PCR - Polymerase chain reaction:

PCR is an enzymatic reaction alows the selective amplification of a defined segment of DNA

- **Genomic DNA**
- **Plasmid**
- **cDNA**
- **Mitochondrial DNA**

One of the most significant applications in molecular biology
Basis for other important methods

Basic PCR, Cloning of PCR product, RT-PCR, RACE, Quantitative PCR, Multiplex PCR, Hot start PCR, Touchdown PCR,PCR sequencing, Real-time RT-PCR (RT_2-PCR).....

PCR - Polymerase chain reaction:

Kary B. Mullis

(1944 -)
The inventor of the DNA synthesis process known as the Polymerase Chain Reaction (PCR). The process is an invaluable tool to today's molecular biologists and biotechnology corporations.

Mullis, born in Lenoir, North Carolina, attended the University of Georgia Tech for his undergraduate work in chemistry, and then obtained a Ph. D. in biochemistry from Cal Berkeley.

In 1983, working for Cetus Corporation, Mullis developed the Polymerase Chain Reaction, a technique for the rapid synthesis of a DNA sequence. The simple process involved heating a vial containing the DNA fragment to split the two strands of the DNA molecule, adding oligonucleotide primers to bring about reproduction, and finally using polymerase to replicate the DNA strands. Each cycle doubles the amount of DNA, so multiple cycles increase the amount of DNA exponentially, creating huge numbers of copies of the DNA fragment.
Mullis left Cetus in 1986. For his development of PCR, he was co-awarded the Nobel Prize in chemistry in 1993.

http://library.thinkquest.org/24355/data/details/profiles/mullis.html

PCR Polymerase chain reaction:

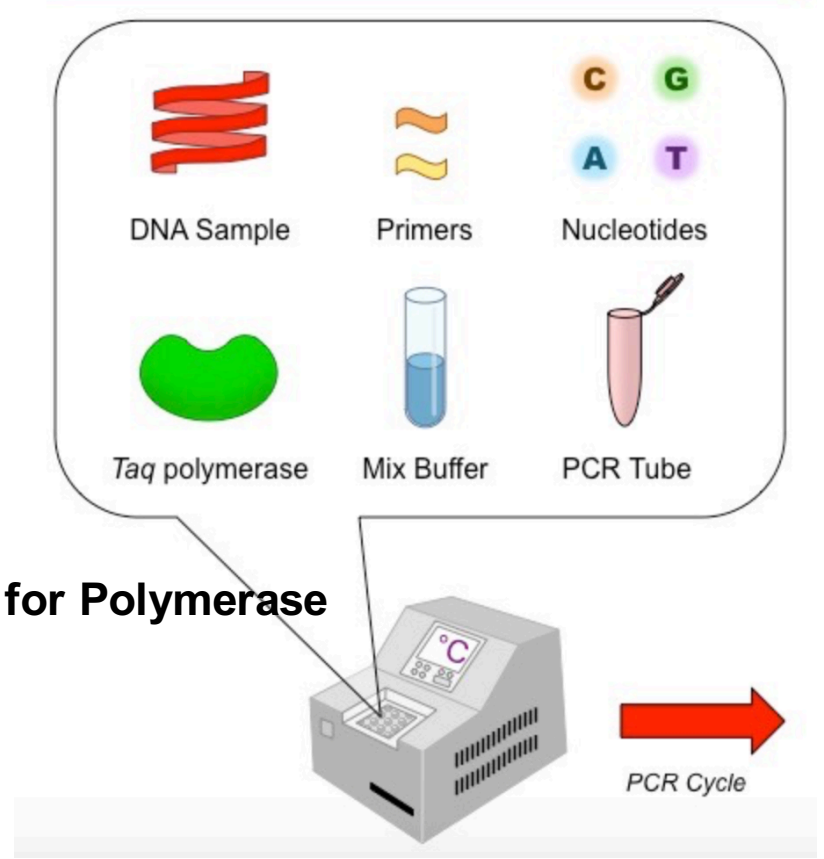
1985: The power of PCR an example

- Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia.
  Science. 1985 Dec 20;230(4732):1350-4.

  - **Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N.**
  - Cetus Corporation, Department of Human Genetics, Emeryville, CA 94608.

PCR Requires the following:

- Template DNA to be amplified
- Pair of DNA primers
- Thermostable DNA polymerase
- dNTPs
- Buffer to maintain pH and to provide Magnesium Ions for Polymerase
-Thermal cycler



PCR Requires the following:

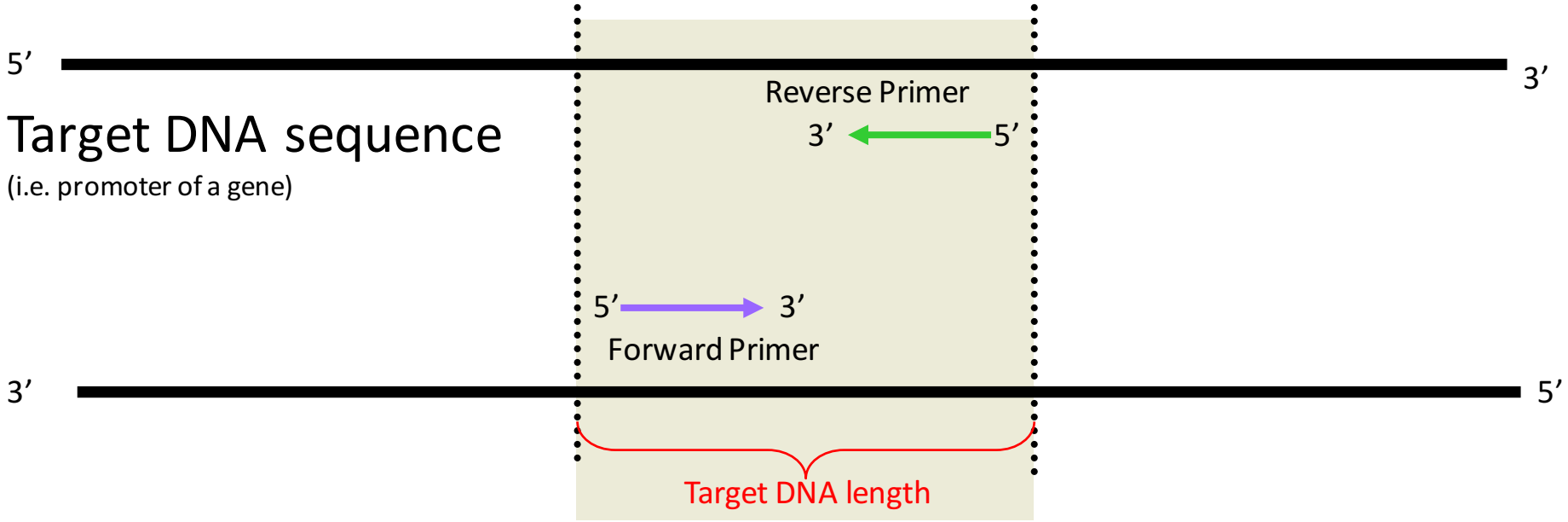
1. Template DNA

- A sequence of DNA that is to be copied - also called *target* DNA.
- PCR can amplify (copy mnay times) a piece of DNA ~50 to >4000 bp
- A part of the sequence must be known to locate 1 **primer** for DNA polymerase on each single DNA strand oriented with the 3'ends oriented towards each other (convergent)
- DNA must be isolated from an organism before it can used
- Any type of DNA can be amplified (bacteria, vertebrates, plants…)
- DNA must be as pure as possible and must not be degraded

PCR Requires the following:

2. A pair of DNA primers

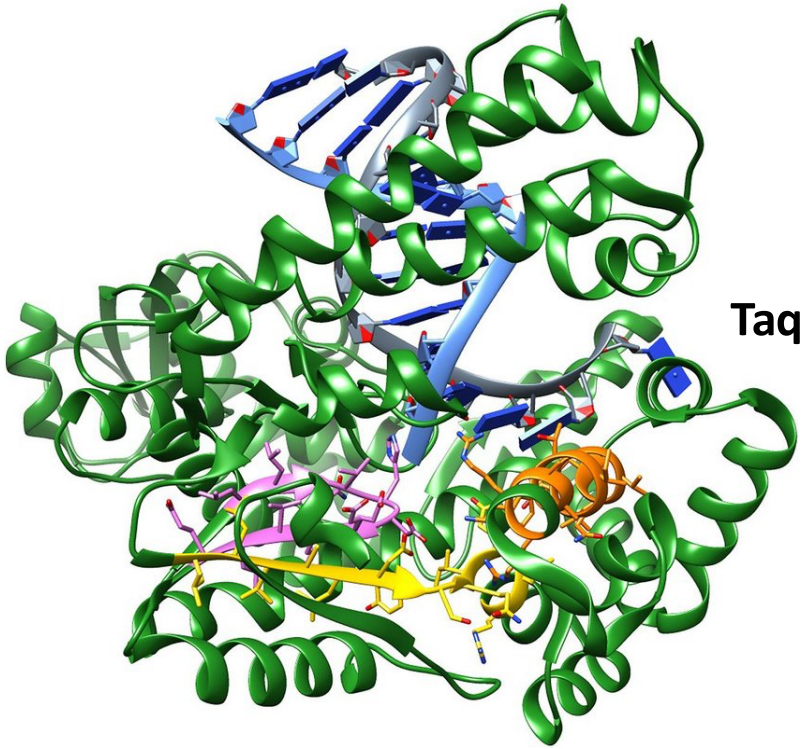
- In the cell (*in vivo*), primers are short RNA strands that serve as a starting point for DNA replication
- In a PCR reaction (*in vitro*), Primers are short synthetic strands of single stranded DNA that exactly match the beginning and the end of the DNA fragment to be amplified.



PCR Requires the following:

3. Thermostable DNA polymerase

- Polymerase builds a new DNA strand in the 5’ to 3’ direction.
- The newly-polymerized molecule is complementary to the template strand and identical to the template's partner strand.
- Most known: Taq polymerase, Pfu polymerase



Taq polymerase

PCR Requires the following:

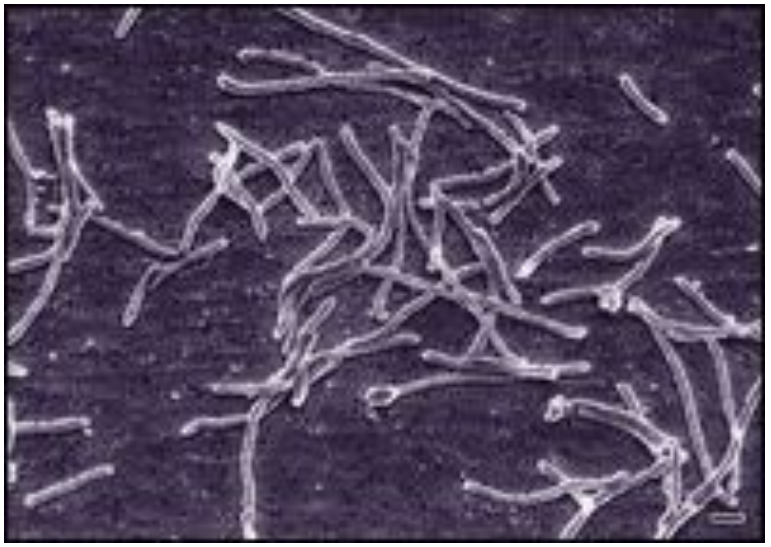
3. Thermostable DNA polymerase

- DNA polymerase must be Thermostable (Heat–stable)
- Temperatures in PCR range between 4°C and 94°C
- Polymerase does not unfold at high temperatures !!
- DNA polymerases purified from thermophil archaebacteria
- Most famous: Taq polymerase, isolated from the bacteria *Thermus aquaticus* (they live in hot springs)
- Taq pol has 5'-3' exonuclease activity, but lacks 3'-5' exonuclease activity (no proofreading)

Processivity of PCR polymerases

<72°C: inefficient DNA synthesis
72°C: ideal temperature for DNA synthesis
>94°C: protein unfolding – no DNA synthesis

60 nucleotides per second at 70 °C
24 nucleotides/sec at 55 °C
1.5 nucleotides/sec at 37 °C
0.25 nucleotides/sec at 22 °C.
0 nucleotides/sec above 90 °C



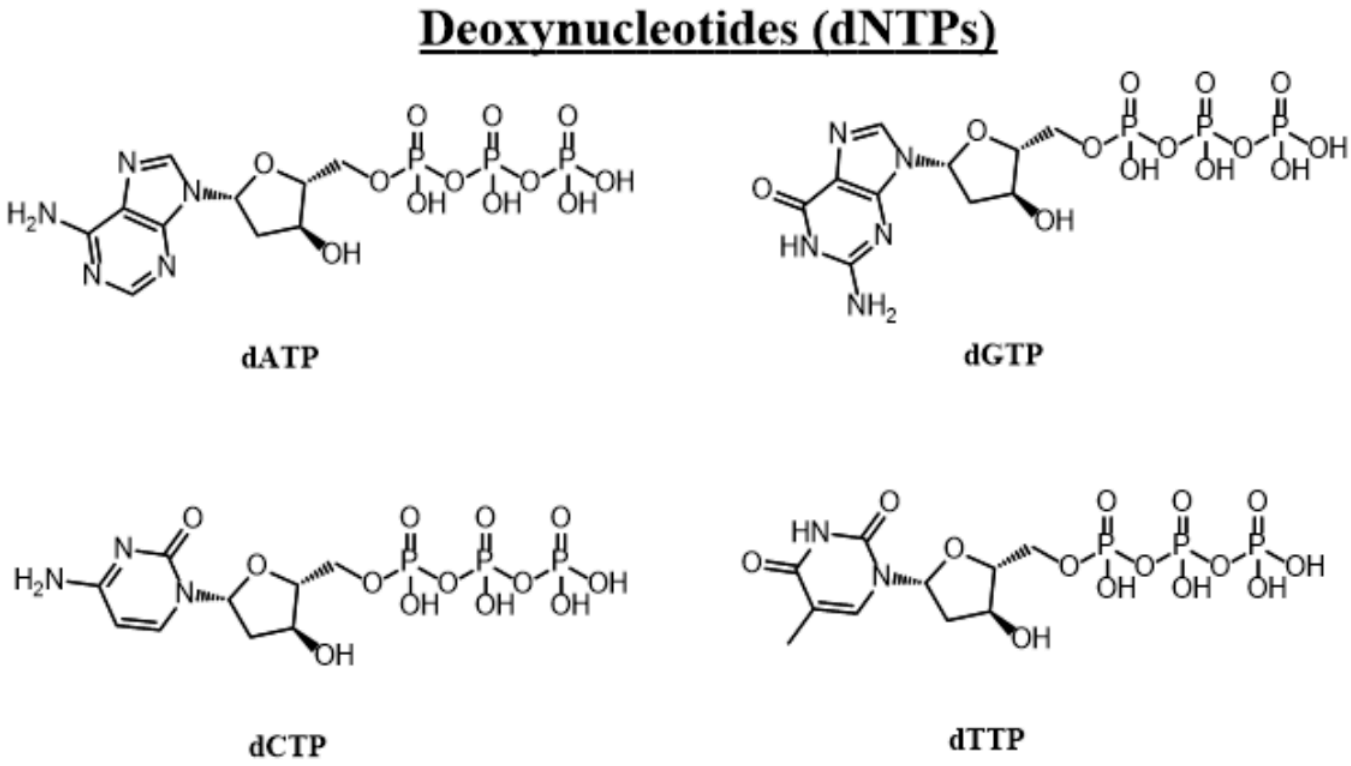
→ During a PCR based amplification of a target DNA, temperatures are shifted to control the activity of DNA polymerase

PCR Requires the following:

3. dNTPs = mix of dATP, dTTP, dCTP, dGTP

- dNTPs (deoxyribonucleotides) are the building blocks in DNA synthesis reaction

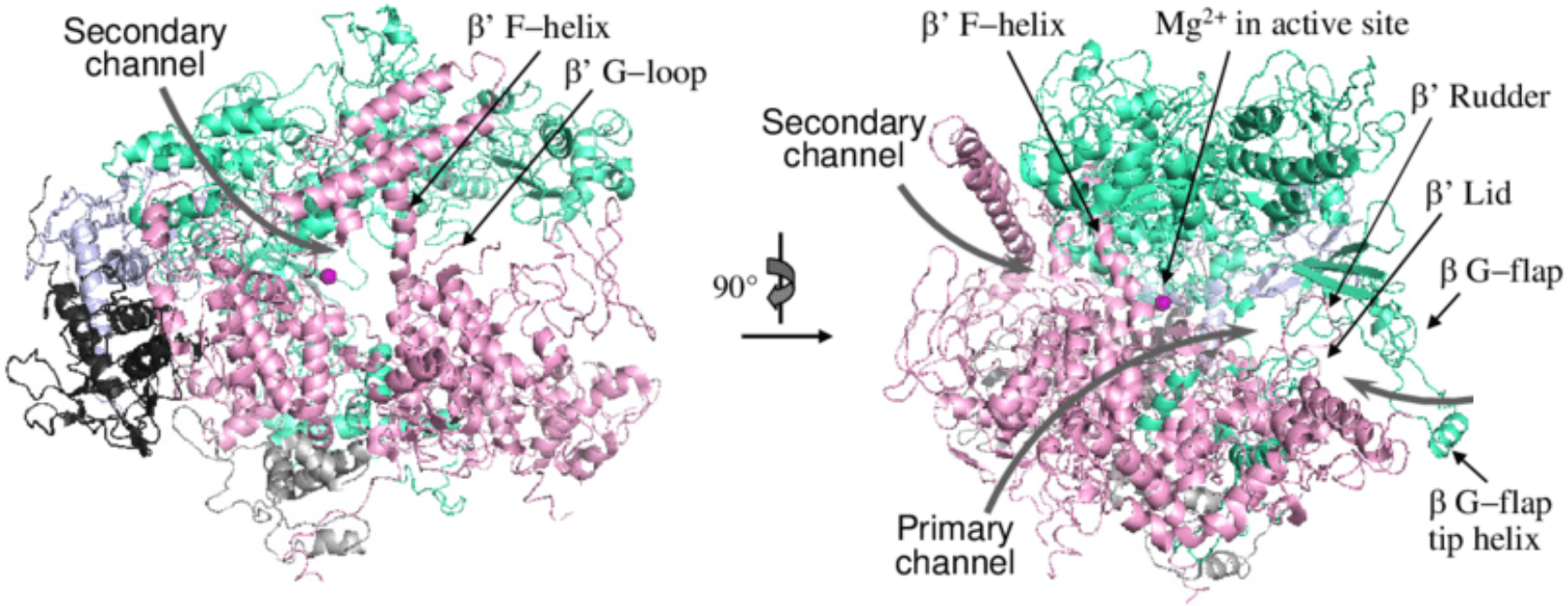
- present as monomers that DNA polymerase will in a polymerization reaction to create a new strand in a template-sequence dependent manner



PCR Requires the following:

5. Buffer

- DNA polymerase needs Mg^{2+} in active center
- The concentration of magnesium ions may need to be optimized with each target and primer combination (too little magnesium could equal little or no PCR product, too much could mean unwanted product….
- Buffer also maintains pH and has salt concentrations ideal for DNA pol function
- Example: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 5mM $Mg2^{+}$



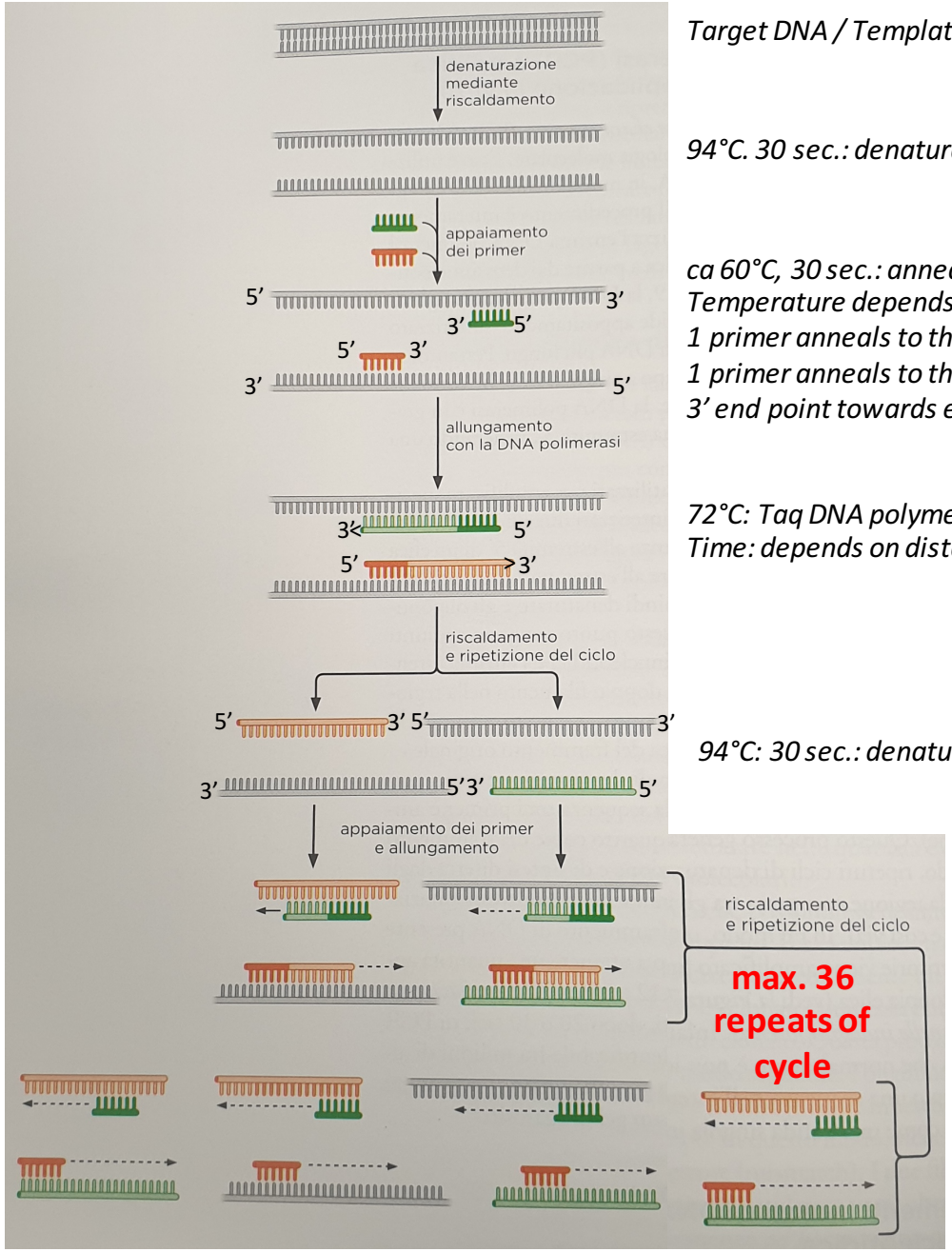
PCR Requires the following:

Components	Volume	Final Concentration
10X PCR buffer minus Mg	10 µl	1X
10 mM dNTP mixture	2 µl	0.2 mM each
50 mM $MgCl_2$	3 µl	1.5 mM
Primer mix (10 µM each)	5 µl	0.5 µM each
Template DNA	1-20 µl	-----
Taq DNA Polymerase (5 U/µl)	0.5 µl	2.5 units
Autoclaved distilled water to	100 µl	

Buffer Composition (10X): 200 mM Tris-HCl (pH 8.4), 500 mM KCl.

One **Unit Taq DNA Polymerase** is defined as the amount of enzyme that incorporates **10 nmol** of total deoxyribonucleoside triphosphates into acid precipitable DNA **within 60 min at +65 °C** under the assay conditions stated above.

Mechansim of PCR:



Target DNA / Template

94°C. 30 sec.: denaturation of double-stranded DNA

ca 60°C, 30 sec.: annealing of oligonucleotides (primers) to specific sites on the target DNA
Temperature depends on the length and sequence of the oligonucleotide
1 primer anneals to the sense strand
1 primer anneals to the anti-sense strand
3' end point towards each other

72°C: Taq DNA polymerase bind 3'end of primer and starts synthesis 3'→ 5' using dNTPs
Time: depends on distance between 3' ends of primers; ***typically 1min per 1000 nucleotides***

94°C: 30 sec.: denaturation of double-stranded DNA

ca 60°C, 30 sec.: annealing of oligonucleotides (primers) to specific sites on the target DNA ***AND*** *the newly synthesized DNA strands*

72°C: DNA synthesis

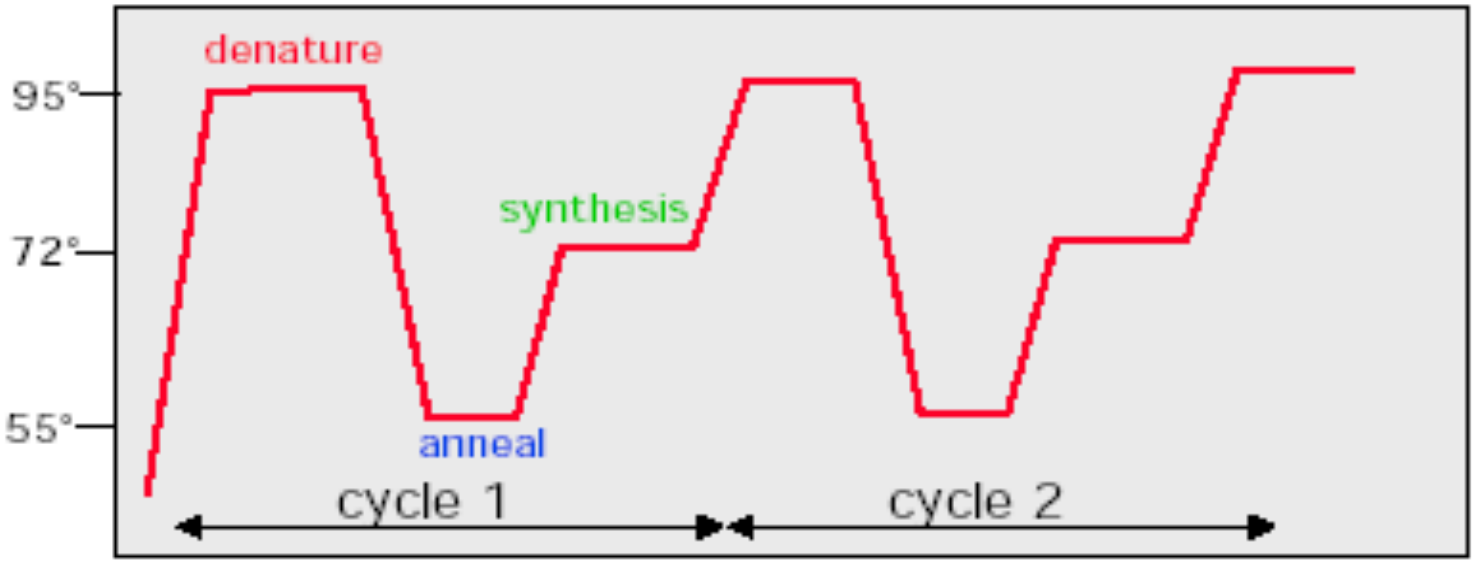
94°C: 30 sec.: denaturation of double-stranded DNA

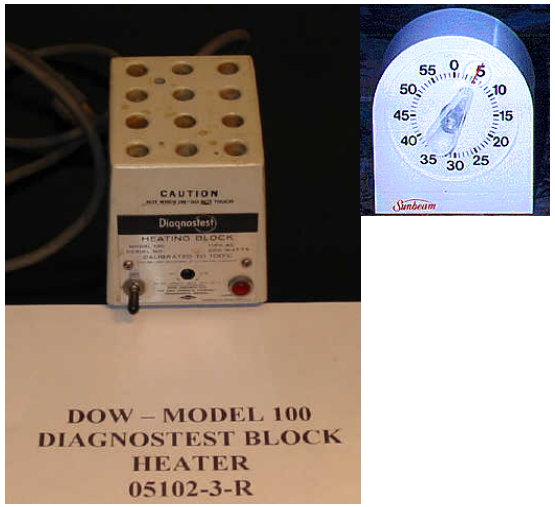
ca 60°C, 30 sec.: annealing of primers

72°C: DNA synthesis

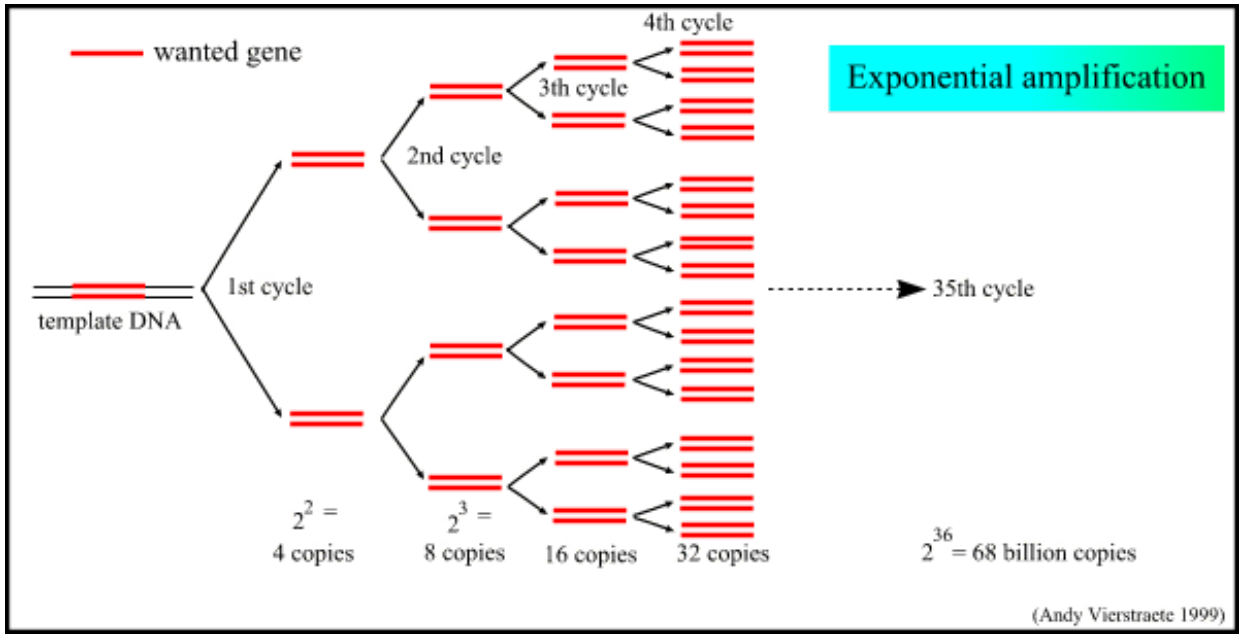
Mechansim of PCR:

PCR is organized in cycles during which the DNA amplification process takes place. This process requires a cyclic change in temperature and is carried out automatically by special machines (termocyclers), which produce PCR amplicons in up to 36 cycles in an hour





Mechansim of PCR:

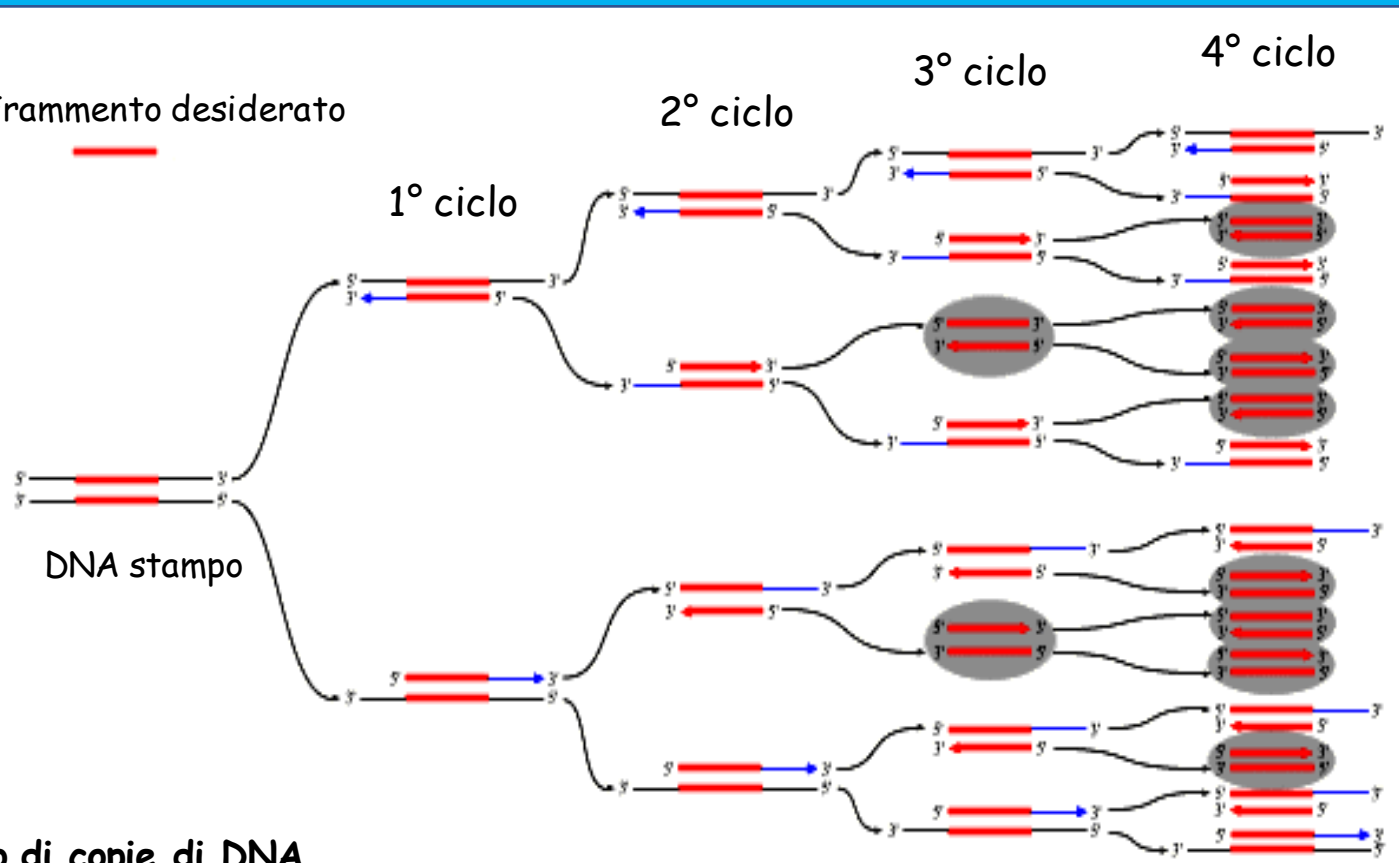


$$Y = N2^n$$

Y= numero molecole di DNA amplificato
N= numero molecole di DNA di partenza
n= numero dei cicli di PCR

Numero di cicli	Numero di molecole di amplificati
1	2
2	4
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1.024
11	2.048
12	4.096
13	8.192
14	16.384
15	32.768
16	65.536
17	131.072
18	262.144
19	524.288
20	1.048.576
21	2.097.152
22	4.194.304
23	8.388.608
24	16.777.216
25	35.544.432
26	67.777.216
27	134.217.728
28	268.435.456
29	536.870.912
30	1.073.741.724

Mechansim of PCR:

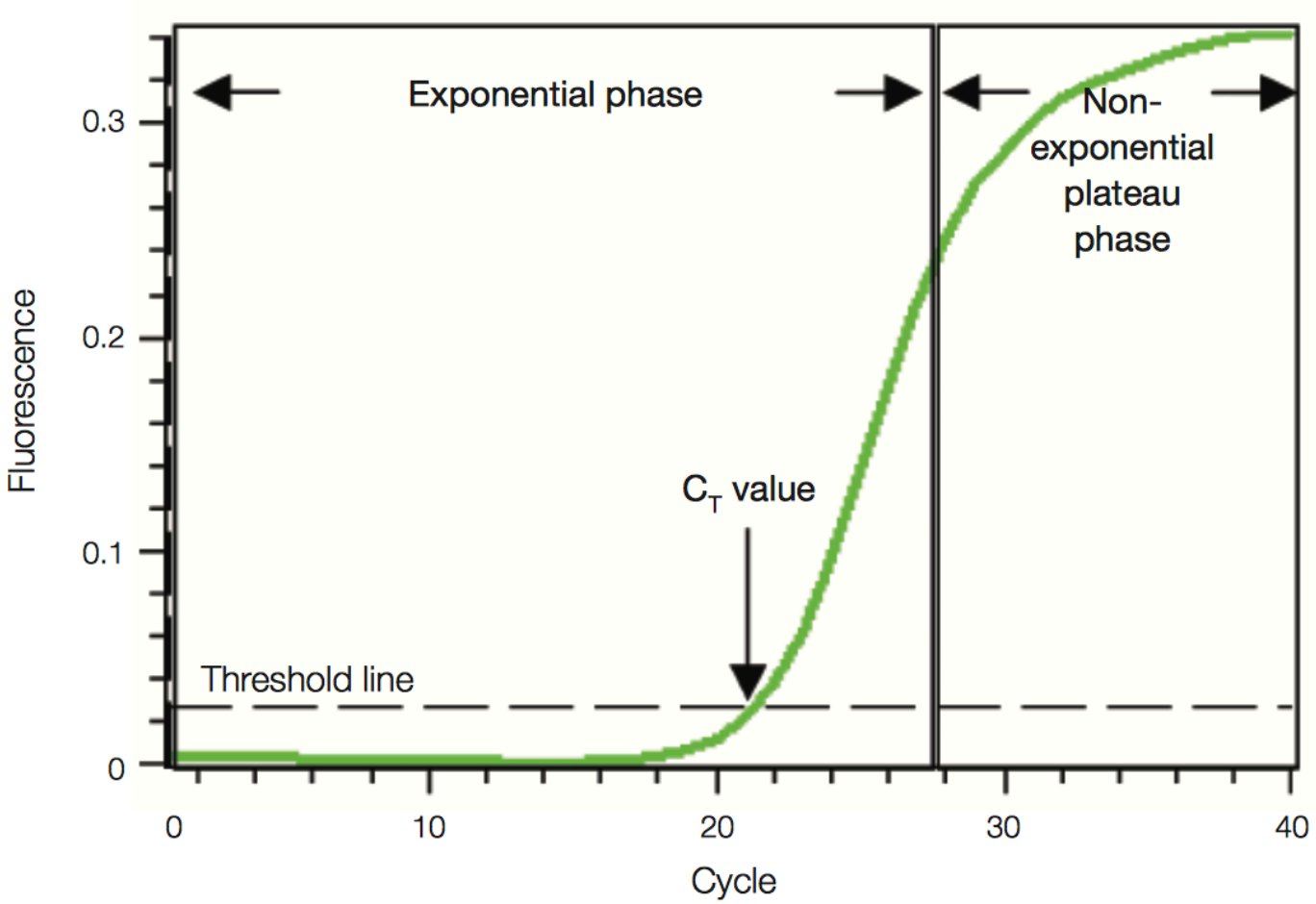


Amplificazione esponenziale

Numero di copie di DNA contenenti il frammento desiderato	$2^1 = 2$	$2^2 = 4$	$2^3 = 8$	$2^4 = 16$	2^{35}
Numero di copie di DNA della corretta lunghezza contenenti il frammento desiderato	0	0	2	8	

I primi 4 cicli della PCR in dettaglio

Amplification of DNA during PCR



PCR Primer Design

Primers need to be carefully designed and target sites in DNA need to be selected with caution

Basic important factors

- Specificity of targeting (primer target sites should be UNIQE in PCR template DNA)
- Length of PCR oligos (18-25 bp)
- GC content of oligos (circa 60%);
- Melting temperature «**Tm**», also called **annealing temperature** of both oligos should be similar (ideal: 60°C)
- avoid secondary structures in single oligo nucleotide
- Avaid paring between the 2 prmers
- The last nucleotide should ideally be G/C. 3 H-bond with template → stabilizes start site for DNA synthesis by DNA polymerase.

Experts can consider other factors for optimizing PCR amplification

PCR primer design:

Details: **Lenght: min. 16 bp; standard 18 – 22 nucleotides**

16 bp statistically present 1x in $4+10^{16}$ bp → length of genome $3x10^9$

T_m primer 1: IDEALLY THE SAME LIKE: T_m primer 2

How to calculate T_m : Depends on length and sequence context of oligo

$$T_m = 4(G + C) + 2(A + T) = °C$$

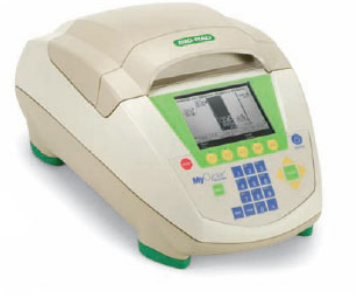
Lab-rule T annealing ⟹ ~ 2-5°C below lower primer Tm

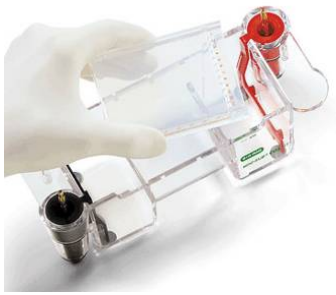
Highly different Tm result assymetric amplification of template strands

For Tm calculation, more info + examples see:

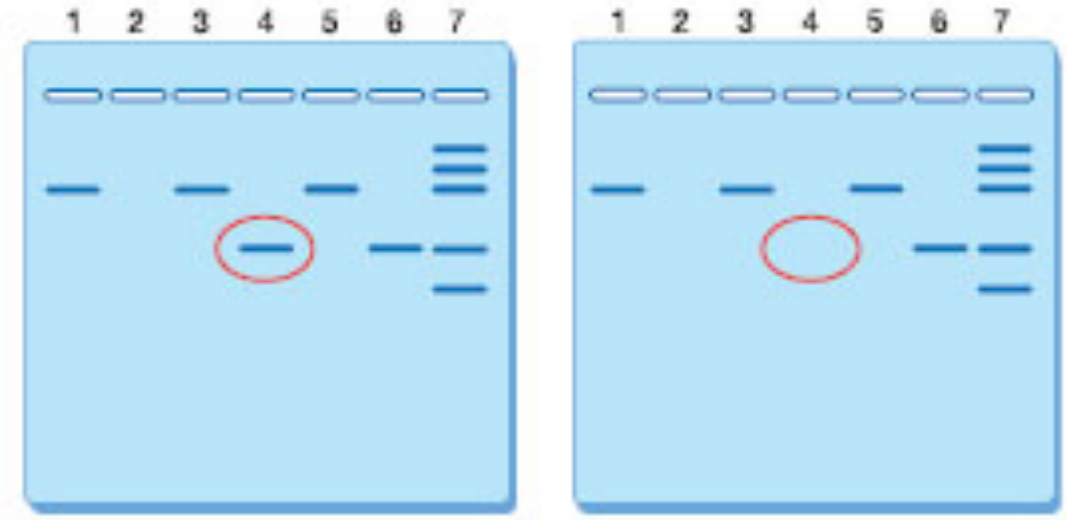
https://www.austincc.edu/mlt/mdfund/mdfund_unit9assignmentsMeltingTemperature.html

Visualization of PCR products by agarose gel electrophoresis:





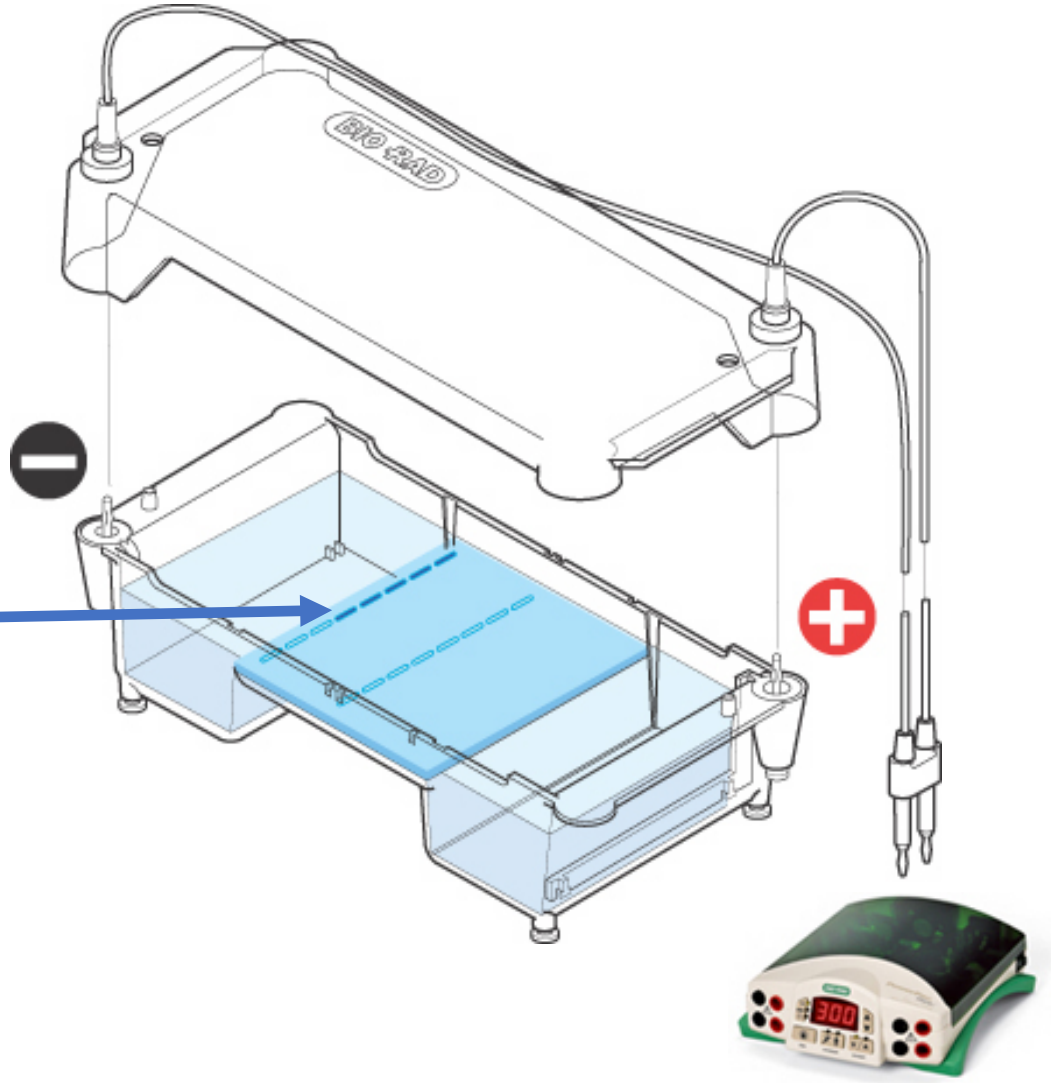
- *After thermal cycling, tubes are taken out of the PCR machine.*
- *Contents of tubes are loaded onto an agarose gel.*
- *DNA is separated by size using an electric field.*
- *DNA is then stained.*
- *PCR products are visible as different "bands".*



Visualization of PCR products by agarose gel electrophoresis:

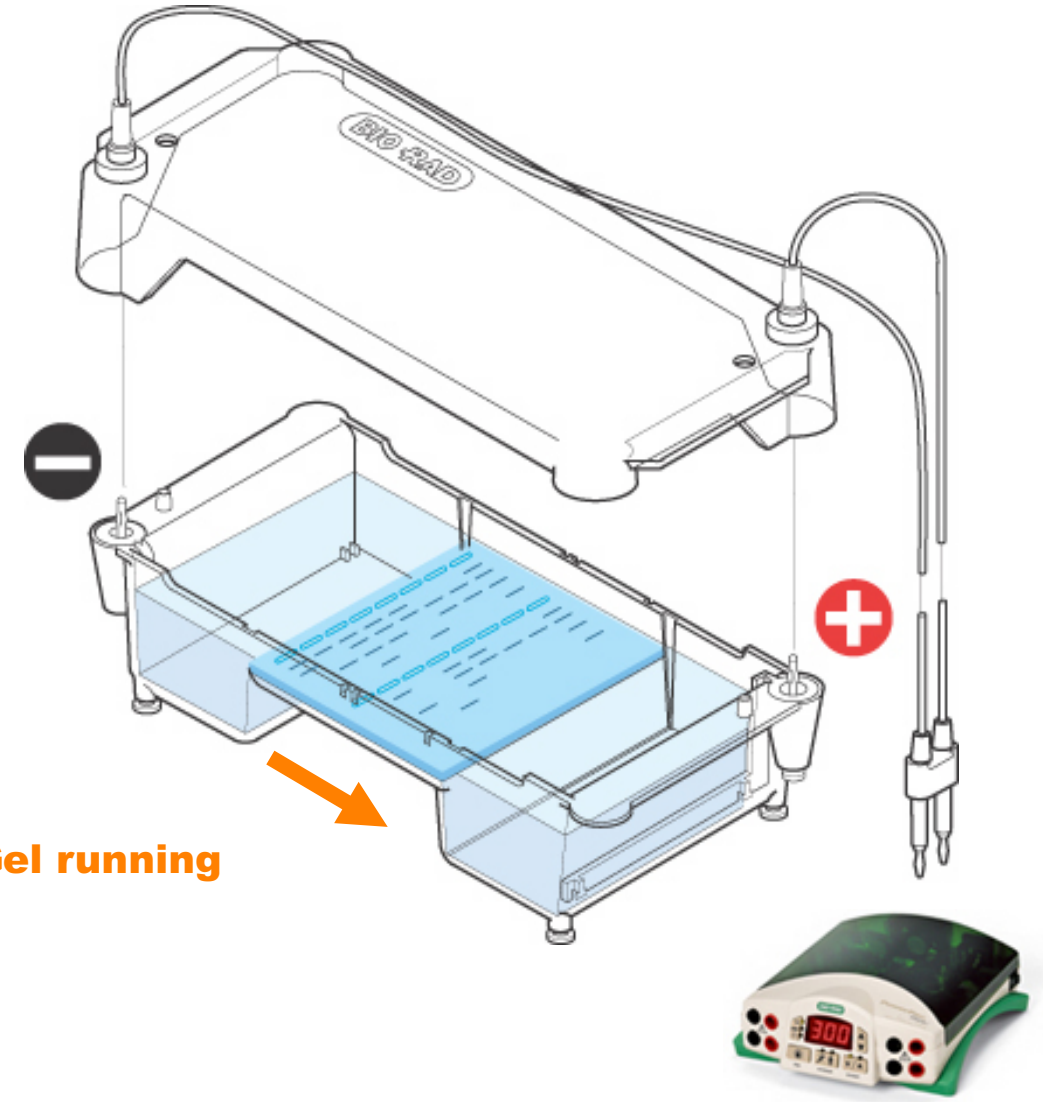
- Make Agarose gel and add dye that visualizes DNA

- Load PCR reactions into slots

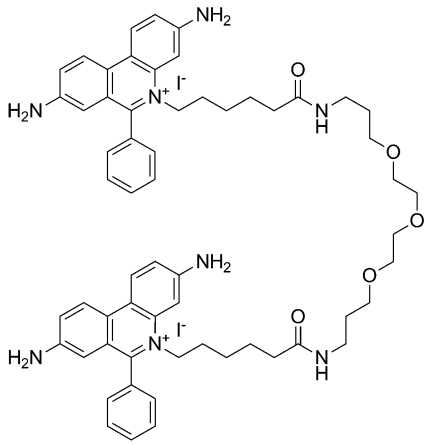


Visualization of PCR products by agarose gel electrophoresis:

Apply current

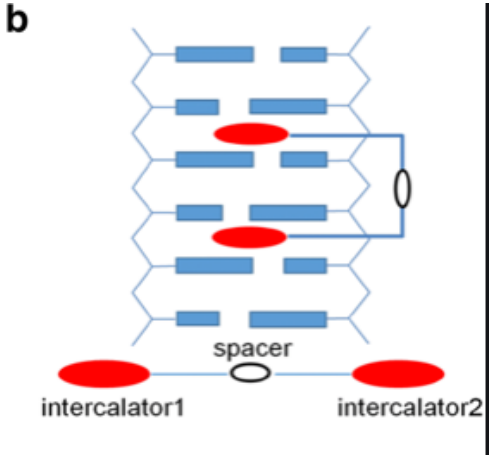


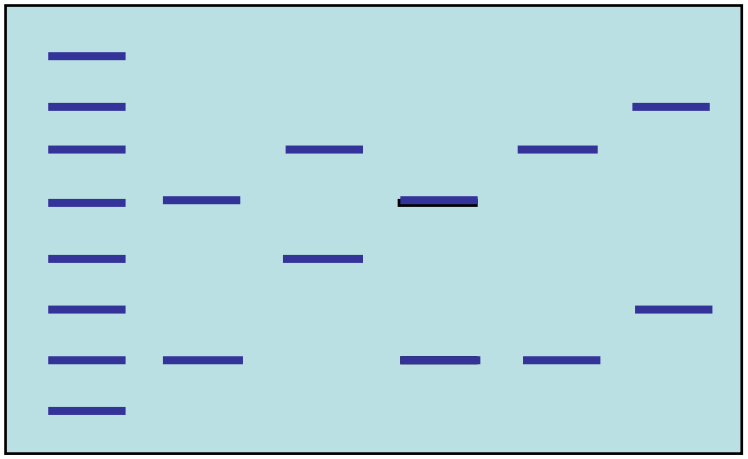
Visualization of PCR products by agarose gel electrophoresis:

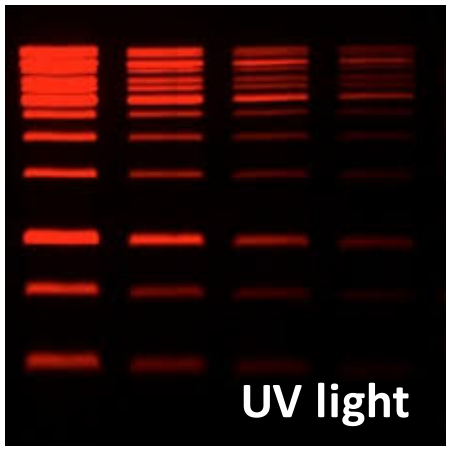


The gel contains DNA dye ("GelRed") that intercalates into DNA when passing through the gel

+ UV light







Bands can be compared against each other, and to known size-standards, to determine the presence or absence of a specific amplification product.

PRODUCT SHEET FOR COMPONENTS OF A PCR-KIT

***Taq* DNA Polymerase PCR**

Cat. No. 18067-017

The following protocol serves as a general guideline and a starting point for any PCR amplification. Optimal reaction conditions (incubation times and temperatures, concentration of *Taq* DNA Polymerase, primers, $MgCl_2$, and template DNA) may vary and need to be evaluated by the customer.

1. Add the following components to a DNase/RNase-free 0.5-ml microcentrifuge tube sitting on ice. Scale the reaction volumes as needed. Prepare a master mix for multiple reactions, to minimize reagent loss and to enable accurate pipetting.

Components	Volume	Final Concentration
10X PCR buffer minus Mg	10 µl	1X
10 mM dNTP mixture	2 µl	0.2 mM each
50 mM $MgCl_2$	3 µl	1.5 mM
Primer mix (10 µM each)	5 µl	0.5 µM each
Template DNA	1-20 µl	-----
Taq DNA Polymerase (5 U/µl)	0.5 µl	2.5 units
Autoclaved distilled water to	100 µl	

2. Mix contents of tube and overlay with 50 µl of mineral or silicone oil.
3. Cap tubes and centrifuge briefly to collect the contents to the bottom.
4. Incubate tubes in a thermal cycler at 94°C for 3 minutes to completely denature the template.
5. Perform 25-35 cycles of PCR amplification as follows:

Denature	94°C for 45 seconds
Anneal	55°C for 30 seconds
Extend	72°C for 1 minute, 30 seconds

6. Incubate for an additional 10 minutes at 72°C and maintain the reaction at 4°C. The samples can be stored at -20° until use.
7. Analyze the amplification products by agarose gel electrophoresis and visualize by ethidium bromide staining.