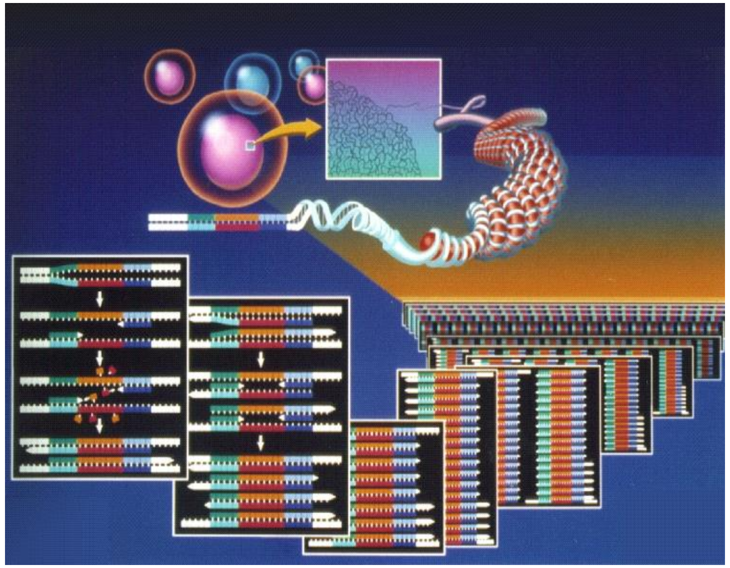
Week 4. PCR



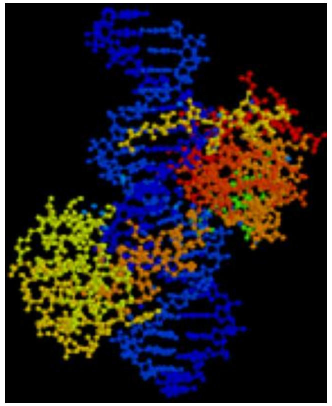
Polymerase Chain Reaction

Department of Microbiology

Lecture Content

- *Definition*
- *History*
- *Intracelluler DNA replication*
- *PCR contents*
- *PCR basic principles*
- *Where to use PCR?*
- *Advantages and disadvantages*
- *PCR optimisation*
- *PCR inhibitors and enhancers*
- *Troubleshooting*

PCR definition



- *in-vitro* enzymatic synthesis of the copies of a specific DNA fragment by the help of small oligonucleotides called “primers”
- *in-vitro* amplification of nucleic acids
- DNA photocopy
- samanlıkta iğne aramak yerine samanlıktaki iğnelerin sayısını çoğaltmak

Short History of PCR:

- **1971** *Khorana et al.* Developed a method replicating a specific region of an double-stranded DNA by the help of two DNA synthesis primers 3' ends of which are opposed to each other
- **1983** Kary Mullis (Cetus firm) developed PCR

Kary B. Mullis

- **1985** First report of *PCR with a DNA polymerase I Klenow fragment (Saiki et al., 1988).*
- **1988** First application of PCR by the help of first time use of Taq polymerase
- **1993** *Kary Mullis awarded Nobel Prize in Chemistry*
- **1993** Licences of PCR technology and Taq polymerase enzymes were bought by the world leader commercial firms

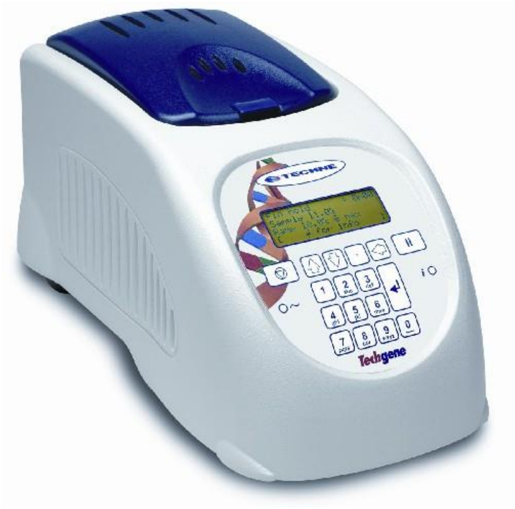
Intracellular DNA replication

- *DNA replication occurs at 37°C*
- Helper proteins like single strand binding proteins are used in replication
- *A RNA primer of 12 nucleotide are formed by an enzyme "primase" in the origin of replication*
- DNA polymerase binds to this enzyme and synthesis DNA by binding nucleotides to 3' end

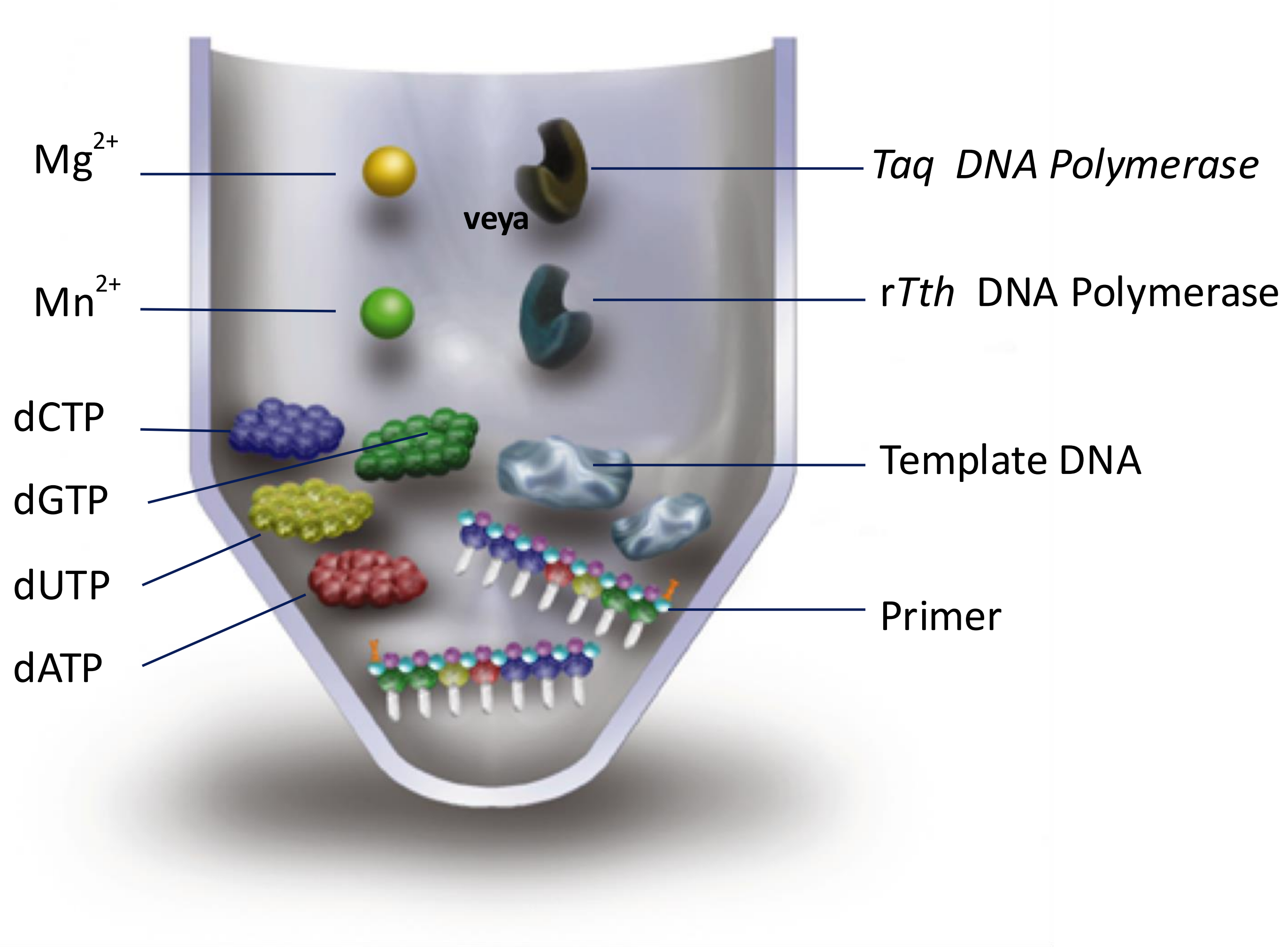
PCR

- **Reaction content**
  - Reaction buffer
  - Template DNA
  - Primers
    - Forward ve reverse
  - Nucleotides
    - DNA synthesis
  - Polymerase
    - Taq DNA polymerase
  - $MgCl_2$
    - Enyzme activity

- **Temperature control**
  - PCR equipment (thermal cycler)
  - Automatic regulation of temperature by steps and cycles



PCR mix contents

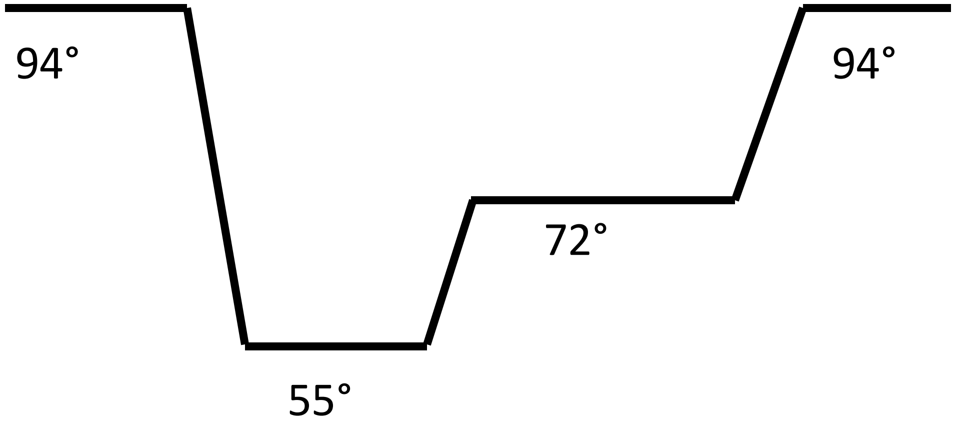


Characteristics of Primers

- 18-30 nucleotides
- F and R primers should have similar Tm values
- G+C content ~ 50%
- Primer should end with G or C at the 3′ end
- Primers should not end with A or T
- Sequences those could form hairpins should be inhibited
- Primers should not be complementary to each other

PCR

- **How does PCR work?**:
  - Separation of two strands from each other (94^{o}C)
  - Annealing of Primers (55^{o}C)
    - Beginning of Replication
  - Extension (polymerisastion) (72^{o}C)
    - = replication
  - Repeat for 20-30 times (cycles)



CYCLE PARAMETRES

Denaturation; 93°C - 95°C

30 sec – 1 min

Annealing; 37°C - 65°C

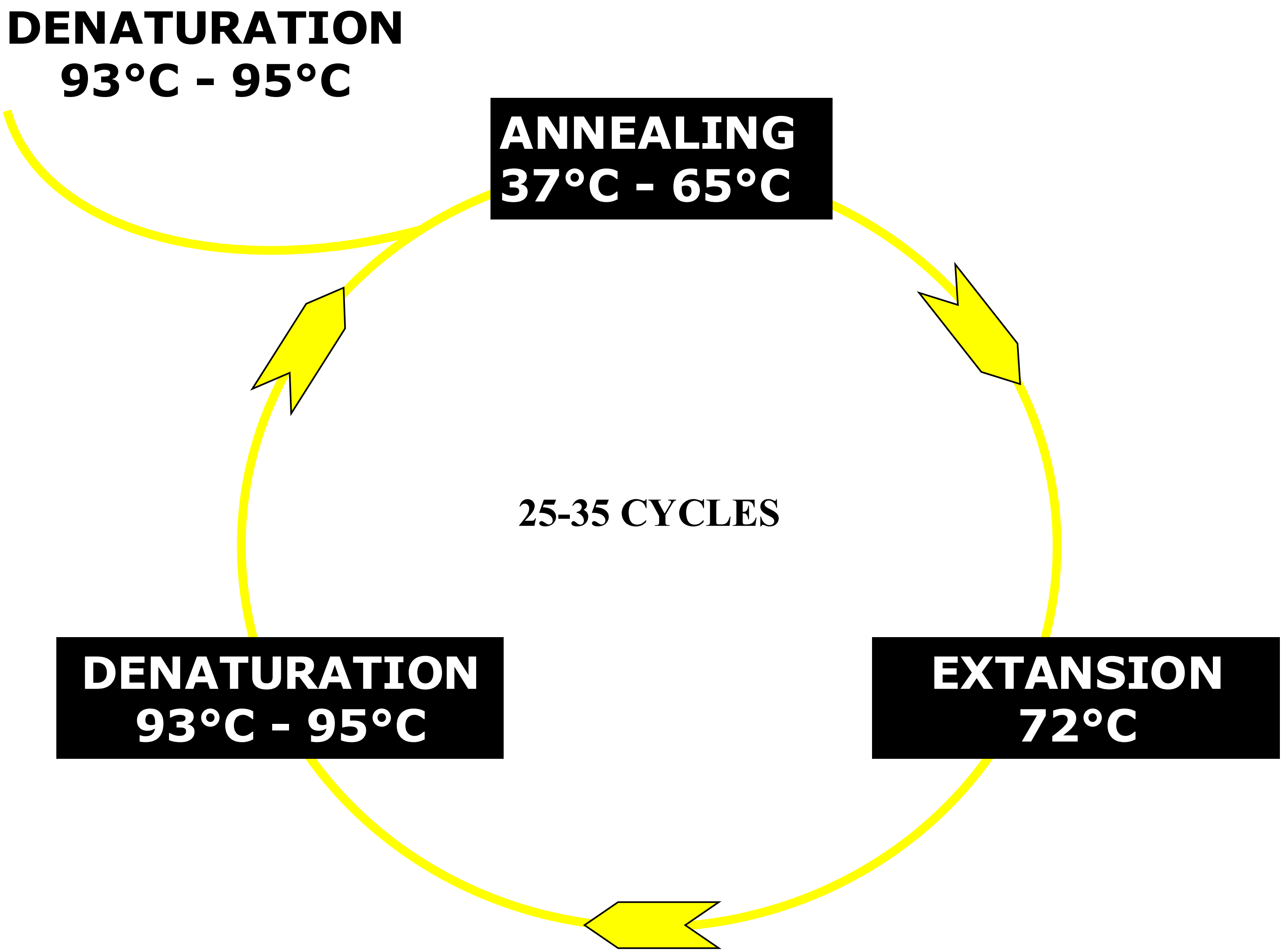
30 sec – 1 min

Extension; 72°C

1 min
(For every 500 bp DNA add + 30 sec)

25-35 cycles

Final exstantion 2-10 min


DENATURATION
93°C - 95°C
ANNEALING
37°C - 65°C
25-35 CYCLES
DENATURATION
93°C - 95°C
EXTANSION
72°C

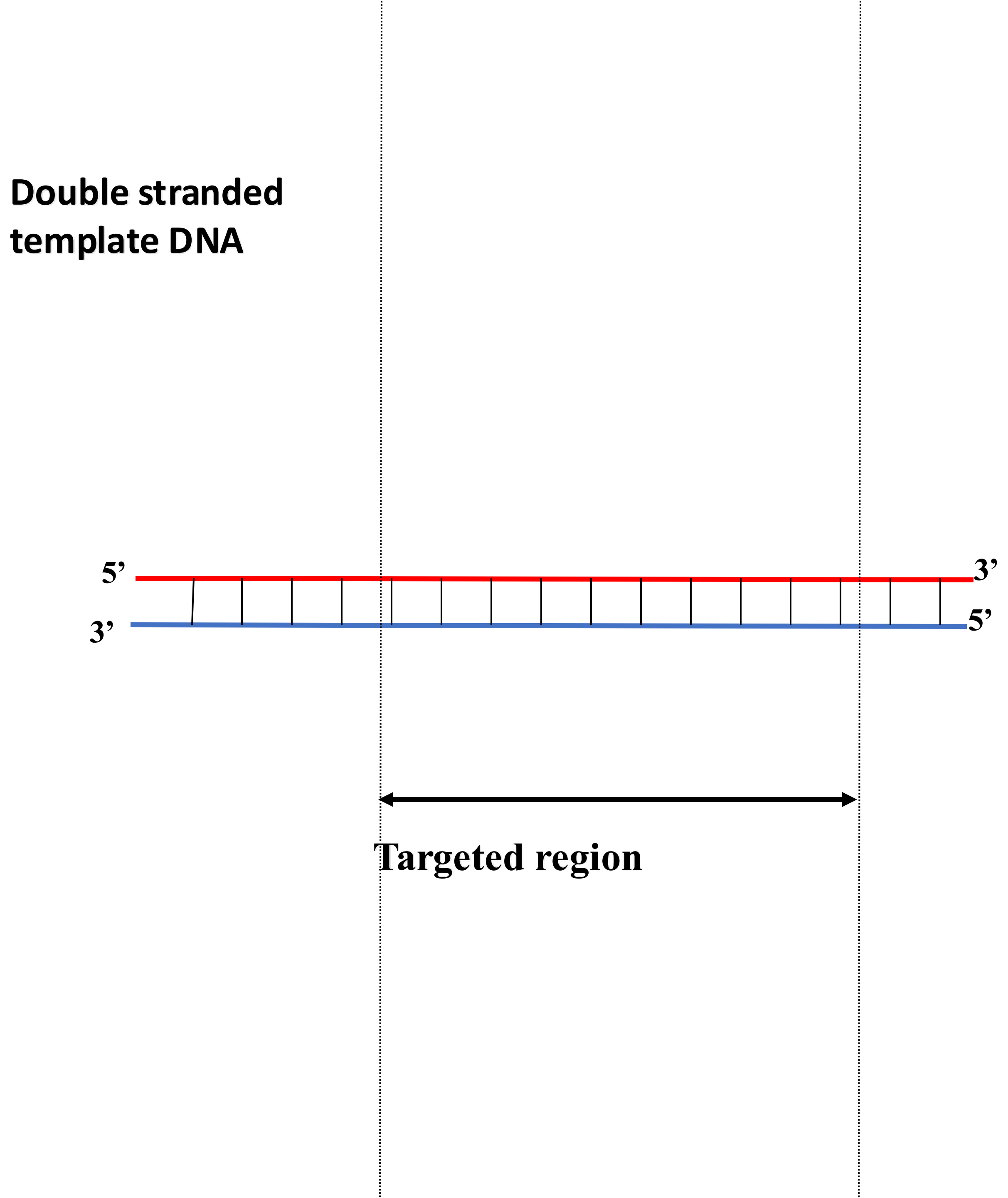
Basic PCR conditions

- 25-50-100 µl final reaction volumes
- Template DNA 1-1000ng
- Primers 10-20 pmols
- 10 mM Tris-CL pH 9.0, 50 mM KCl (10xPCR buffer)
- $MgCl_2$ 0.5-3.0 mM
- final concentration of each dnTPs would be 200 µM dNTP'ler: dATP, dGTP, dTTP, dCTP
- 1 unit Taq polymerase enzyme

REACTION MIXTURE

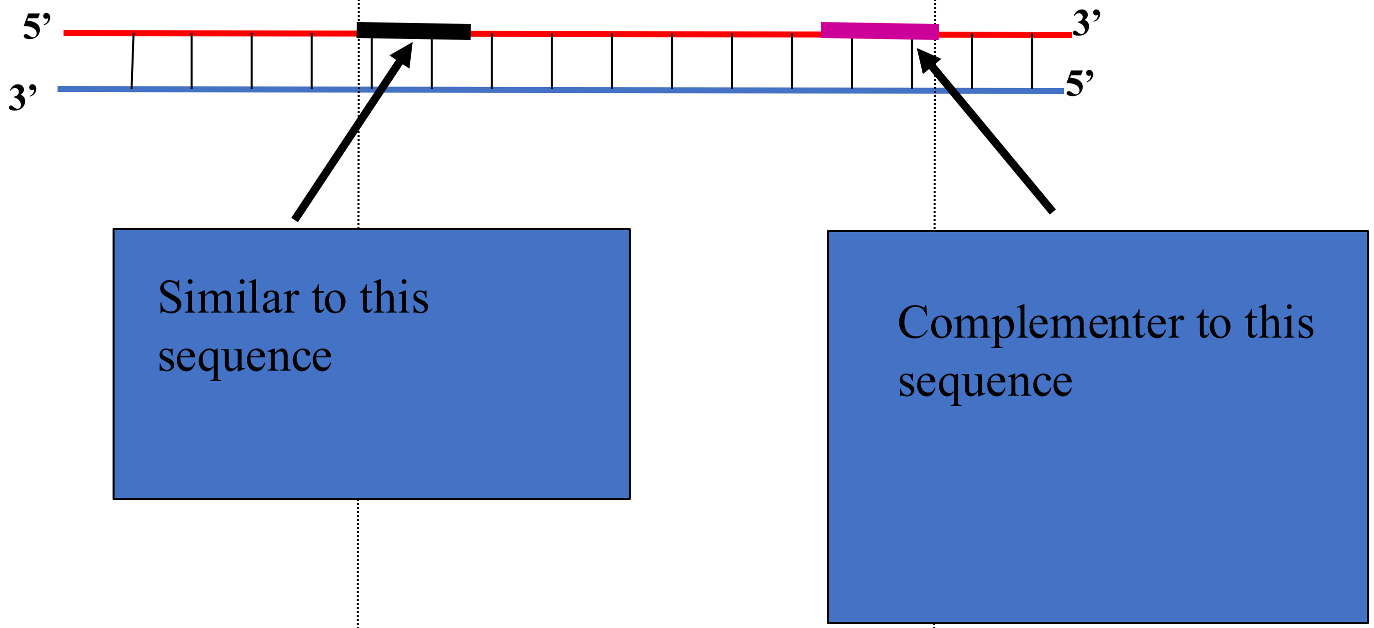
25 or 50⌠l final volume Eppendorf (0.2 ml) tube

COntents	Volumes	Final Concentration
10 X PCR Buffer	**5⌠l**	**1X**
10 X dNTPs (2mM)	**5⌠l**	**200⌠M**
Forward primer (10pmols/⌠l)	**5⌠l**	1⌠M (50pmols/50⌠l)
Reverse primer (10pmols/⌠l)	**5⌠l**	1⌠M (50pmols/50⌠l)
Genomic DNA template	**2⌠l**	**1⌠g**
Taq polymerase (2U/⌠l)	**0.5⌠l**	**1 unit**
H_2O (to 50⌠l Final volume)	**27.5⌠l**	

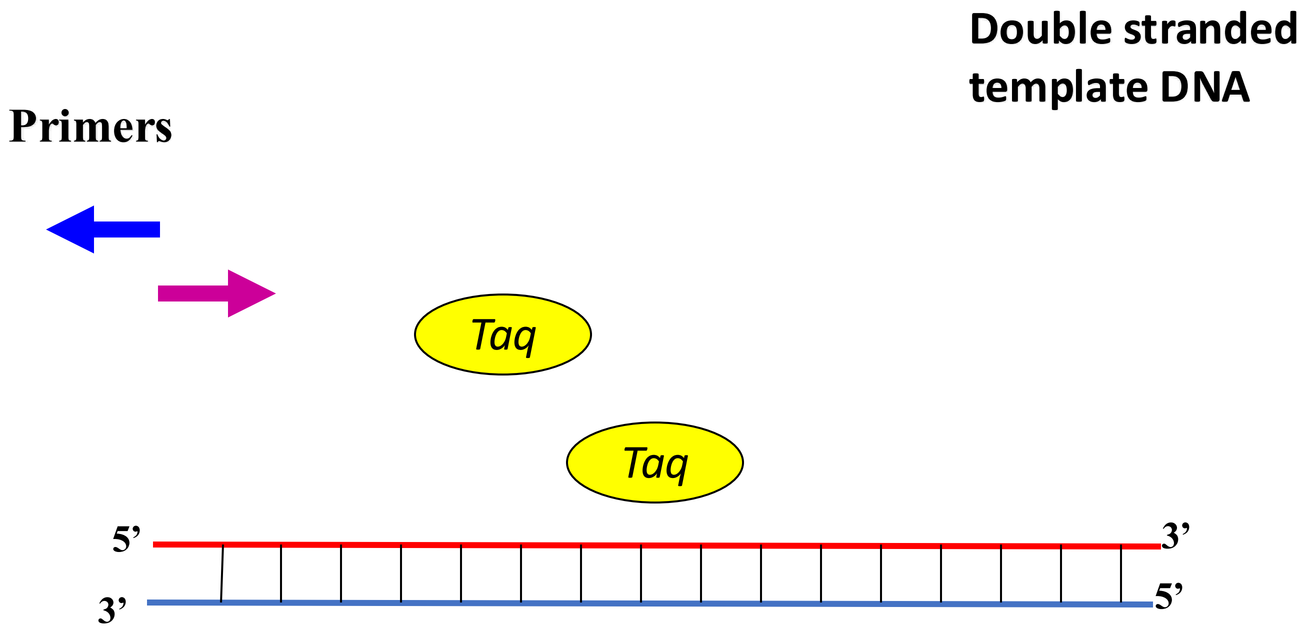

Double stranded
template DNA
5’
3’
3’
5’
Targeted region

Double stranded template DNA

Synthesize the primers!

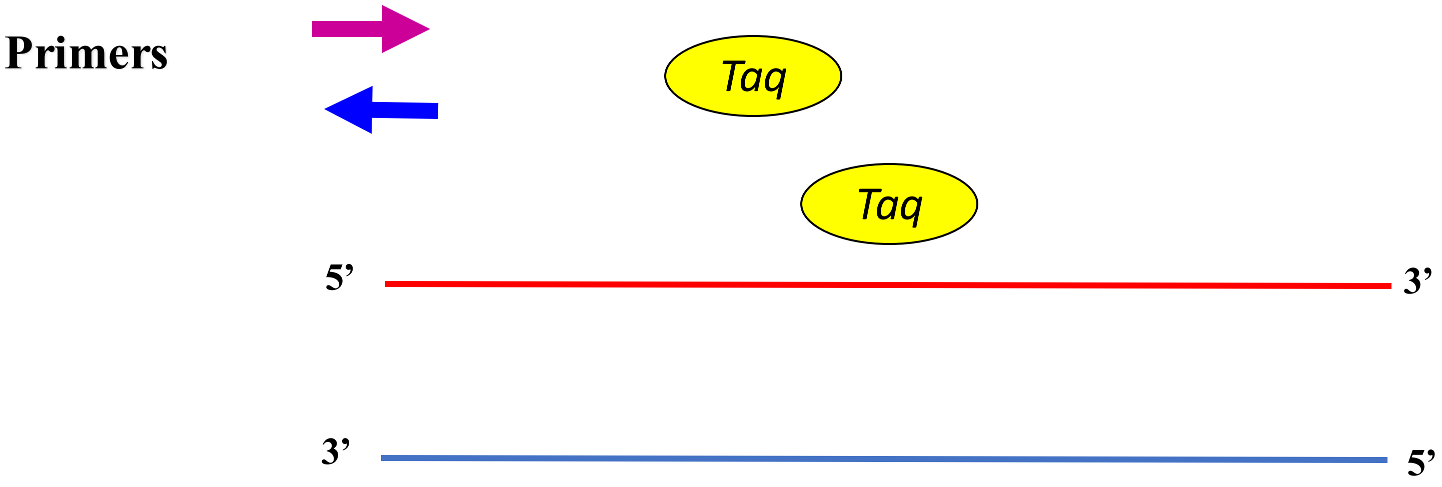


PCR CYCLE 1



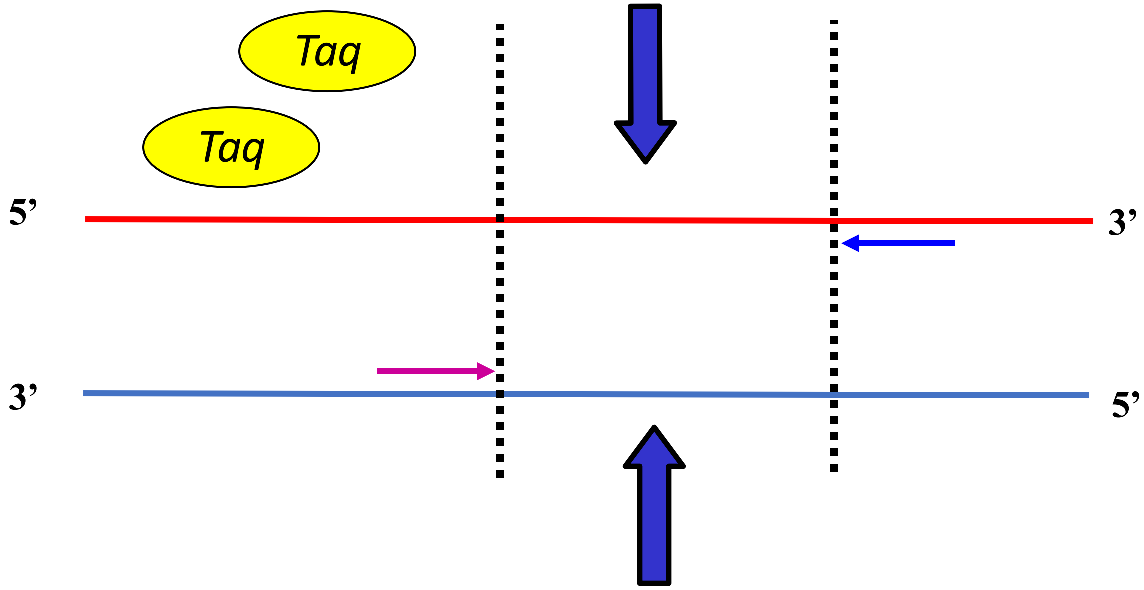
PCR CYCLE 1

Denaturation at
95^{0}C
Strands separated



Taq polymerase has a termostabile characteristic

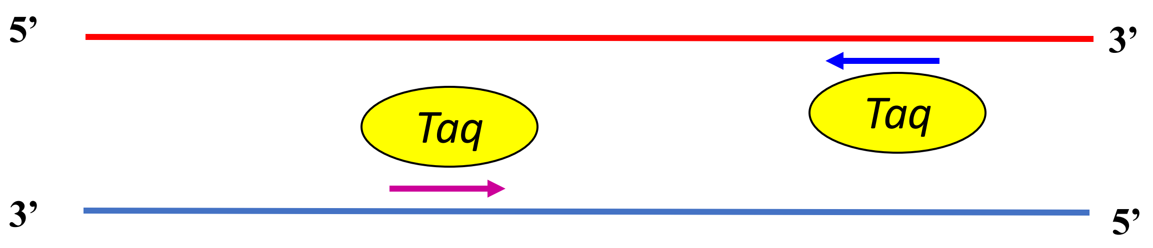
PCR CYCLE 1



Forward primer anneals to upper strand
Reverse primer anneals to lower strand

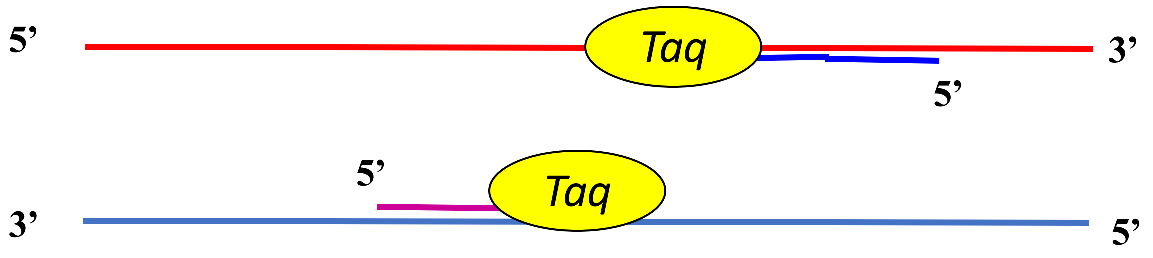
PCR CYCLE 1

Annealing
~55^{0}C
***Taq* anneals to**
primer-strand
complexes



PCR CYCLE 1

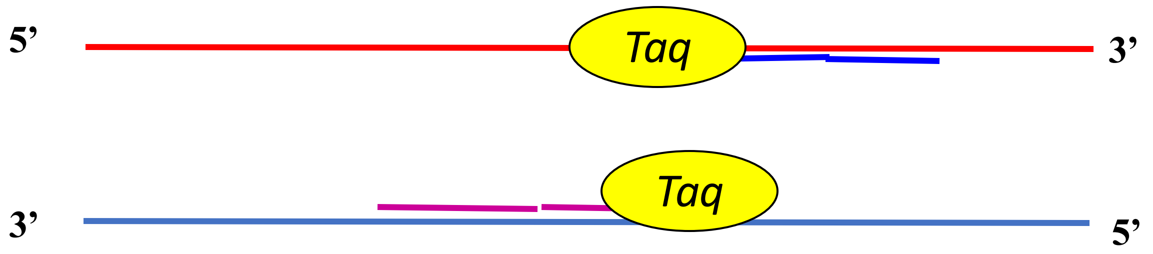
Extension
72°C
By the help of dNTPs
Taq copies DNA



Taq synthesizes DNA in the direction 5’ to 3’

PCR CYCLE 1

Extension
72^{o}C
By the help of dNTPs
Taq copies DNA



Taq synthesizes DNA in the direction 5’ to 3’

PCR CYCLE 1

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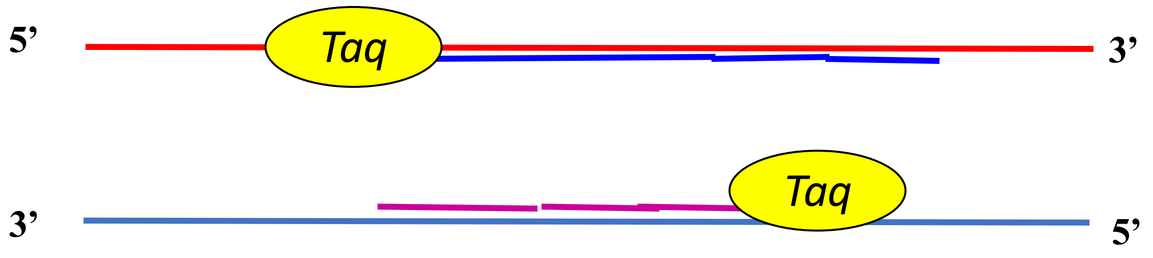
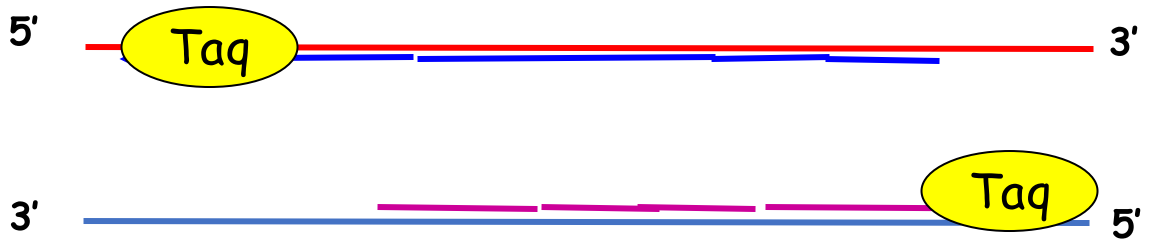


Taq synthesizes DNA in the direction 5' to 3'

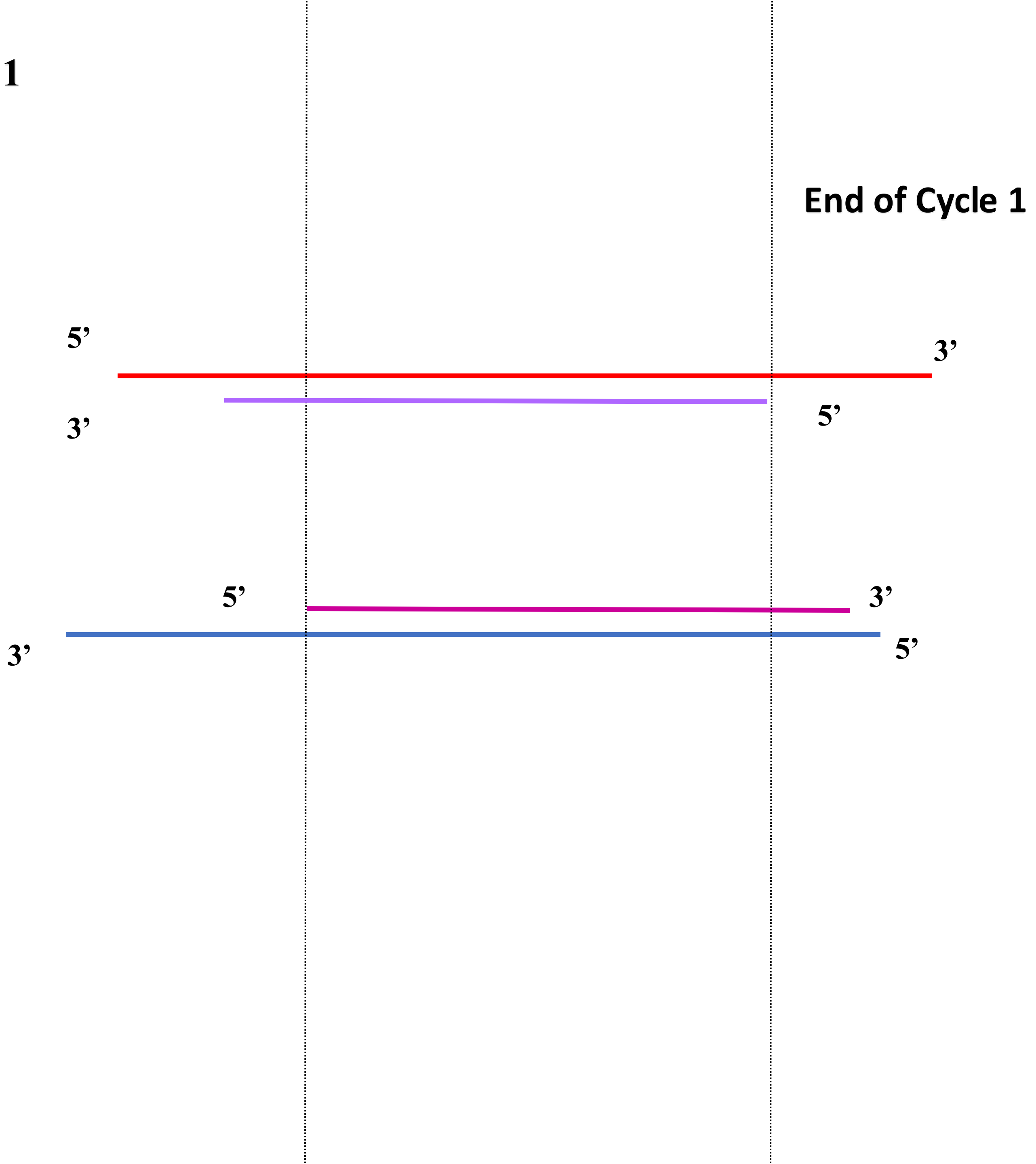
PCR CYCLE 1

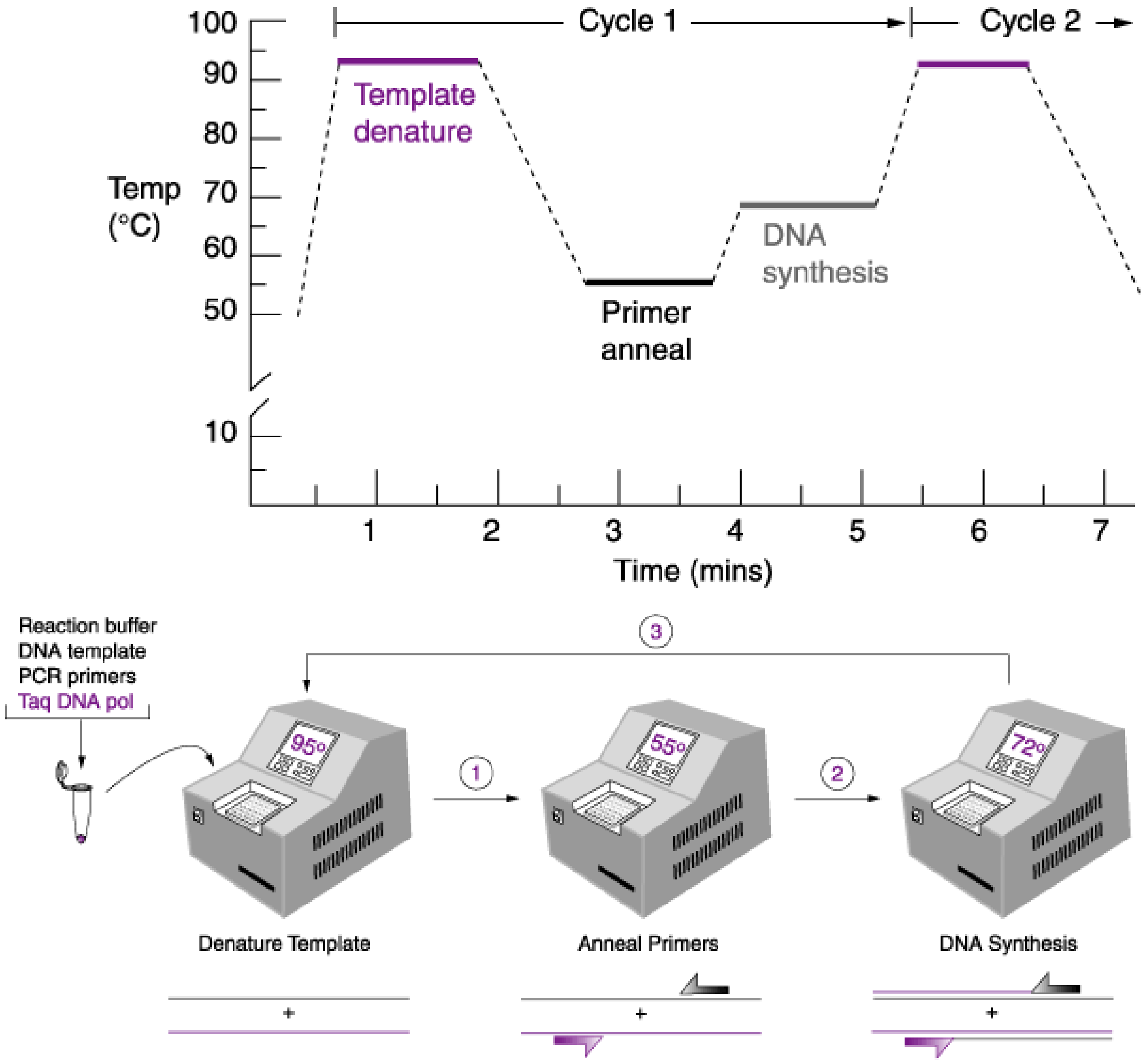
Extension
72ºC
By the help of dNTPs
Taq copies DNA



Taq synthesizes DNA in the direction 5' to 3'

PCR CYCLE 1

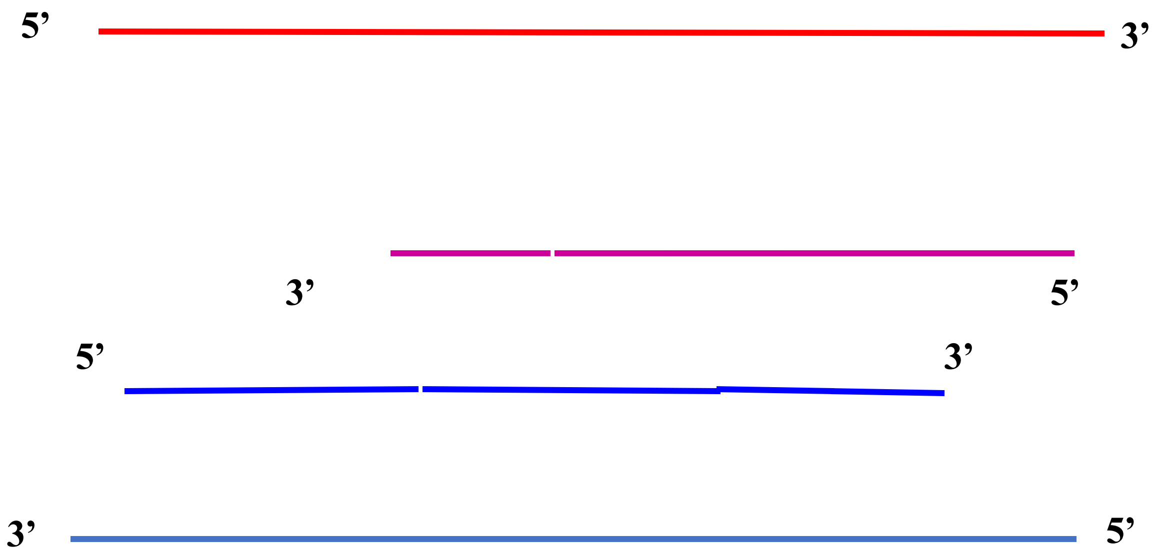




Cycle 1
Cycle 2
100
90
80
70
60
50
10
Temp
(°C)
Template
denature
Primer
anneal
DNA
synthesis
1
2
3
4
5
6
7
Time (mins)
Reaction buffer
DNA template
PCR primers
Taq DNA pol
1
2
3
95°
55°
72°
Denature Template
Anneal Primers
DNA Synthesis
+
+
+

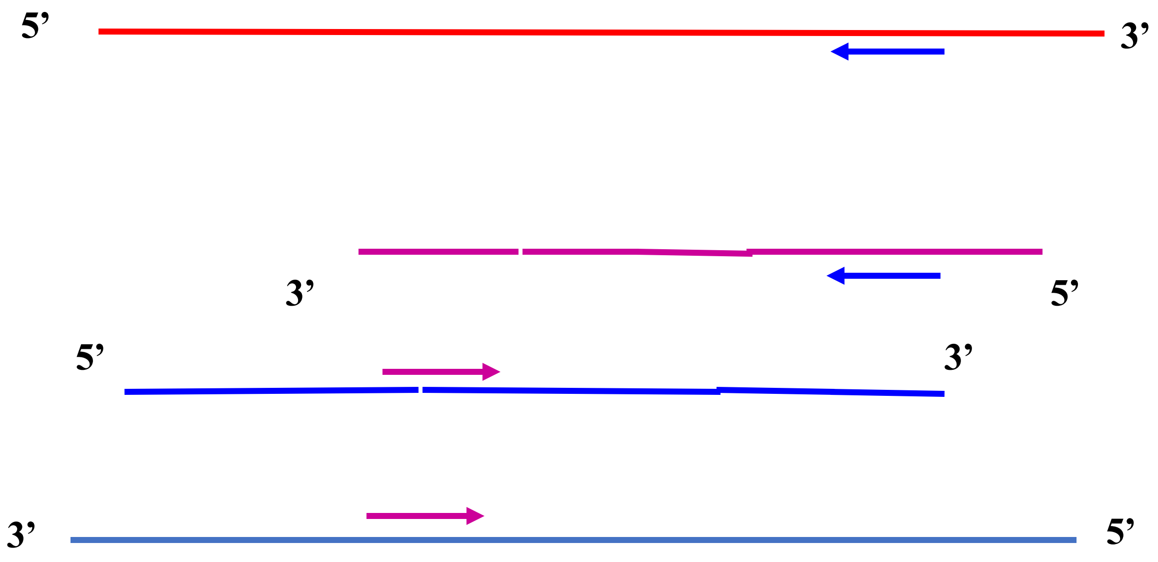
PCR CYCLE 2

Denaturation at
95^{0}C
Strands separated



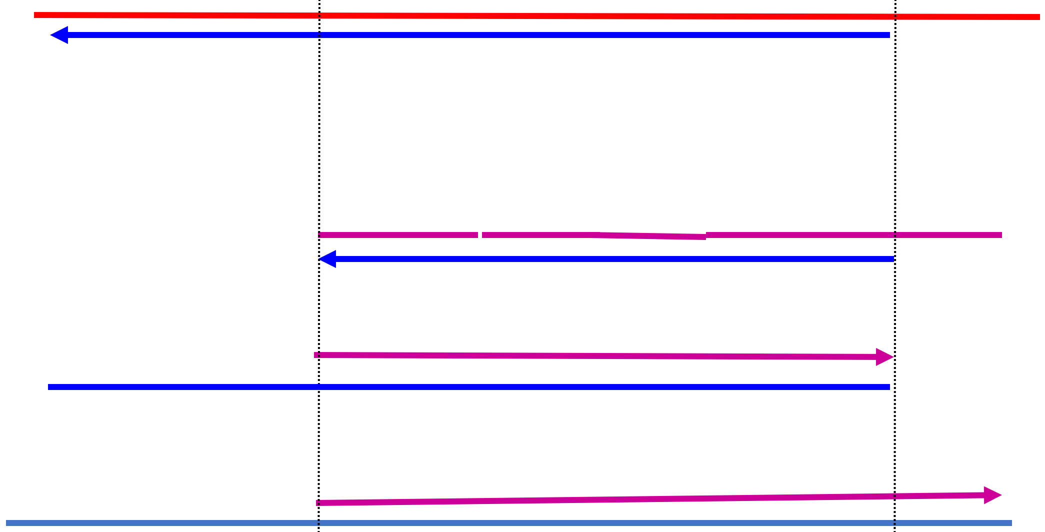
PCR CYCLE 2

Binding of primers at ~55^{0}C

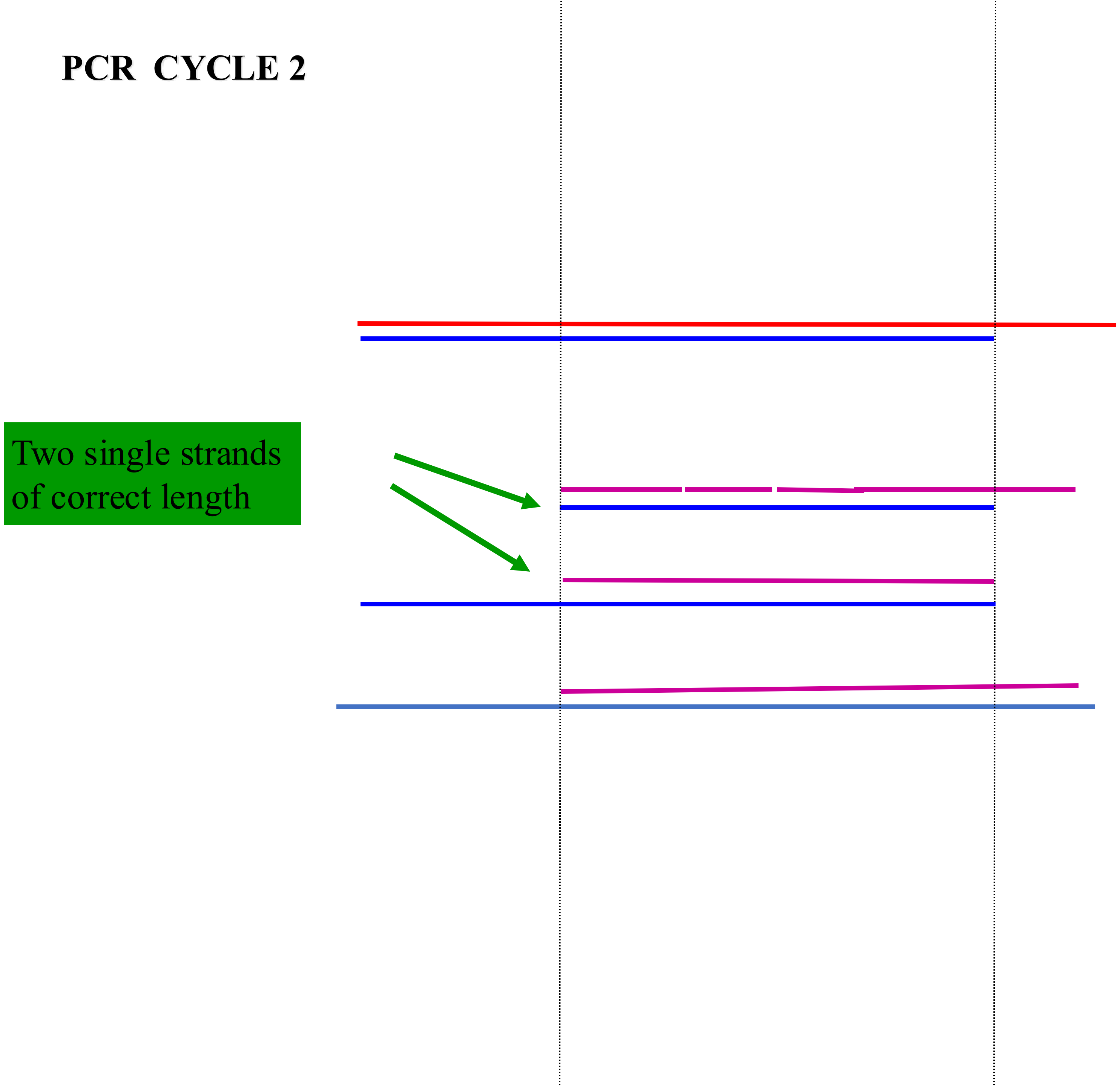


PCR CYCLE 2

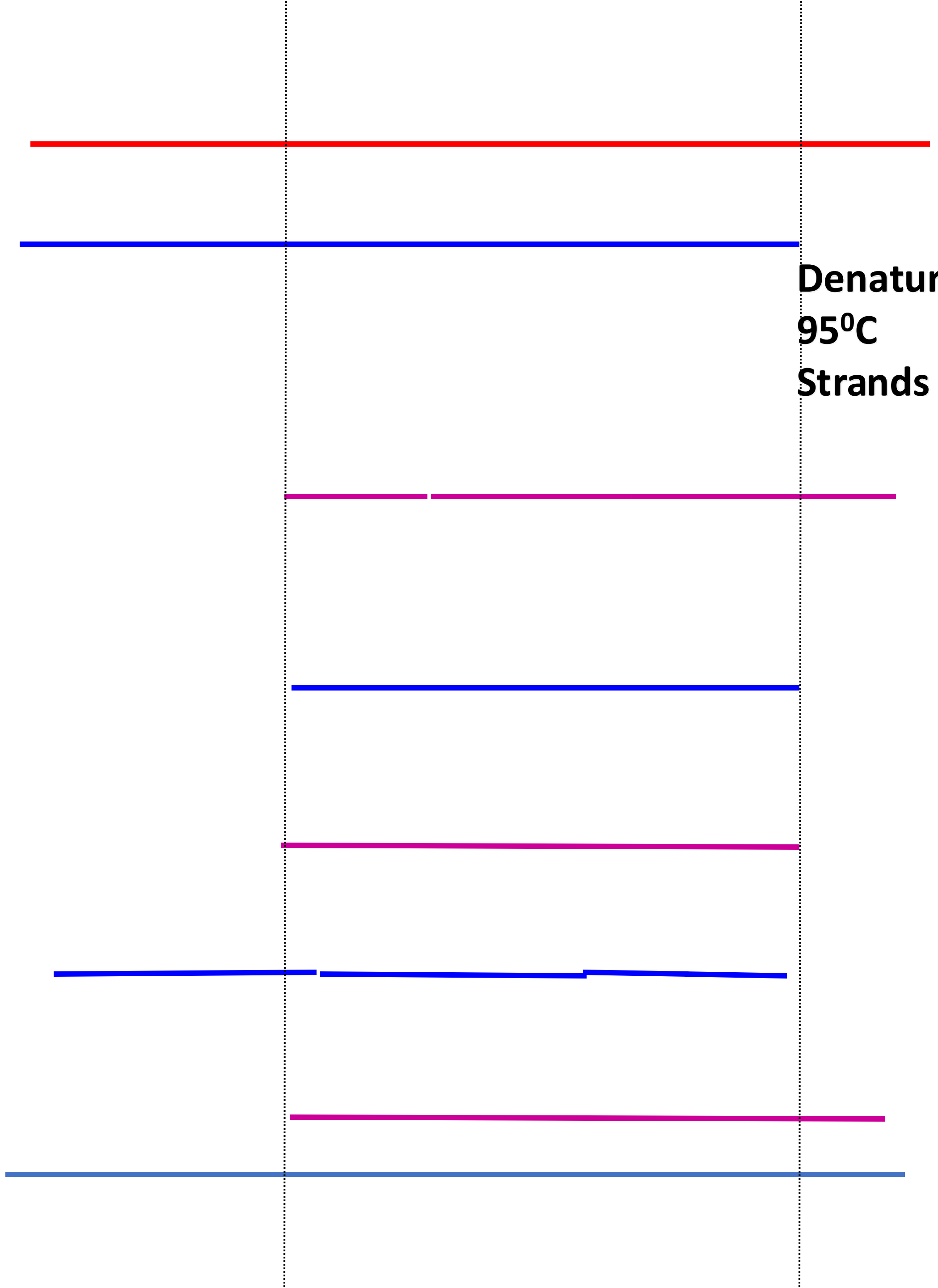
Extension at ~72^{0}C



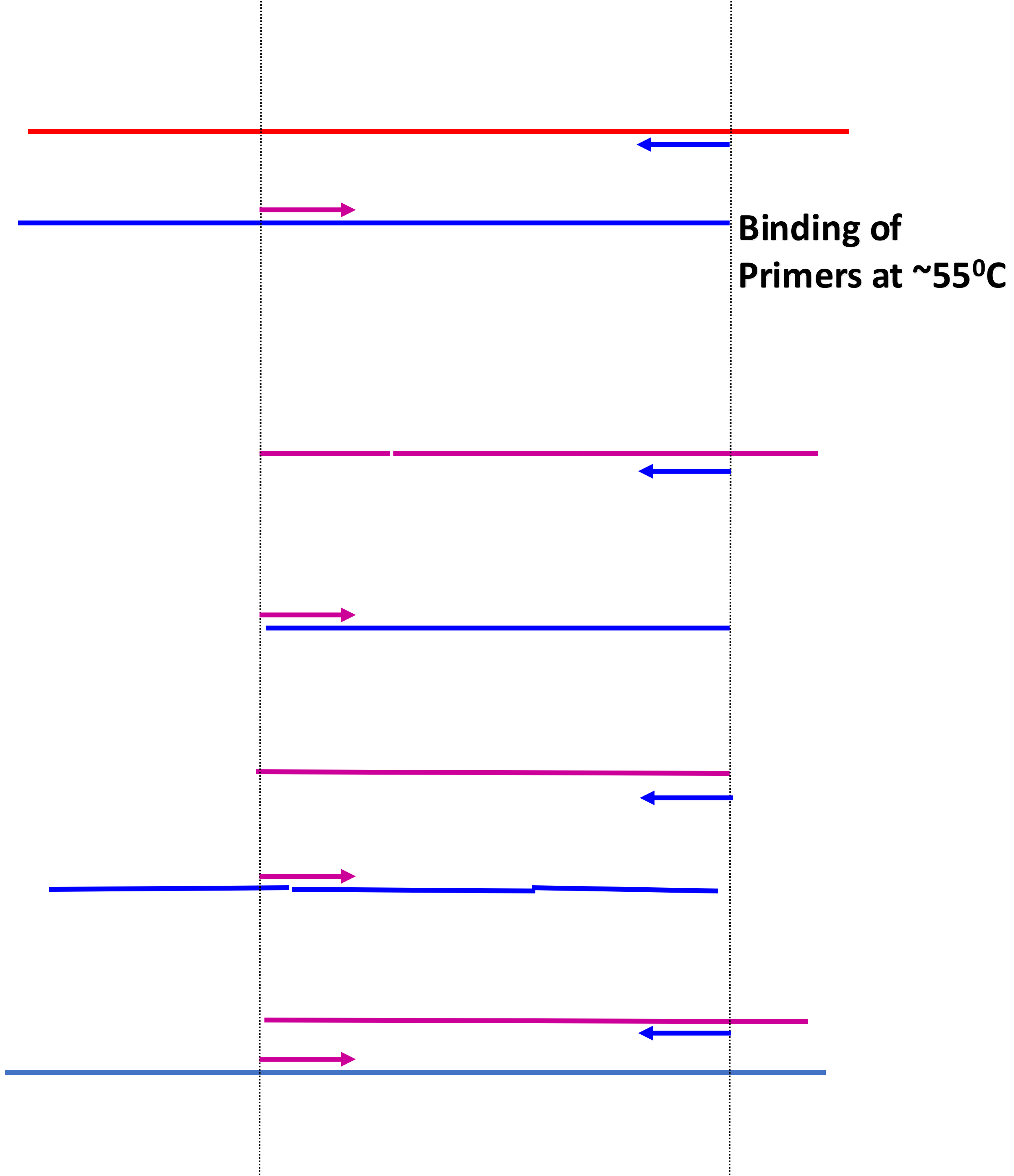
PCR CYCLE 2



PCR CYCLE 3

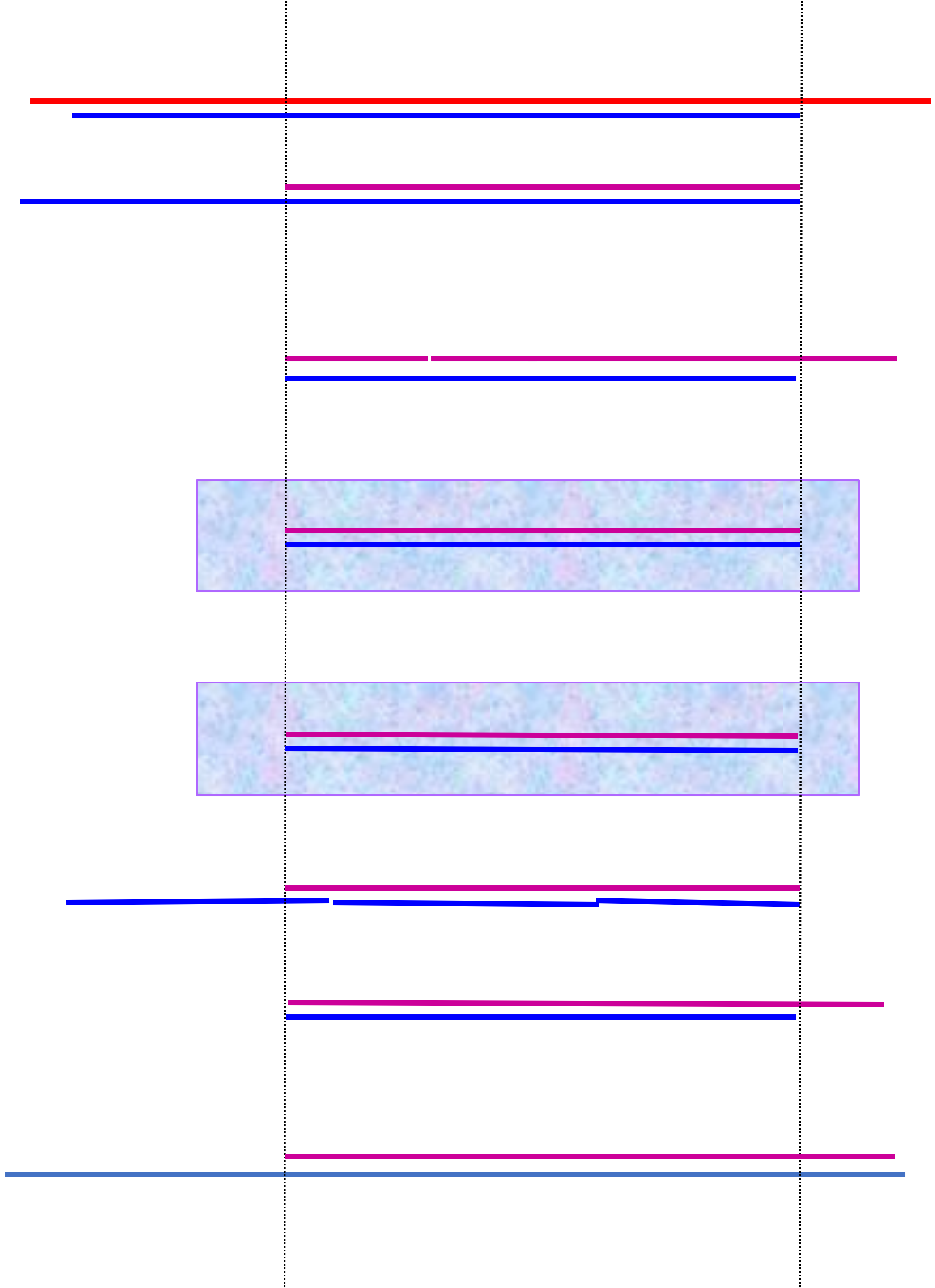


PCR CYCLE 3



PCR CYCLE 3

Extension
72^0C



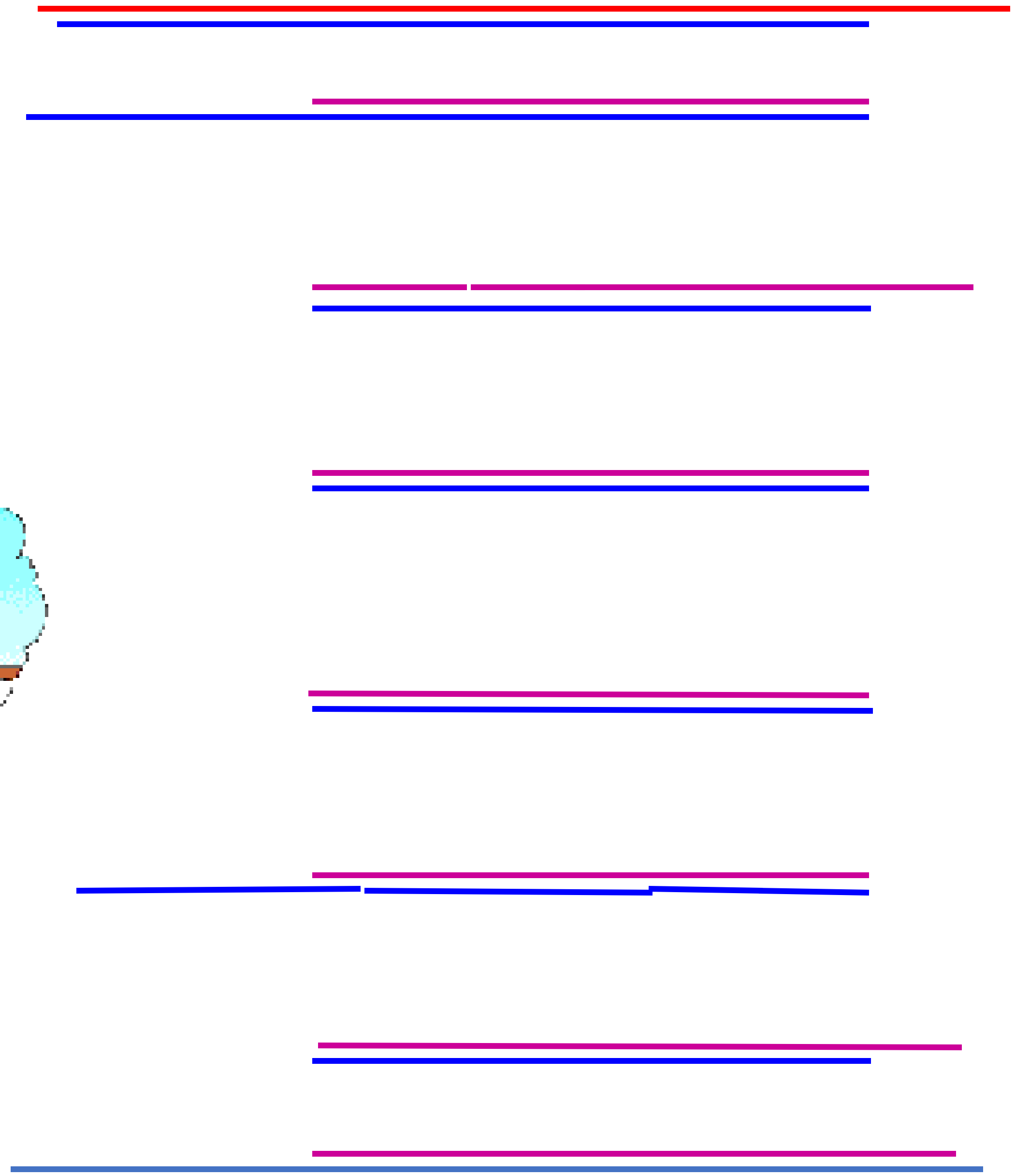
3. Cycle is the first cycle when the first products of targeted length

From this cycle on amplication proceeds exponentially

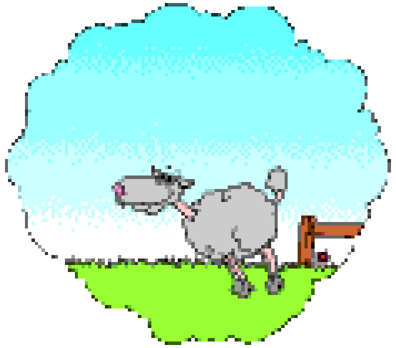
Amplimers synthesized according to equation mentioned below:

After n number of PCR cycles where exponential amplification
$No(1+Y)^{n-1}$ target copy will be formed!

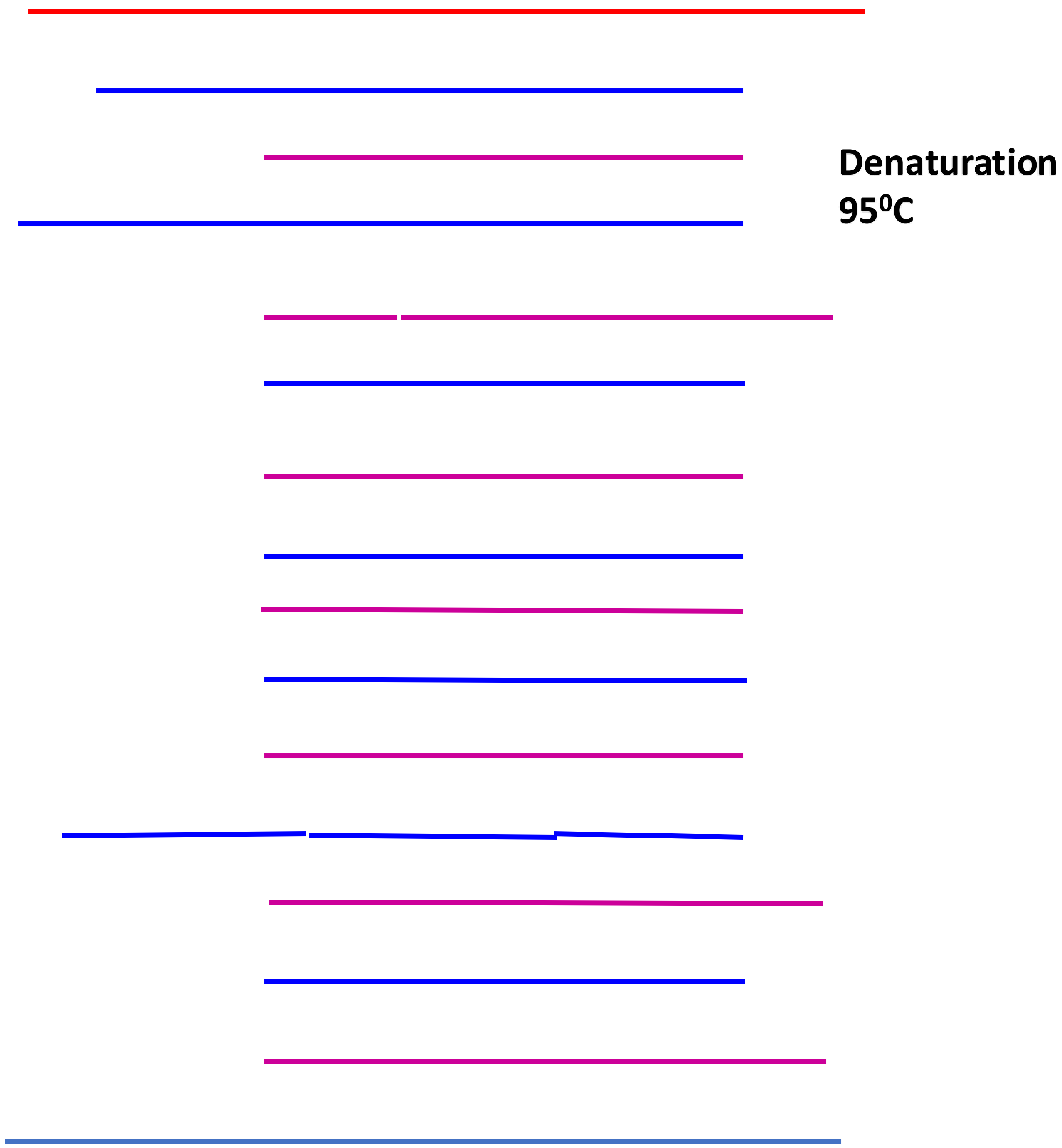
No	*starting number of DNA targets*
Y	*Efficiency of PCR reaction*
n	cycle number



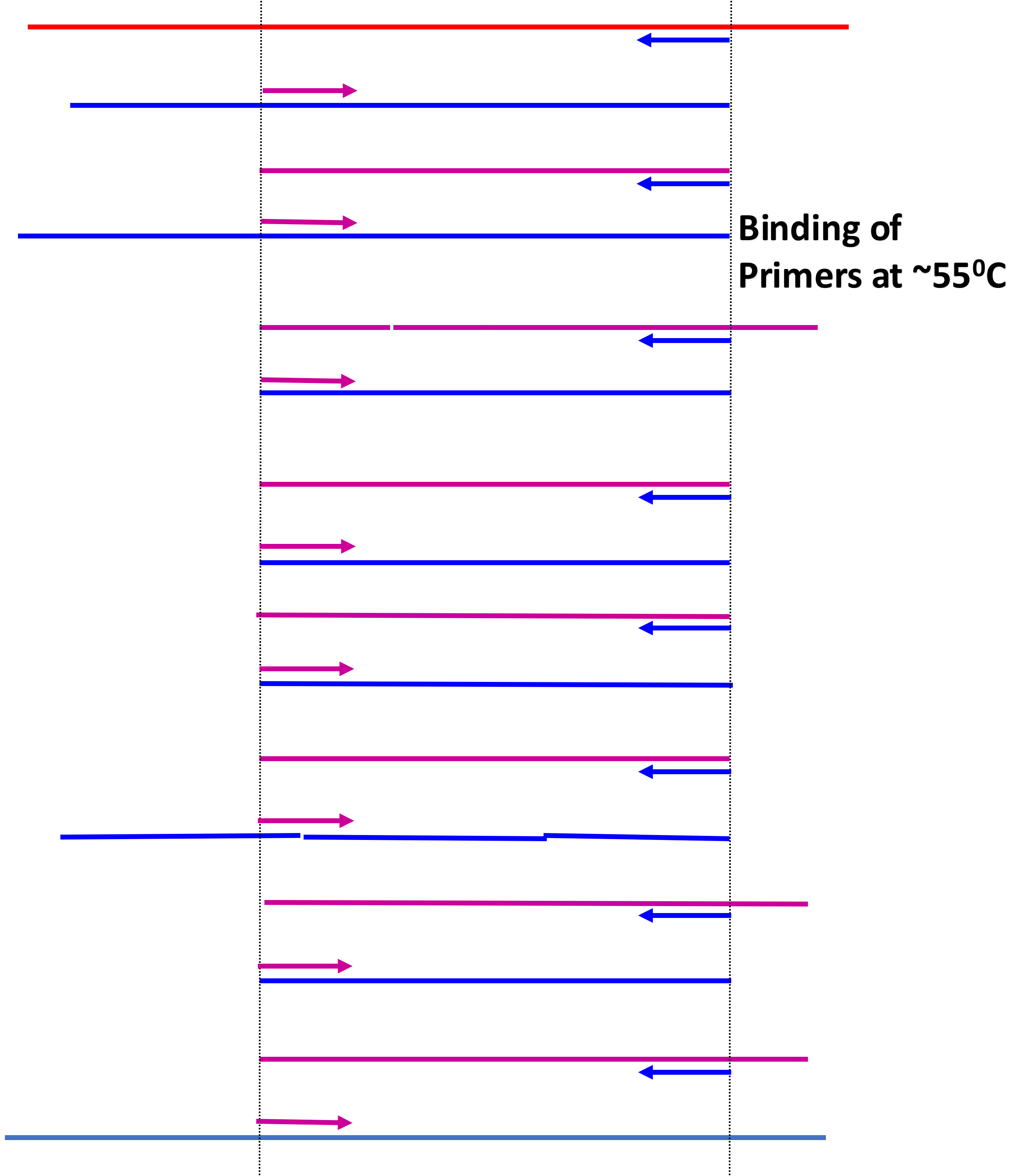
END OF
PCR CYCLE 3



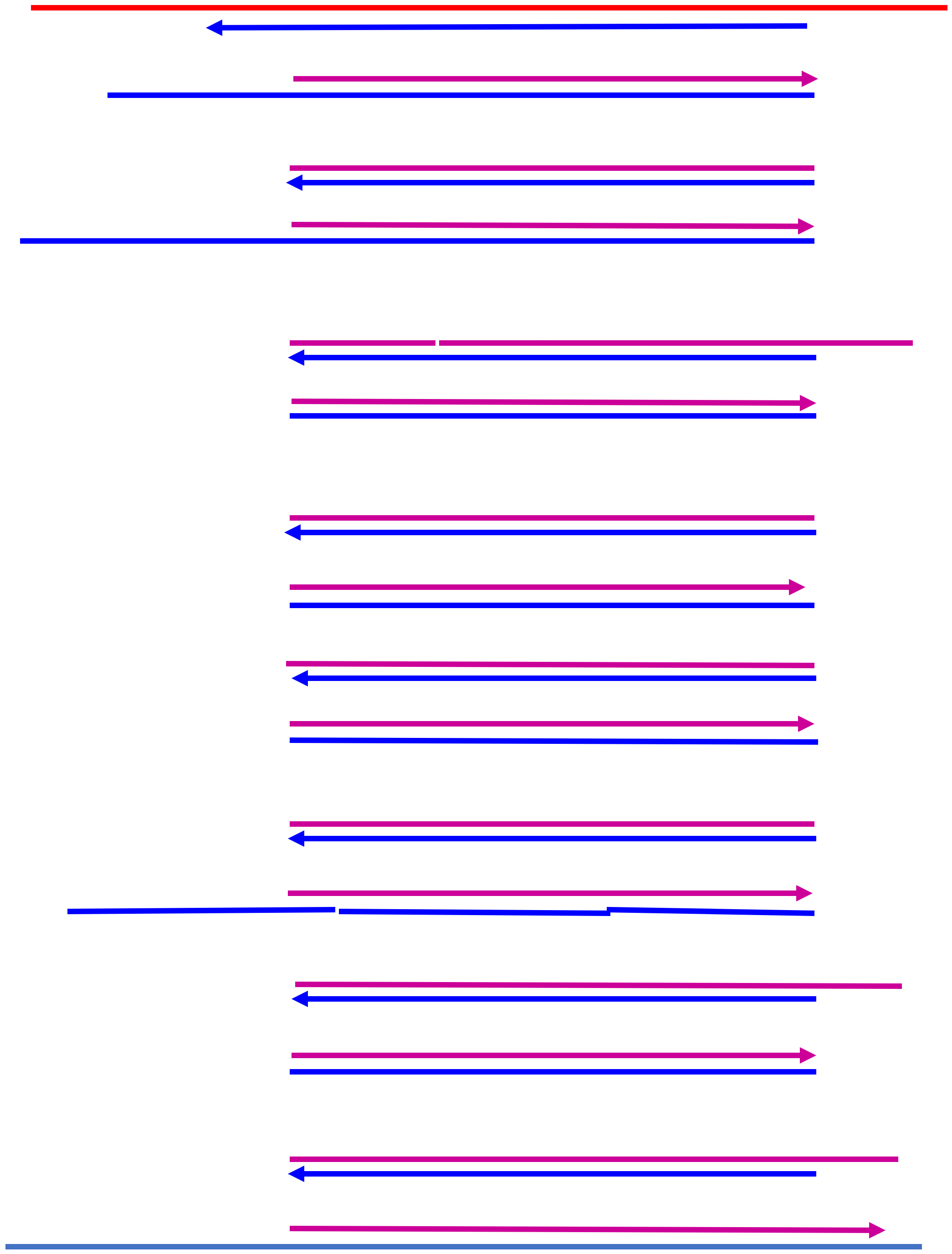
PCR CYCLE 4



PCR CYCLE 4

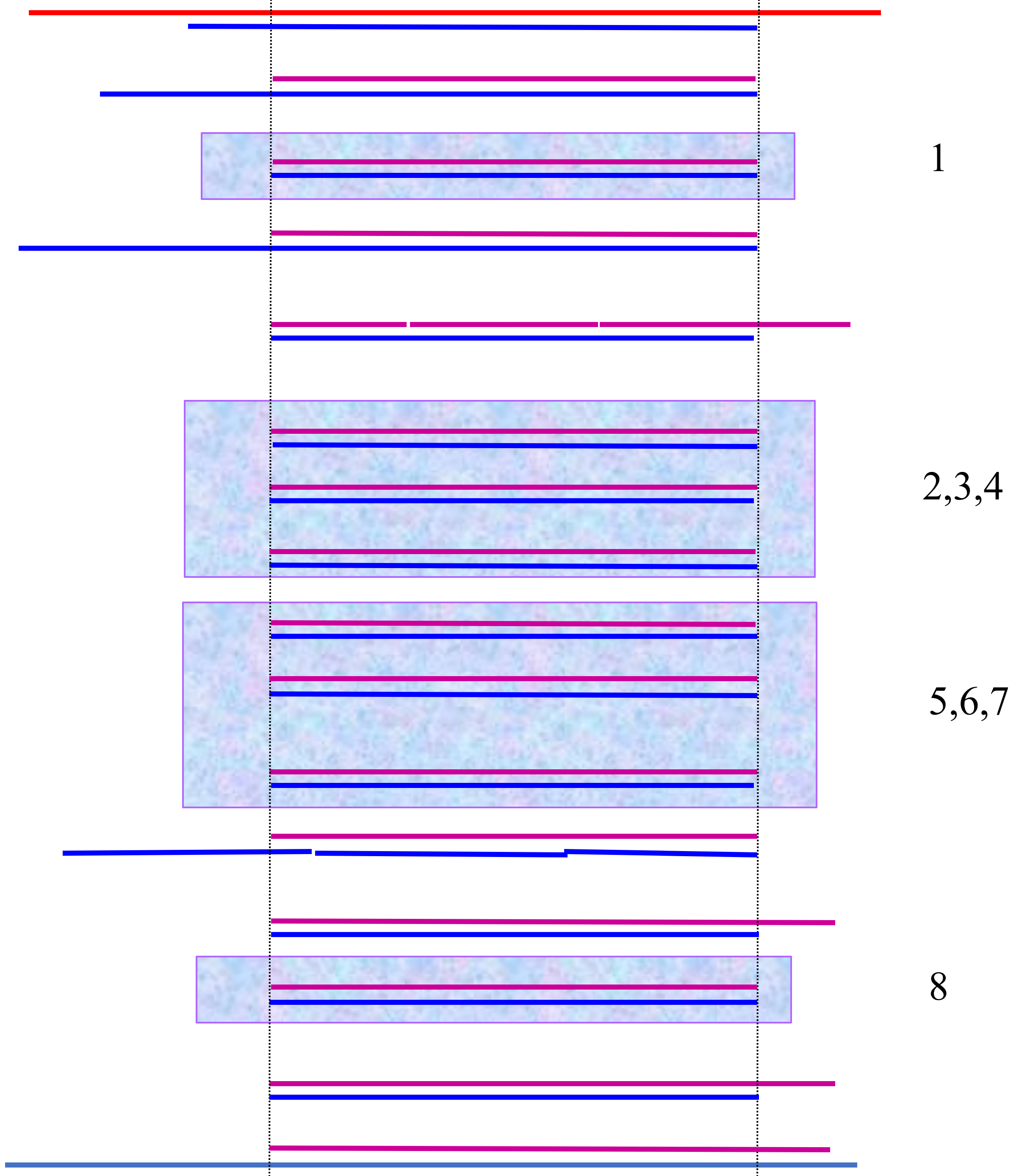


PCR CYCLE 4



Extension
72^0C

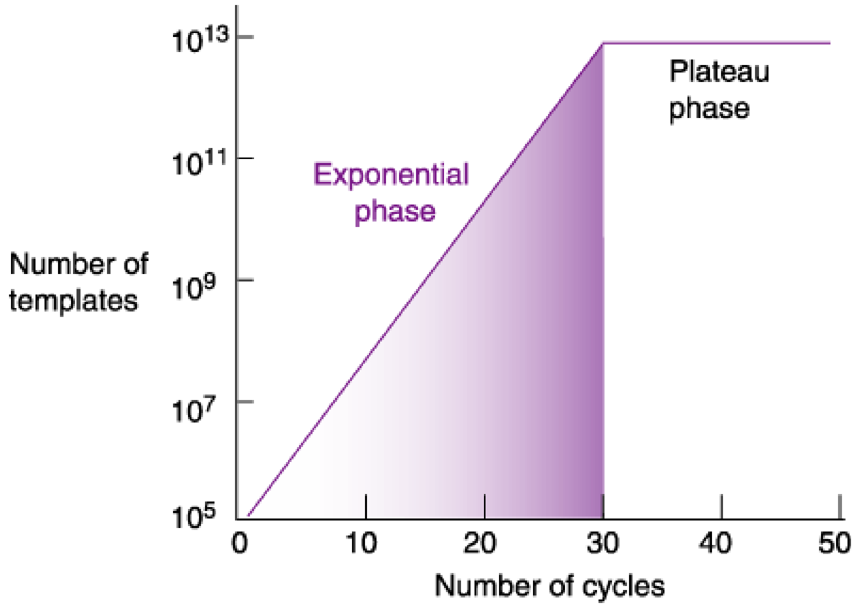
PCR CYCLE 4

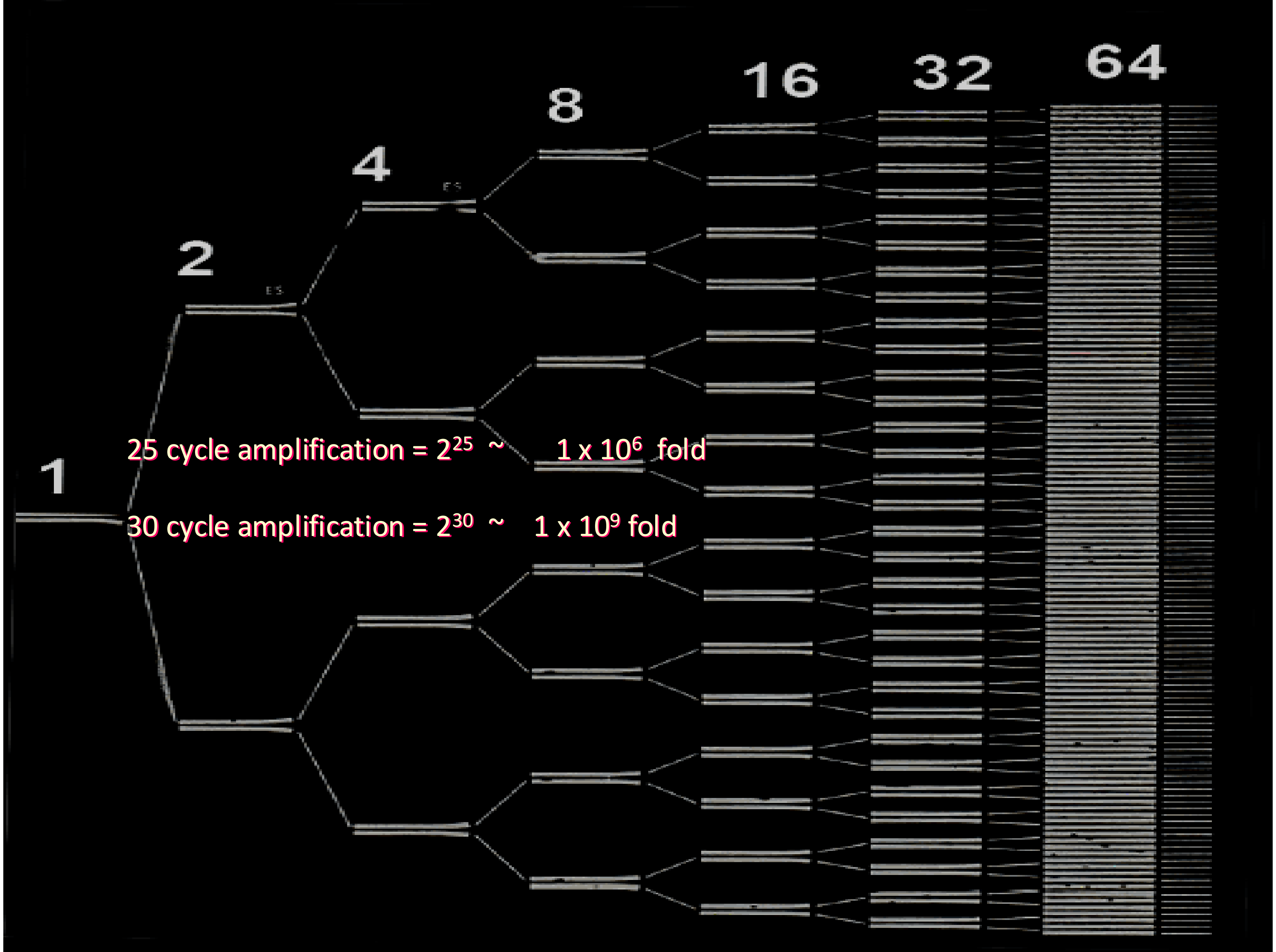


Lets presume that only 1 DNA target exists

- 4. cycle $1(1+1)^3=8$
- 5. cycle $1(1+1)^4=16$
- 6. cycle $1(1+1)^5=32$
- After a definite number of cycles PCR efficiency decreases.

$No(1+Y)^{n-1}$



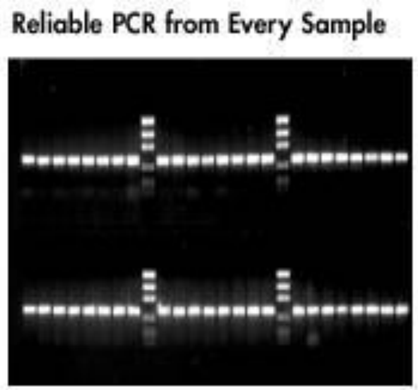


1
2
4
8
16
32
64
ES
ES
25 cycle amplification = 2^25 ~ 1 x 10^6 fold
30 cycle amplification = 2^30 ~ 1 x 10^9 fold

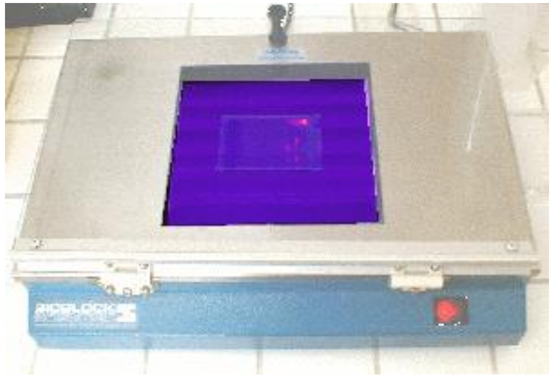
PCR

Agarose gel electrophoresis

3-4 hours



Final product



UV imaging

ALWAYS SHOULD BE REMEMBERED!

PCR is a very sensitive technique– DNA contamination with an unwanted DNA could be significant!

Always add negative controls to the reaction!

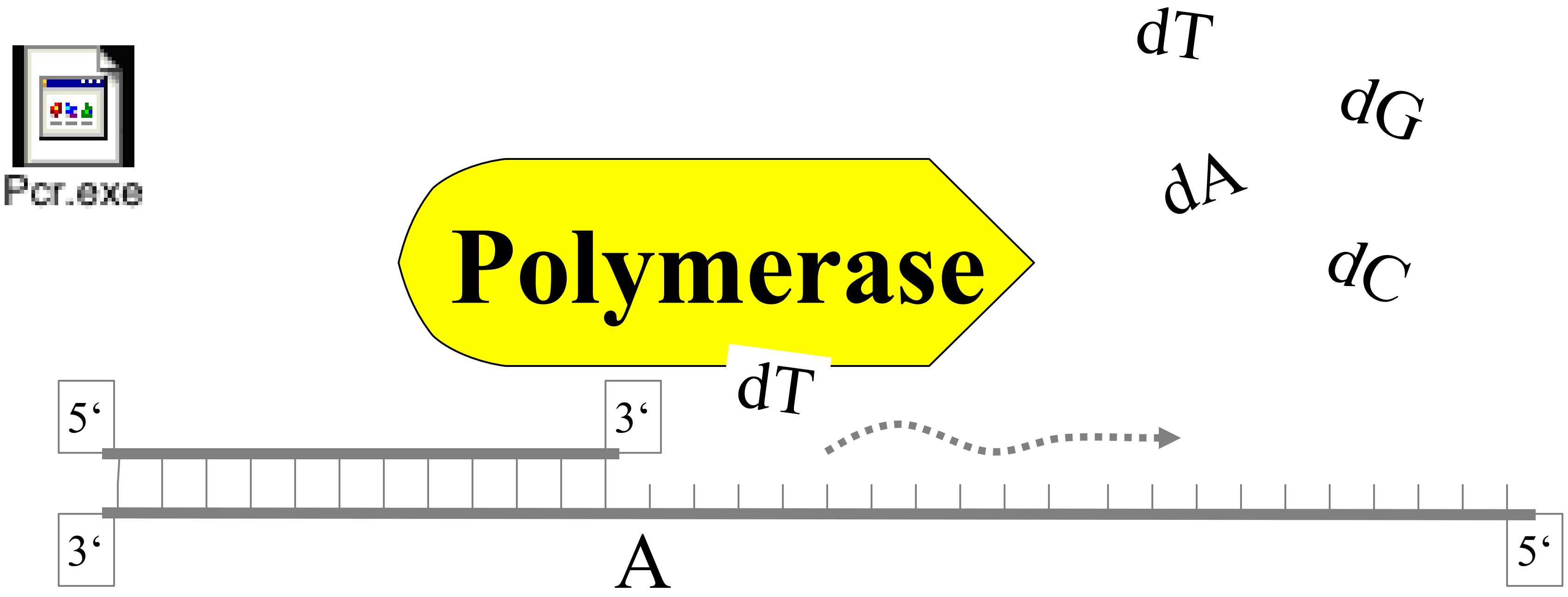
Always add positive controls to the reaction!

Use appropriate filtered pipets and pippet tipds

Perform PCR in separate units

Use laminar flows with UV lambs

Polymerisation



- **Nucleofilic effect**
- **phosphodiester bonds cathelysis**

PCR is used for;

- Cloning of gene or gene fragments
- Genetic diagnosis – Detection mutations
- Maternity-Paternity Tests
- DNA sequence analysis
- Forensic identification
- Determination of quality control of industrial products
- Determination of appropriate tissue type for tissue transplantation
- Determination of polymorphism in between species
- Molecular typing
- Detection of pathogens

PCR for forensic identification:

Example 3: Multilocus Fingerprinting

Multilocus fingerprinting to match trace evidence from a crime with suspects. Which suspect matches the specimen?

Maternity/Paternity Testing:

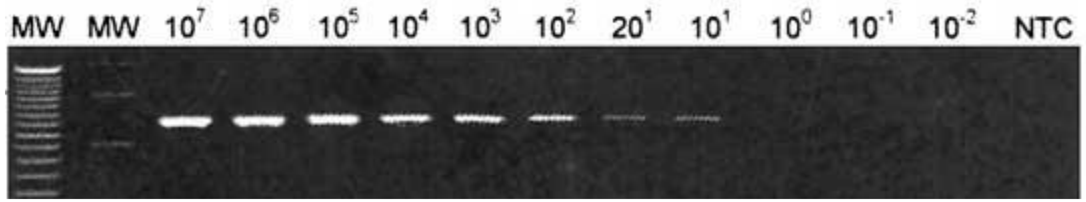
Example 2: Multilocus Fingerprinting

Microsatellite fingerprinting to establish parentage. The probe, $(CAG)_5$, recognizes a large number of loci. Examine the bands detected in DNA from the child that are not detected with DNA from the mother. Which male is the biologic father of the child?

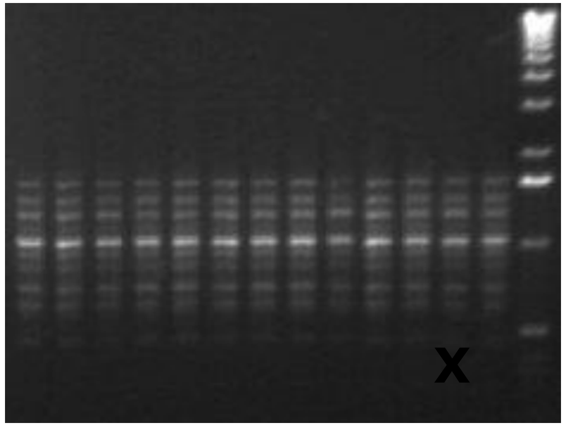
Advantages and Disadvantages of PCR!

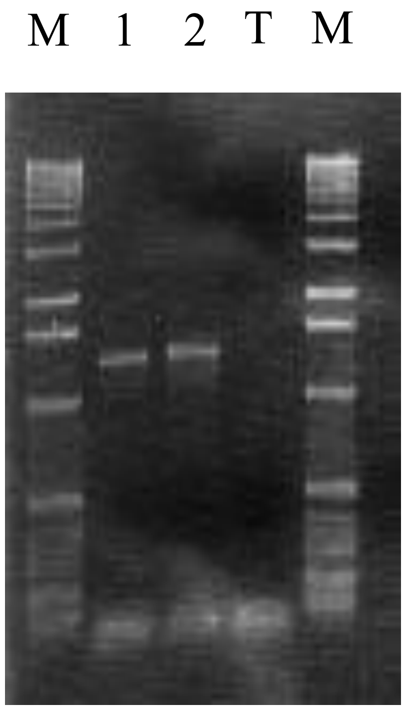
- High sensitivity and specificity!
- Fast detection and identification!
- Could detect inanimate (dead) agents!
- Could detect acid-fast and environment fragile agents!
- Could detect slow growing bacteria
- Provide the probability of later sophisticated studies (typing, sequence analysis, clonning) olanak sağlaması

- Still cannot replace isolation in definitive diagnosis!
- False-positiveness due to cross-contamination!
- Requires lab infrastructure!
- Requires well trained personnel!
- High expendature costs!



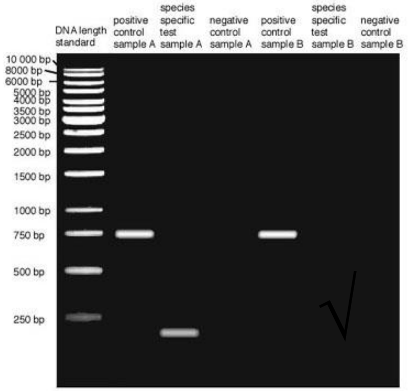
template DNA concentration



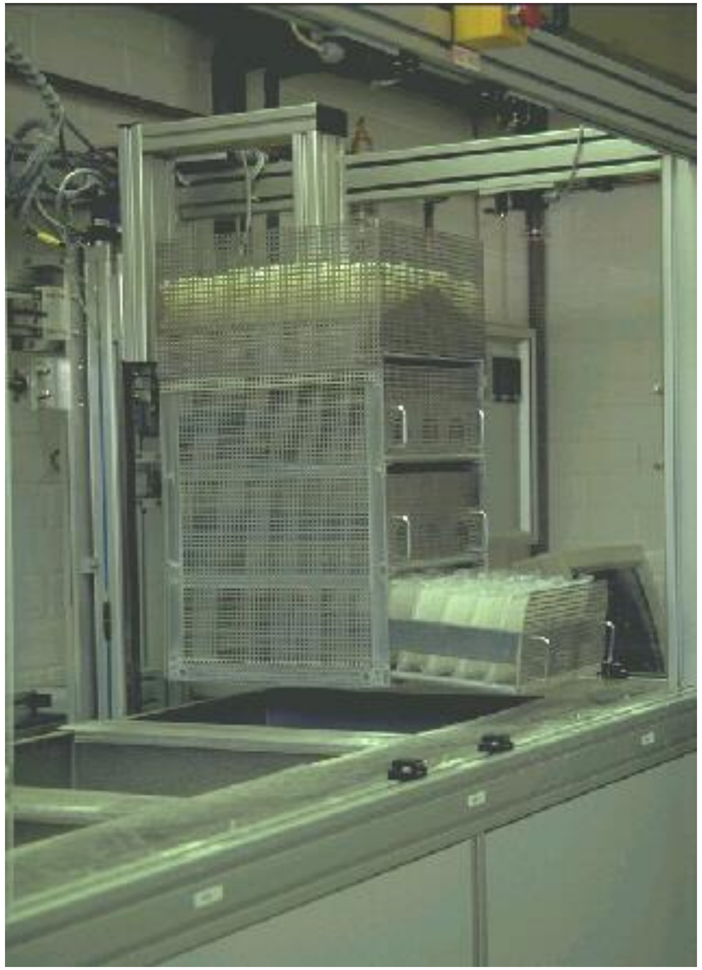


PCR products

Artefactual products



Thermal Cycler Models



Portative Molecular Biology Laboratories!!!