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

- Detection of infectious disease and pathogens
- Detection of non-infectious disease and mutations (cancer, genetic disease)
- Forensic analysis
- Cloning of DNA
- Biodiversity
- Determination of expression levels

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:

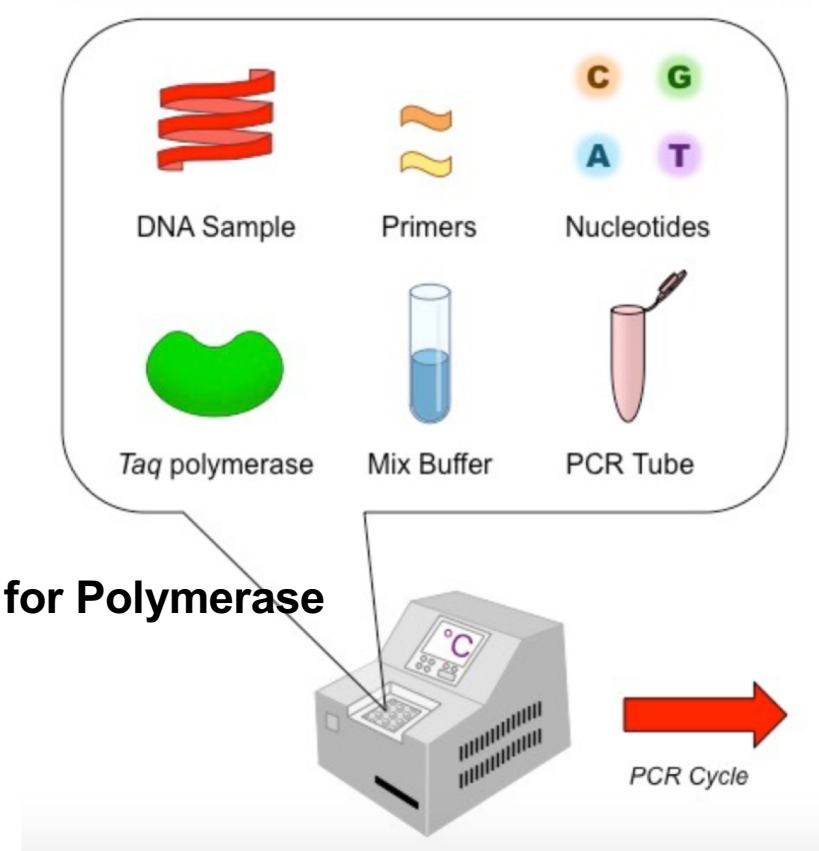
1985: The power of PCR: an example

- 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:

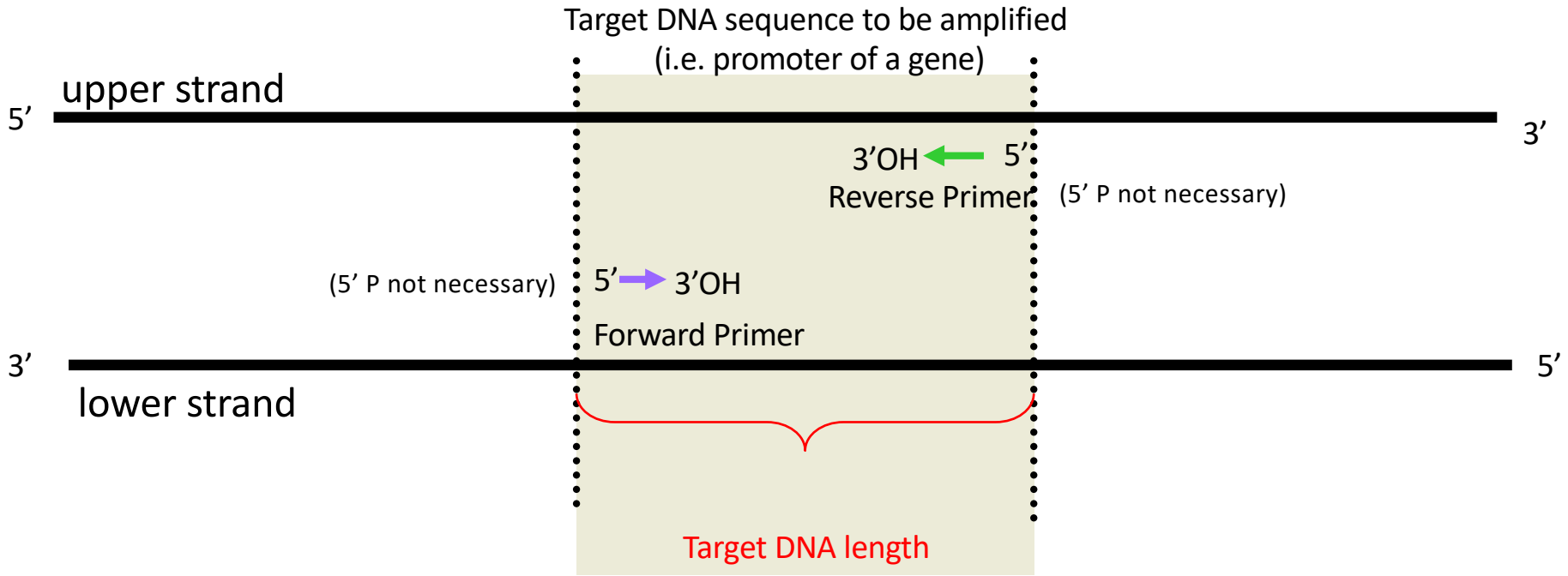
1. Template DNA

- A sequence of DNA that is to be copied - also called *target* DNA or DNA *template*.
- PCR can amplify (copy many times) a piece of DNA (amplification range: ~50 to 30000 nt)
- Lateral segments of the sequence to be amplified must be known to locate a **primer** for DNA polymerase; on each DNA single strand, 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 fragmented/degraded

PCR Requires the following:

2. A pair of DNA primers

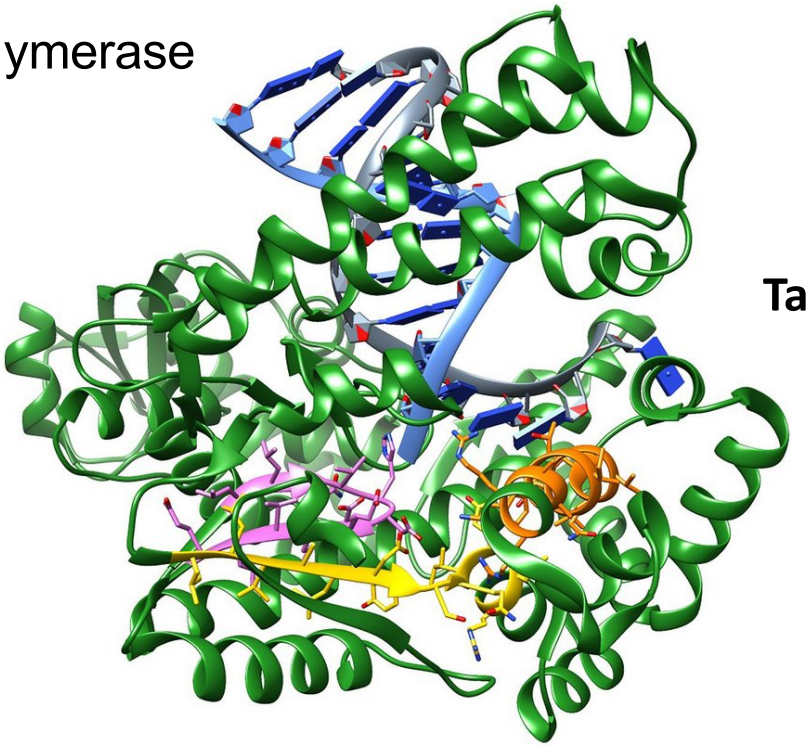
- In the cell (*in vivo*), **primers** are short RNA strands that serve as a starting point for DNA replication
- In a PCR reaction (*in vitro*), **Primers** are short synthetic strands of single stranded DNA (**oligonucleotides**) that exactly match the beginning and the end of the DNA fragment to be amplified. Primer length: ca. 18-25nt
- 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, the 5'P of primers are 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 archaebacteria
- 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* (lives in hot springs)
- Taq pol has 5'-3' DNA polymerase activity and 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

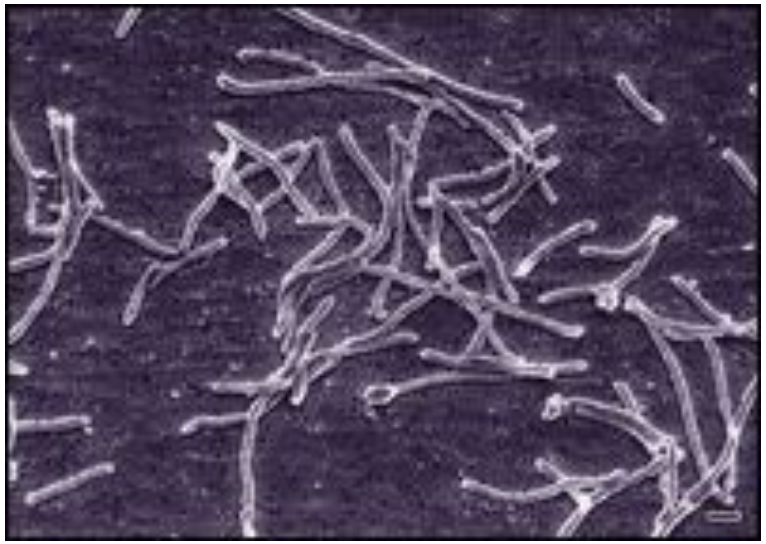
<72°C: inefficient DNA synthesis

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

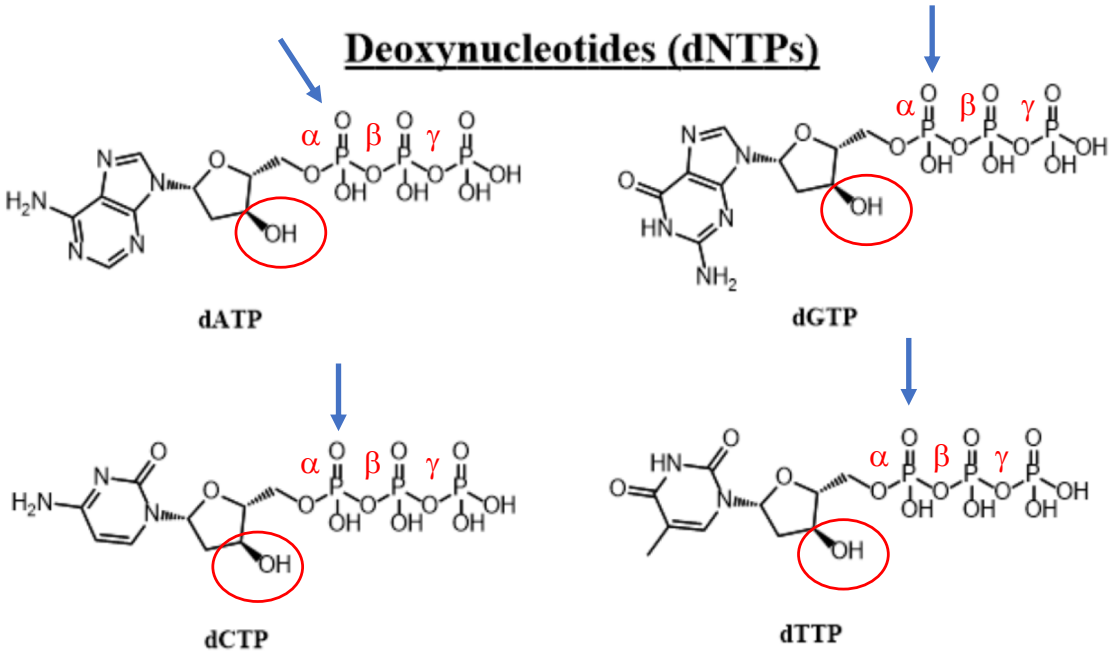
PCR Requires the following:

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

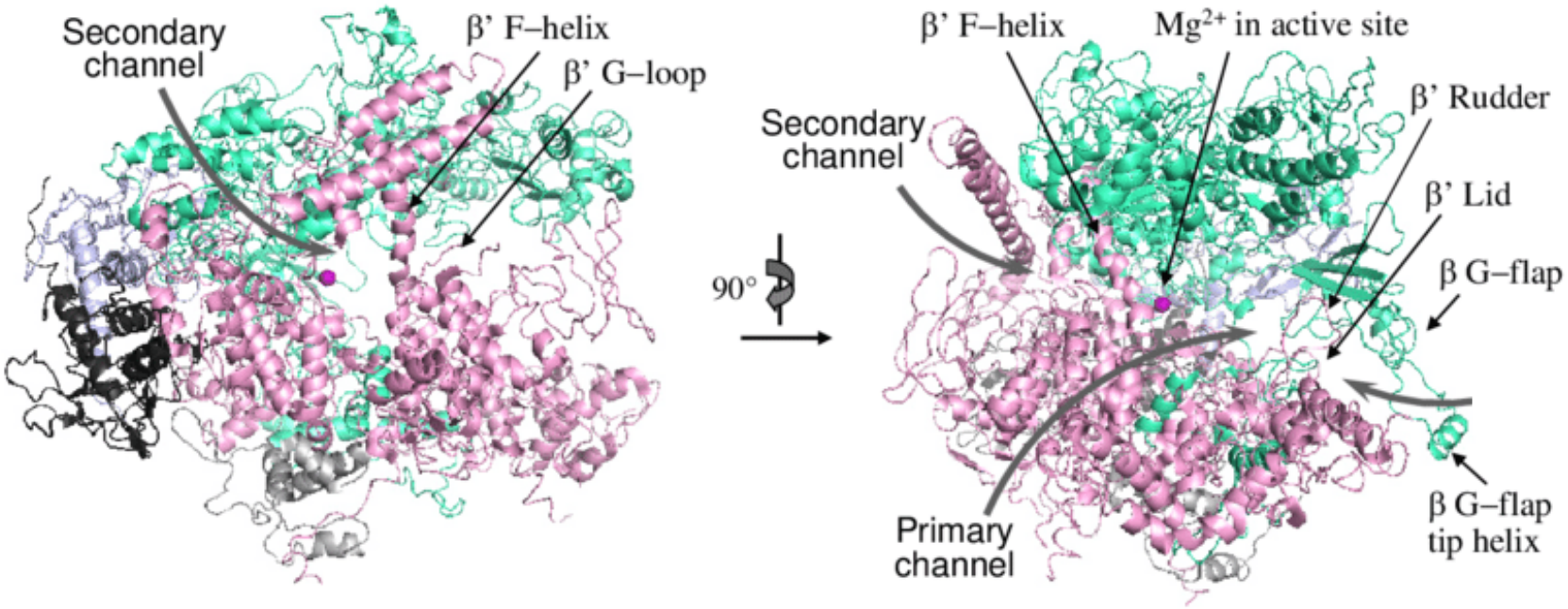
!!!! remember during DNA synthesis the alpha positioned phosphate will be fused with 3'OH of last nucleotide (most 3' located) positioned in the neo-synthesized DNA filament



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+}



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. Optional; not done anymore
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.

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	

ca. 500 fold more

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

«Unit» : describes the activity of an enzyme under in-vitro condition:

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.

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	

1 PCR reaction

EASY: only 1 PCR reaction

1. Autoclaved destilled water: 74,5µl
2. 10x Buffer minus Mg): 10 µl
3. $MgCl_2$ (50mM): 3 µl
4. dNTP (10nM): 2,0 µl
5. Primer 1 (10uM): 2,5 µl
6. Primer 2 (10uM): 2,5 µl
7. Template DNA (10ng/ul) : 5,0 µl
8. Taq: 0,5 µl

Totale: 100µl

WHAT TO DO WHEN HAVING SEVERAL DNA SAMPLES FOR PCR?

- Sample are different (come from different sources)
- PCR mix is always the same (same primer pairs, etc..)

↓

MAKE MASTER MIX OF PCR REAGENTS

PCR Requires the following:

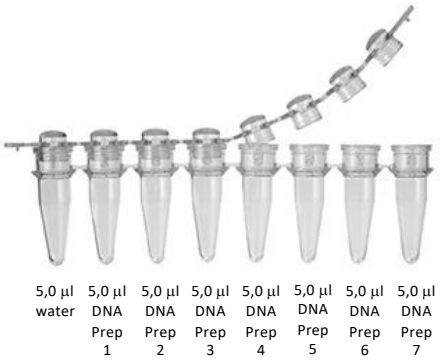
Components	Volume	Final Concentration
10X PCR buffer minus Mg	10 µl	1X
10 mM dNTP mixture	2 µl	0.2 mM each
50 mM $MgCl_2$	3 µl	1.5 mM
Primer mix (10 µM each)	5 µl	0.5 µM each
Template DNA	1-20 µl	-----
Taq DNA Polymerase (5 U/µl)	0.5 µl	2.5 units
Autoclaved distilled water to		

EXAMAPLE:

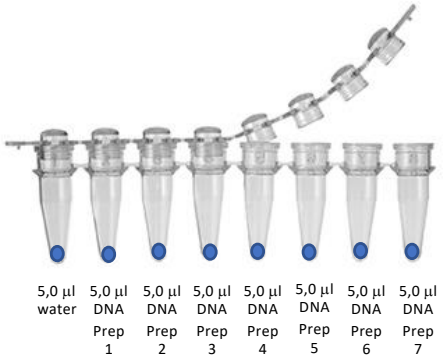
8 reactions in parallel:

7 genomic DNA preps from different patients

1 negative control (H_20) --> no template no amplification



PREPARATION OF MASTER MIX



" 1 PCR reaction"

1. Autoclaved destilled water: 74,5µl
2. 10x Buffer minus Mg): 10 µl
3. $MgCl_2$ (50mM): 3 µl
4. dNTP (10nM): 2,0 µl
5. Primer 1 (10uM): 2,5 µl
6. Primer 2 (10uM): 2,5 µl
7. NO Template DNA
8. Taq: 0,5 µl

Totale: 95µl

multiply by 8,5

" 8,5x master mix"

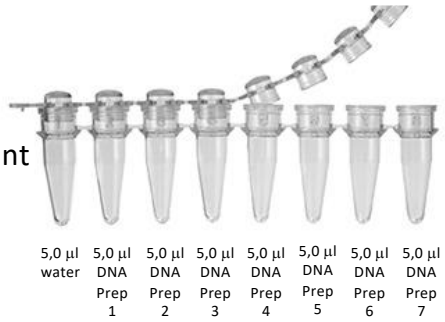
1. Autoclaved destilled water: 633,25µl
2. 10x Buffer minus Mg): 85 µl
3. MgCl2 (50mM): 25,5 µl
4. dNTP (10nM): 17 µl
5. Primer 1 (10µM): 21,25 µl
6. Primer 2 (10µM): 21,25 µl
7. NO Template DNA
8. Taq: 4,25 µl

Totale: 850µl

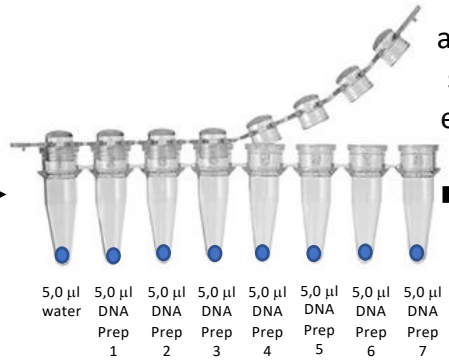
PCR Requires the following:

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	

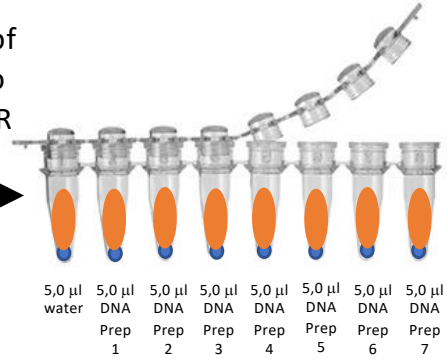
8 reactions:
7 genomic DNA preps from different patients
1 negative control (H_20)



add 5µl of sample to empty PCR tube



add 95µl of sample to empty PCR tube



Advantage of master mix approach??

- Less pipetting
- More precise
- Conditions in different tubes are more reproducible (same PCR mix used)

"8,5x master mix"

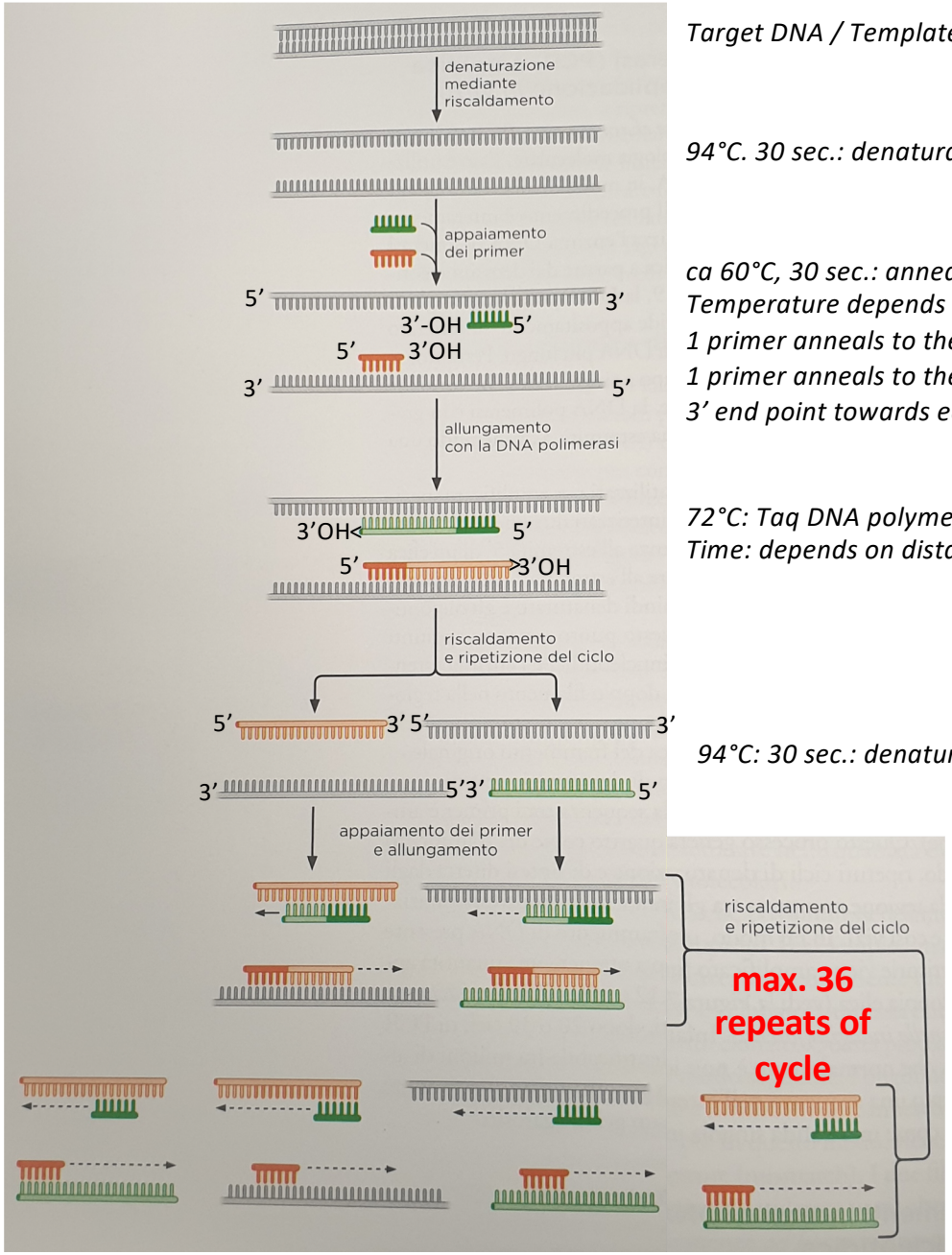
1. Autoclaved destilled water: 633,25µl
2. 10x Buffer minus Mg): 85 µl
3. MgCl2 (50mM): 25,5 µl
4. dNTP (10nM): 17 µl
5. Primer 1 (10µM): 21,25 µl
6. Primer 2 (10µM): 21,25 µl
7. Template DNA: put directly in PCR tube
8. Taq: 4,25 µl

Totale: 850µl

Setup of PCR

1. Add 5 µl of DNA (or water) to PCR tube
2. Add 95 µl of Mastermix to PCR tube
3. Close strip and start PCR

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

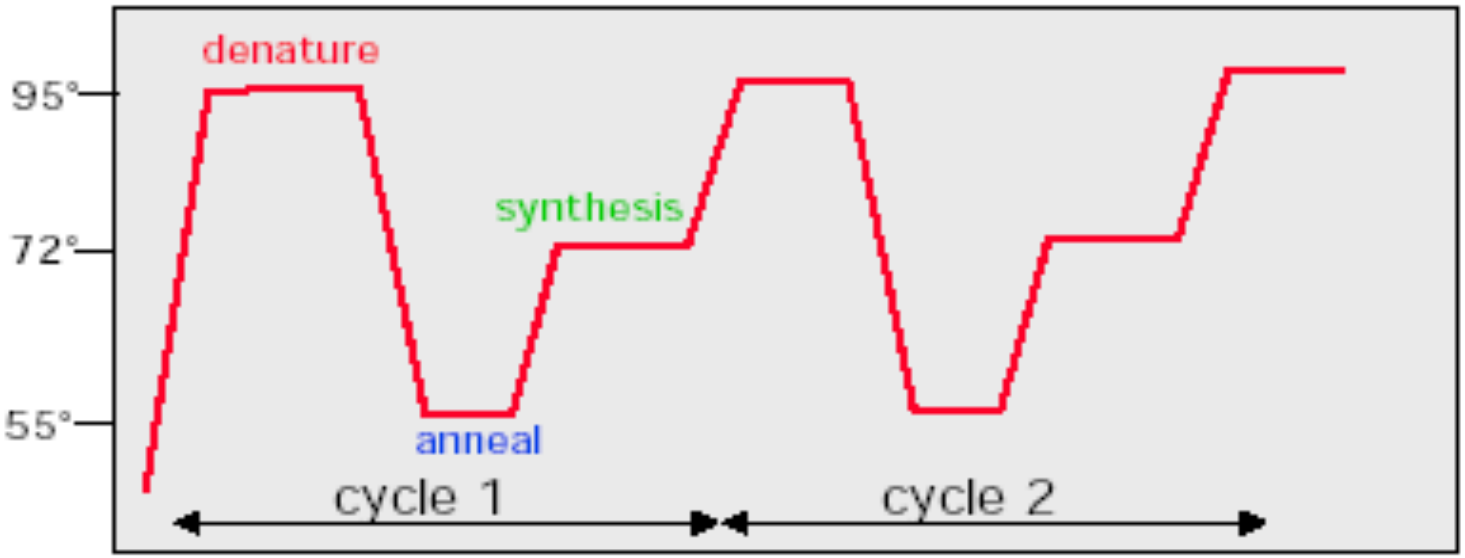
94°C: 30 sec.: denaturation of double-stranded DNA

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



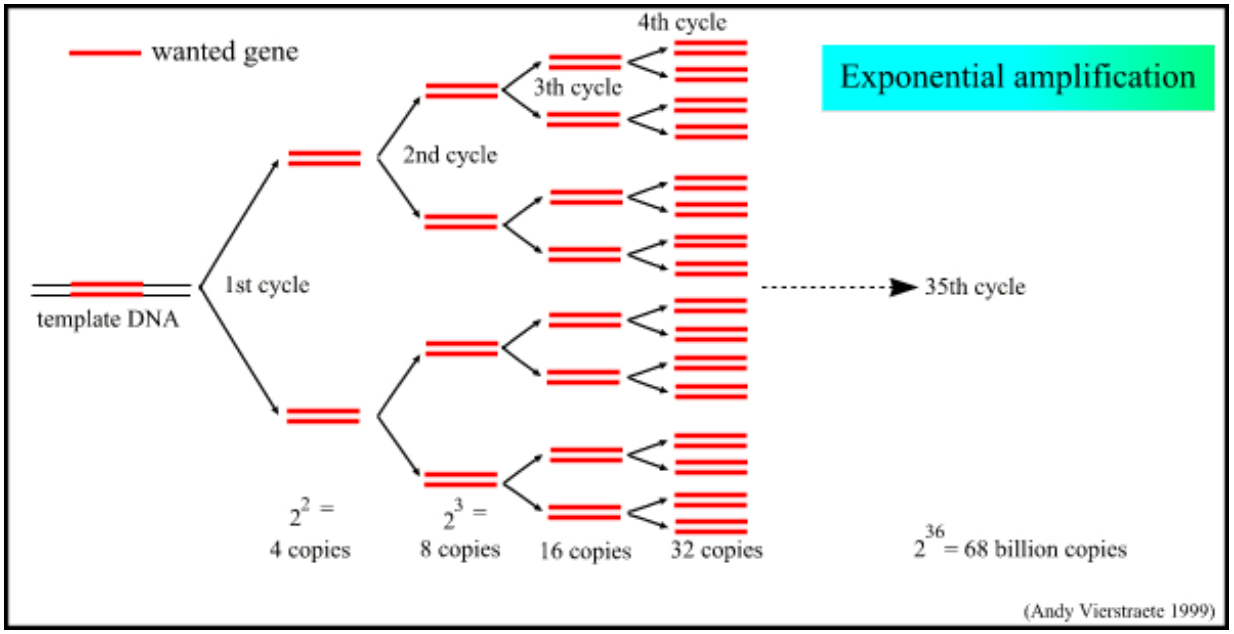
Note: Taq processivity:

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

PERFECT EXPLANATION OF PCR IN A MOVIE

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

Mechansim of PCR:



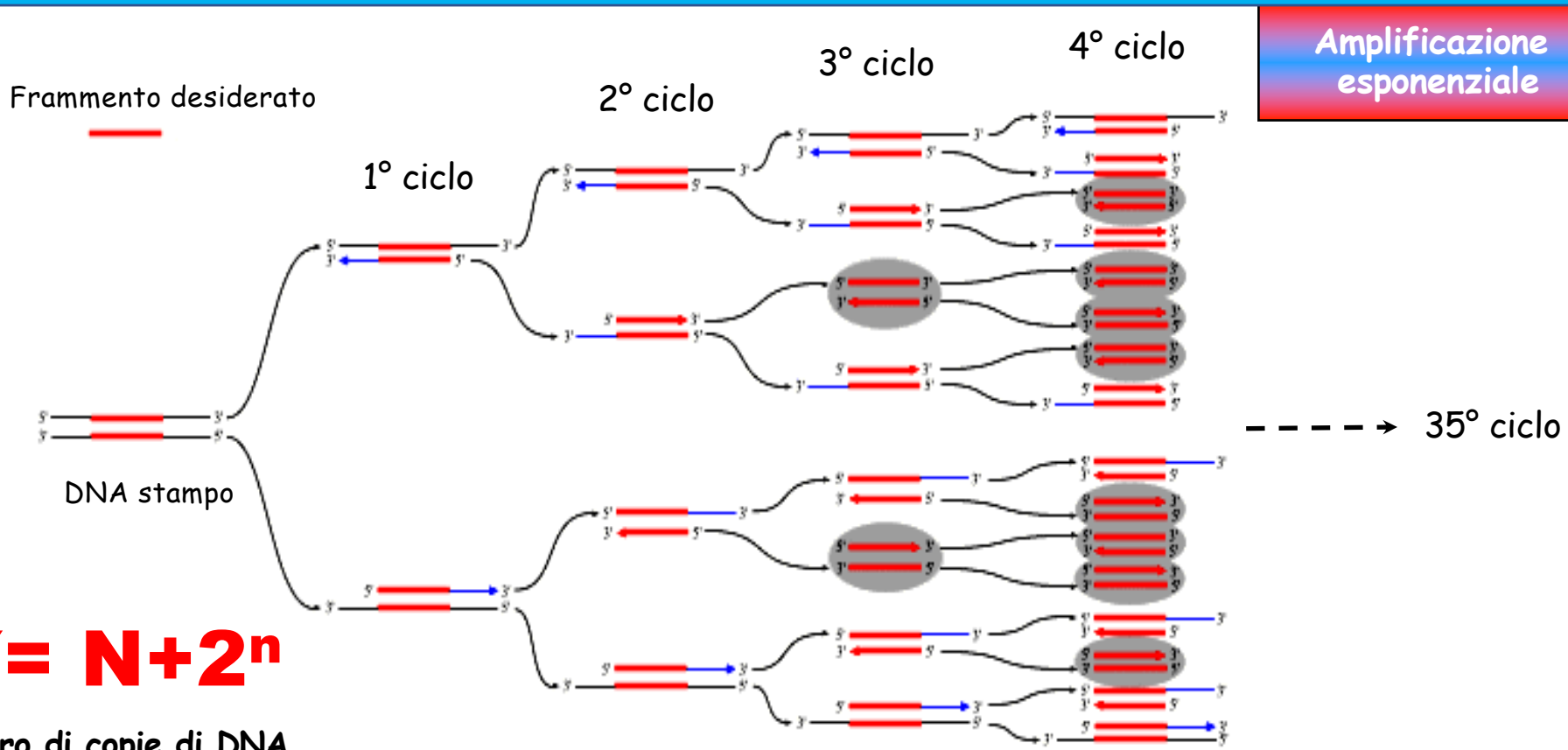
1 molecole di DNA di partenza:

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

$$Y = N * 2^n$$

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

Mechansim of PCR – I primi 4 cicli in dettaglio:



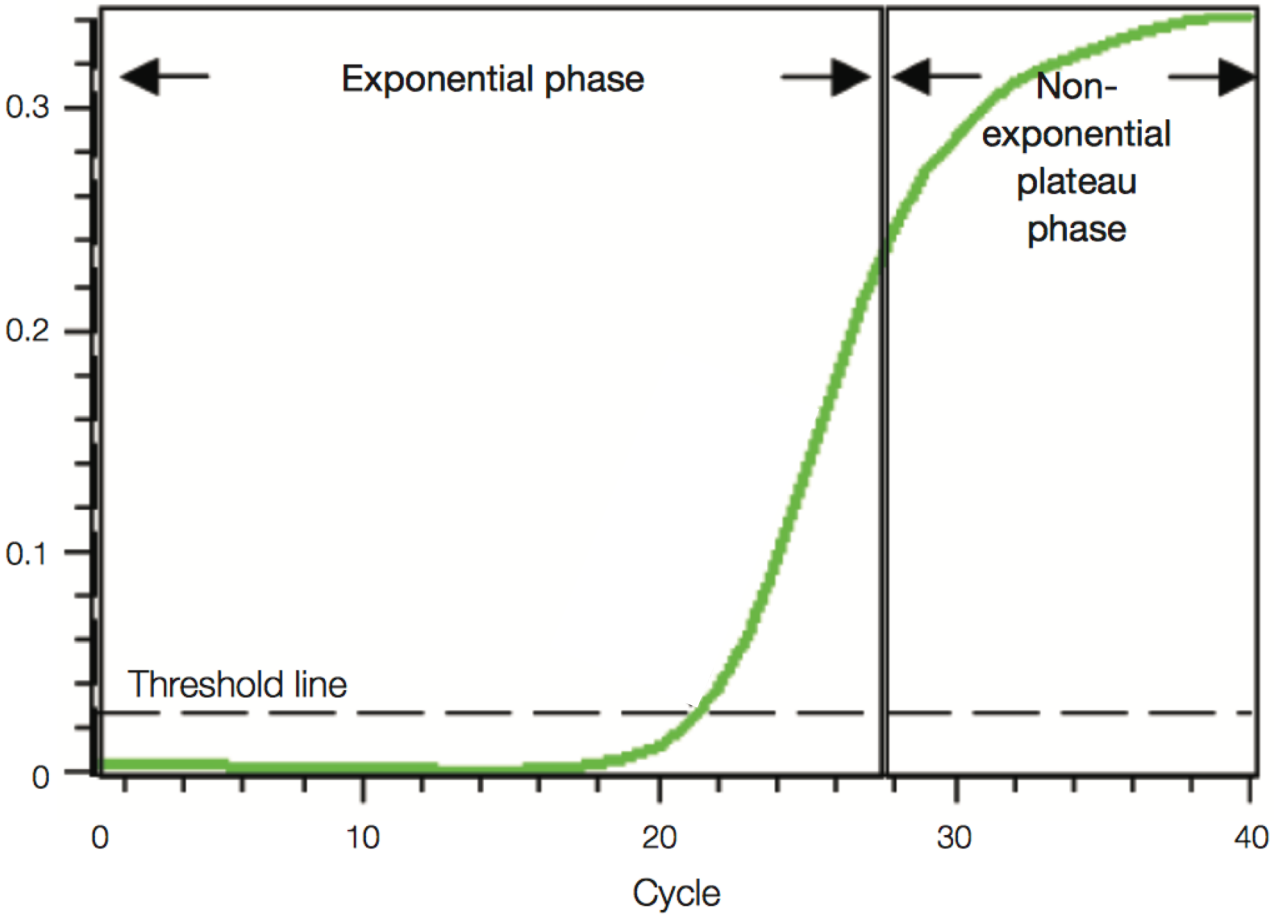
Amplificazione esponenziale

$Y = N+2^n$

Numero di copie di DNA contenenti il frammento desiderato

$1x2^1 = 2$ $1x2^2 = 4$ $1x2^3 = 8$ $1x2^4 = 16$ $1x2^{35}$

Amplification of DNA during PCR



For standard application:
max. cycle number is typically: 36

Exponential phase: During the exponential phase, ***no factor is limiting****, and the amplification products accumulate at a steady rate.*
Plateau phase: 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 lead to the so-called "plateau phase" were no additional amplification can be achieved.*

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 (for example in the human genome if your use a genomic DNA preparation from human cells)
- Length of PCR oligos (18-25 bp)
- GC content of oligos (circa 60%);
- Melting temperature «$\mathbf{T_m}$», also called **annealing temperature** of both oligos should be similar (**ideal: 60°C**)
- avoid secondary structures in single oligo nucleotide (PCR primers)
- Avaid base paring between the 2 primers
- The most 3'located nucleotide should ideally be G or C. 3 H-bond with template → stabilizes start site for DNA synthesis by DNA polymerase.

Experts can consider additinal other factors for optimizing PCR amplification

PCR primer design:

Lenght: min. 16 bp; standard 18 – 25nucleotides

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

→ Unique primer-target site interaction (theoretical)

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: Temperature for annealing of primers to template in PCR reaction → **~ 2-5°C below lower primer Tm**

Tm calculated = 62°C → set annealing temperature in PCR program @ 60°C

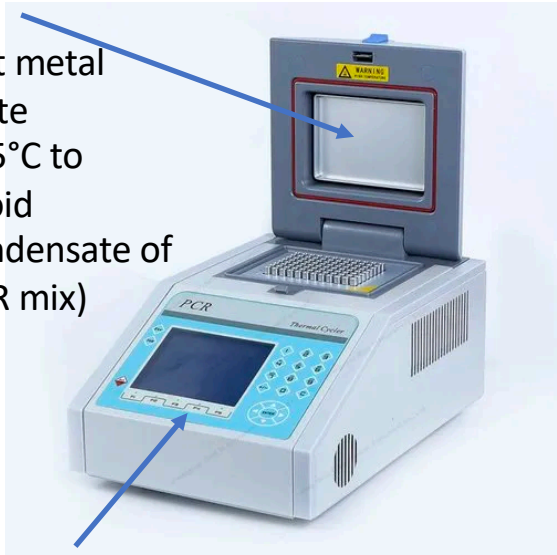
Highly different T_m result assymetric amplification of template strands

For T_m calculation see:

http://insilico.ehu.es/tm.php?formula=basic

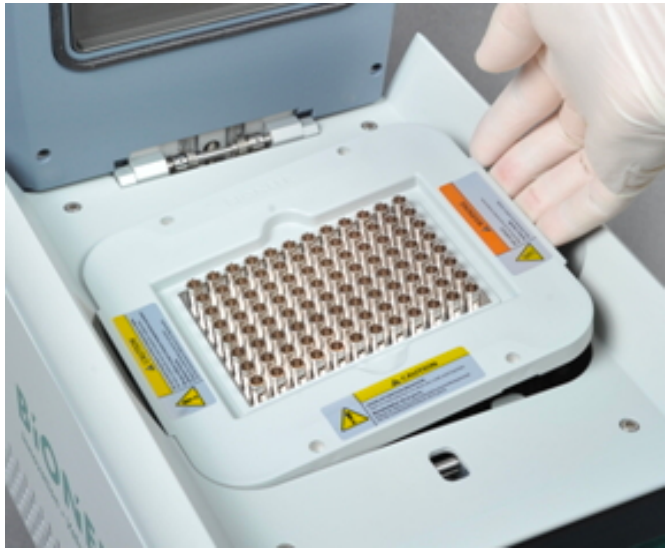
Let’s have a look at a PCR machine (thermocycler)

Thermocycler



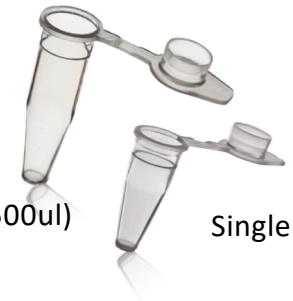
Display and tool to program
PCR reaction (temperature and time)

Heating block



Heating block

PCR strips (500ul)



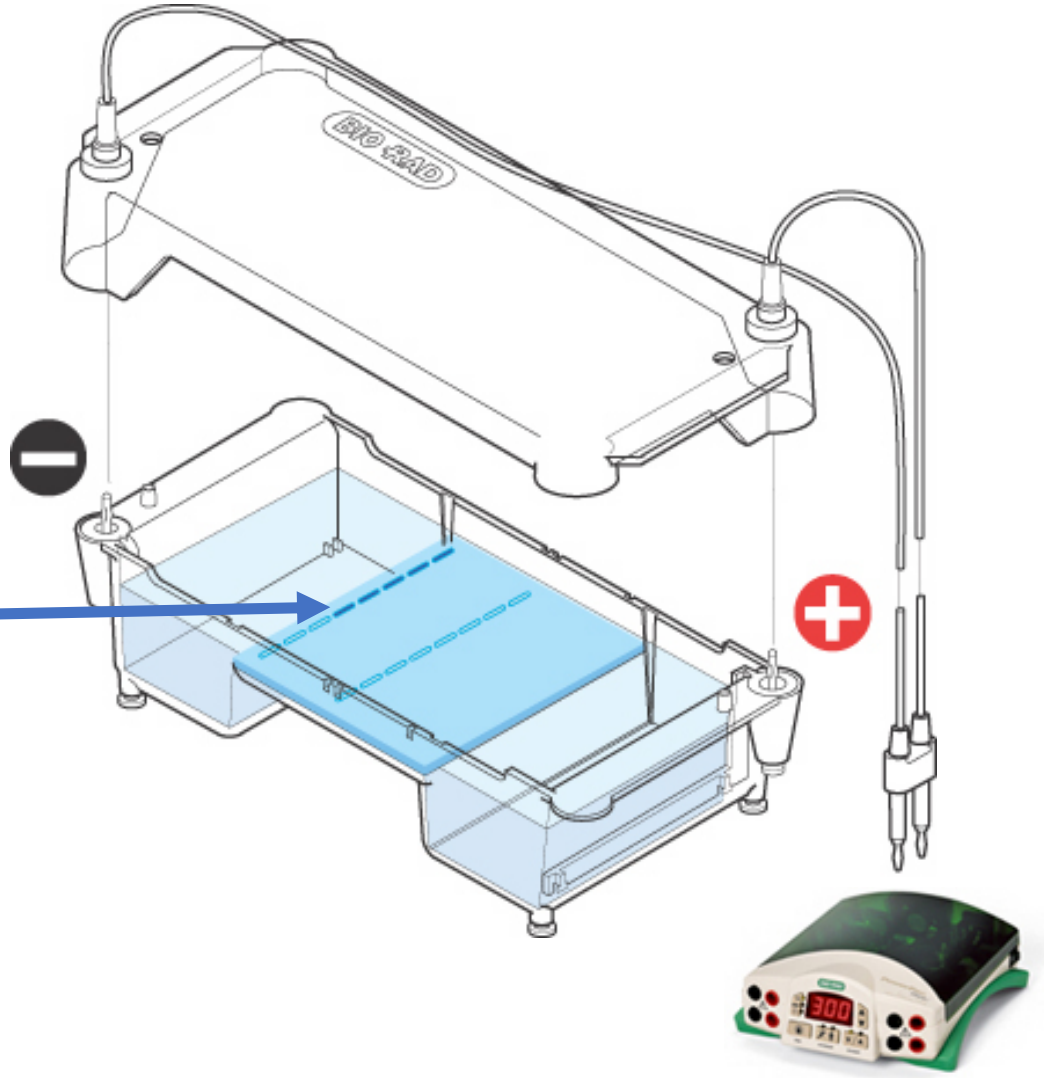
Standard tube
Not for PCR(1500ul)

Single PCR tube (500ul)

Visualization of PCR products by agarose gel electrophoresis:

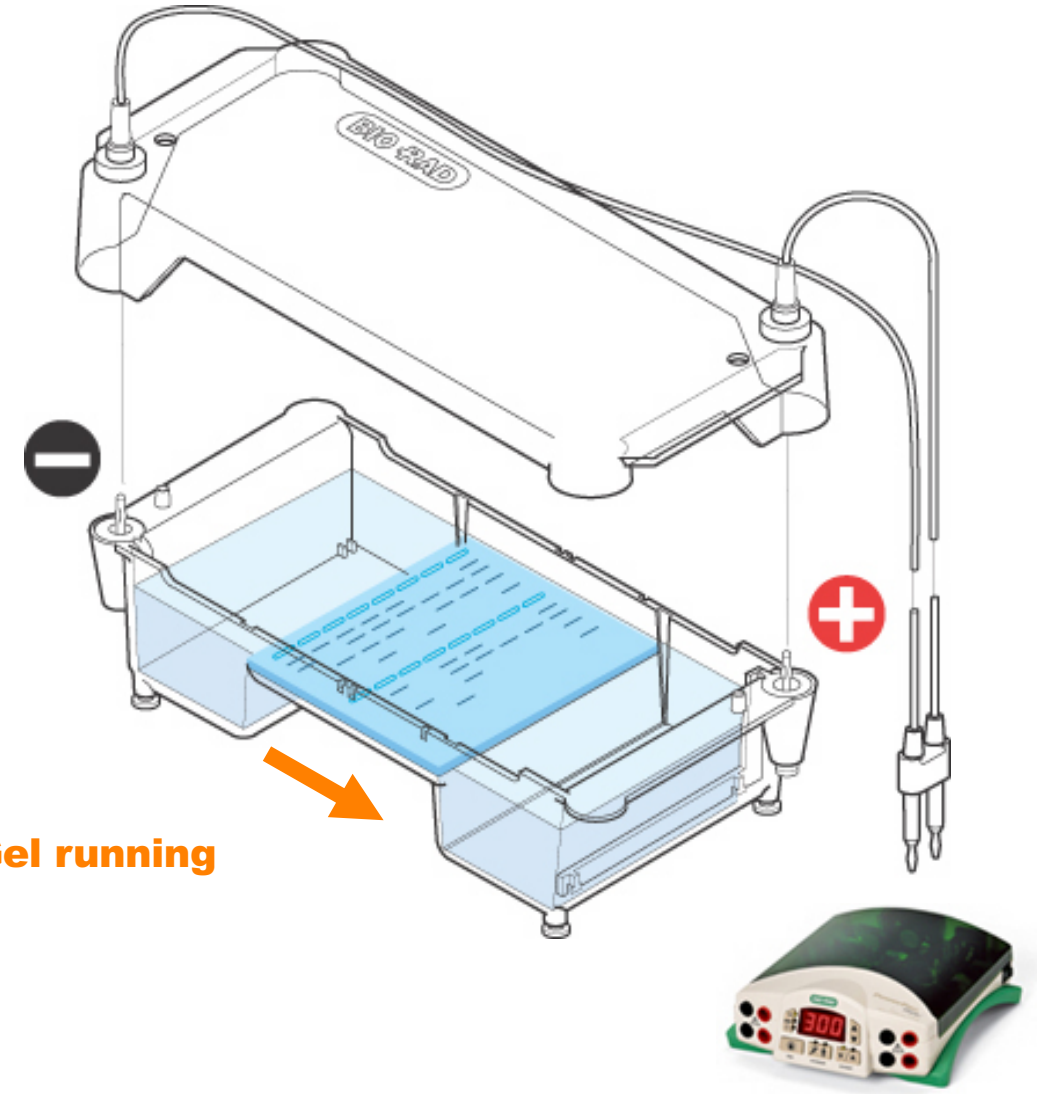
- Make Agarose gel and add dye that visualizes DNA

- Load PCR reactions into slots

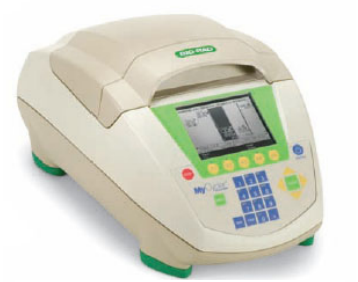


Visualization of PCR products by agarose gel electrophoresis:

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 (all or fraction)*
- *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 increasing cycle numbers that bring PCR to plateau phase.

Example: results indicate that plateau phase is reached at cycle number 30 = end point

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.