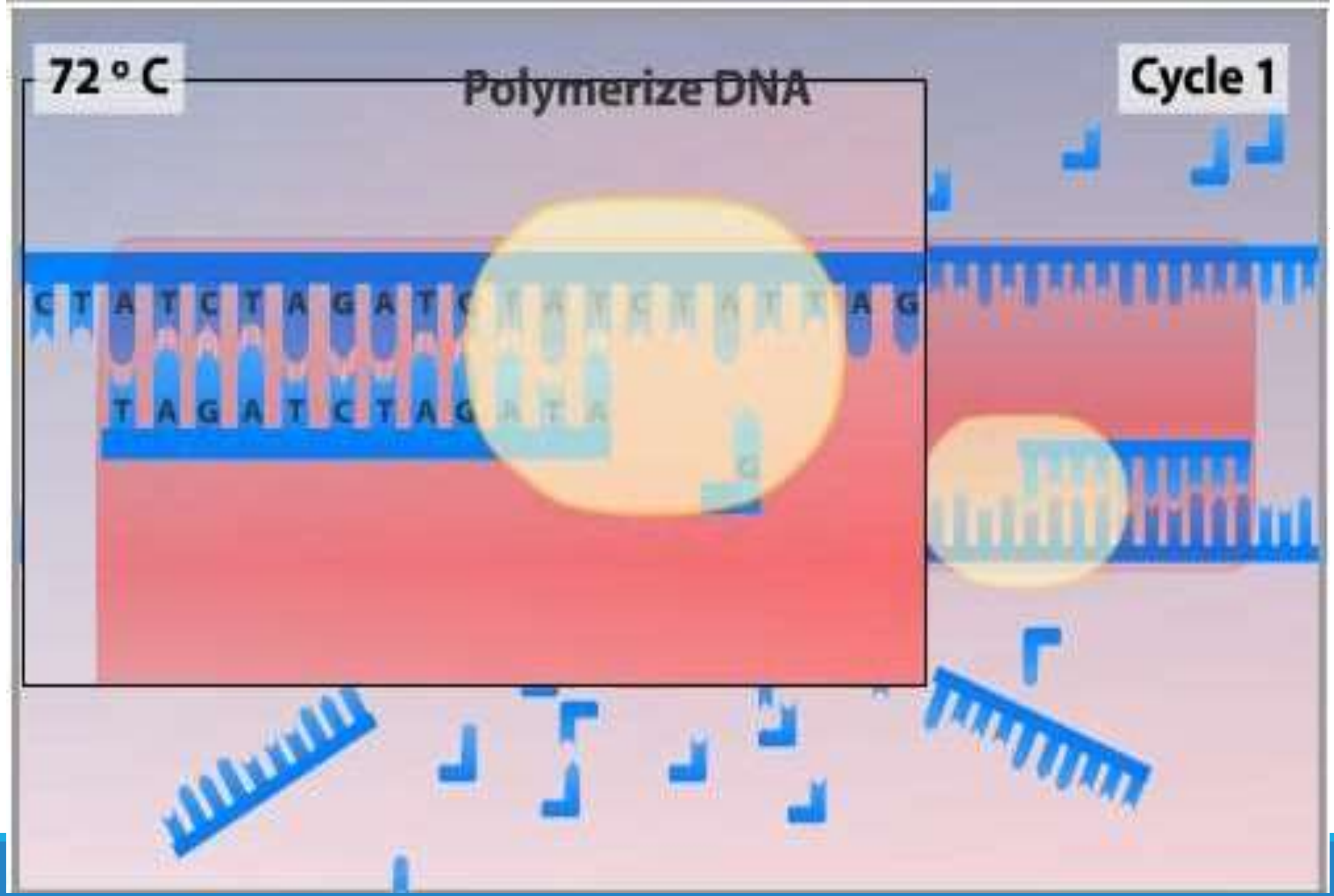

60 ° C
Denature DNA
Cycle 1

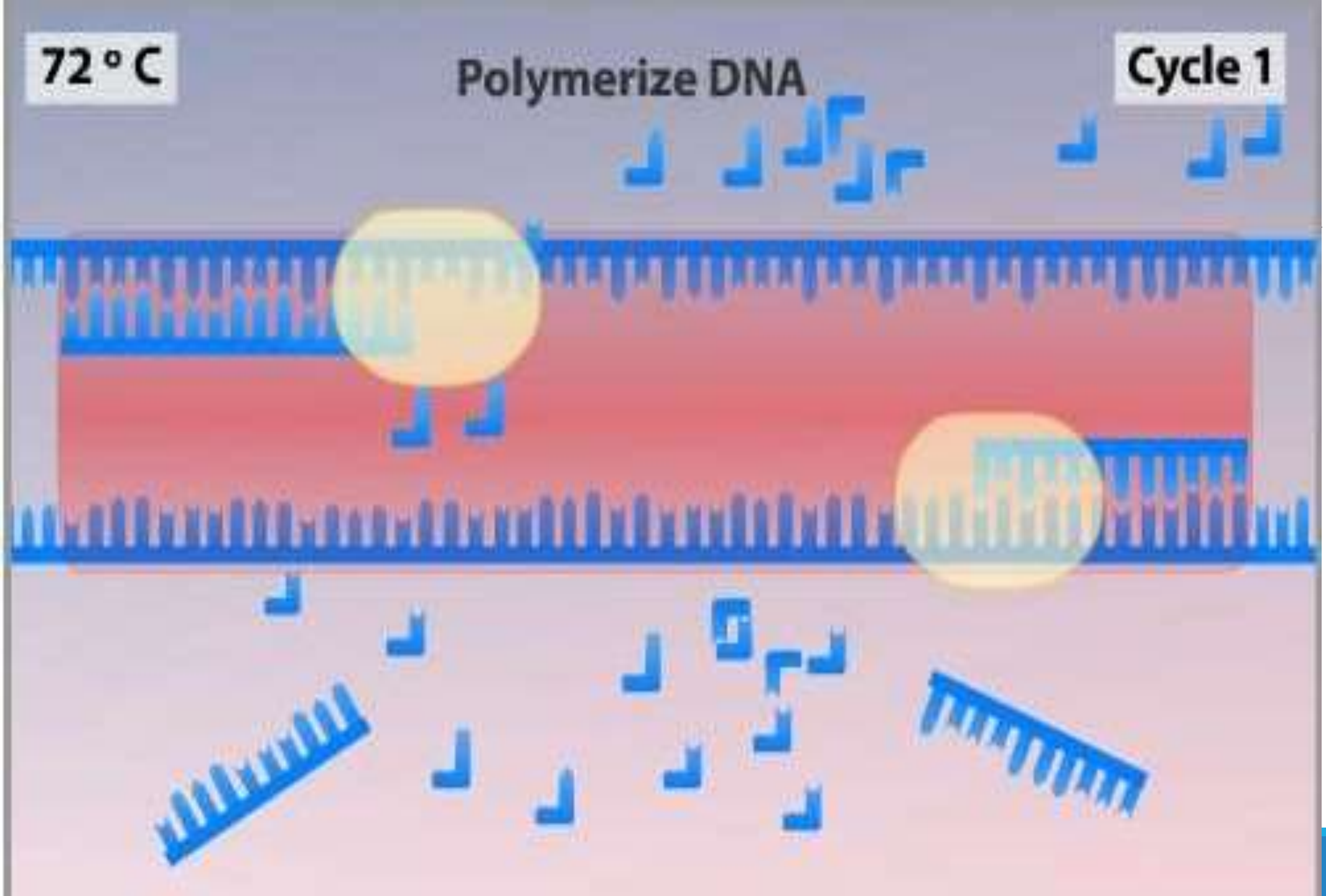

60 ° C
Anneal Primers
Cycle 1

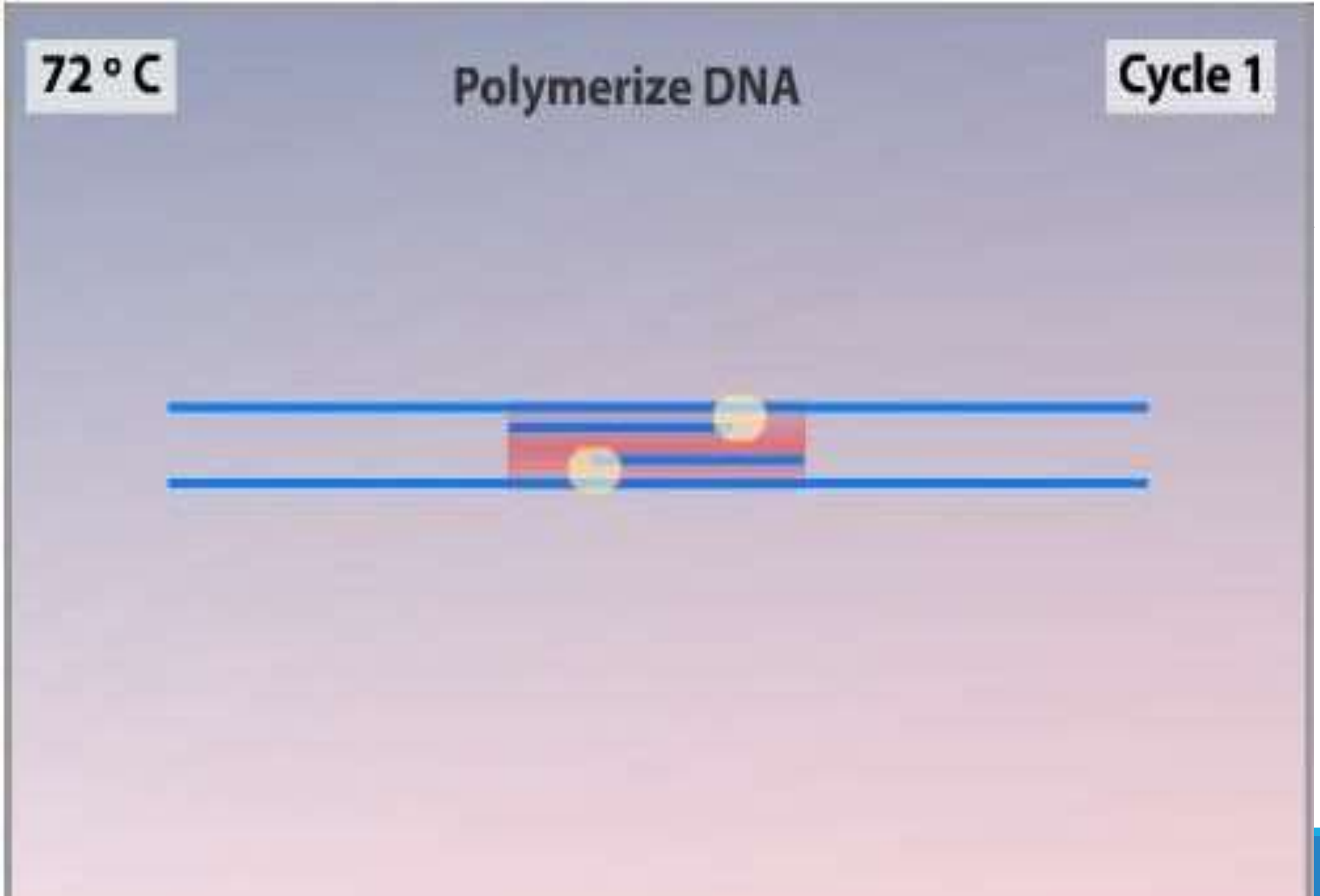

60 ° C
Anneal Primers
Cycle 1
C T A T C T A G A T C T A T C T A T T A G
T A G A T C T A G A
Hydrogen bonds
5'
3'


72 ° C
Polymerize DNA
Cycle 1
C T A T C T A G
T A G A T C
T T A G
Taq polymerase


72 ° C
Polymerize DNA
Cycle 1
C T A T C T A G A T
T A G A T C T A


72 ° C
Polymerize DNA
Cycle 1
C T A T C T A G A T C
T A G A T C T A G

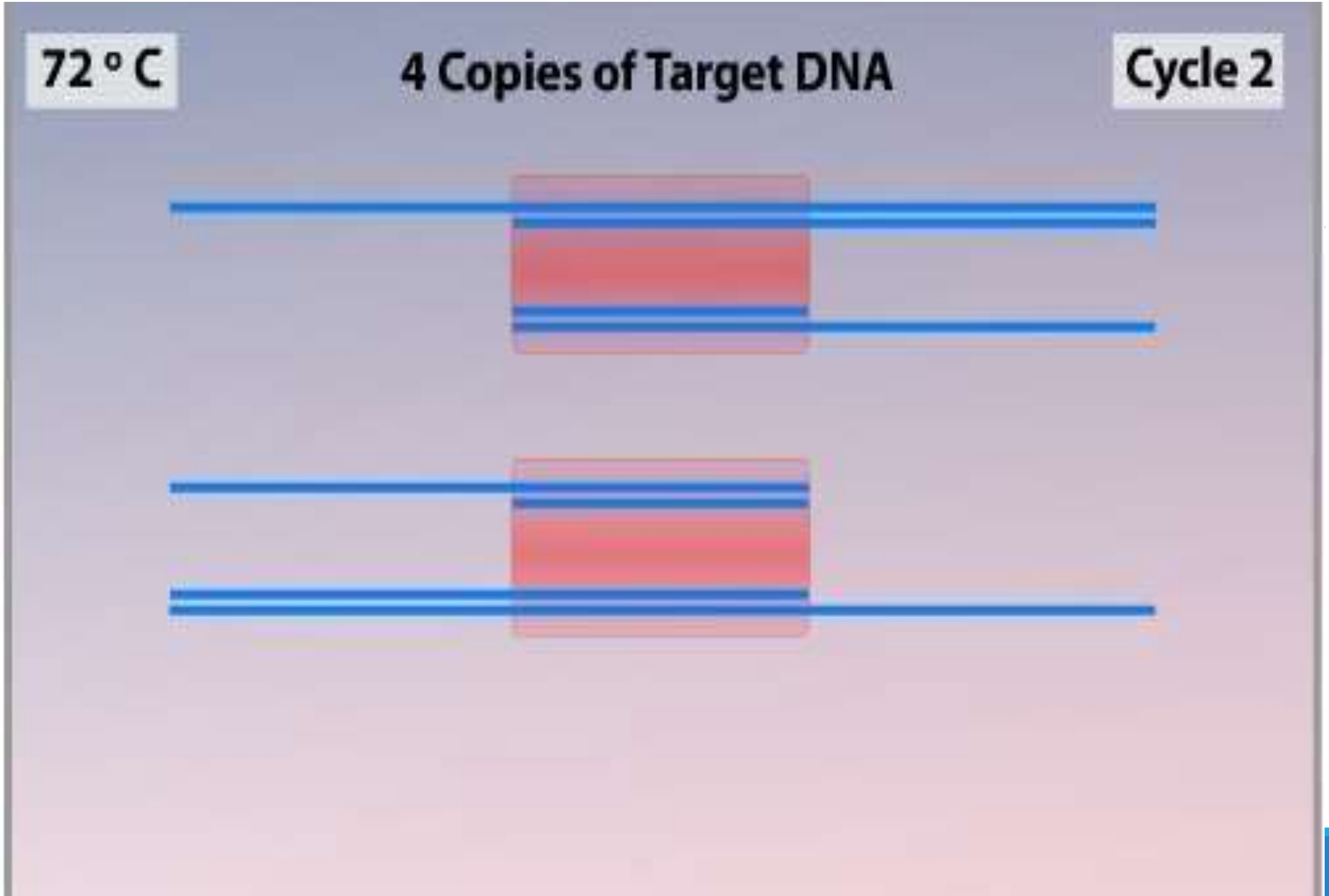

72 ° C
Polymerize DNA
Cycle 1

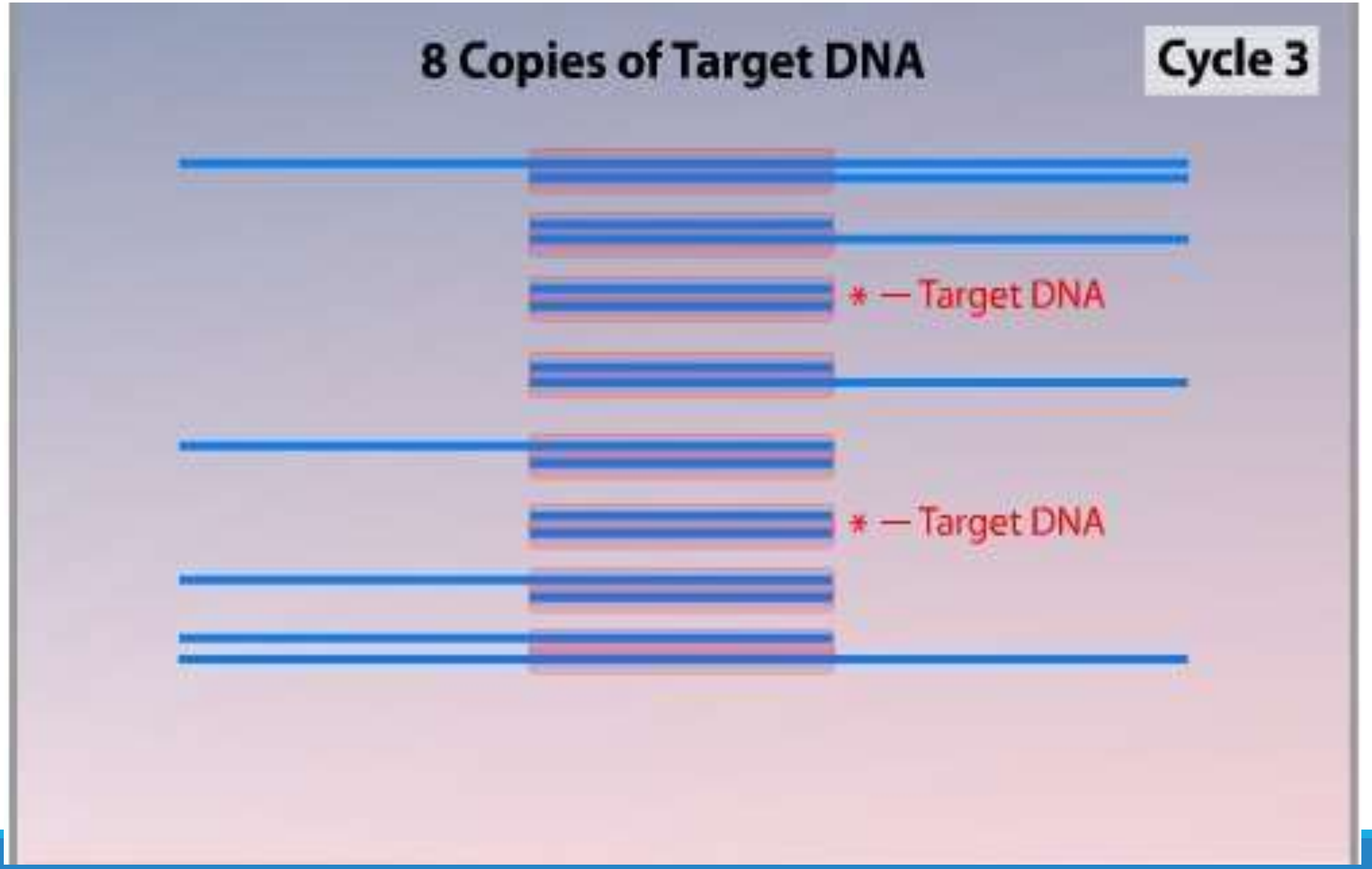

72 ° C
Polymerize DNA
Cycle 1

72 ° C

Polymerize DNA

Cycle 1

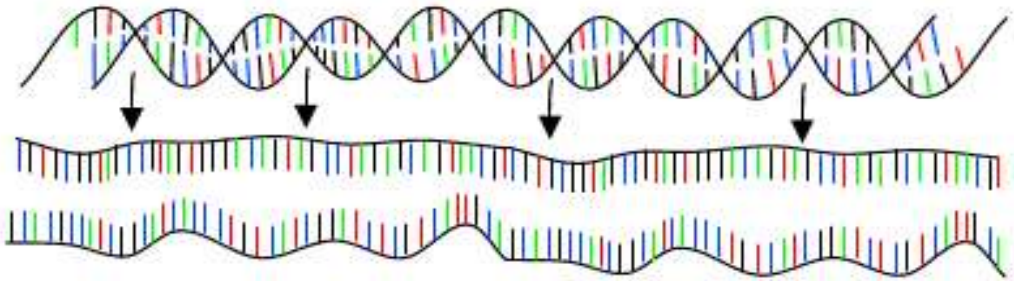

72 ° C
4 Copies of Target DNA
Cycle 2


8 Copies of Target DNA
Cycle 3
* — Target DNA
* — Target DNA


16 Copies of Target DNA
Cycle 4

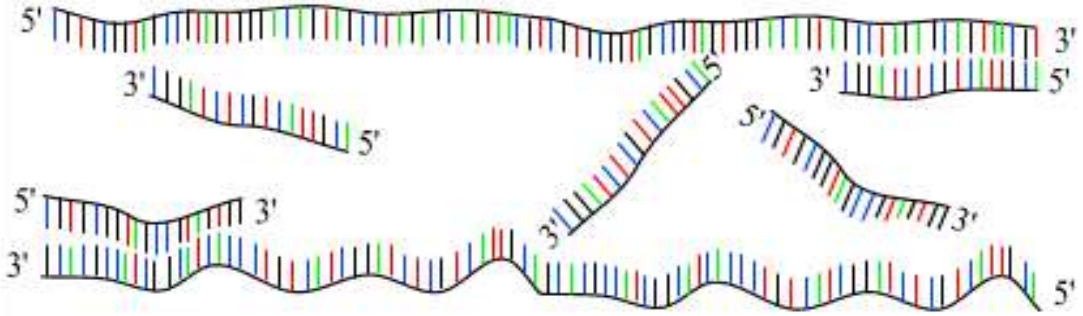
PCR : Polymerase Chain Reaction

30 - 40 cycles of 3 steps :



Step 1 : denaturation

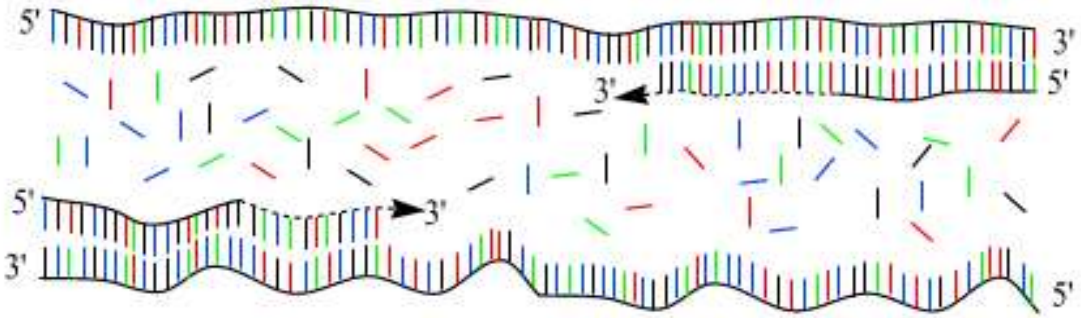
1 minut 94 °C



Step 2 : annealing

45 seconds 54 °C

forward and reverse primers !!!



Step 3 : extension

2 minutes 72 °C

only dNTP's

(Andy Vierstraete 1999)

- A reaction that runs for 30 cycles will amplify 2^{30}, or one billion fold.

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i.e. No of copies = $2^{\text{no of cycles carried out}}$

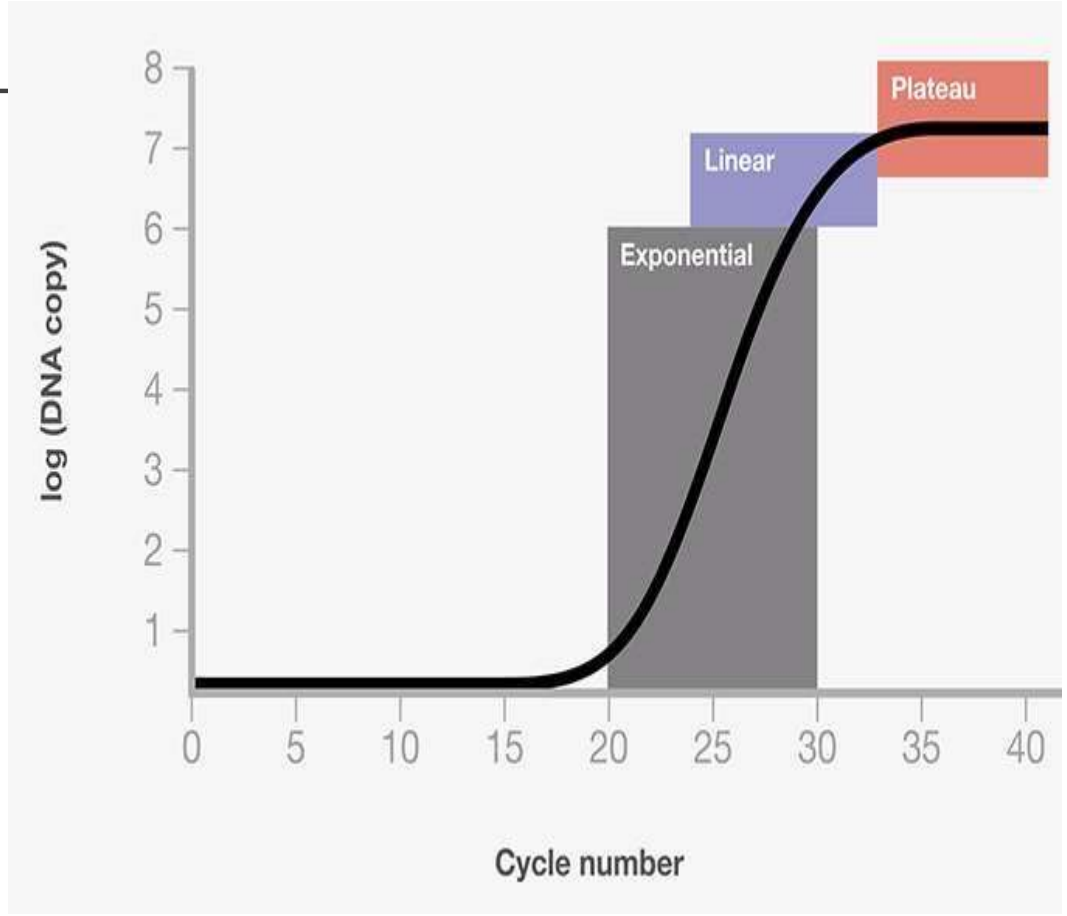
- Most PCR assays plateau after 30 cycles.
- The plateau occurs when:
  - The reagents are depleted
  - The products re-anneal
  - The polymerase is damaged
  - Unwanted products accumulate
  - Substrate excess

Stages in PCR:

The efficiency of PCR are affected by limiting factors. Thus, the entire PCR process can further be divided into three stages based on reaction progress:

- *Exponential amplification*: At every cycle, the amount of product is doubled (assuming 100% reaction efficiency). After 30 cycles, a single copy of DNA can be increased up to 1 000 000 000 (one billion) copies.

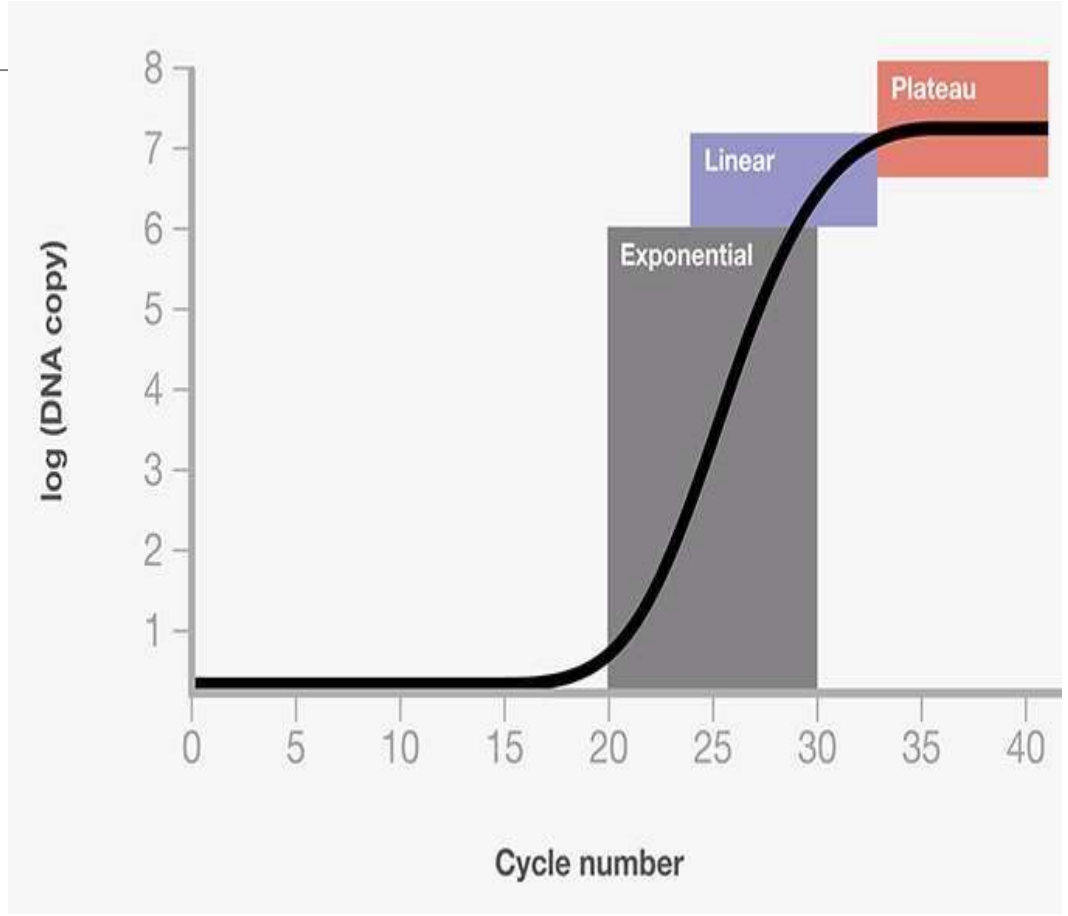
**The reaction is very sensitive: only minute quantities of DNA must be present.



Stages in PCR:

- *Leveling off stage*: The reaction slows as the DNA polymerase loses activity and as consumption of reagents such as dNTPs and primers causes them to become limiting.

- *Plateau*: No more product accumulates due to exhaustion of reagents and enzyme.



Why “Polymerase chain reaction***”

??

It’s a mean of selectively amplifying a particular segment of DNA.

The segment may represent a small part of a large and complex mixture of

DNAs. It act as molecular photocopier.

It is DNA replication in a test tube.

Components of PCR

I. DNA template that contains the DNA region (target) to be amplified.

II. Two primers that are complementary to the 3’ ends of each of the sense and anti- sense strand of the DNA target.

III. Taq polymerase or another Thermo-stable DNA polymerase with a temperature optimum at around 70 °C.

IV. Deoxynucleoside triphosphates(dNTPs)

V. Buffer solutions, (Tris, ammonium ions and/or potassium ions, magnesium ions)

Components of PCR

I. DNA template that contains the DNA region (target) to be amplified.

- Any source of tissue is used to PCR-based diagnosis because the technique does not require the presence of intact, high molecular weight DNA.
- DNA-PCR can be performed from formalin-fixed, paraffin-embedded tissue blocks.
- RNA-PCR : bone marrow smears.
- It is difficult to perform PCR on unmanipulated whole blood because the hemoglobin inhibits the activity of the *Taq* polymerase.

Components of PCR

I. DNA template that contains the DNA region (target) to be amplified.

- Dried blood
- Semen stains
- Vaginal swabs
- Single hair
- Fingernail scrapings
- Insects in Amber
- Egyptian mummies
- Buccal Swab
- Toothbrushes

Components of PCR

I. DNA template that contains the DNA region (target) to be amplified.

II. Two primers that are complementary to the 3’ ends of each of the sense and anti-
sense strand of the DNA target.

- These can be purchased from a variety of different companies.
- The oligos are usually 18–25 bases in length.
- Short DNA fragments containing sequences complementary to the target region
- No complementarity at the 3' ends of the primer pair because this may result in the primers annealing to each other rather than to the target DNA, with the creation of a so called primer-dimer product rather than the desired product.
- It is essential to have large molar excess of primers to ensure sufficient amounts throughout the exponential progression of target
- Introduction
- processing

Components of PCR

I. DNA template that contains the DNA region (target) to be amplified.

II. Two primers that are complementary to the 3’ ends of each of the sense and anti-sense

III. strand of the DNA target.

Polymerase or another Thermo-stable DNA p

temperature optimum at around 70 °C.

- They are derived from microorganisms that live in high-temperature environment (hot springs).

These includes :

- **Thermus aquaticus (Taq),**
- Thermus thermophiles (Tth) and
- Thermotoga maritime (Tma)

Components of PCR

I. DNA template that contains the DNA region (target) to be amplified.

II. Two primers that are complementary to the 3’ ends of each of the sense and anti-sense strand

III. of the DNA target.

Polymerase or another Thermo-stable DNA polymerase with a temperature optimum at around 70 °C.

Catalyzes the elongation of DNA by adding nucleoside triphosphates to the 3’ end of the growing strand.

DNA polymerase can *only* add nucleotides to 3’ end of growing strand. DNA polymerase fidelity is expressed in terms of error rate (ER)

$$ER = \frac{\textit{number of mutations per bp}}{\textit{number of amplicon doublings}}$$

Components of PCR

I. DNA template that contains the DNA region (target) to be amplified.

II. Two primers that are complementary to the 3’ ends of each of the sense and anti-sense strand

III. of the DNA target.

Taq polymerase or another Thermo-stable DNA polymerase with a temperature optimum at around 70 °C.

IV. Deoxynucleoside triphosphates(dNTPs) Like dATP, dTTP, dGTP, dCTP

Components of PCR

I. DNA template that contains the DNA region (target) to be amplified.
II. Two primers that are complementary to the 3’ ends of each of the sense and anti-sense strand of the DNA target.
III. Taq polymerase or another Thermo-stable DNA polymerase with a temperature optimum at around 70 °C.
IV. Deoxynucleoside triphosphates(dNTPs)
V. Buffer solutions

- These are usually supplied along with the Taq polymerase (to provide it a suitable chemical environment for optimum activity and stability.) and consequently manual preparation may no longer be required.
- *bivalent cations*, typically magnesium (Mg) or manganese (Mn) ions; Mg^{2+} is the most common, but Mn^{2+} can be used for PCR-mediated DNA mutagenesis, as a higher Mn^{2+} concentration increases the error rate during DNA synthesis *monovalent cations*, typically potassium (K) ions

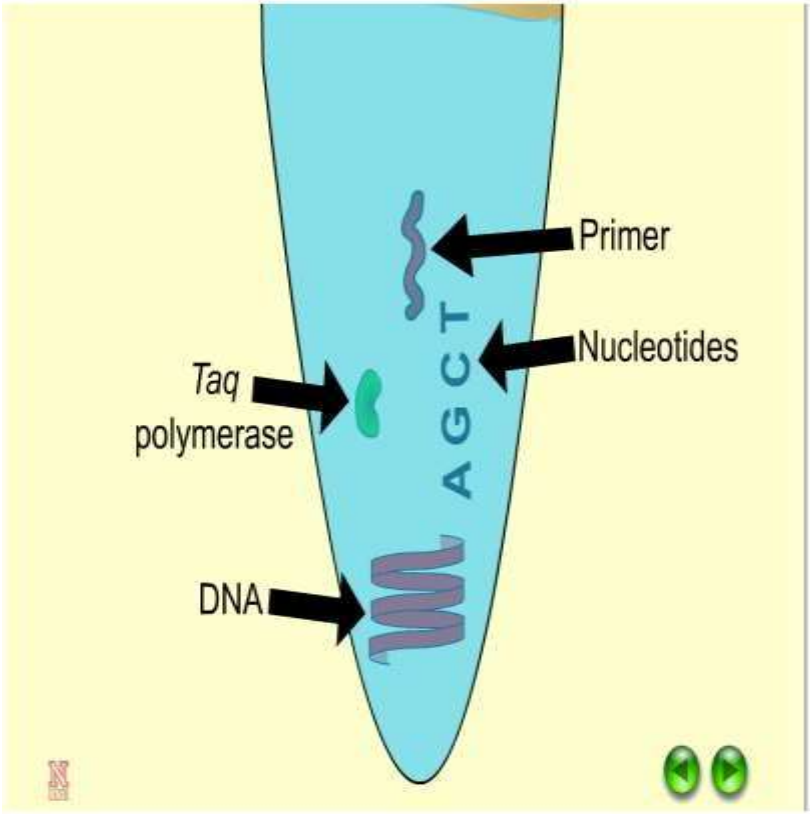
Components of PCR

I. DNA template that contains the DNA region (target) to be amplified.
II. Two primers that are complementary to the 3' ends of each of the sense and anti-sense strand of the DNA target.
III. Deoxynucleoside triphosphates(dNTPs)
III. Taq polymerase or another Thermo-stable DNA polymerase with a temperature optimum at around 70 °C.
V. Buffer solutions

Three different buffers can be prepared as follows:

- *×10 PCR buffer I*: 100 mmol/l Tris-HCl, pH 8.3, 500 mmol/l KCl, 15 mmol/l $MgCl_2$, 0.1% (w/v) gelatine, 0.5% (v/v) NP40, and 0.5% (v/v) Tween 20.
- *×10 PCR buffer II*: 670 mmol/l Tris, pH 8.8, 166 mmol/l $(NH_4)_2SO_4$, 25 mmol/l $MgCl_2$, 670 µmol/l Na_2EDTA, 1.6 mg/ml bovine serum albumin (BSA), and 100 mmol/l β mercaptoethanol. This buffer is used in conjunction with 10% dimethyl sulphoxide (DMSO) in the final reaction mixture.
- *×10 PCR buffer III*: 750 mmol/l Tris, pH 8.8, 200 mmol/l $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20. A solution of 25 mmol/l $MgCl_2$ is also prepared and added separately to the PCR reaction.

PCR thermocycler



PCR Thermocycler

Selection criteria for primers

- Primers are selected by virtue of their position - flanking the sequence of interests.
- The ratio of G+C: A+T in the primer (of 15 to 30 base pairs) should range between
  40% and 60% ie 2:3.
- No long stretches of polypurines or polypyrimidines
- In any PCR reaction, there should be large molar excess of primers to ensure sufficient amounts throughout the exponential progression of target production.
- **Primer melting temperatutre (Tm) = 4 (G+C) + 2 (A+T)**

-Temperature at which one half of the DNA duplex will dissociate to single strand

-Range 52 – 58^{o} C

- The PCR is commonly carried out in a reaction volume of 10– 200 µl in small reaction tubes (0.2–0.5 ml volumes) in a thermal cycler.

The Thermal cycler

- The thermal cycler heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction.
- This is done on an automated cycler, which can heat and cool the tubes with the reaction mixture in a very short time.
- The exact temperature and incubation time required for each step depends on factors like :
  - The length of the target DNA and
  - GC content of the primer/template

How do we know gene has been copied or not?

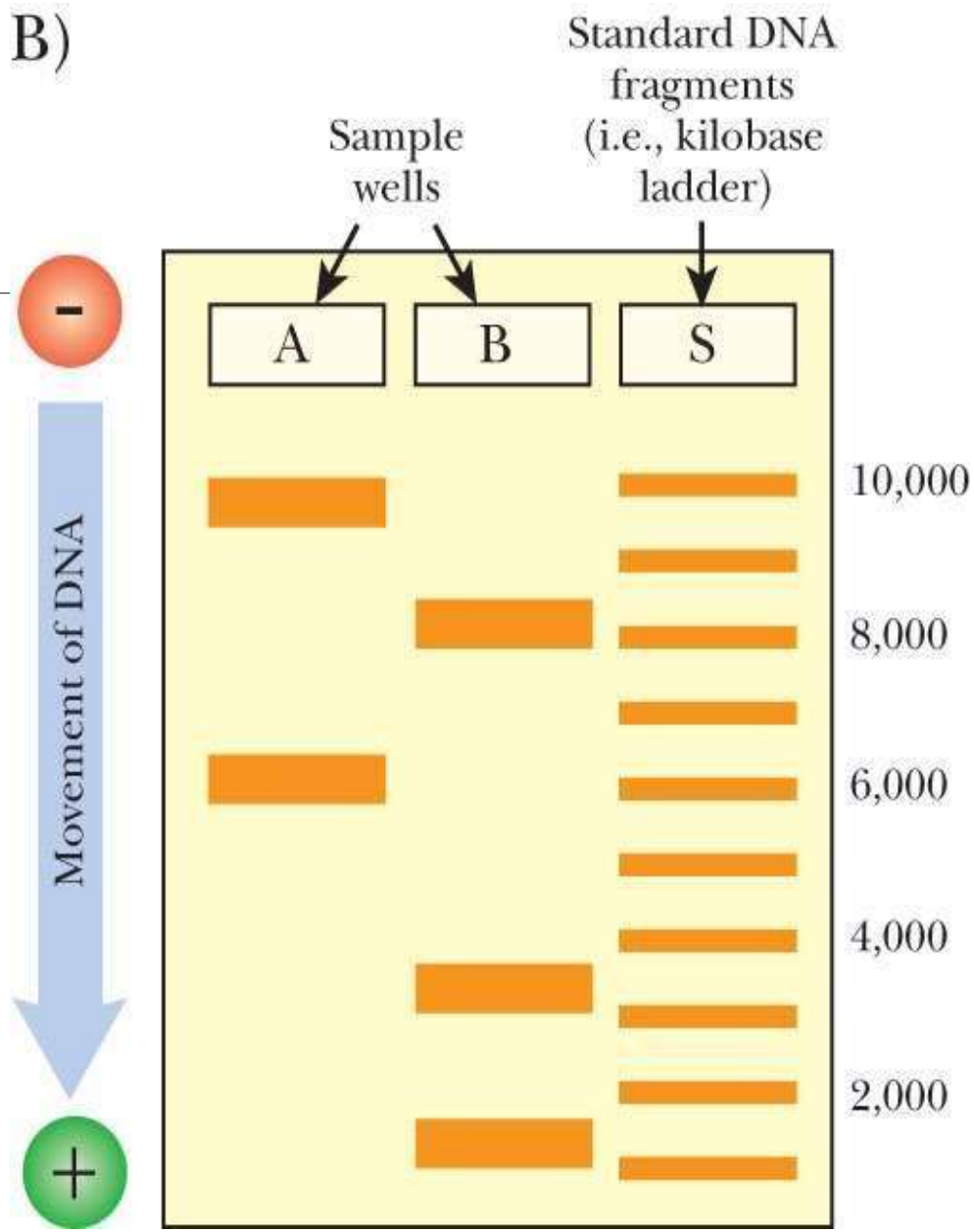
The amplified product obtained at the end of the cycle can be visualized in an electrophoretic gel stained with ethidium bromide.

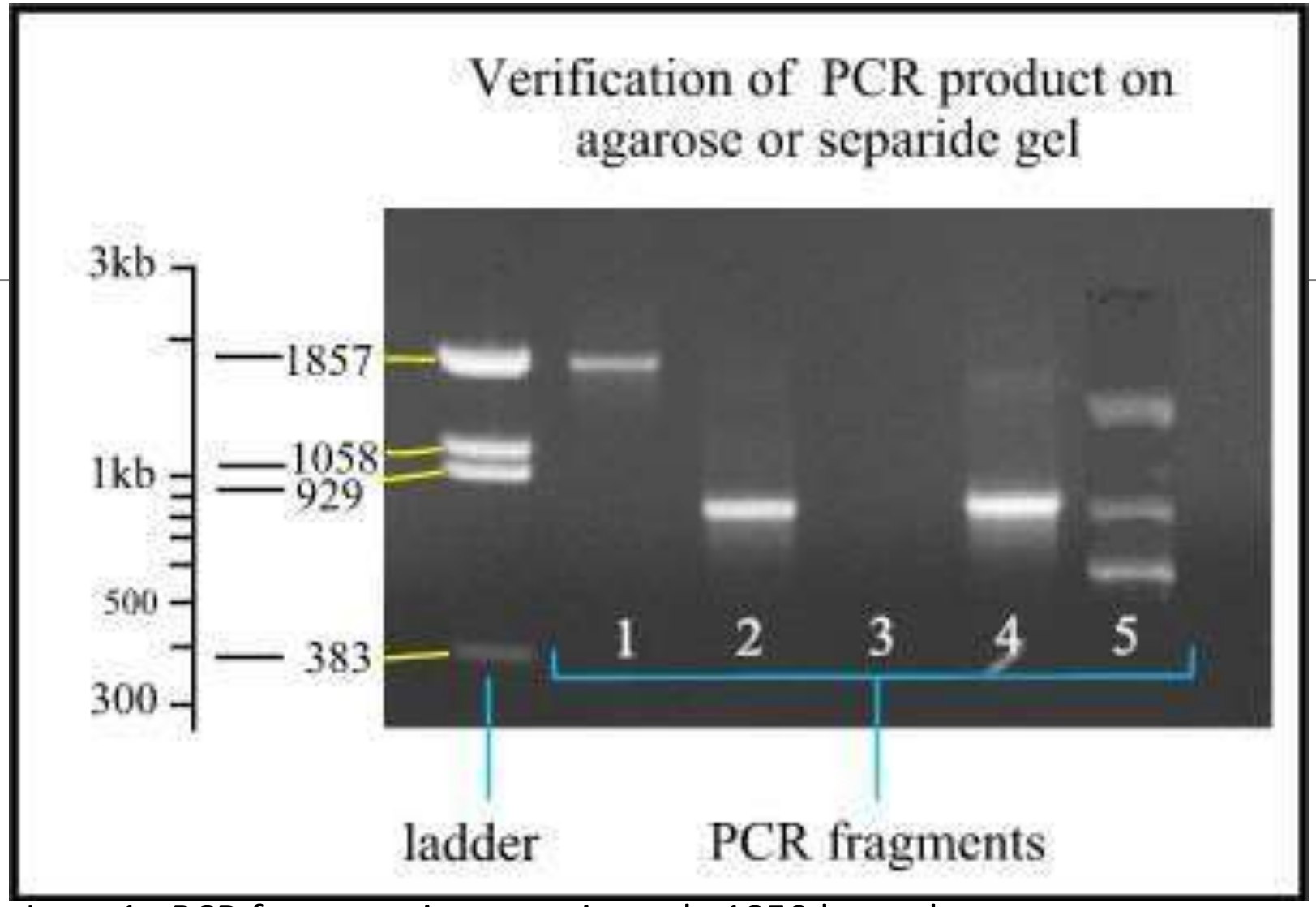
Look for

- Is there a product formed?
- Is the product of the right size ?
- Is there only one band formed or different bands in one lane ?

Why electrophoresis is needed ?

-To separate DNA fragments from each other
-To determine the sizes of DNA fragments
-To determine presence or amount of DNA
-To analyze restriction digestion products





Lane 1 : PCR fragment is approximately 1850 bases long.
Lane 2 and 4 : the fragments are approximately 800 bases long.
Lane 3 : no product is formed, so the PCR failed.
Lane 5 : multiple bands are formed because one of the primers fits different

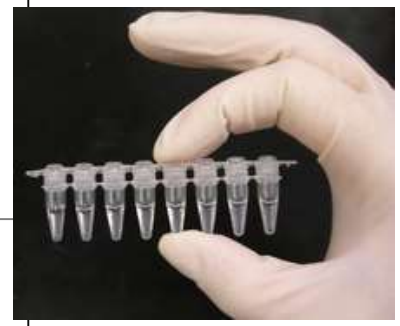
Detailed Procedur e

Prepare a PCR mixture for 20 reactions (with a final volume of 25 µl for each DNA sample)

Stock Solution	Vol (µl)	Final Concentration
×10 PCR buffer III	50	×1
25 mmol/l $MgCl_2$	40	2.0 mmol/l
10 mmol/l dNTP	10	0.02 mmol/l
10 µmol/l Primer (1)	20	0.04 µmol/l
10 µmol/l Primer (2)	20	0.04 µmol/l
5 u/µl Taq polymerase	2	0.02 µ/ml
Water	358	
Final volume	*500*	

Add the Taq polymerase last, mix well and pulse-spin in a microcentrifuge to bring down the contents of the tube.

*Aliquot 24 µl of the PCR reaction mix into each tube.
Add 1 µl of template DNA at approximately 0.05 ng/ml into all the reaction tubes.



Place the tubes in a PCR machine (thermocycler), using a heated lid, Programmed for three steps (already d

While the PCR program is running, a 1.5% agarose** minigel is prepared

Visualise the DNA on an ultraviolet (UV) transilluminator and take a photograph.

Load the gel and run at a constant voltage of 100 V for 1 hour in ×0.5 TBE buffer. A molecular size marker should be included to establish the size of the amplified fragment; these are commercially available.

To check if the amplification has been successful, add 1 µl of tracking dye to a 10-µl aliquot of the PCR reaction mixture, being careful not to pipette the mineral oil overlaying the PCR reaction.

Amplification methods

- The molecular diagnostic laboratory depends upon amplification technique- to study the small amounts of nucleic acids that are of interest in clinical samples.
- The human genome is so large that it is difficult to detect small changes in one small
part of the genome.
- Techniques that increases the amount of the target nucleic acid or the detect the signals of a unique sequence of interest are referred to as amplification methods.

- Target amplifications – The nucleic acid region around the area of interest is copied many times by in vitro methods.

- Signal amplifications – The amount of target stays the same, but the signal is increased by several methods including *sequential hybridization of branching nucleic acid structure* and *continuous enzyme action on substrate that may be recycled*.

Post - PCR

Following PCR, the amplified product is processed and the DNA is subjected to agarose or polyacrylamide gel electrophoresis.

- In DNA fingerprinting,

  -used in criminal forensics, PCR-amplified DNA from the crime scene is compared to amplified DNA from suspects.

- In cloning experiments,

  -the amplified DNA is typically further purified and ligated into the desired vector.

- In DNA sequencing experiments,

  -the amplified DNA (usually radioactively labeled) is run on a thin polyacrylamide sequencing gel.

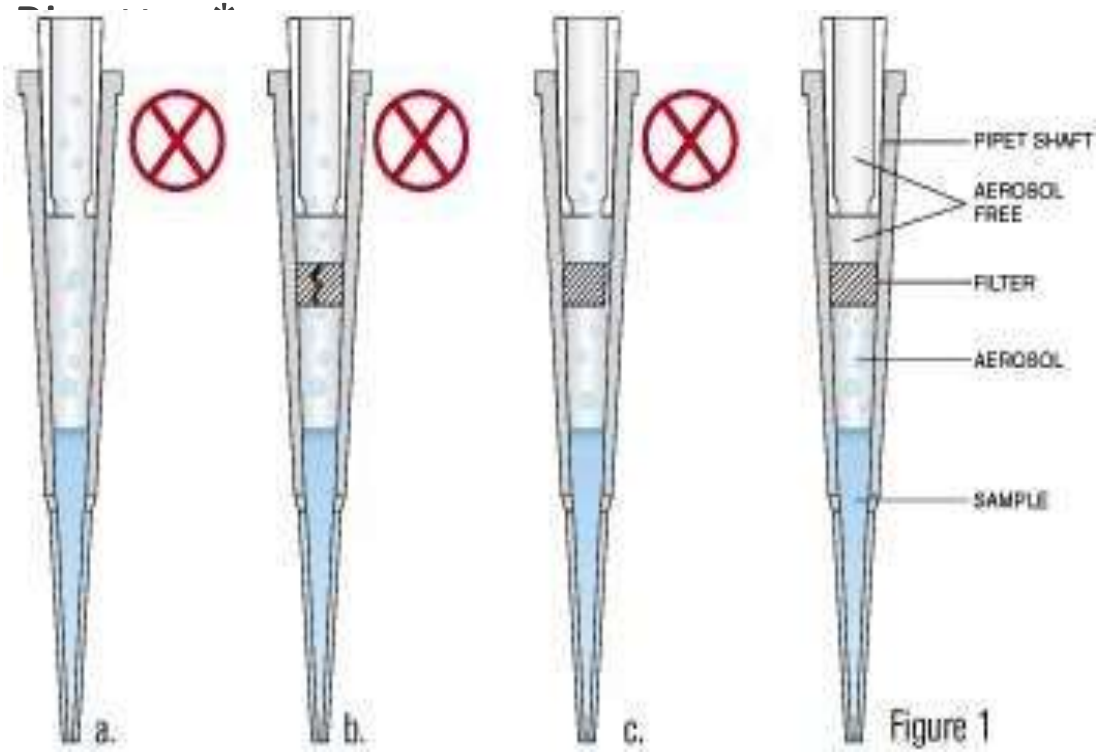
Quality control in PCR

- Prevention of contamination in PCR
- Highly sensitive – a small amount of contamination can
  easily produce a false positive results.
- The greatest potential of contamination comes from the amplification product (Amplicon).
- Amplicon can contaminate reagents, pipettes and glassware.

To avoid this

Physical methods

1) Barrier pipette tips to prevent aerosols / using Aerosol-Resistant



2) Frequently Changing Glove, Aseptic Cleaning Technique, Dedicated Consumables and Equipment

3) Unidirectional Workflow

- PCR mix reagents and samples that may contain templates for PCR should be prepared in the pre-PCR room only.

- Tubes that have undergone amplification in the post-PCR room contain amplicons (amplified template) and should never, under any circumstances, be opened or introduced in the pre-PCR room.*

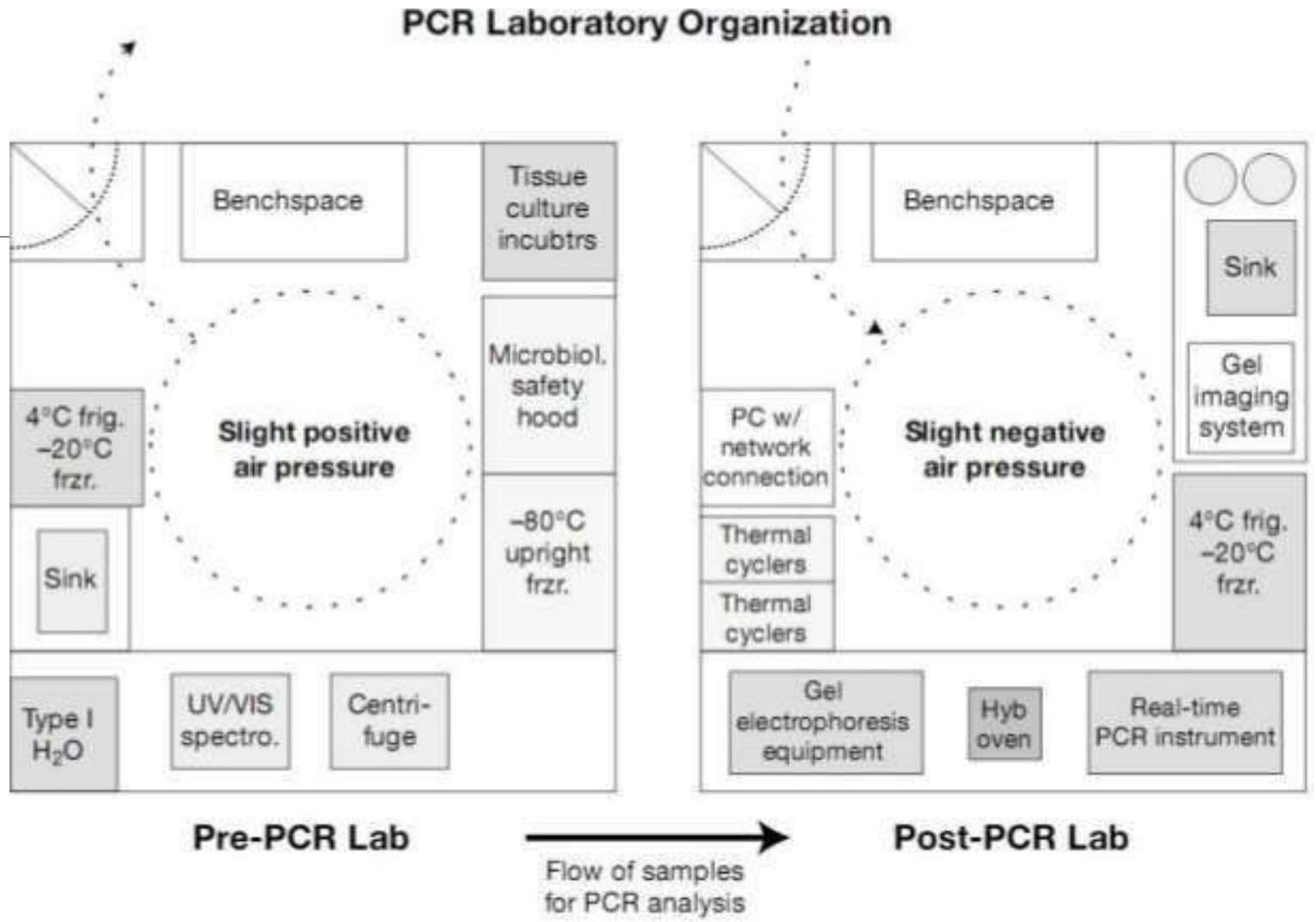
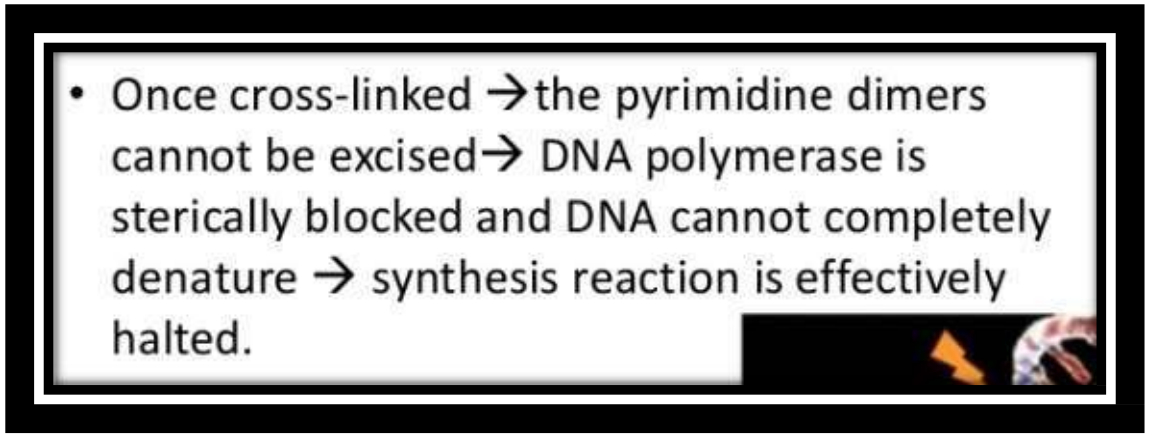


FIGURE 2. Organization of a PCR laboratory with separate pre- and post-PCR rooms.

Prevention of contamination Chemical methods

1) UV photolinking-

Adjacent pyrimidines on a DNA strand can be cross linked when exposed to UV light of 254 nm. Effective for bigger amplicons (>700bp)



Prevention of contamination Chemical methods

1) UV photolinking-

Adjacent pyrimidines on a DNA strand can be cross linked when exposed to UV light of 254 nm. Effective for bigger amplicons (>700bp)

2) Uracil-DNA-glycosylate (UDG) –

- During PCR dU substituted for dT in a DNA strand. In the final product there is dU instead of dT.
- So Before any new sample is processed, it first exposed to UDG enzyme.
- If UDG comes across any U- containing DNA strands, The U's are cleaved, leaving the strand with gaps. These abasic strands in next PCR cannot be amplified.

Prevention of contamination in PCR

3) Irradiation and addition of photoreactive chemicals :

It can make DNA uavailable for further amplification, or ‘sterile’ by cross linking the
complementary strands together or forming monoadducts

-Psoralenes and its derivatives

4) Nuclease treatment –Endonucleases, DNAse1 and exonuclease III

5) Chemical decontamination

-Hydroxylamine hydrochloride

-Hcl or sodium hypochlorite

Things to try if PCR does not work

IF NO PRODUCT OF CORRECT SIZE IS FORMED

i. Check DNA quality
ii. Reduce annealing temperature
iii. Increase magnesium conc.
iv. Use different thermostable enzymes
v. Make new primer stock

IF EXTRA SPURIOUS PRODUCT BAND PRESENT

i. Increase annealing temperature
ii. Reduced magnesium conc.
iii. Reduce number of cycles
iv. Try different enzymes

Advantages over conventional analysis

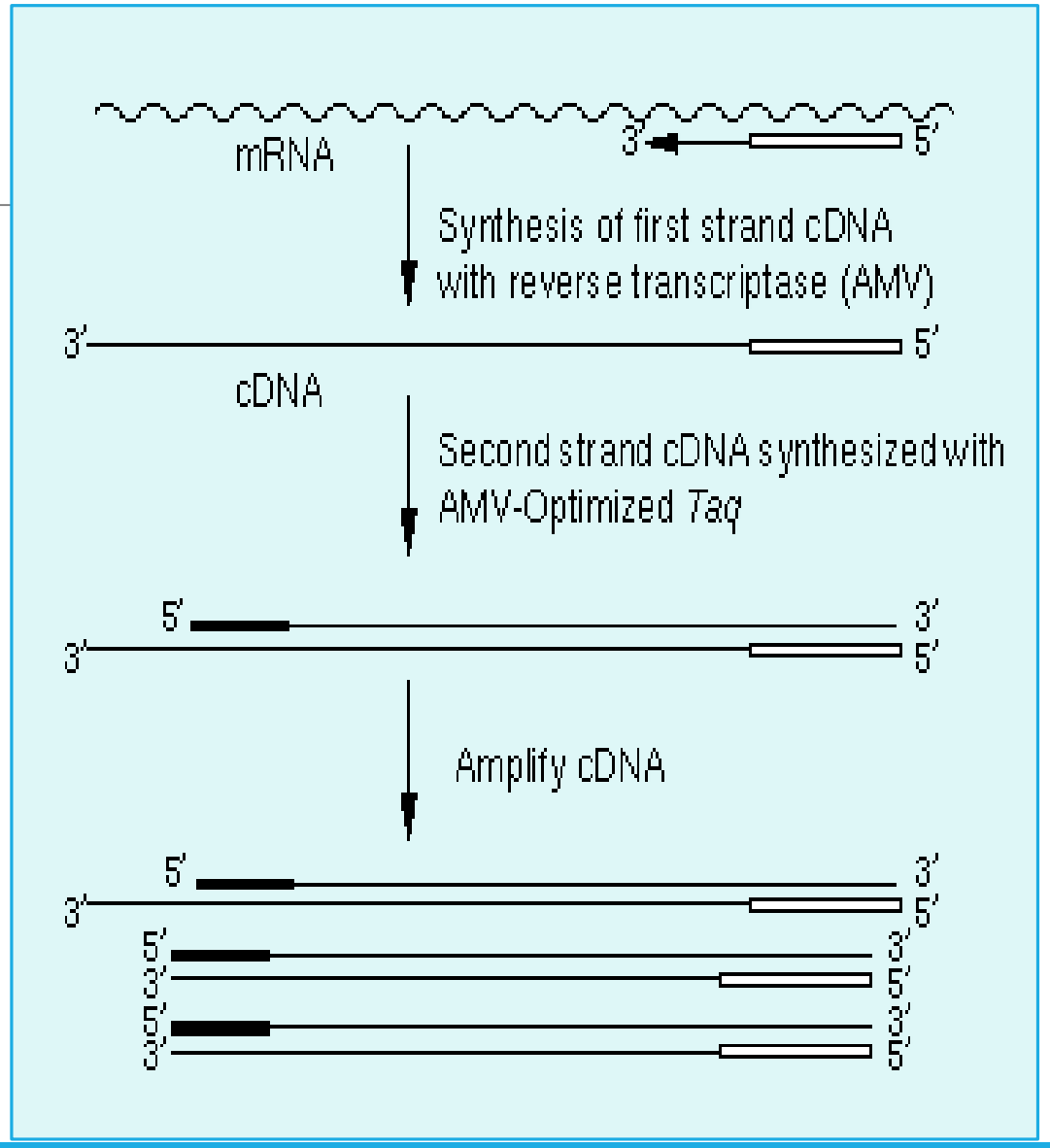
- Rapidity of the Assay – Few hours versus days.
- Use of automated thermal cyclers.
- No universal requirement for radioisotopes – Products can be directly visualized on ethidium bromide stained gel.
- Low level of starting DNA/RNA analyte – Picogram or nanogram versus micrograms.
- Sensitivity – at least 2 to 3 orders of magnitude less target sequence can be detected, even within a background of non target sequence.

Variants of PCR

RT-PCR: (ReverseTranscription PCR)

It is a method used to amplify, isolate or identify a known sequence from a cellular or tissue RNA.

- The PCR is preceded by a reaction using reverse transcriptase to convert RNA to cDNA.
- The cDNA thus formed serves as a template for subsequent PCR reaction.
- The Tth DNA polymerase is ideal as it also has RT activity thus overcoming the requirement for the use of 2 different enzymes.
- It can be performed on RNA extracted from minute volumes of whole blood and



2) In-Situ PCR:

- PCR is performed within intact cells, whether within tissue sections or on cytologic preparation.
- It can be used to detect specific DNA or RNA sequences *within cells* and has the ability to detect *a single copy of a specific gene*.
- The amplified product may be visualized by microscopy by using riboprobes or with direct incorporation of labeled nucleotides during the actual PCR reaction.
- Primarily used for the detection of *viral infections, such as HIV or HPV, in particular cell types.*

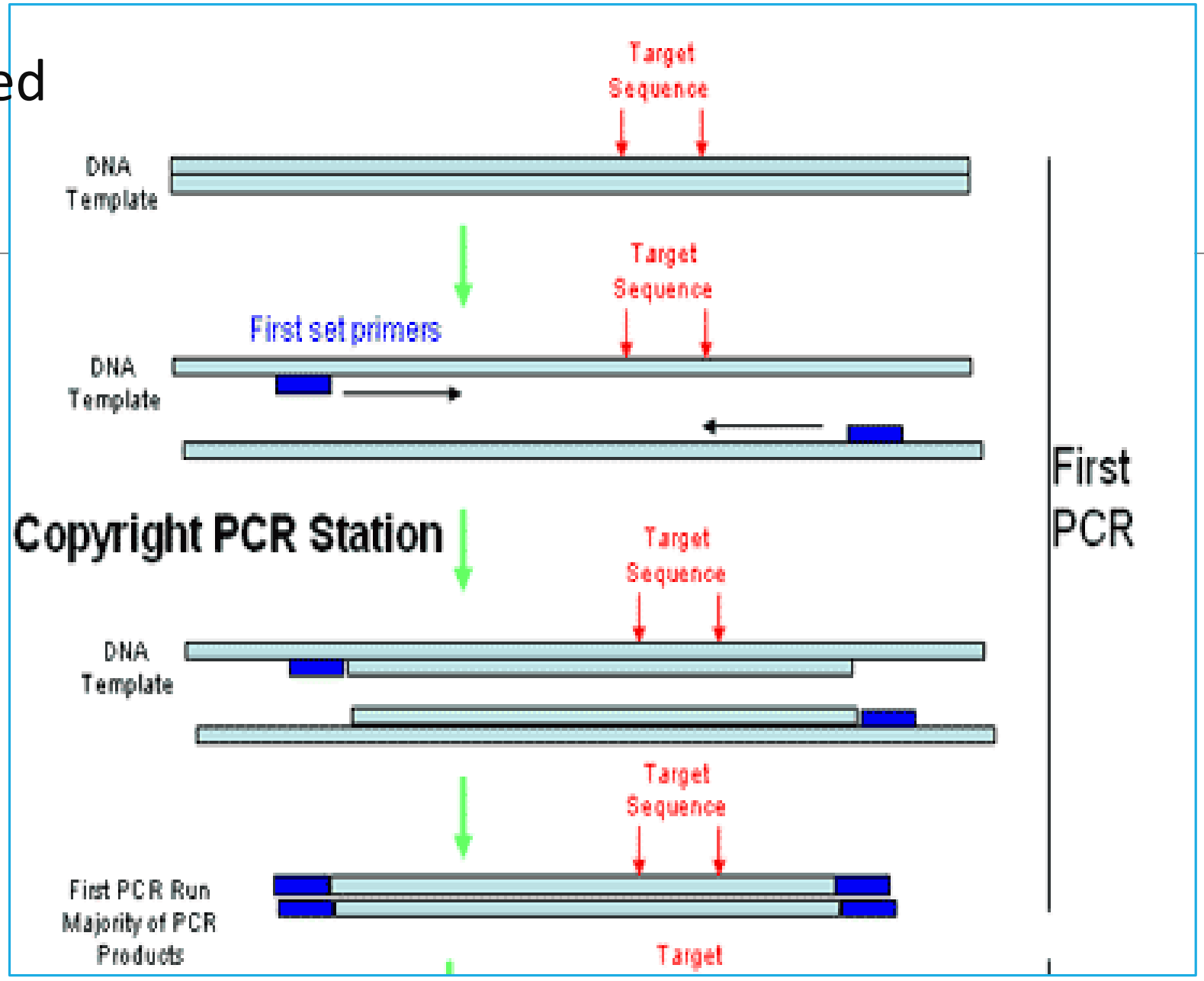
Labels used – Biotin, Digoxygenin and Fluroscein

False positive results – Fixed paraffin wax embedded tissue

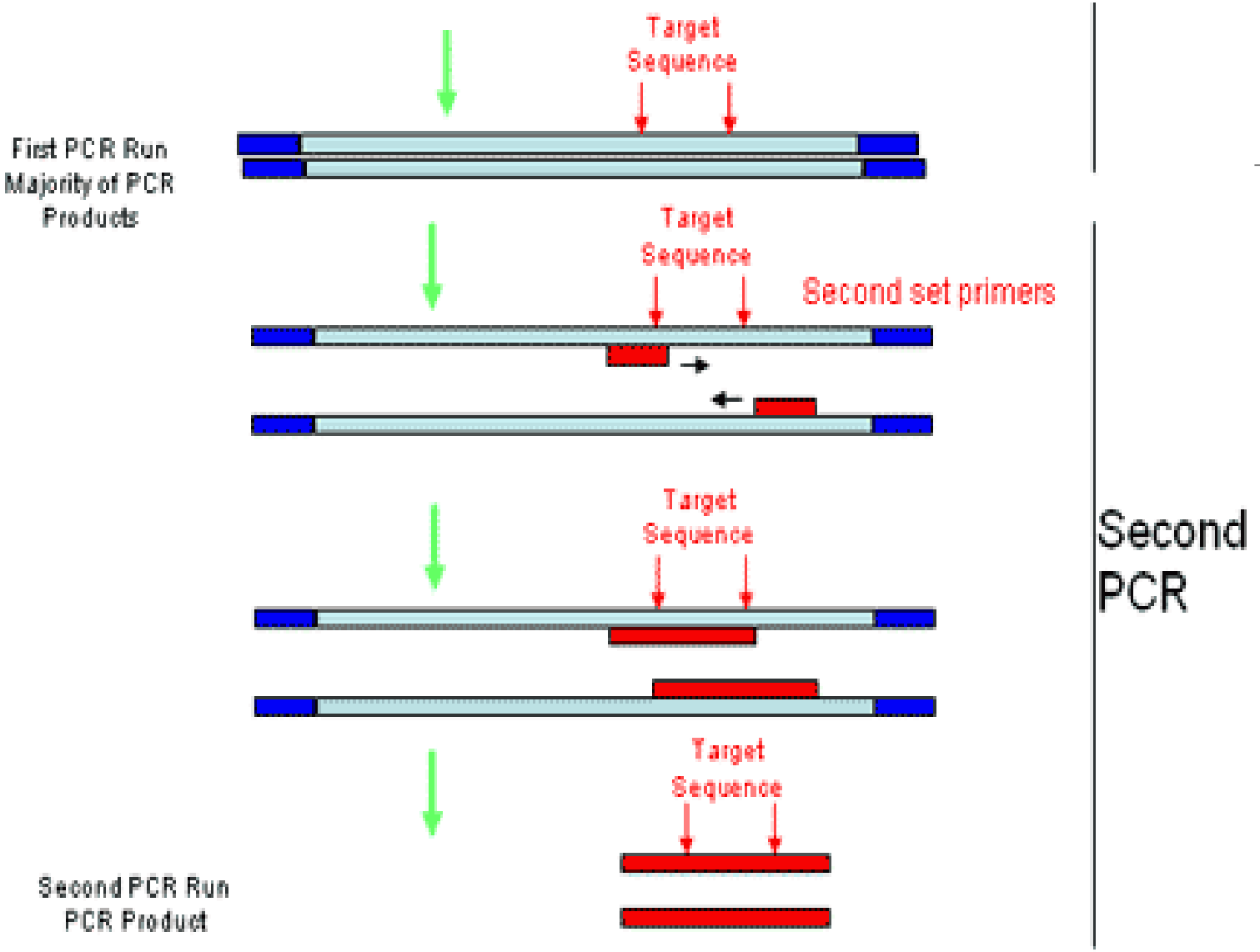
3) Nested PCR:

- It increases the specificity of DNA amplification, by reducing background due to non-specific amplification of DNA.
- Two sets of primers are being used in two successive PCRs.
- In the first reaction, one pair of primers is used to generate DNA products, which besides the intended target, may still consist of non-specifically amplified DNA fragments.
- The products are then used in a second PCR with a set of primers whose binding sites are completely or partially different from and located 3' of each of the primers used in the first reaction.
- Advantage:
  - Very low probability of non specific amplification
  - Important control for many experiments.

3) Nested PCR:


Target
Sequence
DNA
Template
Target
Sequence
First set primers
DNA
Template
Copyright PCR Station
Target
Sequence
DNA
Template
Target
Sequence
First PCR Run
Majority of PCR
Products
Target
First
PCR

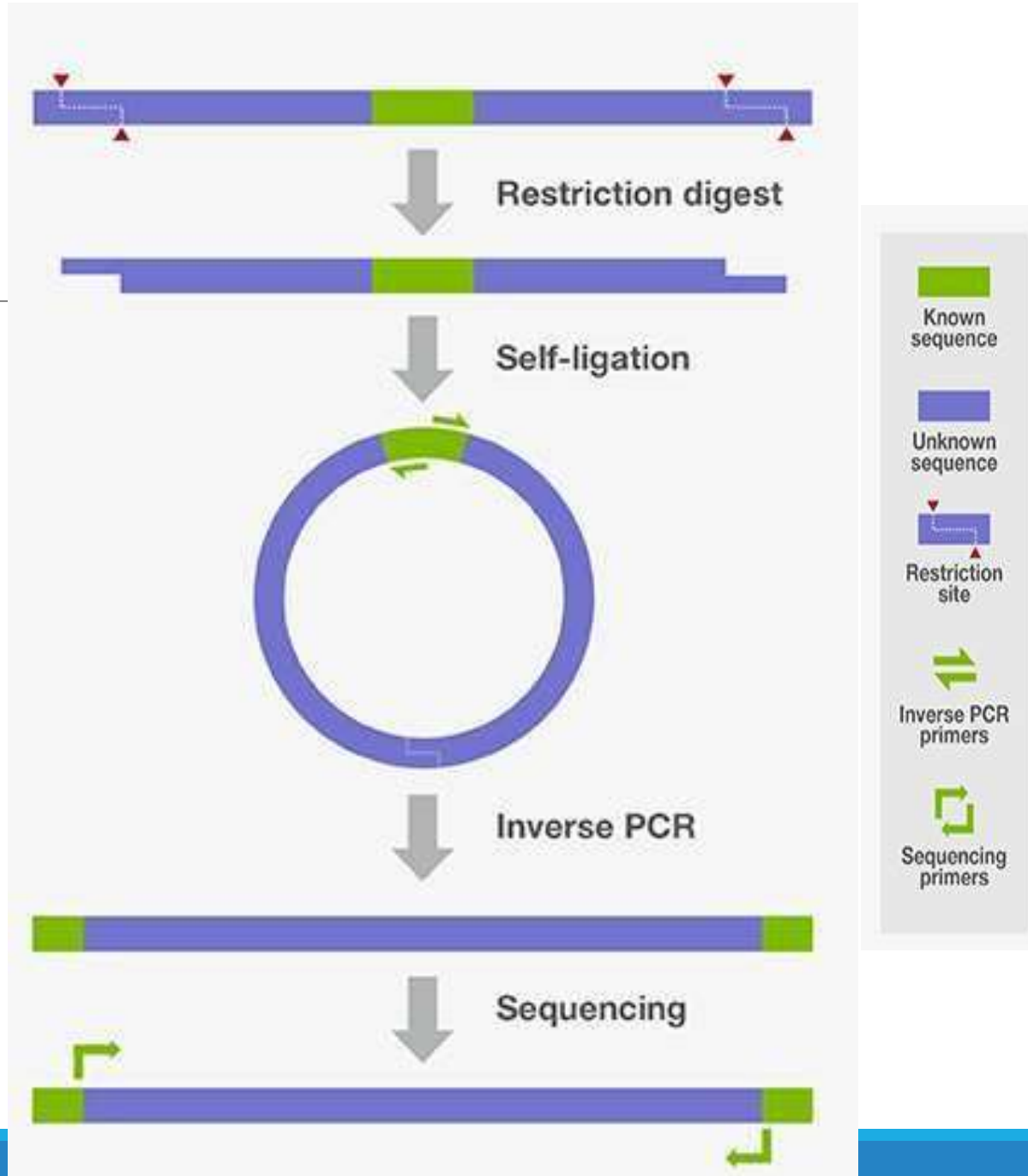
3) Nested PCR:


Target Sequence
First PCR Run
Majority of PCR Products
Target Sequence
Second set primers
Target Sequence
Second PCR
Target Sequence
Second PCR Run
PCR Product

4) Inverse PCR

- Amplification of DNA of unknown sequence that flanks DNA of known sequence occurs.

- A fragment of DNA is circularized by ligation and then amplified away from rather than towards the known DNA sequence.
- DNA that flanks the known DNA sequence is amplified.
- resulting in known sequences at either end of the unknown sequence)
- Used for
  - *analyzing viral integration sites as well as regulatory elements adjacent to genes, genome*



5) Single-cell PCR:

- With the enormous amplification power of PCR, the technique is

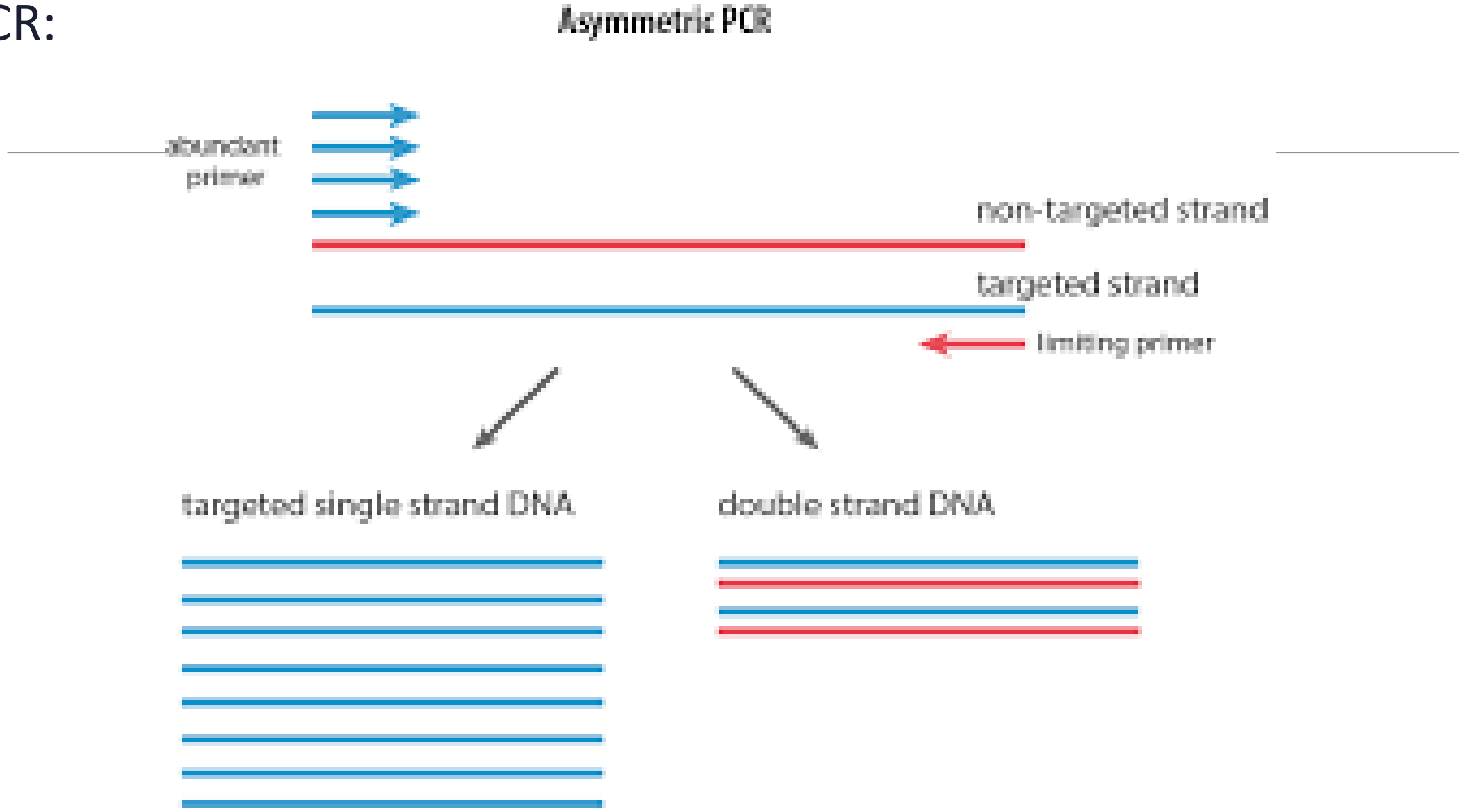
  applied at the level single cell.

- It has been performed in the analysis for the cystic fibrosis gene in a single cell removed from a human eight-celled embryo.
- Sperm analysis.
- Evaluation of gene expression in single Reed-Sternberg cells in an attempt to delineate the origin of this cell.

6) Asymmetrical PCR:

- Technique that produces ssDNA by using 2 primers of vastly different concentrations in the ratio of 50-100:1
- dsDNA is produced exponentially in the initial PCR cycles.
- Once the primer in the lower concentration has been depleted, priming only occurs with the remaining primer, resulting in the linear expansion of ssDNA.

6) Asymmetrical PCR:

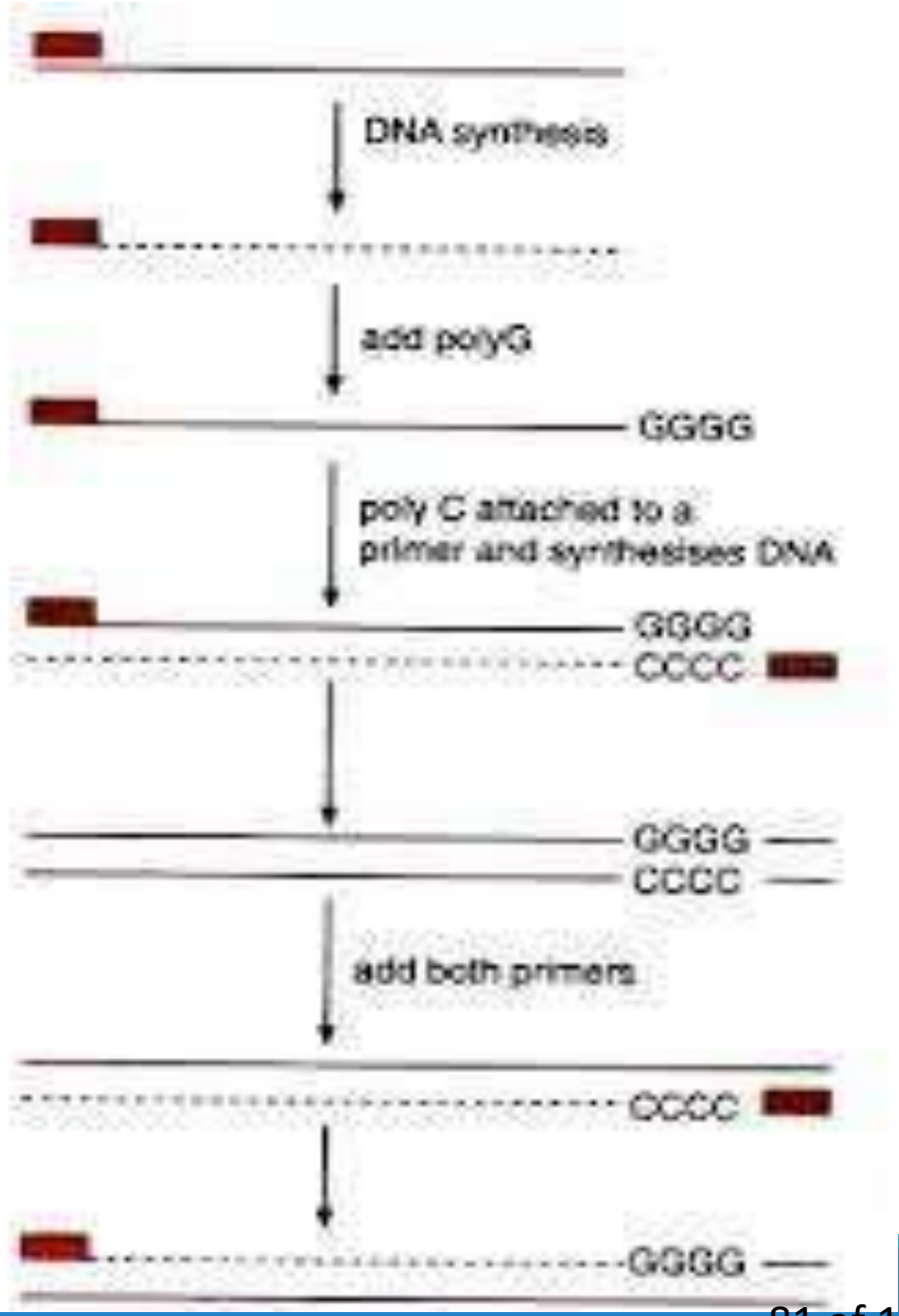


6) Asymmetrical PCR:

- Technique that produces ssDNA by using 2 primers of vastly different concentrations in the ratio of 50-100:1
- dsDNA is produced exponentially in the initial PCR cycles.
- Once the primer in the lower concentration has been depleted, priming only occurs with
  the remaining primer, resulting in the linear expansion of ssDNA.
- ssDNA is preferred for DNA sequencing and hybridization probing where amplification of only one of the two complementary strands is required.
- A recent modification on this process, known as *L*inear-*A*fter-*T*he-*E*xponential-PCR (LATE- PCR), uses a limiting primer with a higher melting temperature (Tm) than the excess primer to maintain reaction efficiency as the limiting primer concentration decreases mid-reaction.

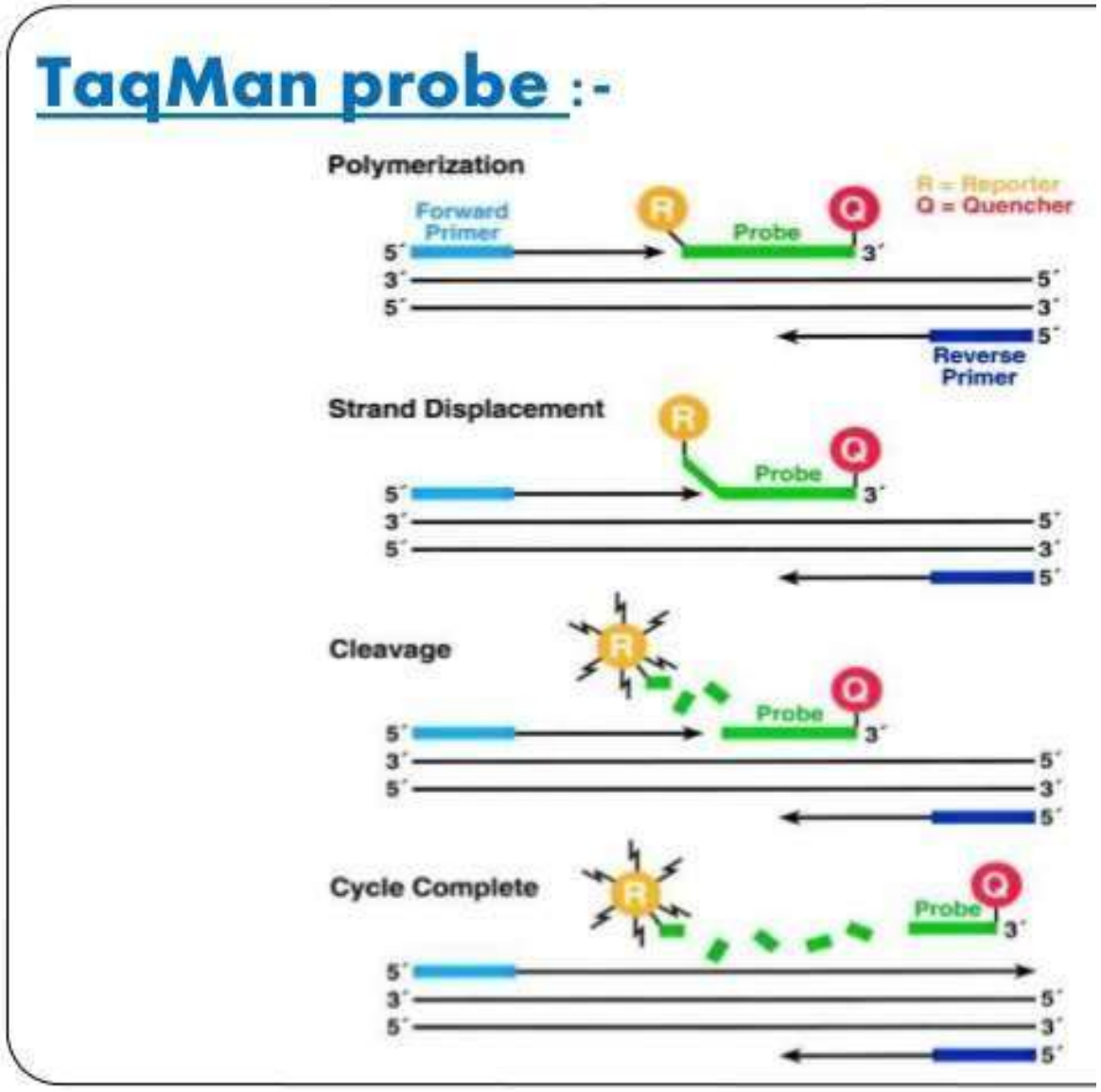
7) Anchored PCR

- Amplification of DNA or RNA where only one end of the sequence of interest is known.
- Utilizes only one primer instead of two primers.
- Only one strand will be copied first after which a poly G tail will be attached at the end of the newly synthesized strand
- This newly synthesized strand with poly G tail at its 3' end will then become template for the daughter strand synthesis.
- Anchor primer with which a poly-C sequence is linked to complement with poly-G of the template.
- Conventional PCR can now be performed.



8) Real Time PCR (is Quantative assay)

- is commonly used to determine whether a
  - DNA sequence is present in a sample and
  - the number of its copies in the sample.
- *Quantitative PCR* has a very high degree of precision.
- Quantitative PCR methods use fluorescent dyes, such as Sybr Green, EvaGreen or fluorophore-containing DNA probes, such as TaqMan, to measure the amount of amplified product in real time.
- It is also sometimes abbreviated to RT-PCR (*real- time* PCR) but this abbreviation should be used only for reverse transcription PCR.
- qPCR is the appropriate contractions for quantitative PCR



8) Real Time PCR

(use)

- **Diagnostic uses – To diagnose infectious diseases**

- **Microbiological uses -** Used by microbiologists in the fields of food safety, food spoilage and fermentation and for the microbial risk assessment of water quality.

- **Uses in research -** used to provide quantitative measurements of gene transcription.

9) Allele-specific PCR:

10)Assembly PCR or Polymerase Cycling Assembly (PCA)

11) Convective PCR

12) Digital PCR (dPCR)

13) In silico PCR (digital PCR, virtual PCR, electronic PCR, e-PCR)

14) Helicase-dependent amplification:

15) Methylation-specific PCR (MSP)

16) Multiplex ligation-dependent probe amplification (MLPA)

17) Multiplex-PCR: It consists of multiple primer sets within a single PCR mixture to produce amplicons of varying sizes that are specific to different DNA sequences.

- By targeting multiple genes at once, additional information may be gained from a single test-run that otherwise would require several times the reagents and more time to perform.
- Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction, and amplicon sizes. That is, their base pair length should be different enough to form distinct bands when visualized by gel electrophoresis.

Applications of PCR

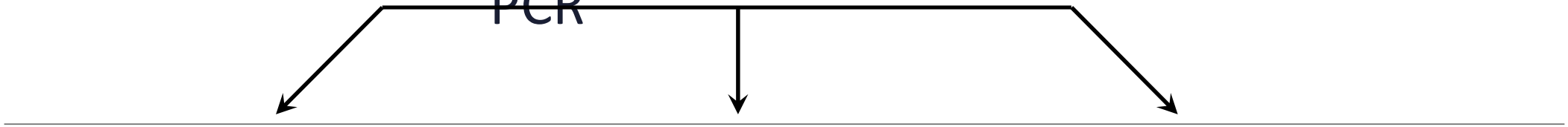
Basic Research

- Mutation screening
- Drug discovery
- Classification of organisms
- Genotyping
- Molecular Archaeology
- Molecular Epidemiology
- Molecular Ecology
- Bioinformatics
- Genomic cloning
- Site-directed mutagenesis

Applied Research

- Genetic matching
- Detection of pathogens
- Pre-natal diagnosis
- DNA fingerprinting
- Gene therapy

Applications of PCR



Molecular Identification

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

Sequencing

- Bioinformatics
- Genomic cloning
- Human Genome Project

Genetic Engineering

- Site-directed mutagenesis
- Gene expression studies

Application

I. Neoplasia
    i) Hematological malignancies
    ii) Non-hematological malignancies
II. Infectious diseases
III. Inherited genetic diseases
IV. Identity determination
    i) Transplantation
    ii) Forensic testing
    iii) Parentage testing

Hematological malignancies

1) Antigen Receptor gene rearrangement

- Earliest step in the maturation of B and T cells is the rearrangement of
  - their respective antigen receptor genes,
  - the immunoglobulin gene in the B lymphocytes and
  - the T cell antigen receptor gene in T lymphocytes.
- The recombination site of the antigen receptor gene is amplified using PCR.
- But *in clonal lymphcytes, the sequence amplified will be identical in every cell and PCR product will be uniform in size.*
- Discrete band will be formed on gel electrophoresis compare to smear pattern formed in a polyclonal lymphocyte population.

2) Assignment of lineage

- In Hematologic malignancies, the lineage of cells, which was unclear based on morphology and immunophenotypic studies, have had lineage assigned to them based on finding clonal antigen receptor gene rearrangement.
- The finding of immunoglobulin heavy chain gene rearrangements in "common" acute lymphoblastic leukemia and hairy cell leukemia was used to firmly establish these leukemias as being of B-cell lineage.
- Cross-lineage rearrangements are not uncommon.
- Should always be evaluated in the context of conventional morphology, cytochemistry and imunophenotyping.

- In addition to providing answers to availability of much shorter periods of time- formalin fixed, paraffin embedded tissue can be used for PCR analysis.
- Southern Blot analysis has the ability to detect a single neoplastic cell in the background of 25-50 normal cells, PCR can detect one neoplastic cell admixed with 100,000 normal cells.

Molecular Cytogenetics

- Recurrent chromosomal translocations characterize the acute myeloid and lymphoid leukemias.
- The identification of particular fusion gene accomplished by reverse-transcription polymerase chain reaction (RT-PCR) amplification, followed by detection of the specific PCR products by gel electrophoresis.
- T perform PCR on DNA is dependent on the knowledge of the sequence of the genes
that flank the break points of chromosomal translocation.

Translocation in hematological malignancies amenable to PCR

Disease	Translocation	Genes involved
Chronic myelocytic leukemia	t(9;22)	abl-bcr
Acute lymphoblastic leukemia	t(9;22)	abl-bcr
Follicular Non-Hodgkin's Lymphoma	t(14;18)	IgH-bcl2
Anaplastic large cell lymphoma	t(2;5)	ALK-NPM
AML (FAB M2)	t(8;21)	eto-aml-1
AML (FAB M3)	t(15;17)	pml-rarα
AML (FAB M4E)	inv(16)	CBFβ-MYHII

PCR – PROGNOSTIC INFORMATION

- In paediatric, ALL,the presence of t(1;19,),t(9; 22) or t(4;11)is associated with - a poor prognosis.
- In adult, acute non-lymphoblastic leukemia, t(8;21),t(15;17) and inv(16) abnormalities - favourable outcome.
- In patients with diffuse large cell NHL, *bcl-6* rearrangements-Good prognosis, while
  bcl-2 rearrangements - poor outcome.

Mutational analysis

- The molecular analysis of mutations has also provided diagnostic and prognostic insights in hematologic malignancies.
- Mutation of *ras*-genes have been suggested to be an indication of evolution of myelodysplastic syndromes into acute leukemia and when found in ALL, are predictive of an increased risk of relapse.
- Mutation of the *c-fms* protooncogene are more commonly seen in acute non-lymphoblastic leukemia of monoblastic lineage.

Non-hematologic malignancies

- Soft tissue sarcomas have been recently identified to have numerous nonrandom and specific chromosomal translocation.
- RT-PCR assays of these translocations are particularly useful in reaching accurate diagnosis that are otherwise difficult to make using histological and immunophenotypic criteria.
- PCR based mutation and polymorphism detection systems has demonstrated that p53 function is lost in approx 50% of all cancers and that loss of function is caused by point mutations.

Translocation in soft tissue tumors amenable to RT-PCR

Diseases	Translocation	Gene involved
Ewing's sarcoma	t(11;22)	EWS-FLI1
Peripheral Neuroectodermal tumour	t(21;22)	EWS-ERG
Desmoplastic small round cell tumour	t(11;22)	EWS-WT1
Alveolar Rhabdomyosarcoma	t(2;13) t(1;13)	PAX3-FKHR PAX7-FKHR
Clear Cell carcinoma	t(12;22)	EWS-ATF-1
Myxoid liposarcoma	t(12;16	CHOP-TLS

- The frequent occurrence of p53 mutations in cancers can found in a number of clinical scenarios.
- In patients with cancer of the lung, tumor cells can be detected in the sputum in situations where cytologic evaluation failed to detect tumor cells.
- Local recurrence in patients of squamous cell carcinoma with microscopically negative surgical margins occurs.
- The advent of assessment of “molecular margins” through a molecular evaluation of p53 mutation may dramatically refine the prediction of local recurrence of the tumor

Infectious diseases:

- Characterization and detection of infectious disease organisms have been revolutionized by PCR.
- The earliest tests for detection of HIV infection relied on the presence of antibodies to the virus circulating in the bloodstream.
- However, antibodies don't appear until many weeks after infection, maternal antibodies mask the infection of a newborn.

- Tuberculosis is difficult to sample from patients and slow to be grown in the laboratory.
- PCR-based tests have allowed detection of small numbers of disease organisms (both live or dead), in convenient samples.
- Detailed genetic analysis can also be used to detect antibiotic resistance, allowing immediate and effective therapy.
- Disadvantage - their ability to detect only one organism at a time and their lack of amenability to antimicrobial sensitivity testing.

- The spread of a disease organism through populations of domestic or wild animals can be monitored by PCR testing.
- The recent emergence and global spread of the swine flu virus (H1N1) can reliably be diagnosed using Real-Time, Polymerase Chain Reaction (RT-PCR) technology and can also discriminate the H1N1 influenza virus from other strains.
- Furthermore, this test specifically amplifies and characterizes the viral genetic material, enabling rapid detection of new viral strains as they evolve.

PCR for blood grouping

- Predict blood group phenotypes from DNA to determine fetal blood groups.
- When a pregnant woman has a blood group antibody with the potential to cause
  haemolytic disease of the newborn
- The usual source of fetal DNA is amniocytes, obtained by amniocentesis.

Inherited genetic diseases

- PCR can be used for detection of mutations and screening of inherited disorders.
- Prospective parents can be tested for being genetic carriers, or their children might be tested for actually being affected by a disease.
- DNA samples for Prenatal testing can be obtained by amniocentesis, chorionic villus sampling.
- PCR analysis is also essential to Pre-implantation genetic diagnosis, where individual
cells of a developing embryo are tested for mutations.

- The most common defect in cystic fibrosis is 3 base pair deletion resulting in the loss of single amino acid, can be detected using pre amplification of the flanking sequence by PCR. And difference in size between the normal and the mutant allele can be resolved by using polyacrylamide gel electrophoresis.
- Duchenne's muscular dystrophy and Becker's Muscular dystrophy have deletion of dystropin gene can be rapidly screened using Multiplex PCR amplification.
- Sickle cell anaemia diagnosis use direct analysis, almost always requiring PCR amplification of that part of the β-gene involved.

Identity determination

- PCR technique can be used to assign identity in several areas of relevance to pathology.
- Three areas in which they are best established are in the context of

1) Transplantation
2) Forensic pathology
3) Parentage testing

Transplantation

- Tissue antigen matching is critical to ensure sustained engraftment and prevention of graft-versus-host-disease and to optimize reconstitution of the immune system.
- Matching of HLA gene product is traditionally performed using complement mediated immunologic assays and mixed lymphocyte reaction.
- Molecular typing at the HLA-D loci has been shown to supersede the conventional methods at several level.

Parentage testing

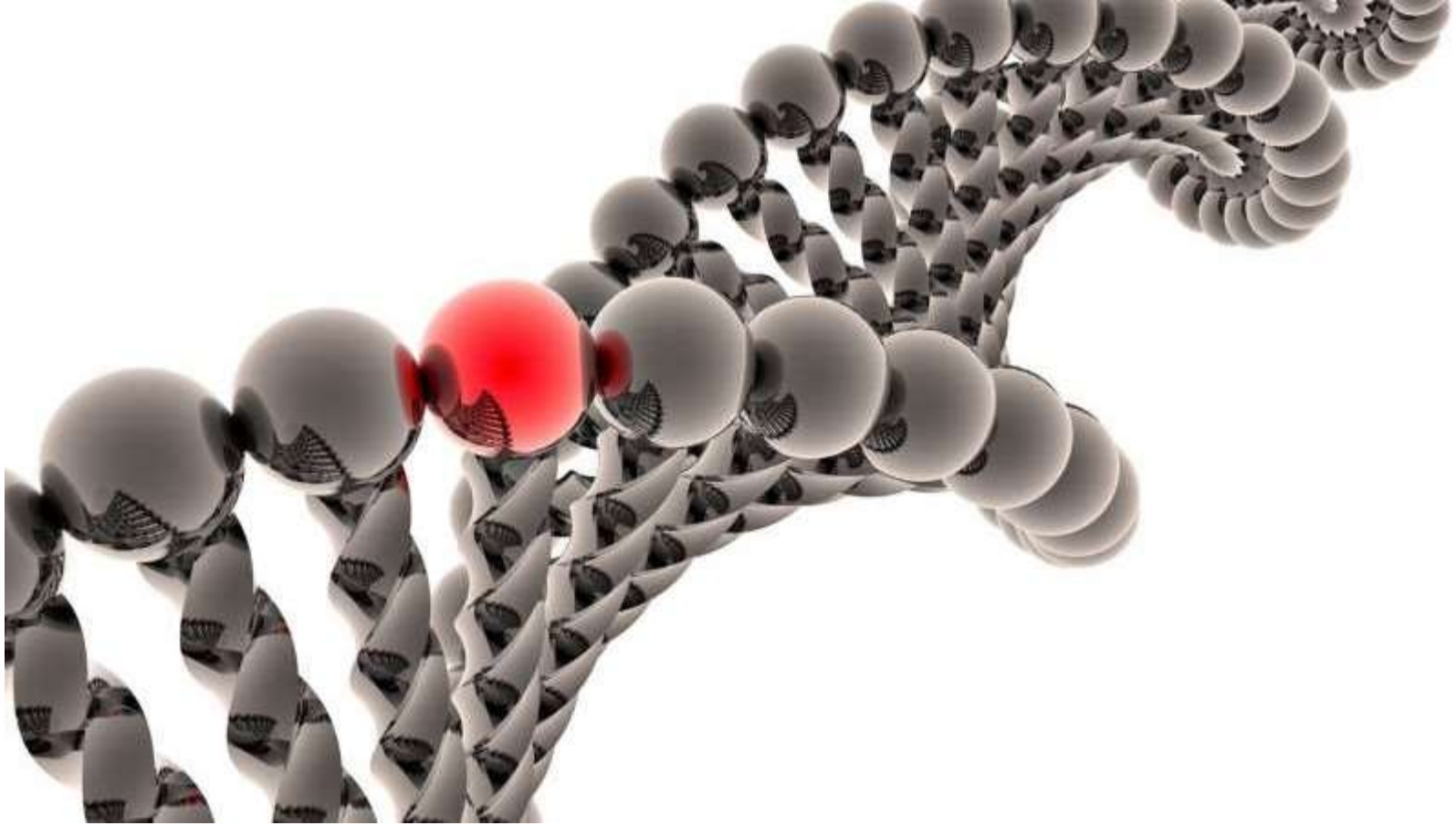
- Conventional serologic and protein polymorphism testing has been used to exclude but not establish relationship reliable in
- DNA typing for parentage testing is based on the fundamental principle that each individual inherits one allele, for any given locus, from each parent.
- Methodologies used are identical to those used in forensic testing.

The future

- *Sample preparation time is being reduced* with the availability of a variety of DNA/RNA extraction kit.
- *PCR enables the rapid preparation of virtually any tissue sample ranging from fixed*, paraffin embedded tissue to a drop of blood obtained with needle stick.
- *Automation of PCR* has meant improvement in reliability of results and a decrease in hands-on-time.
- *Hand-held diagnostic units* may become available for use at the bedside and in
- The selection of therapy for many disease process will be made on the basis of specific molecular lesion.
- The choice of antibiotic could be based on the DNA typing of drug-resistant micro organisms.
- Targeted gene therapy for cancer is being intensively developed.
- Identity testing at the molecular level will continue to have a significant influence in the legal system and

Economic

- It is very difficult to measure the cost-benefit ratio for this technology because-
  - It gives Precise diagnosis before the initiation (if intervention) therapy. It will definitely save time and avoid additional diagnostic test or ineffective treatments.
  - The ability to perform diagnostic molecular examination on small tissue samples may have measurable effect by lessening the extent of a surgical procedure required to obtain diagnostic material.



THANK YOU..!!

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A Review of Probability

A COIN THROW

The probability of a heads (H) or a tails (T) is always 0.5 for every throw. What is the probability of getting this combination of tails in a row?

Event		Probability	
Tails		0.5	= 0.5
T,T		0.5 x 0.5	= 0.25
T,T,T		0.5 x0.5 x 0.5	= 0.125
T,T,T,T,T		$(0.5)^5$	= 0.03125
T,T,T,T,T,T,T,T,T,T,T	$(0.5)^{11}$	= 0.0004883	
T,T,T,T,T,T,T,T,T,T,T,T,T,T,T	$(0.5)^{16}$	=0.00001526	

So it become increasing unlikely that one will get 16 tails in a row (1 chance in 65536 throws). In this same
way, as the primer increases in size the chances of a match other than the one intended for is highly unlikely.

Probability in Genetics

- There are 4 bases in the DNA molecule A,C,G,T
- The probability of encountering any of these bases in the code is 0.25 (1/4)
- So let us look at the probability of encountering a particular sequence of bases

Event		Probability	
A		0.25	= 0.25
A,T		0.25 x 0.25	= 0.0625
A,T,A		0.25 x0.25 x 0.25	= 0.015625
A,T,A,G,G	$(0.25)^5$		= 0.0009765
A,T,A,G,G,T,T,T,A,A,C	$(0.25)^{11}$		= 0.000002384
A,T,A,G,G,T,T,T,A,A,C,C,T,G, G,T	$(0.25)^{16}$		=0.0000000002384

So it become increasing unlikely that one will get 16 bases in this particular sequence (1 chance in 4.3 billion). In this same way, one can see that as the primer increases in size, the chances of a match other than the one intended for is highly unlikely.

DNA extraction from tissue for PCR

Cells which are to be studied need to be collected

Breaking the cell membranes open to expose the DNA along with the cytoplasm within (cell lysis).

- Lipids from the cell membrane and the nucleus are broken down with detergents and surfactants.
- proteins by protease.
- RNA by RNase

treated with concentrated salt solution to make debris such as broken proteins, lipids and RNA to clump together.

Centrifugation of the solution, which separates the clumped cellular debris from the DNA.

- **Ethanol precipitation** usually by ice-cold ethanol or isopropanol. Since DNA is insoluble in these alcohols, it will aggregate together, giving a *pellet* upon centrifugation. Precipitation of DNA is improved by increasing of ionic strength, usually by adding sodium acetate.
- **Phenol–chloroform extraction** in which phenol denatures proteins in the sample. After centrifugation of the sample, denaturated proteins stay in the organic phase while aqueous phase containing nucleic acid is mixed with the chloroform that removes phenol residues from solution.
- **Minicolumn purification** that relies on the fact that the nucleic acids may bind (adsorption) to the solid phase (silica or other) depending on the pH and the salt concentration of

Application in Sickle cell disease

The presence of Hb S and thus the presence of the sickle cell gene can

be determined by Hb HPLC or sickling test.

- in prenatal- performed at 10 weeks of pregnancy, to identifying sickle cell anaemia and haemoglobin S/β thalassaemia,
- to confirm the diagnosis of sickle cell anaemia in a neonate.

Application in Sickle cell disease

Sample requirements

- From each parent, 10 ml of blood in EDTA or heparin is required.
- If restriction fragment length polymorphism (RFLP) linkage analysis is required, the following additional samples are needed: blood from either a homozygous normal or affected child, or from a heterozygous child and one set of grandparents, or, if no child is available, blood from both sets of grandparents.
- The samples must be carefully and clearly labelled and the family tree must be drawn. Particulars of all haematological tests must be given.
- Chorionic villus samples must be dissected free of any maternal tissue and sent by urgent overnight delivery in tissue culture medium or, preferably, in a special buffer obtainable from the DNA diagnostic laboratory. Amniotic fluid samples (15–20 ml are needed) must be received within 24 h of collection. If a longer transit time is unavoidable, the amniocytes should be resuspended in tissue culture medium.
- precise ethnic origin of family members so that optimal use is made of the DNA available for diagnosis.
- Whenever possible, DNA analysis of the child's globin genes should be carried

Application in Sickle cell disease

- The sickle cell mutation, Glu7Val in codon 7 of the β globin gene, results in the loss of a Bsu36 I restriction enzyme site that is present in the normal *HBB* gene.
- It is therefore possible to detect the mutation directly by restriction enzyme analysis of a DNA fragment generated by PCR.
- A pair of primers are used to amplify axons 1 and 2 of the β globin gene and the products of the PCR are digested with Bsu36 I. The loss of a Bsu36 I site in the sickle cell gene gives rise to an abnormally large restriction fragment that is not seen in normal individuals.
- This mutation can also be detected by ARMS PCR using two reactions per sample,

one specific for the mutant allele and the other for the wild-type allele.

- The 3'-end nucleotide of the mutation-specific primer should be specifically complementary to the mutation, while the primer specific for the wild- type sequence should contain a nucleotide complementary to the wild-type sequence at this end. A common reverse rimer allows the production of an amplification band similar in size in the two reactions, hence the use of two reaction tubes per sample. Primers pecifically used for this analysis are listed below.
- Homozygosity for the mutation will produce amplification in the mutation PCR reaction, while the absence of the mutation will result in amplification in the wild-type PCR only.
- Heterozygous samples will have amplification in both reactions.

How can I design primer for unknown DNA sequence?

- Use reference sequence, as mammalian genome is nearly decoded completely.

- If not available,
  - find the sequences of the closest relatives
  - Put them all together in FASTA format in a .txt document and do a multiple alignment using Clustal Omega. It's best to do these alignments using mRNA/cDNA transcripts or complete coding sequences (cds) because introns will not align well. However, you will need to design primers within exons (not across junctions) if you want to sequence DNA. I do this by using Ensembl to figure out where exons are and using the longest exons as the input to primer design programs (like Primer3). Of course, if you can't get a section long enough for your purposes you will need to use Ensembl to look at how far apart the exons are and choose a pair that give good amplicon length, then design primers in each exon individually. I'd recommend excluding sections of the exon that are not well conserved according to your Clustal alignment.
  - Inverse PCR can be used if Amplification of DNA of unknown sequence that flanks DNA of known sequence has to be done (But it not for designing primer.)