

PCR - Polymerase chain reaction:

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

- **Genomic DNA**
 - **Plasmid**
 - **cDNA**
- **Mitochondrial DNA**
- **All sort of 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 (RT₂-PCR).....

PCR - Polymerase chain reaction:

(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.



Kary B.
Mullis

PCR Polymerase chain reaction:

1985: The power of PCR an example

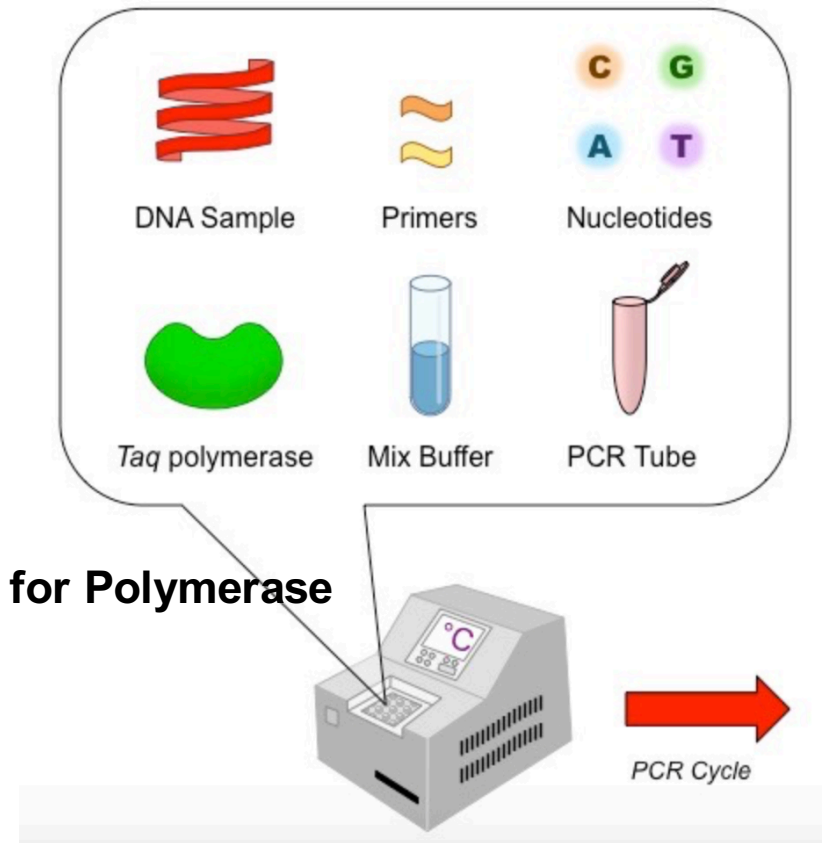
- Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia.

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:

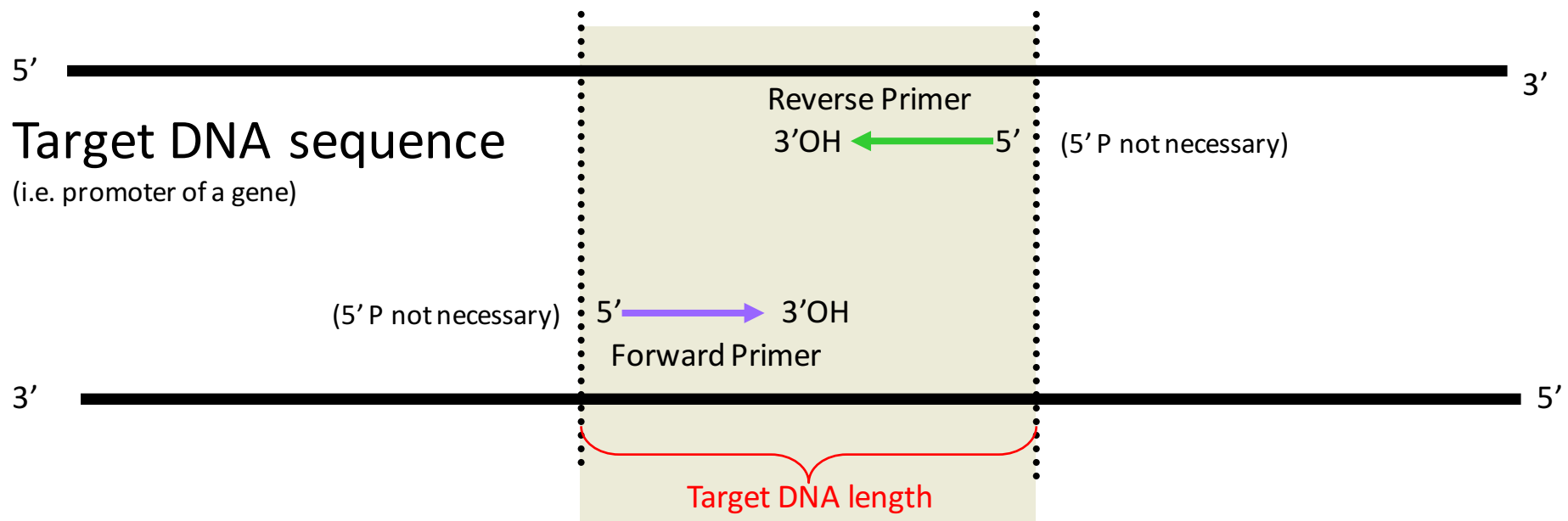
1. Template DNA

- A sequence of DNA that is to be copied - also called *target* DNA.
- PCR can amplify (copy many times) a piece of DNA ~50 to 30000 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 be 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

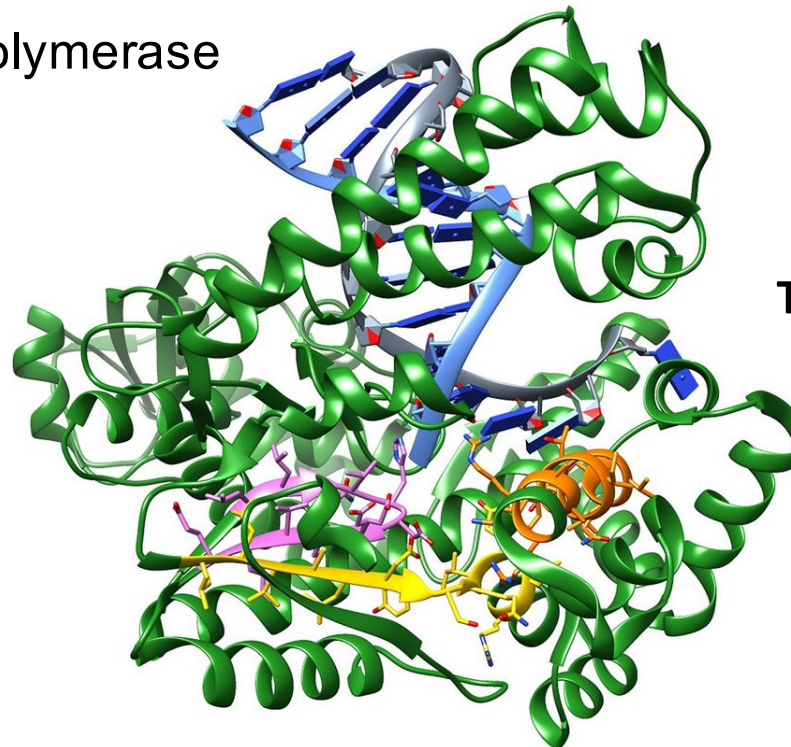
- In the cell (*in vivo*), primers are short RNA strands that serve as a starting point for DNA replication
- In a PCR reaction (*in vitro*), Primers are short synthetic strands of single stranded DNA that exactly match the beginning and the end of the DNA fragment to be amplified.
- 2 PCR primers are required; each pairing to a single strand of template DNA; in convergent orientation (3'OH pointing to each other)
- For amplification 5'P is not required



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

- A polymerases purified from thermophil archaeobacteria
- Polymerase does not unfold at high temperatures !!
- DNA polymerase is thermostable (Heat-stable) – also after heating to 94°C polymerase remains stable
- 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

Temperatures in PCR range between 4°C and 94°C

<72°C: inefficient DNA synthesis

72°C: ideal temperature for DNA synthesis

>94°C: protein unfolding – no DNA synthesis

Note: Taq processivity:

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



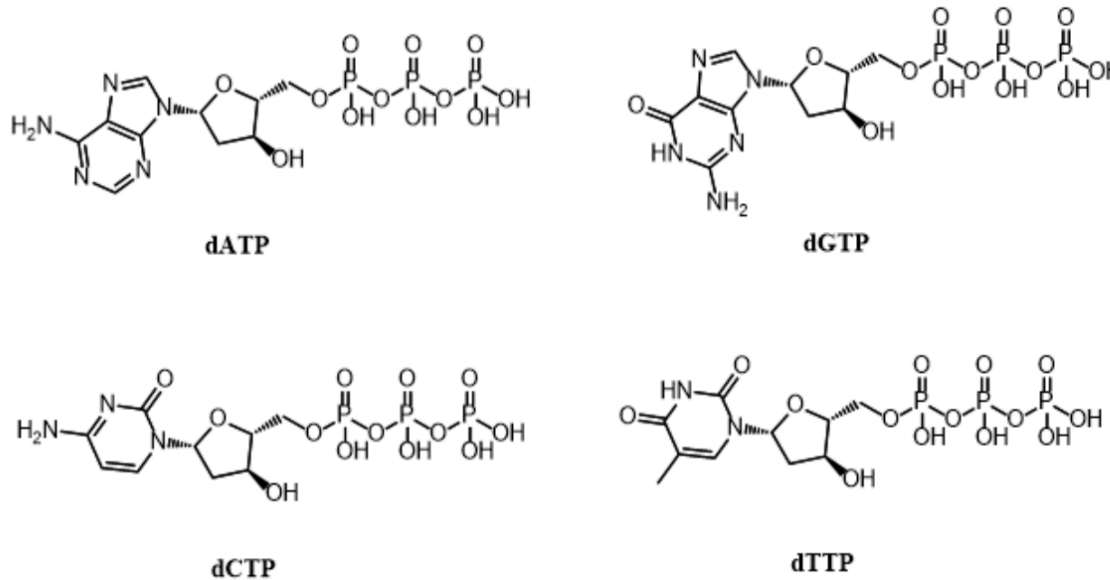
→ During a PCR based amplification of a target DNA, temperatures are shifted to control the activity of DNA polymerase

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 uses in a polymerization reaction to create a new strand in a template-sequence dependent manner
- - remember during DNA synthesis the alpha positioned phosphate will be fused with 3'OH of last nucleotide (most 3' located) positioned in the neo-synthesized DNA filament

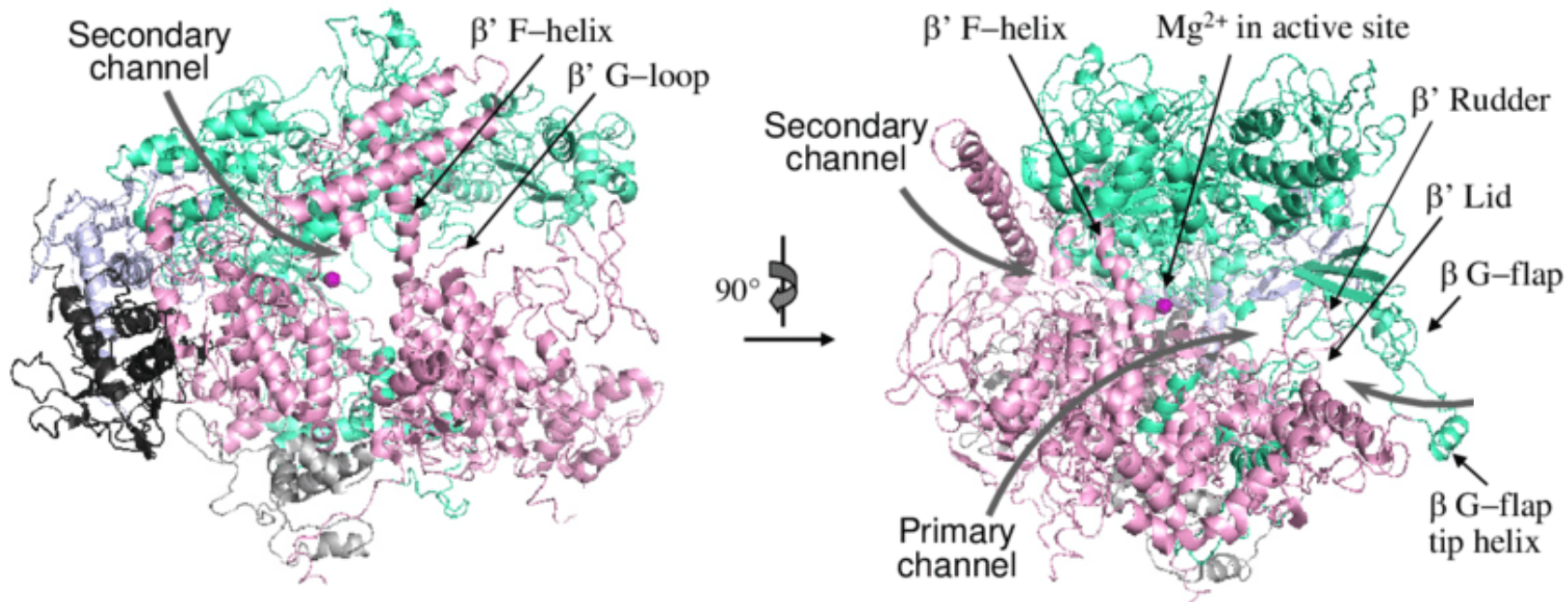
Deoxynucleotides (dNTPs)



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 Mg^{2+}



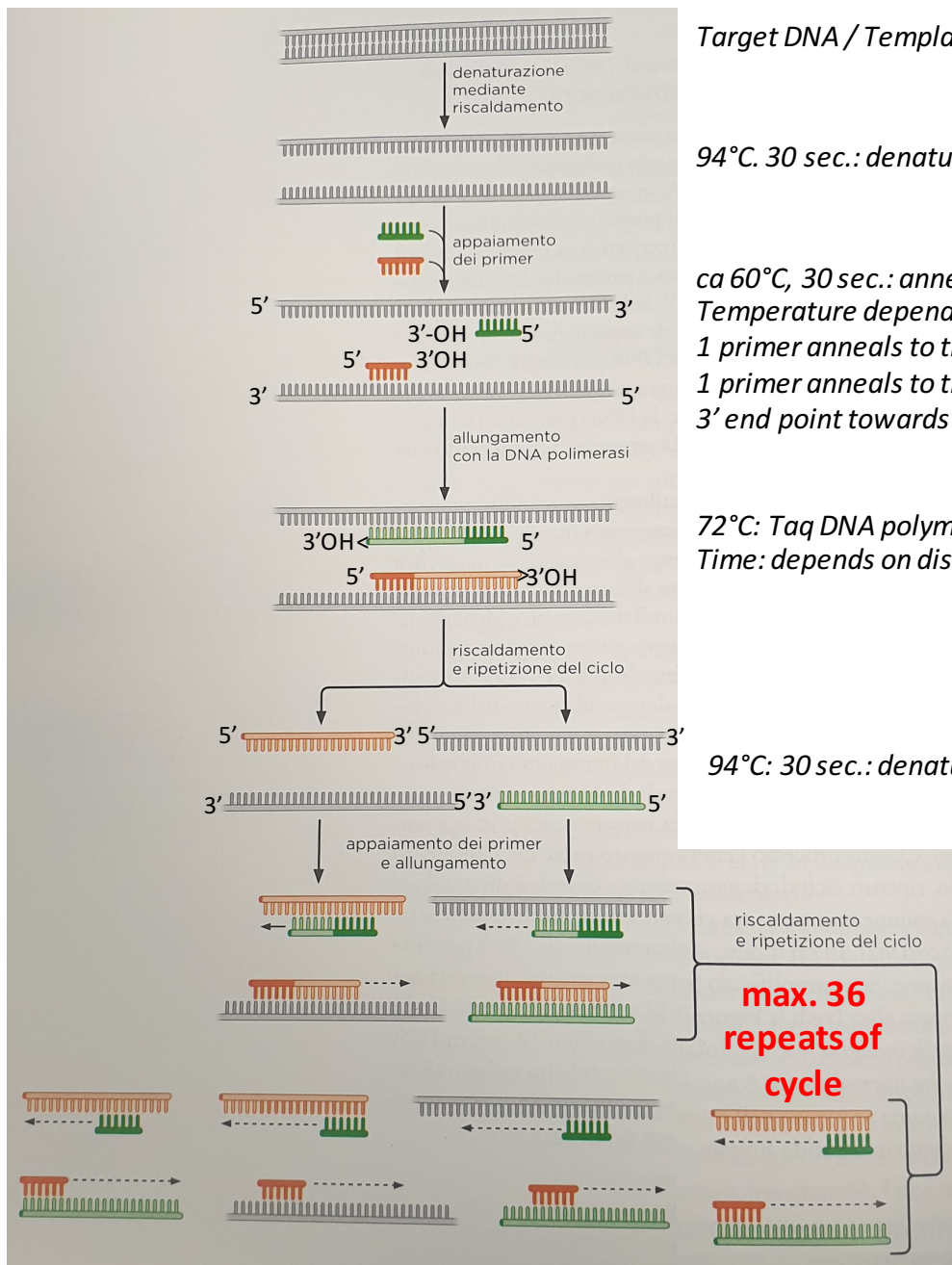
PCR Requires the following:

	<u>Components</u>	<u>Volume</u>	<u>Final Concentration</u>
1000fold more	10X PCR buffer minus Mg	10 μ l	1X
	10 mM dNTP mixture	2 μ l	0.2 mM each
	50 mM MgCl ₂	3 μ l	1.5 mM
	Primer mix (10 μ M each)	5 μ l	0.5 μ M each
	Template DNA	1-20 μ l	-----
	Taq DNA Polymerase (5 U/ μ l)	0.5 μ l	2.5 units
	Autoclaved distilled water to	100 μ l	

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.

Mechanism 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

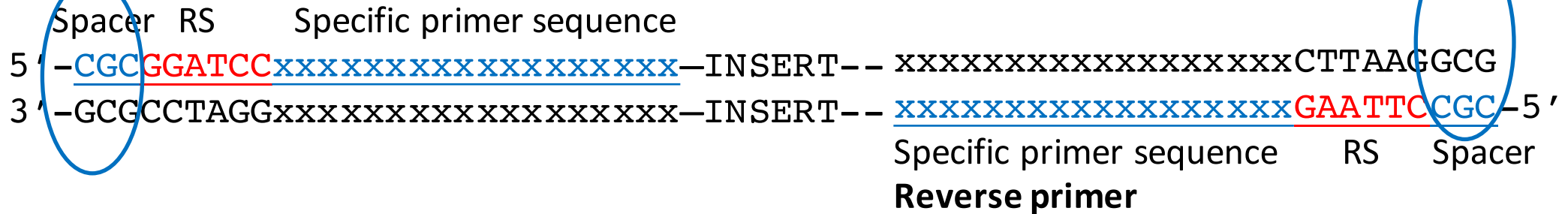
72°C: DNA synthesis

PCR CLONING

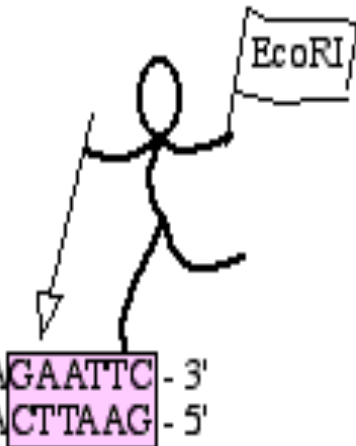
1. Classic PCR cloning

WHY IS A SPACER NEEDED???

Forward primer



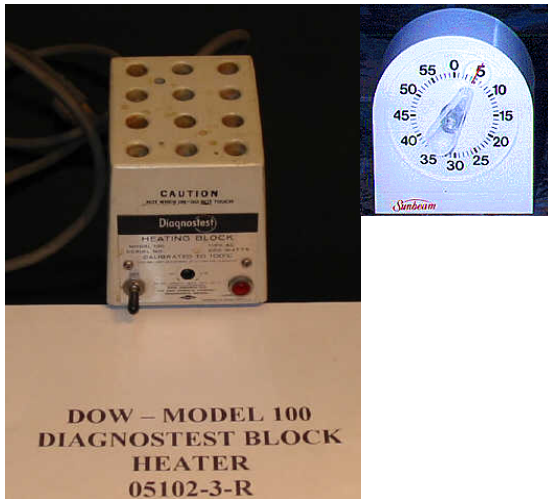
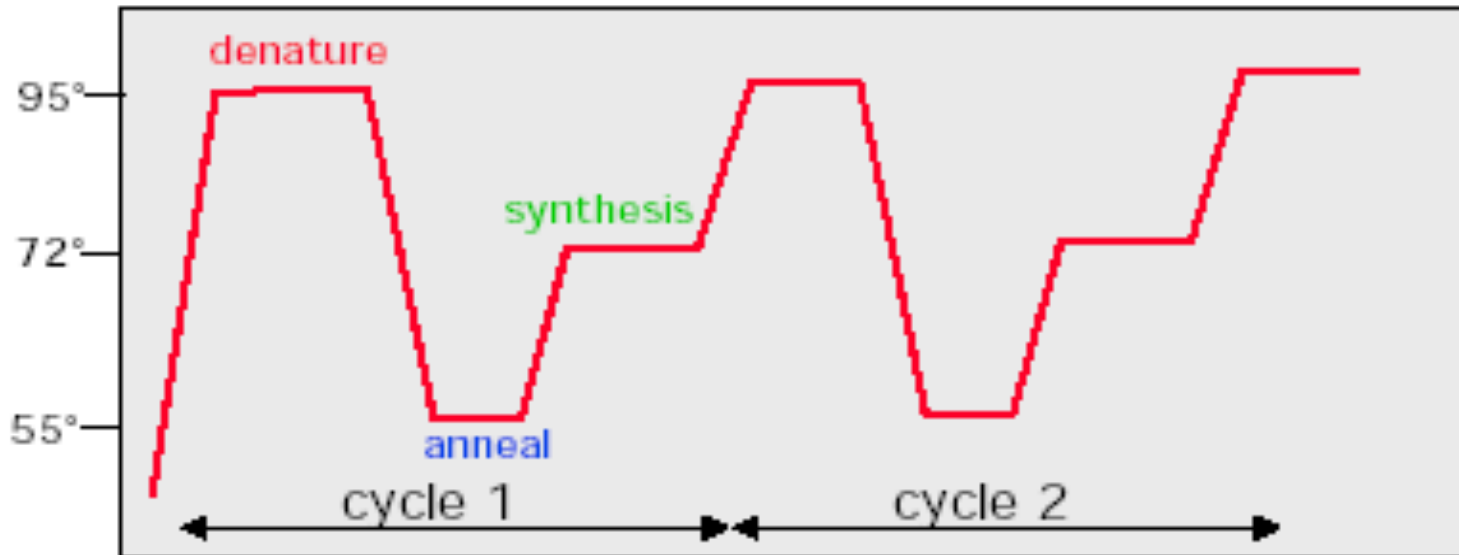
How well do enzymes work at the very ends of DNA molecules?



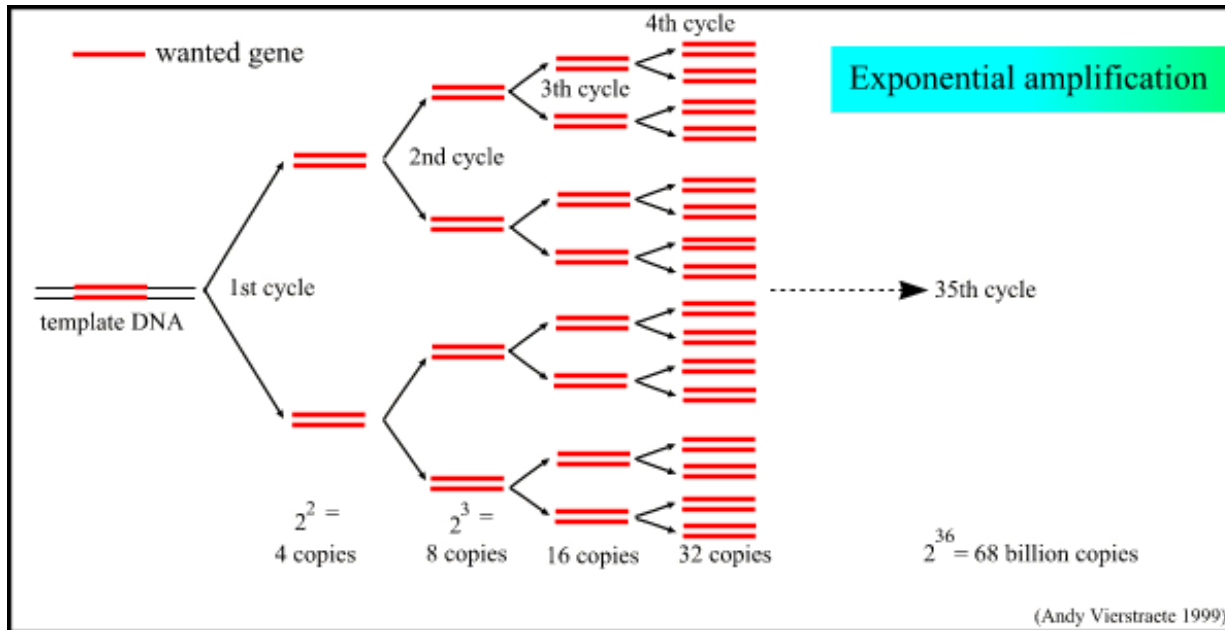
- Many restriction enzymes work poorly on DNA termini
- Catalogues of enzymes provide data on the cutting efficiency of enzymes at the end of DNA molecules.
- Generally, enzymes work better if they have a couple of extra nucleotides at the end – improved interaction with DNA

Mechanism 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 (thermocyclers), which produce PCR amplicons in up to 36 cycles in an hour



Mechansim of PCR:



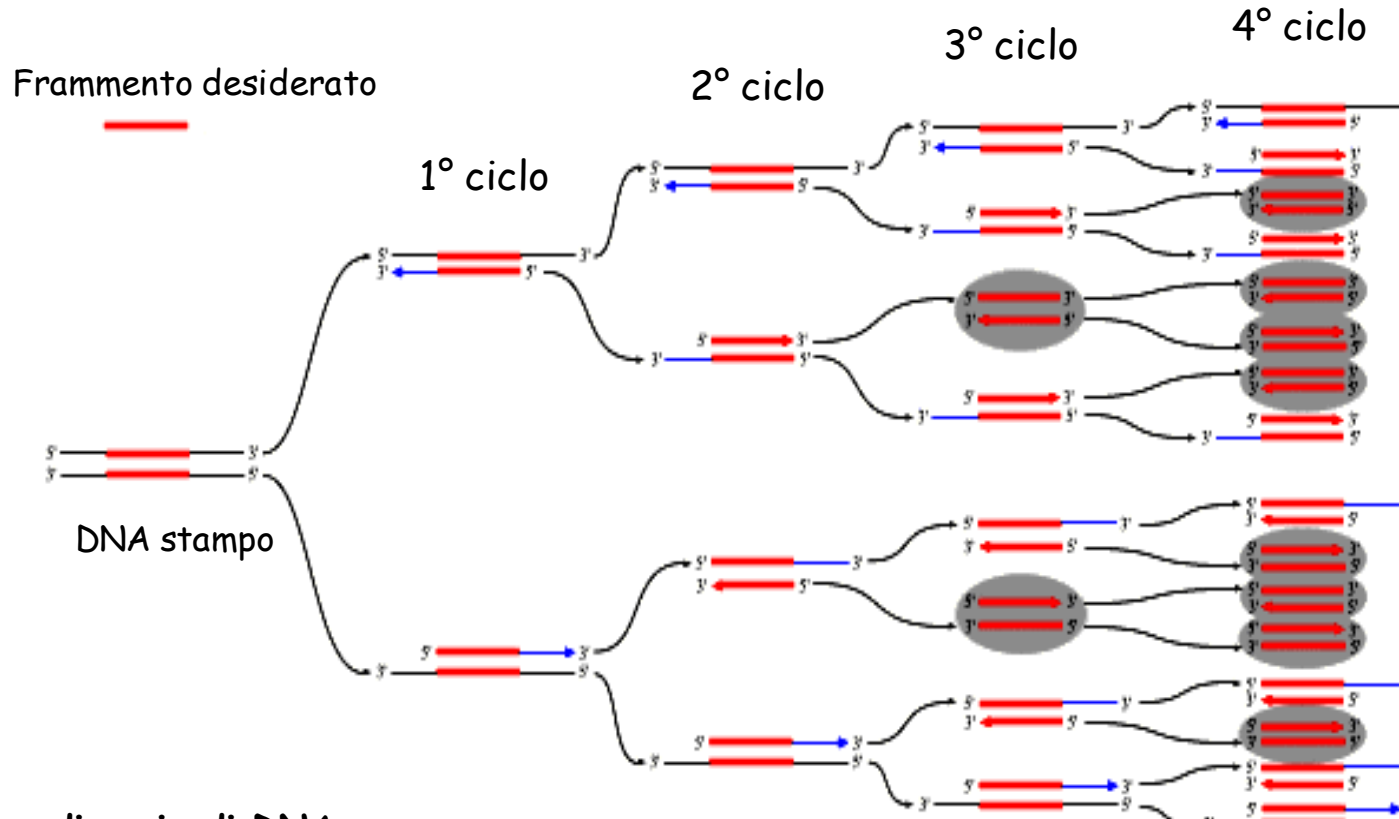
$$Y = N2^n$$

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	33.554.432
26	67.108.864
27	134.217.728
28	268.435.456
29	536.870.912
30	1.073.741.824

Mechanism 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

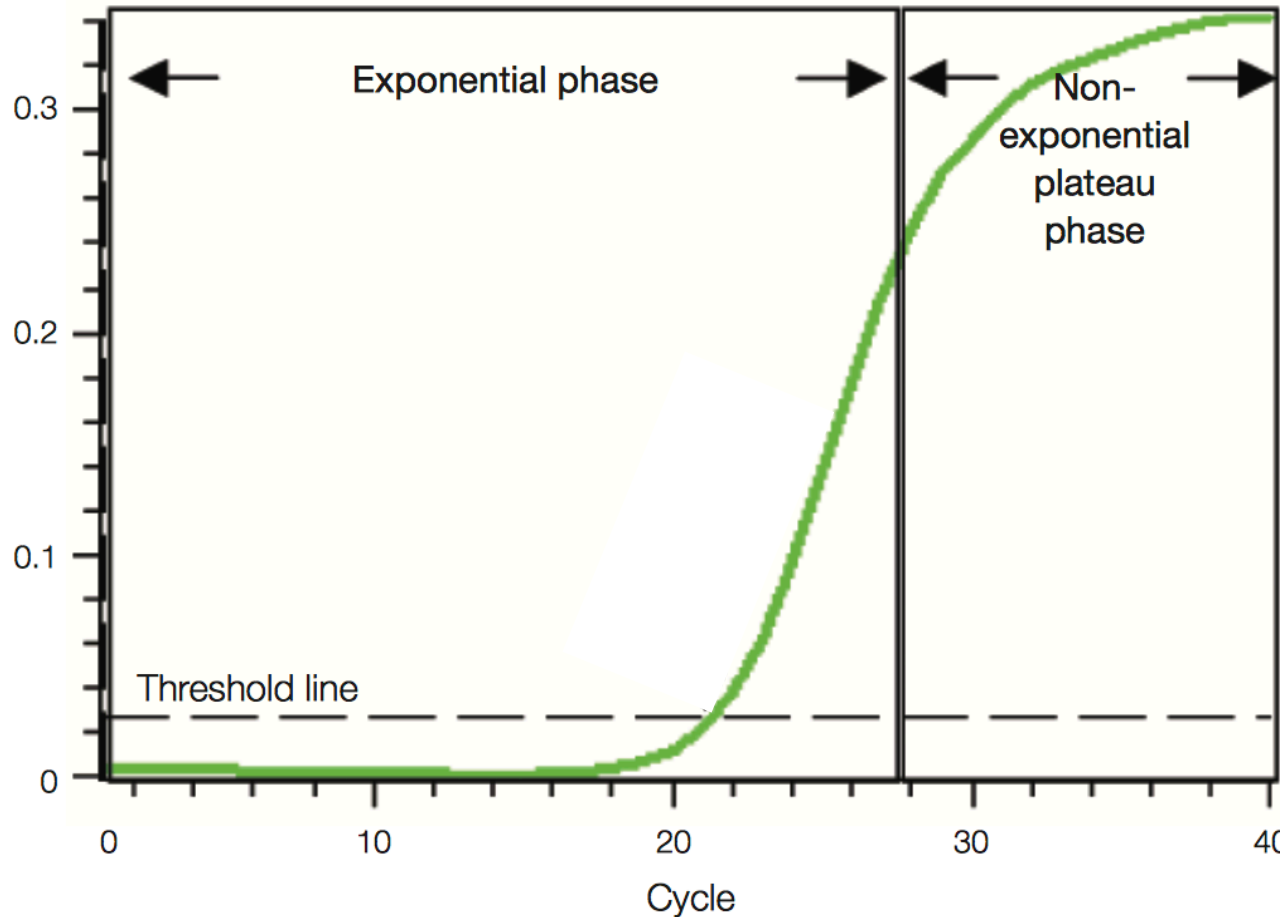
0

2

8

I primi 4 cicli della PCR in dettaglio

Amplification of DNA during PCR



For standard application:
cycle number max. 36

During the exponential phase, no factor is limiting, and the amplification products accumulate at a steady rate. At high cycle numbers the amplified DNA function as inhibitor of DNA polymerase. In addition, reaction components can become limiting, and the efficiency of amplification drops and eventually stops. These reasons can lead to the so-called "plateau phase".

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 **UNIQUE** in PCR template DNA; and DNA preparation)
- ❖ Length of PCR oligos (18-25 bp)
- ❖ GC content of oligos (circa 60%);
- ❖ Melting temperature «**T_m**», also called **annealing temperature** of both oligos should be similar (**ideal: 60°C**)
- ❖ avoid secondary structures in single oligo nucleotide
- ❖ Avoid pairing between the 2 primers
- ❖ 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: Length: min. 16 bp; standard 18 – 22 nucleotides



16 bp statistically present 1x in 4×10^{16} bp \rightarrow length of genome 3×10^9

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

$$T_m = 4(G + C) + 2(A + T) = ^\circ\text{C}$$

Lab-rule: Temperature for annealing of primers to template \Rightarrow $\sim 2\text{-}5^\circ\text{C}$ below lower primer T_m

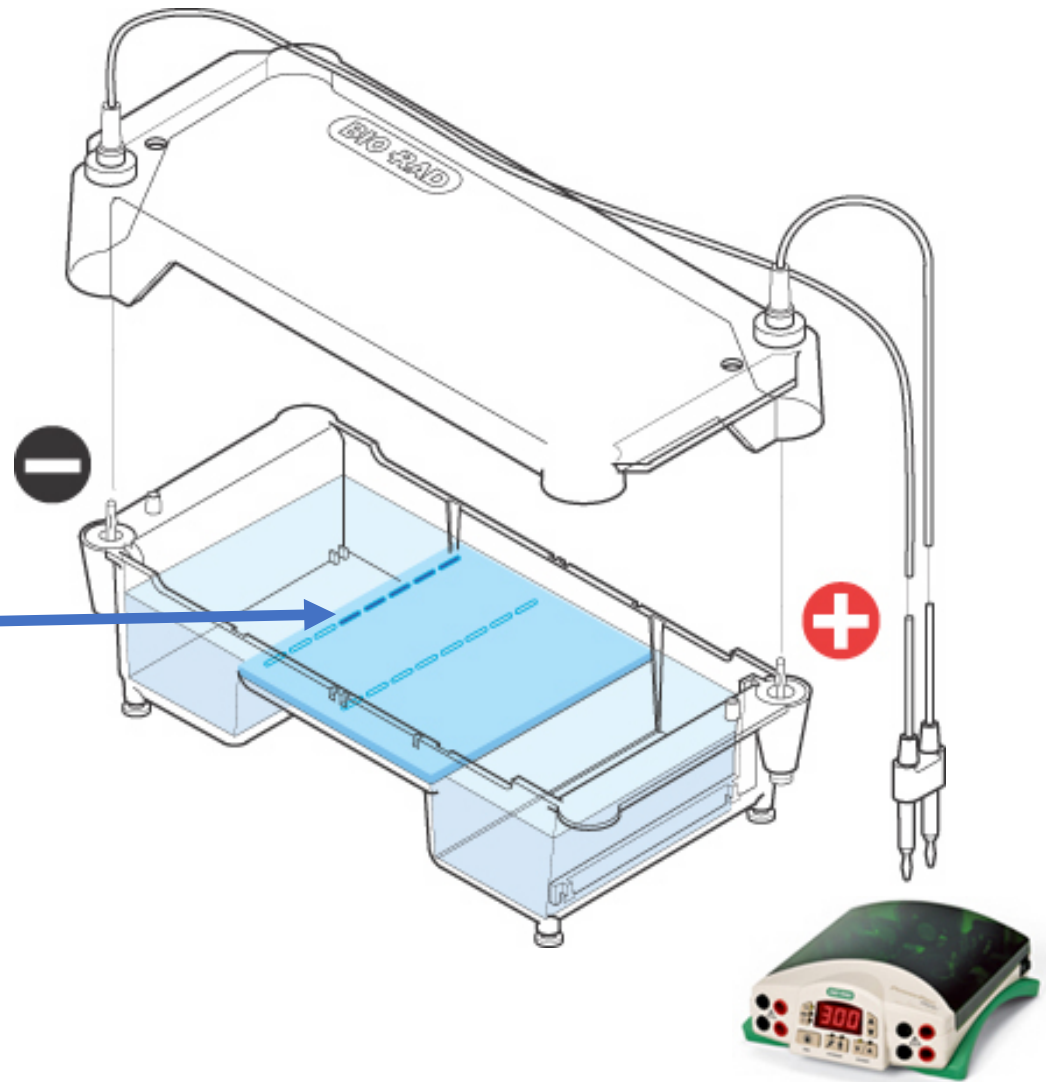
Highly different T_m result asymmetric amplification of template strands

For T_m calculation, more info + examples see:

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

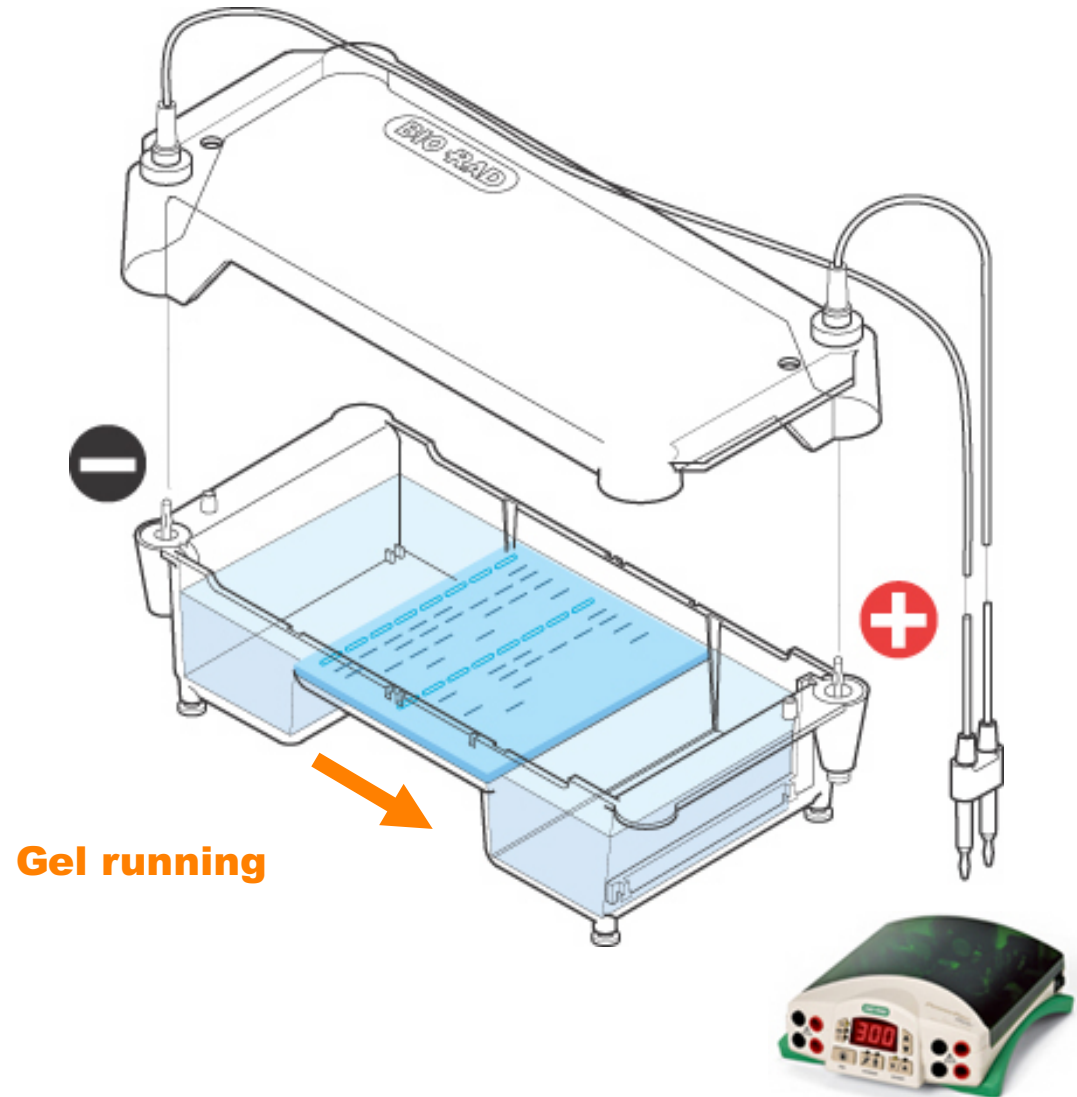
Visualization of PCR products by agarose gel electrophoresis:

- 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:



- *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 “bands”.*

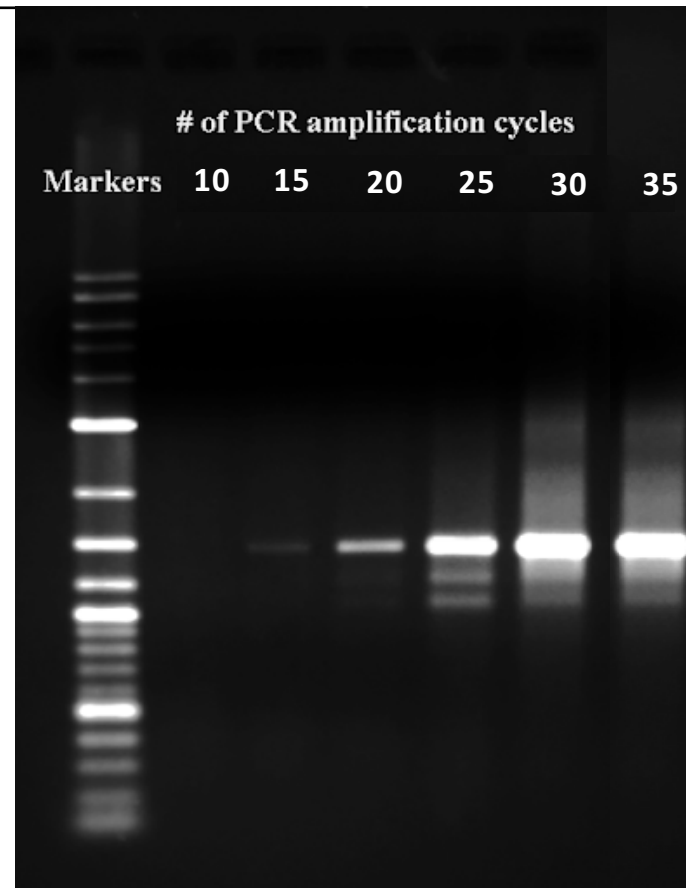


“End point PCR”:

PCR performed with cycle numbers that bring PCR to plateau phase.

Example: results indicate that plateau phase is reached at cycle number 30.

Note: we do not have information on amplification between cycle number 26 and 29. It might be that plateau phase is reached at cycle number 26, 27, 28 or 29.



—PCR product (desired)
—Alternative, non desired PCR products;
—accumulate with increasing cycle number

Taq DNA Polymerase PCR

Cat. No. 18067-017

**PRODUCT SHEET FOR
COMPONENTS OF A PCR-KIT**

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, MgCl₂, 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.

<u>Components</u>	<u>Volume</u>	<u>Final Concentration</u>
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	----
<i>Taq</i> 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.