Fundamentos de Biologia Molecular

Curso de Licenciatura em Biologia
2º Ano, 1º Semestre
Ano Letivo 2021/2022

Componente Teórico-Prática

Ciências ULisboa
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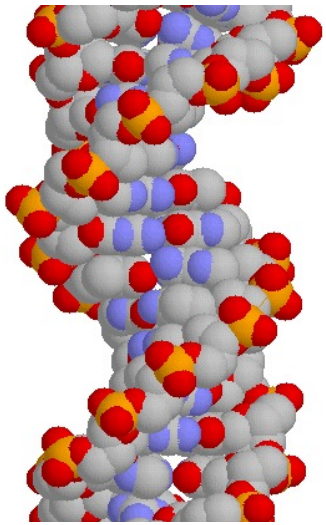
TP2: Polymerase Chain Reaction (PCR)

- DNA structure
- PCR discovery
- Nobel prizes
- PCR
- PCR components
- Setting up a PCR reaction
- Practical applications

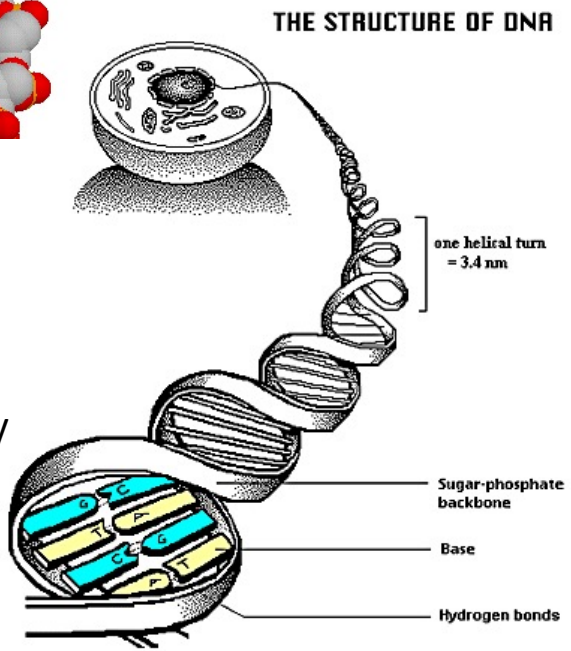
TP2: Polymerase Chain Reaction (PCR)

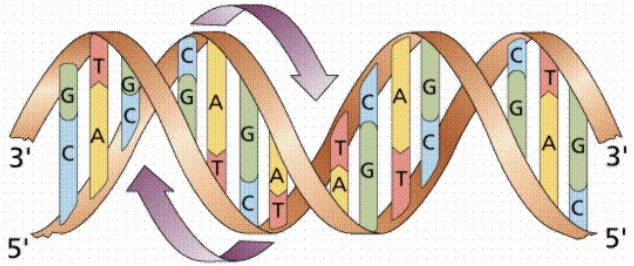
The structure of DNA



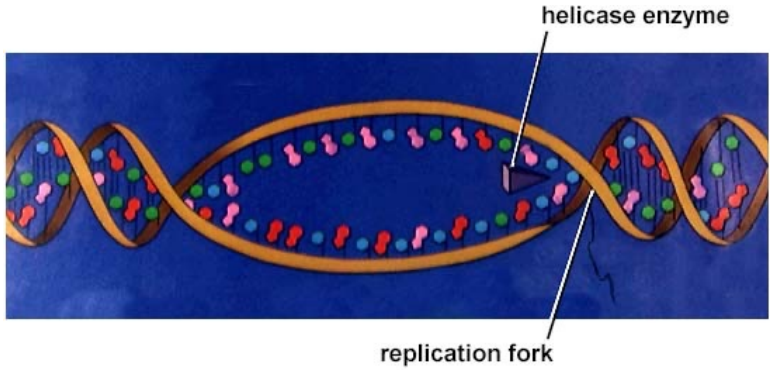
Double Helix

Complementary Base Pairing





Antiparallel Strands



Unzipping

TP2: Polymerase Chain Reaction (PCR)

The structure of DNA

How do we identify and detect a specific sequence in a genome?

- There are a LOT of other sequences in a genome that we're not interested in detecting. (SPECIFICITY)
- The amount of DNA in samples we're interested in is VERY small. (AMPLIFICATION)

How do we identify and detect a specific sequence in a genome?

- Pine: 68 billion bp
- Corn: 5.0 billion bp
- Soybean: 1.1 billion bp
- Human: 3.4 billion bp
- Housefly: 900 million bp
- Rice: 400 million bp
- E. coli: 4.6 million bp
- HIV: 9.7 thousand bp

TP2: Polymerase Chain Reaction (PCR)

Specificity

- The human genome is 3.4 B bp
- If the bases were written in standard 10-point type, on a tape measure...

...The tape would stretch for 8635.73 Km!

Identifying a 500bp sequence in a genome would be like finding a section of this tape measuring only 1.21 meters long!

TP2: Polymerase Chain Reaction (PCR)

Detection

How many molecules do we need to be able to see them?

- We need a minimum of 10 ng of fluorescent stained DNA to detect on agarose gel

URI Genomics & Sequencing Center

Calculator for determining the number of copies of a template

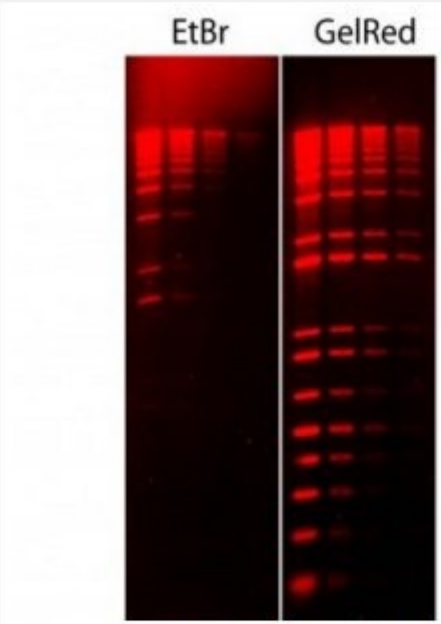
enter amount of DNA (ng): 10

enter length of template (bp): 500

Calculate number of copies!

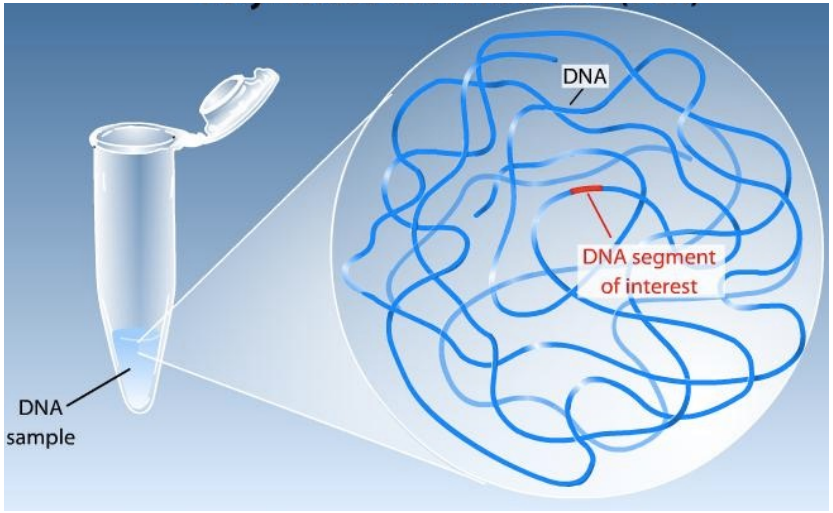
number of copies : 1.85 **x10^** 10

In other words, to "see" a single "gene", the DNA in a sample of 100 cells would have to be multiplied 180 million times!!!!!



Comparison of ethidium bromide (EtBr) and GelRed in precast gel staining in 1% agarose/TBE gel. Two-fold serial dilutions of 1 kb Plus DNA Ladder (Invitrogen) were loaded in the amounts of 200 ng, 100 ng, 50 ng and 25 ng from left to right. Gels were imaged using 300 nm transilluminator and photographed with an EtBr filter.

TP2: Polymerase Chain Reaction (PCR)

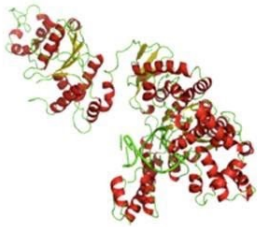


PCR is widely used to amplify target DNA regions of known sequences within a heterogeneous collection of DNA sequences

- Taq DNA polymerase isolation from *Thermus aquaticus* by Brock & Freeze - 1969

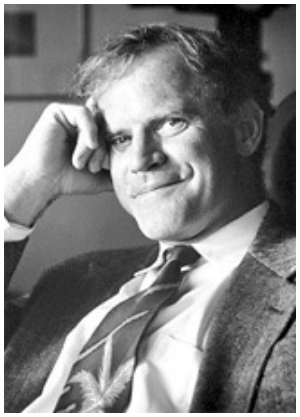
- Kary Mullis, PCR invention - 1988

Habitat of
Thermus aquaticus



Structure of Taq Polymerase

TP2: Polymerase Chain Reaction (PCR)



Kary B. Mullis

- 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.

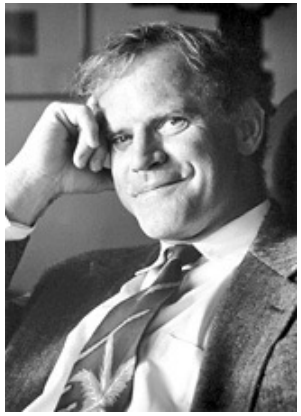
- In 1983, working for Cetus Corporation, Mullis developed the Polymerase Chain Reaction, a technique for the rapid synthesis of a DNA sequence. ***The simple process involved heating a vial containing the DNA fragment to split the two strands of the DNA molecule, adding oligonucleotide primers to bring 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.***

TP2: Polymerase Chain Reaction (PCR)

Michael Smith

- Smith first conceived of ***site-directed mutagenesis*** in the early 1970s and devoted several years to working out the details of the technique. The method provided researchers with a new way to study protein function.
- A protein is a compound made up of strings of amino acids that fold into a three-dimensional structure, and the protein's structure determines its function. Instructions for the amino-acid sequence of a protein are contained in its gene, namely, in the sequence of DNA subunits, called nucleotides, that make up that gene. The amino-acid sequence of a protein, and hence its function, can be modified by inducing mutations in the nucleotide sequence of its gene. Once an altered protein has been produced, its structure and function can be compared to those of the natural protein.
- Smith developed site-directed mutagenesis, a technique that can be used to modify nucleotide sequences at specific, desired locations within a gene. ***This has made it possible for researchers to determine the role each amino acid plays in protein structure and function***. Aside from its value to basic research, site-directed mutagenesis has many applications in medicine, agriculture, and industry.

TP2: Polymerase Chain Reaction (PCR)



Kary B. Mullis

Michael Smith

The Nobel Prize in Chemistry 1993 was awarded "***for contributions to the developments of methods within DNA-based chemistry***" jointly with one half to Kary B. Mullis "for his invention of the polymerase chain reaction (PCR) method" and with one half to Michael Smith "***for his fundamental contributions to the establishment of oligonucleotide-based, site-directed mutagenesis and its development for protein studies***".

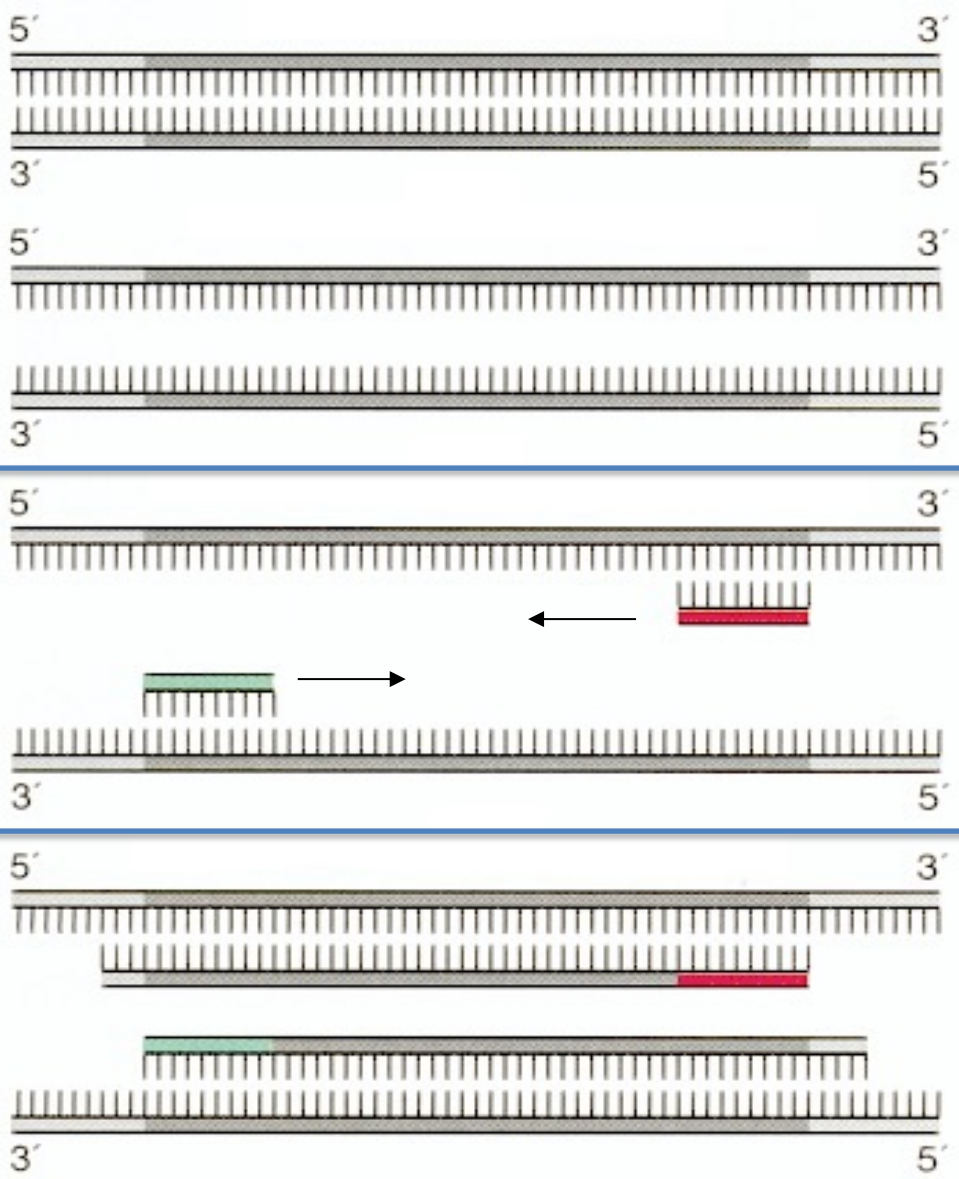
TP2: Polymerase Chain Reaction (PCR)

PCR

- Developed by Kary Mullis in 1985,
- PCR results in the selective amplification of a chosen region of a DNA molecule,
- The borders of the amplified region **have to be known** and two short oligonucleotides have to be synthesized,
- These oligonucleotides, which act as ***primers*** for the DNA synthesis, delimit the region to be amplified.

Exponential copies of template DNA

TP2: Polymerase Chain Reaction (PCR)



Parental strands

Two oligonucleotides (**primers**) that hybridize in opposite strands and flank the target

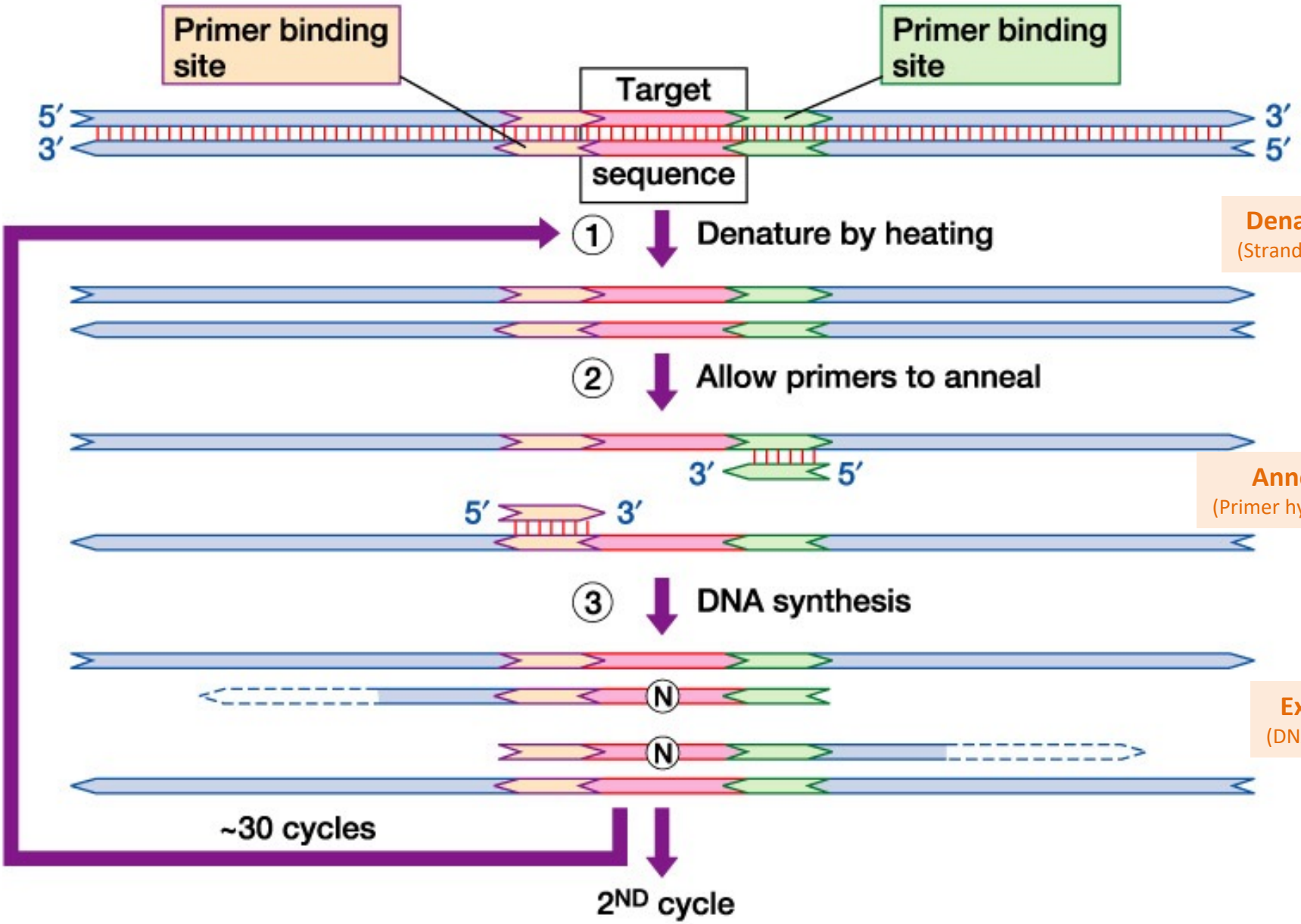
Repetitive cycles:

Template denaturation

Primer annealing

Extension

TP2: Polymerase Chain Reaction (PCR)



TP2: Polymerase Chain Reaction (PCR)

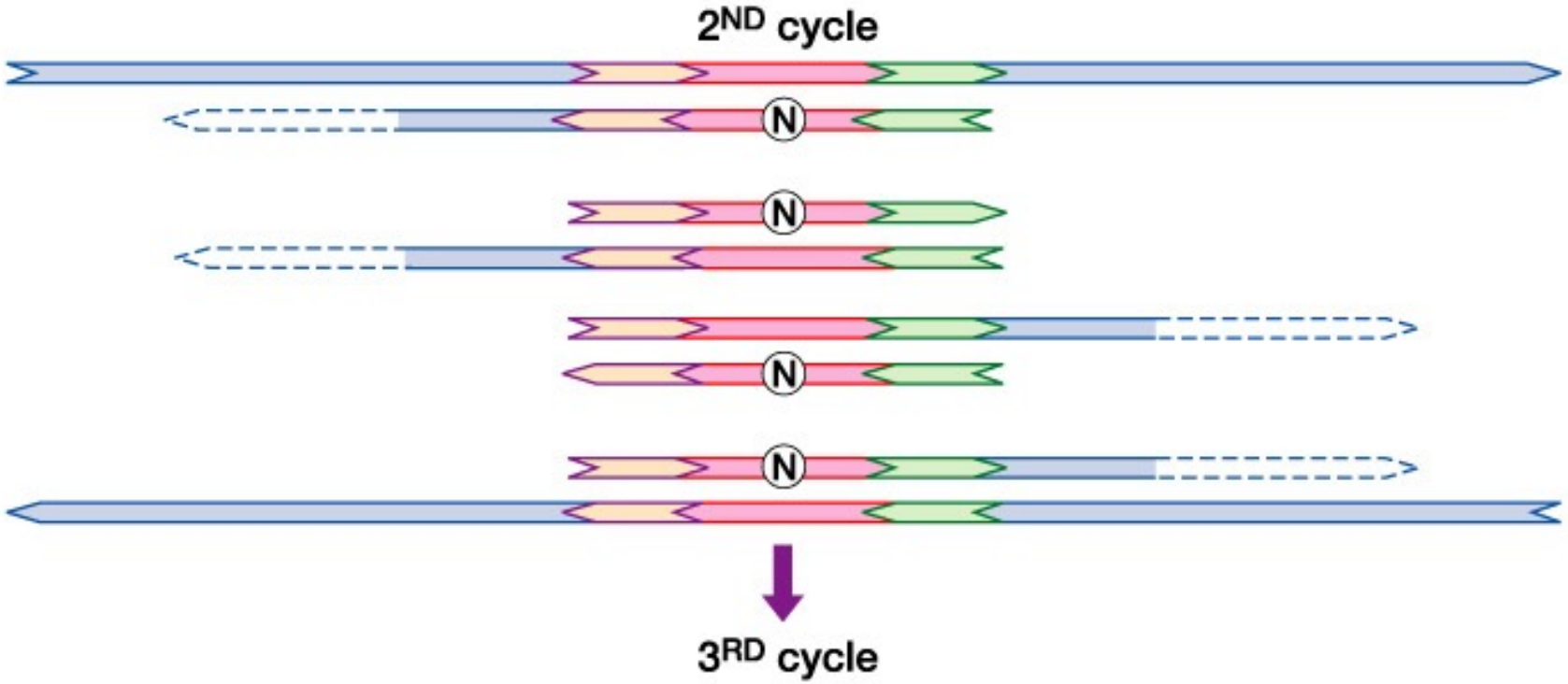
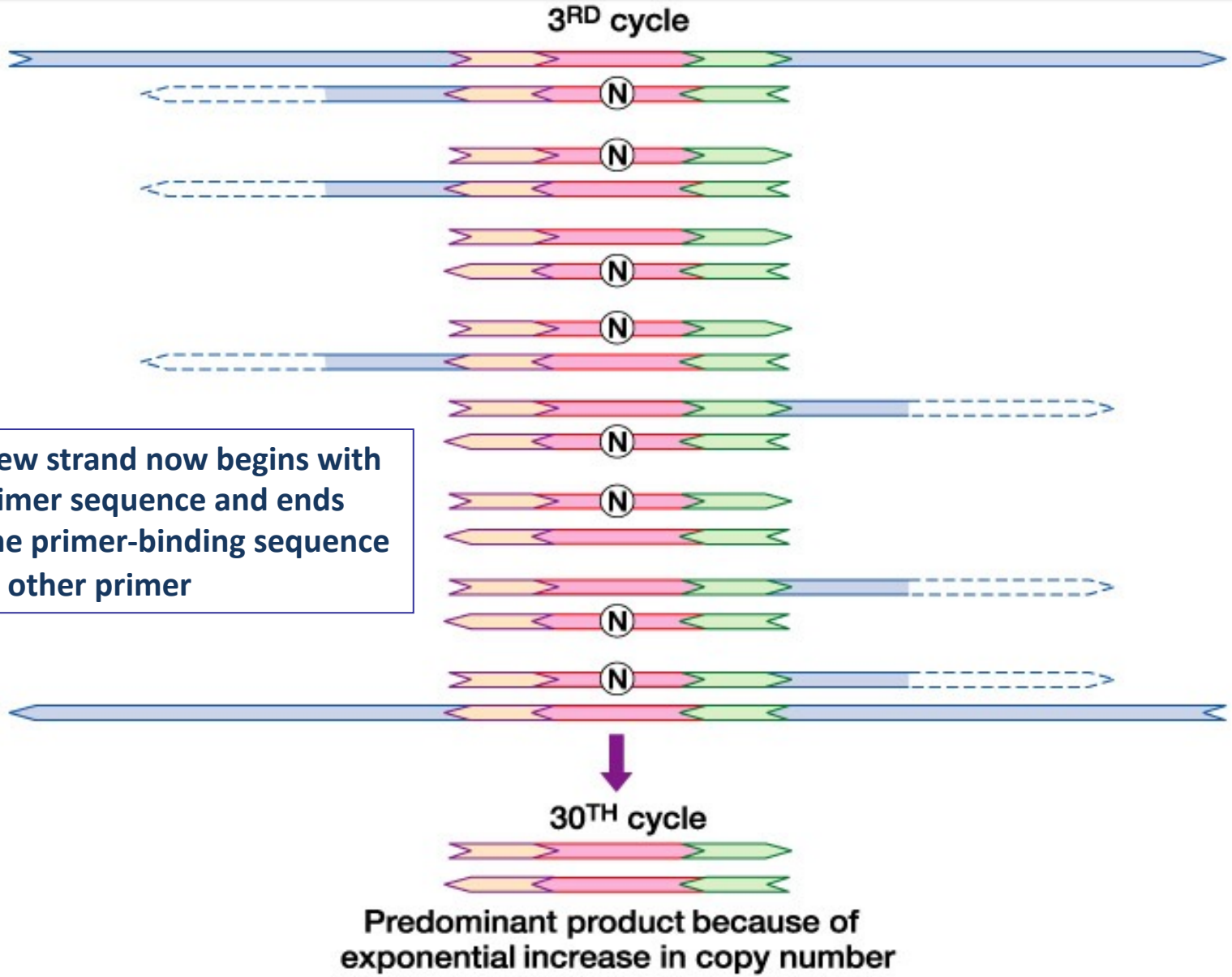


Figure 5-2 part 2 of 3 Human Molecular Genetics, 3/e. (© Garland Science 2004)

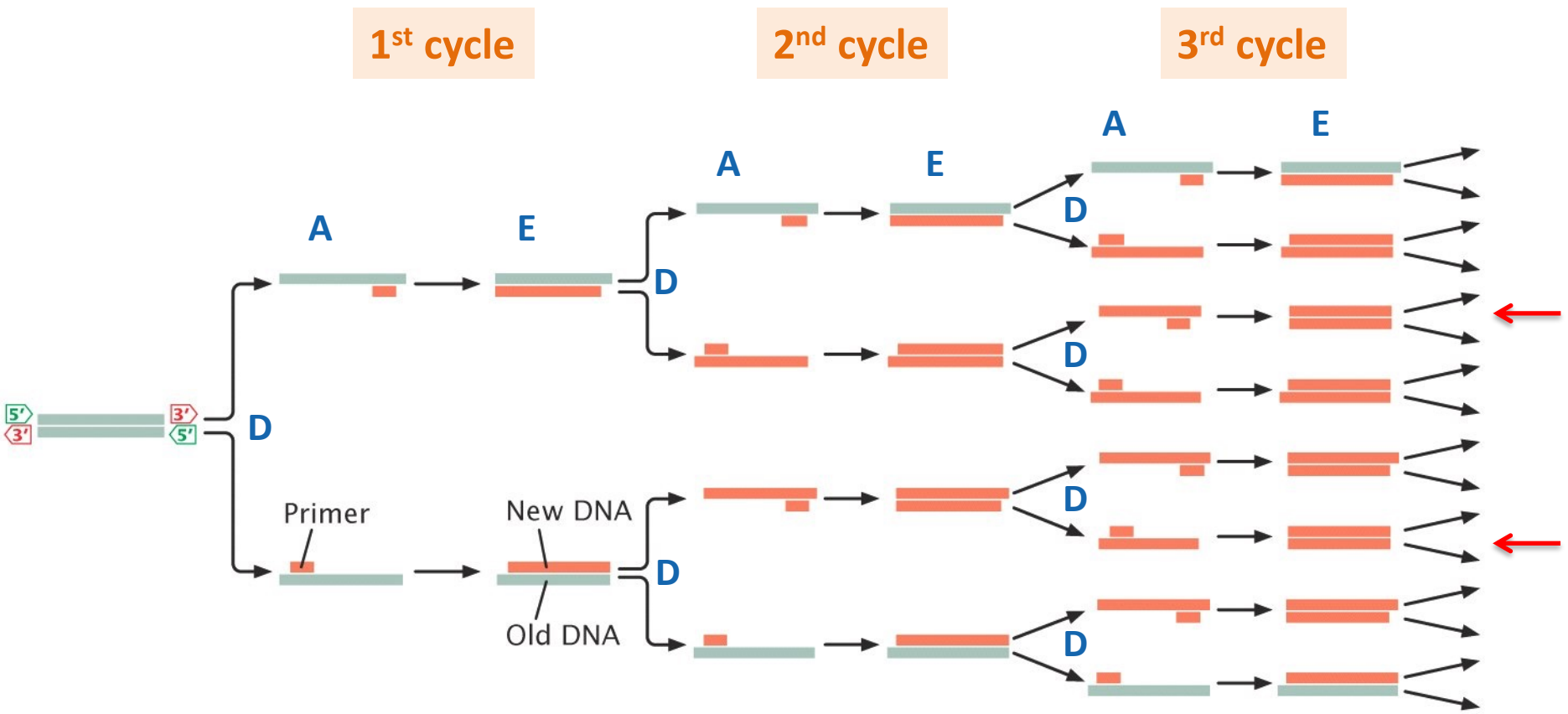
TP2: Polymerase Chain Reaction (PCR)



Each new strand now begins with one primer sequence and ends with the primer-binding sequence for the other primer

Figure 5-2 part 3 of 3, Human Molecular Genetics, 3/e. (© Garland Science 2004)

TP2: Polymerase Chain Reaction (PCR)



In each cycle:
D- denaturation
A- annealing
E- extension

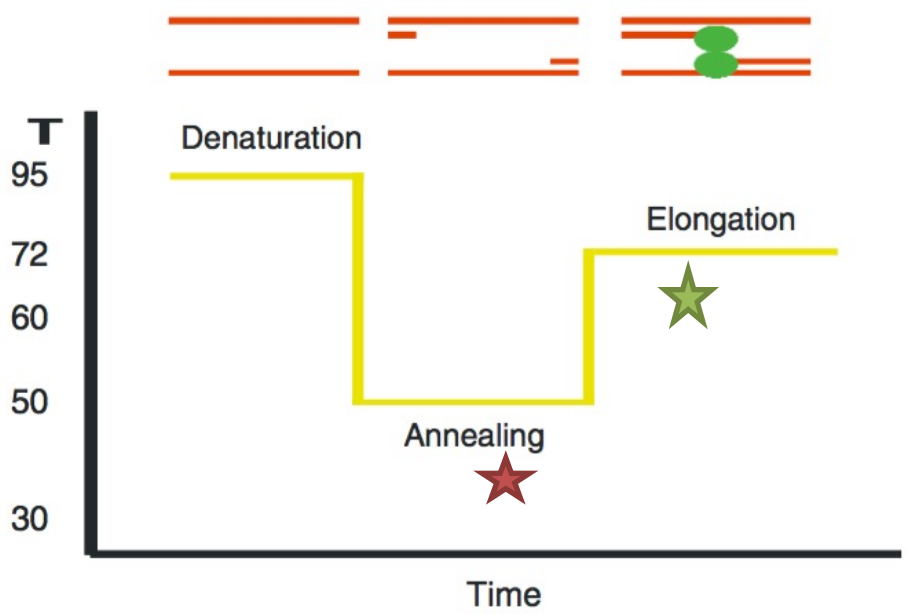
$2^1 = 2$ $2^2 = 4$ $2^3 = 8$

No. of copies/each initial molecule = 2^n
n, no. of cycles

http://openwetware.org/wiki/BME100_f2013:W900_Group17_L4

TP2: Polymerase Chain Reaction (PCR)

PCR



In each cycle:
D- denaturation
A- annealing
E- extension

Fig. 1. The PCR temperature cycle: (1) the temperature is raised to about 95 °C to melt the double stranded DNA, (2) the temperature is lowered to let primers anneal, (3) the temperature is set to 72 °C to let the polymerase extend the primers.

melting temperature (T_m)- dependent

Size- and polymerase- dependent

Kubista et al. 2006, Molecular Aspects of Medicine 27, 95–125

TP2: Polymerase Chain Reaction (PCR)

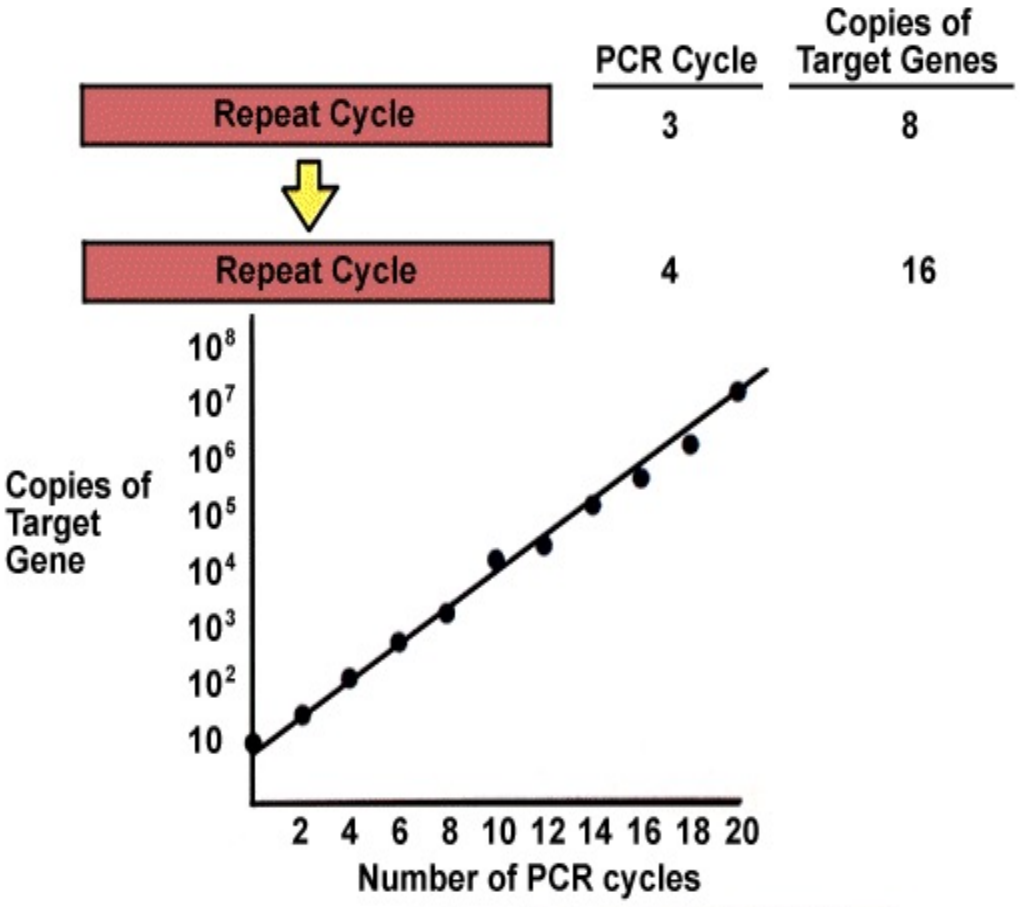


Table 18.5 Number of copies of DNA fragment in PCR amplification

Number of PCR Cycles (*n*)	Number of Double-Stranded Copies of Original DNA (2^n)
0	1
1	2
2	4
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1,024
20	1,048,576
30	1,073,741,824

https://dnalc.cshl.edu/view/15475-The-cycles-of-the-polymerase-chain-reaction-PCR-3D-animation.html

n, no. of cycles

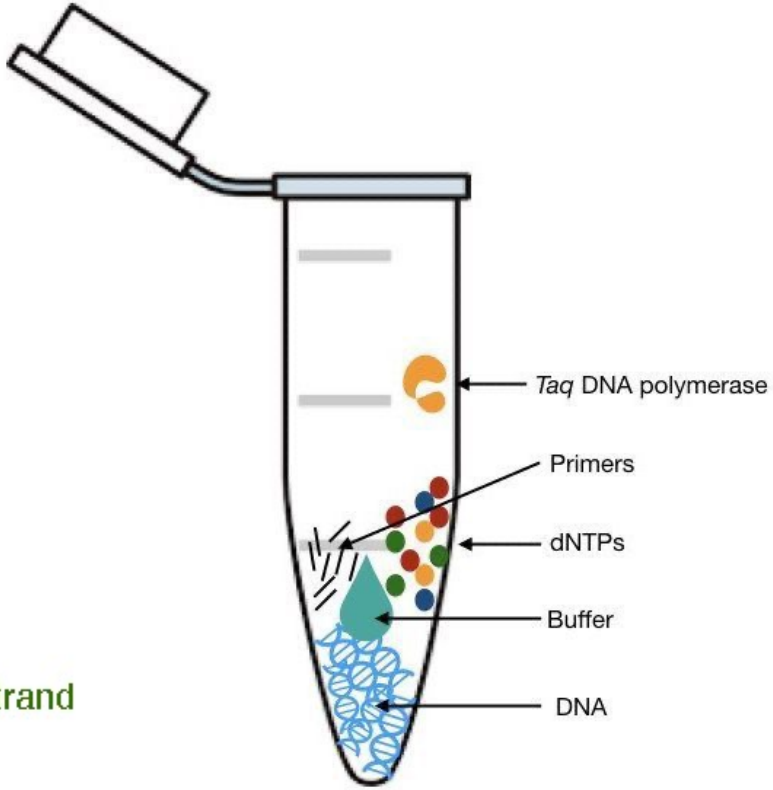
Brock, Molecular Biology of Microorganisms

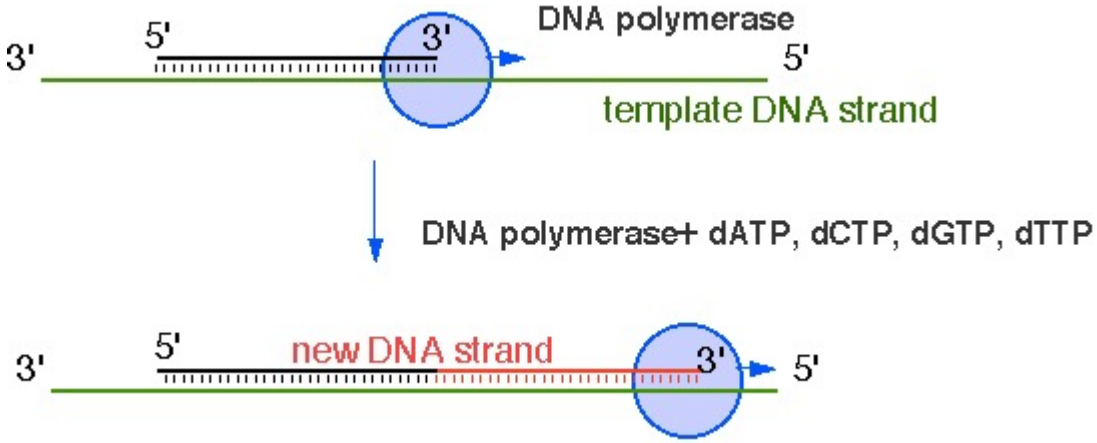
Pierce, Genetics, a conceptual approach

TP2: Polymerase Chain Reaction (PCR)

PCR reaction

- Water
- Buffer
- DNA template
- Primers
- Nucleotides
- Mg^{2+} ions
- DNA Polymerase

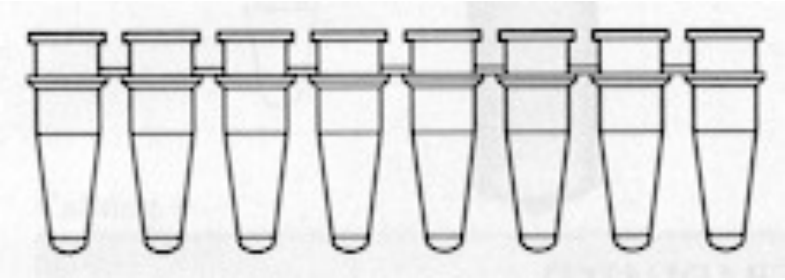




TP2: Polymerase Chain Reaction (PCR)

PCR reaction components

- **Water**
- Buffer
- DNA template
- Primers
- Nucleotides
- Mg^{2+} ions
- DNA Polymerase

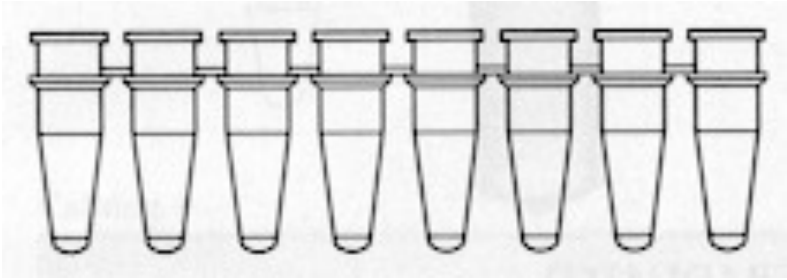


- **Water**
  - The medium for all other components.

TP2: Polymerase Chain Reaction (PCR)

PCR reaction components

- Water
- **Buffer**
- DNA template
- Primers
- Nucleotides
- Mg^{2+} ions
- DNA Polymerase

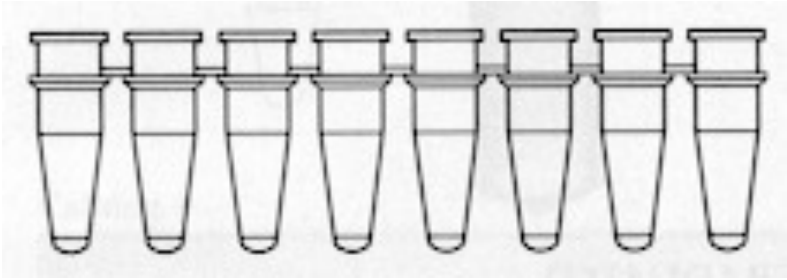


- **Buffer**
  - Stabilizes the DNA polymerase, DNA, and nucleotides
  - 500 mM KCl
  - 100 mM Tris-HCl, pH 8.3
  - Triton X-100 or Tween

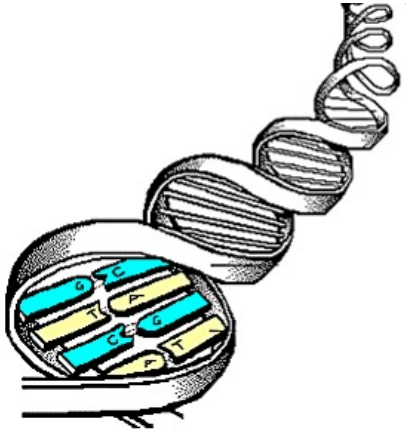
TP2: Polymerase Chain Reaction (PCR)

PCR reaction components

- Water
- Buffer
- **DNA template**
- Primers
- Nucleotides
- Mg^{2+} ions
- DNA Polymerase



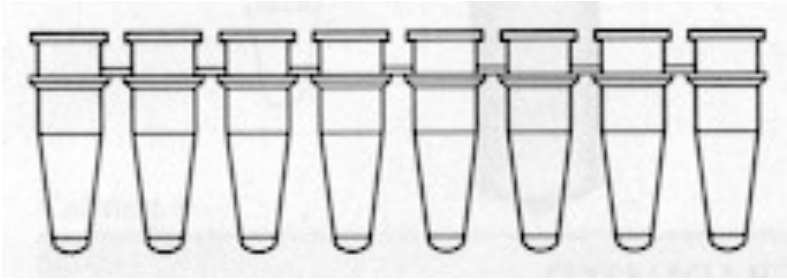
- **DNA template**
  - Contains region to be amplified
  - Any DNA desired
  - Purity not required
  - Should be free of polymerase inhibitors



TP2: Polymerase Chain Reaction (PCR)

PCR reaction components

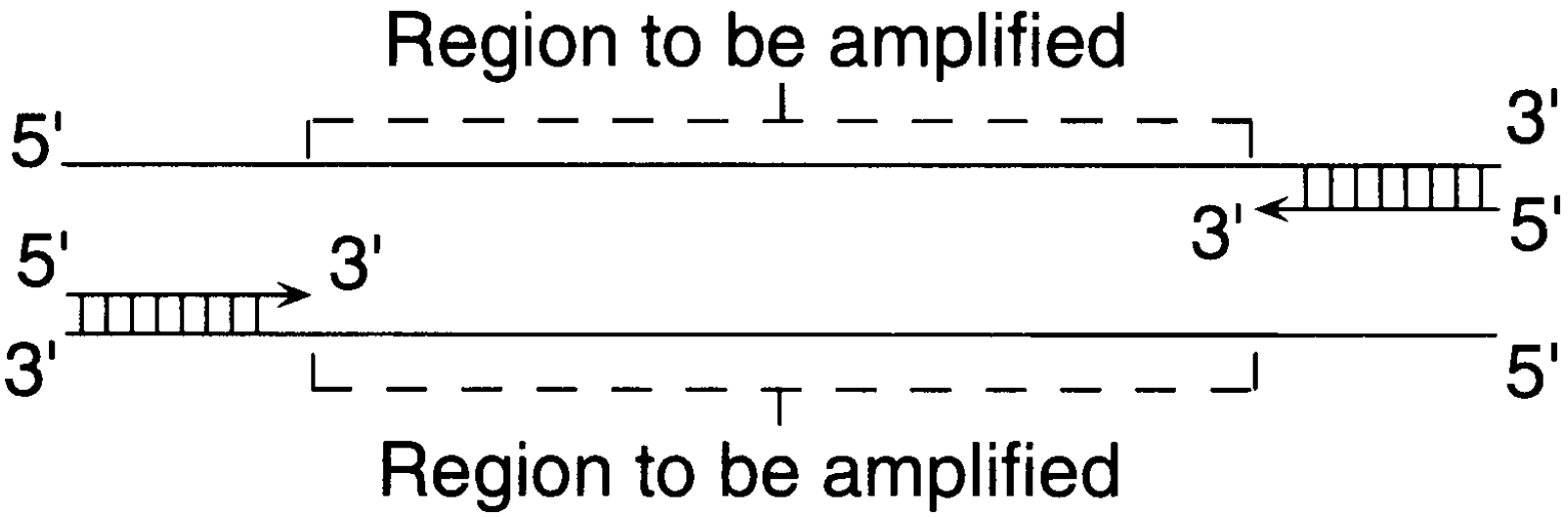
- Water
- Buffer
- DNA template
- **Primers**
- Nucleotides
- Mg^{2+} ions
- DNA Polymerase



- **Primers**
  - Specific for ends of amplified region
  - Forward and Reverse
  - Annealing temperature should be known
    - Depends on primer length, GC content, etc.
  - Length 15-30 nt
  - Conc 0.1 – 1.0 μM (pMol/μl)

TP2: Polymerase Chain Reaction (PCR)

Choosing primer sequences



TP2: Polymerase Chain Reaction (PCR)

Primer design tips

- Typical PCR primers are anything between **18-28 nucleotides in length**
- The **G+C** composition should ideally be similar to that of the desired amplicon and should in general be between **40-60%**
- The calculated Tm for a primer pair should be **balanced**
- Rule of thumb: Tm = 2(A+T) + 4(G+C) and -1.5 °C for every mismatch
- A Tm 55°C -72°C is desired (62-65°C is best)
- Check for complementarity in 3' ends of primer pairs - this leads to primer - dimer artifacts
- Avoid any significant secondary structure within primers i.e. internal palindromic sequences
- Runs of 3 or more C's and G's at 3' ends promote mispriming in G/C rich regions
- Avoid an A and especially a T at the 3' end of a primer
- Avoid any potential mismatches in the 3'end of primers (pay attention when designing degenerate primers)

TP2: Polymerase Chain Reaction (PCR)

Primer design using Primer3

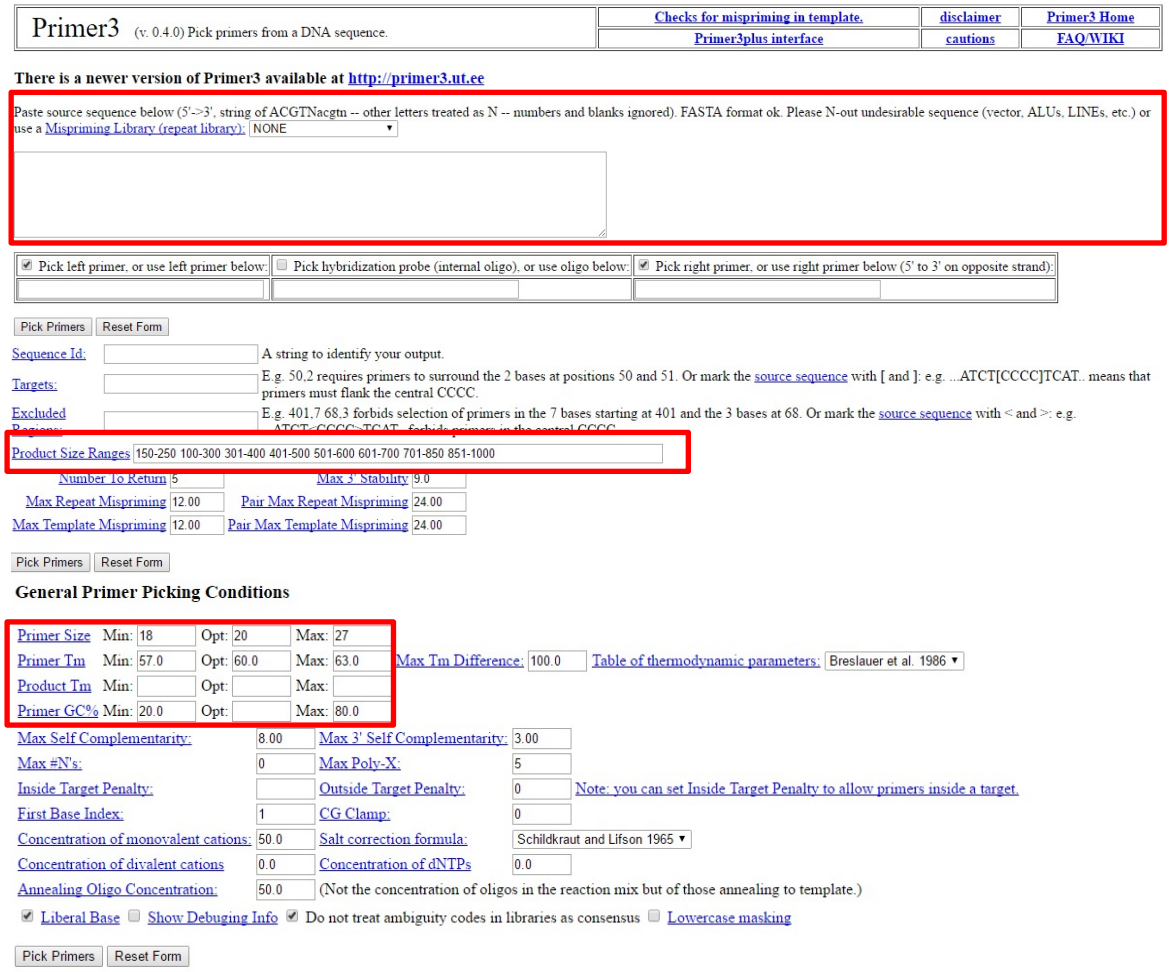
Website: http://bioinfo.ut.ee/primer3-0.4.0/

Place here query sequence for which primers will be designed

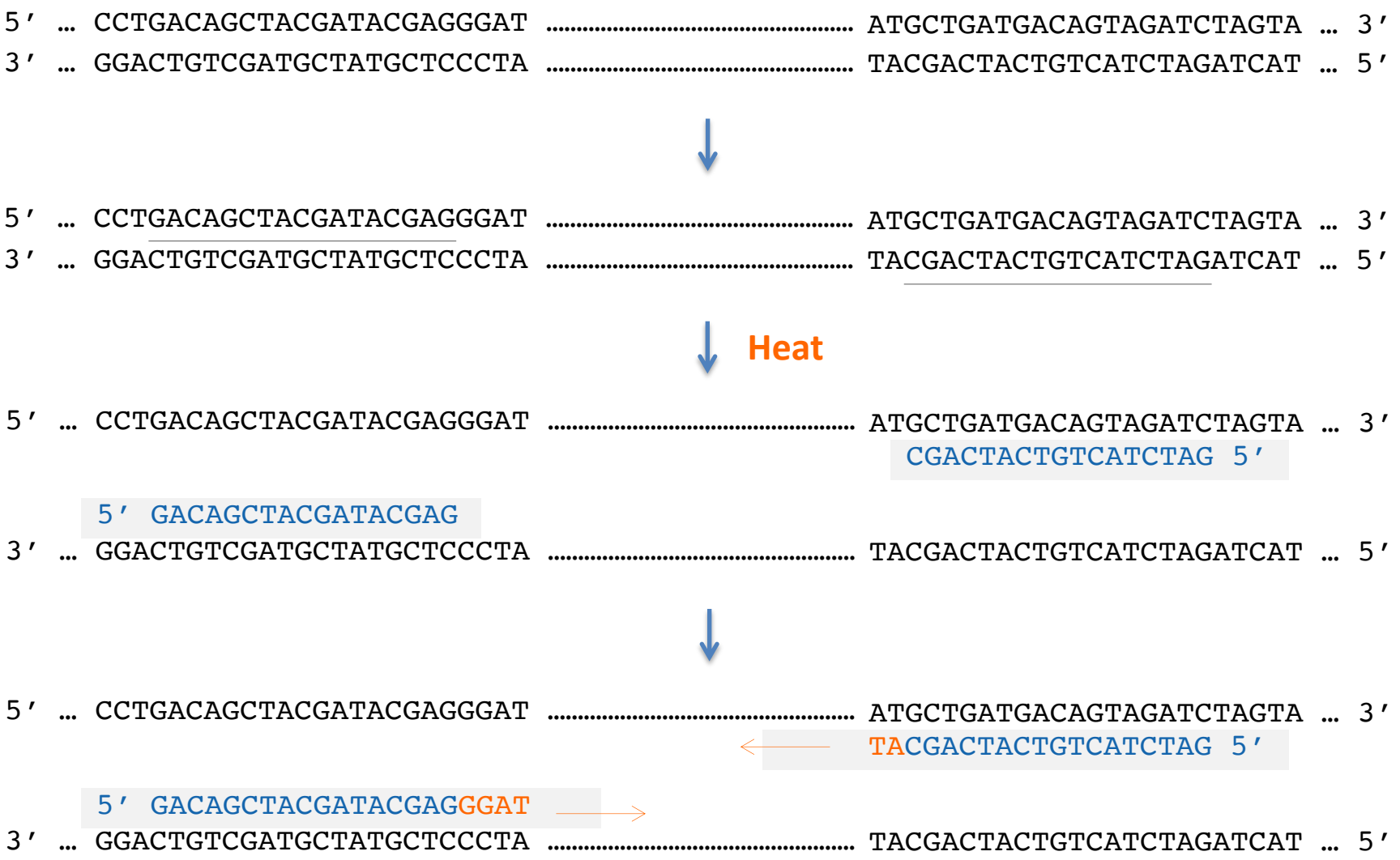
Min-Max size of generated amplicon (also counting with intermediate values)

e.g. – 200-500
Amplicons with size ranging from 200 bp to 500 bp

Min, Max and Opt size/melting temperature and GC content of primers



TP2: Polymerase Chain Reaction (PCR)



TP2: Polymerase Chain Reaction (PCR)

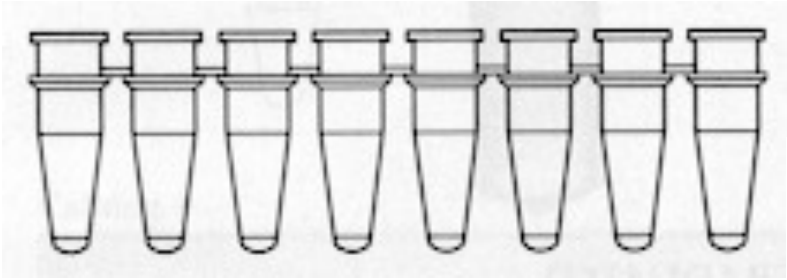
Primer storage

- Oligonucleotides are chemically stable. Left dry, they should be good for years. Once hydrated, they are susceptible to degradation by nucleases. If handled correctly, they should be stable for years.
- Any DNA oligonucleotide can be degraded by microbial or fingertip nucleases.
- Primers should be stored in deionized water or buffered solutions containing EDTA and kept frozen when not in use.
- Make a concentrated stock solution in water or TE (10 mM Tris pH 8.0, 1 mM EDTA). A convenient stock could be 100 µM, stored at -20ºC .
- Stock solutions should be distributed into several tubes for long term storage so that accidental contamination of a tube will not lead to loss of the entire synthesis

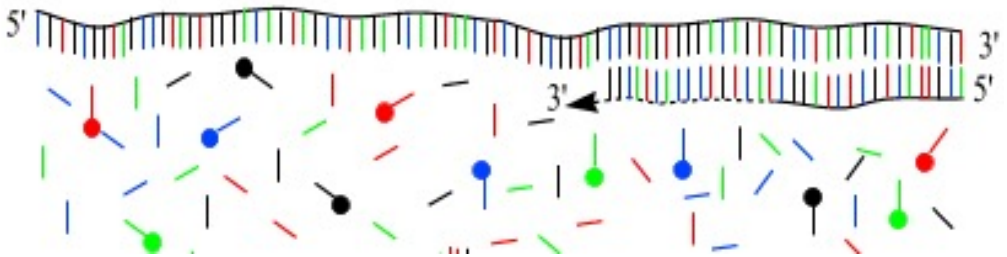
TP2: Polymerase Chain Reaction (PCR)

PCR reaction components

- Water
- Buffer
- DNA template
- Primers
- **Nucleotides (dNTPs)**
- Mg^{2+} ions
- DNA Polymerase

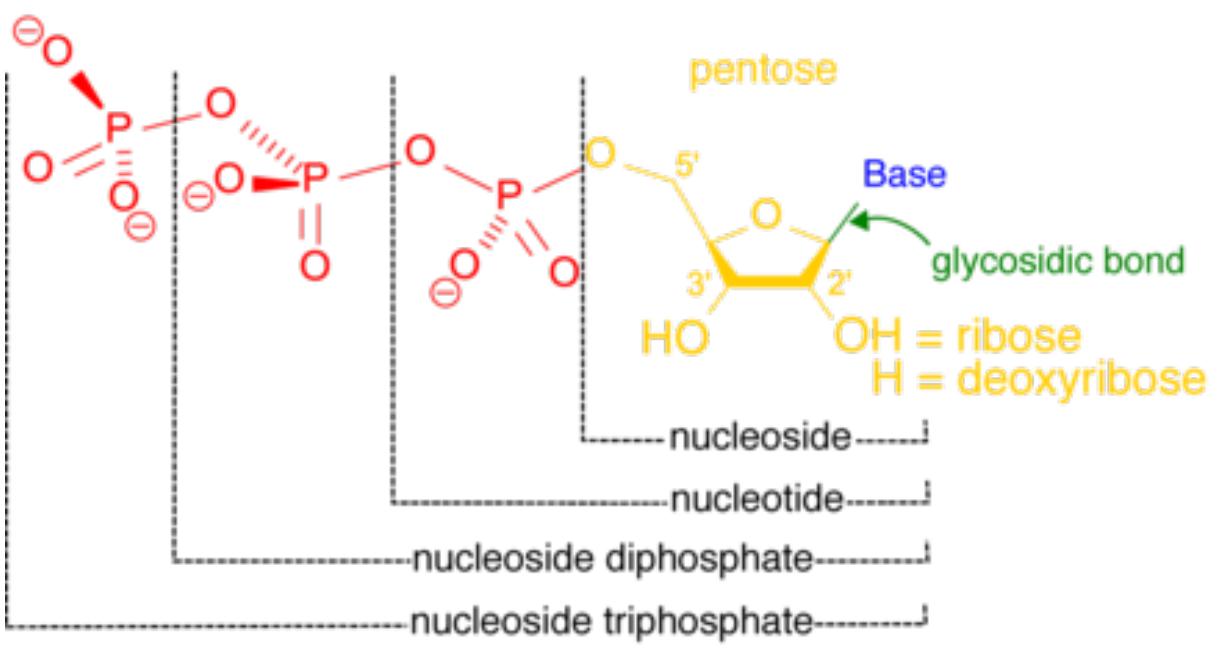


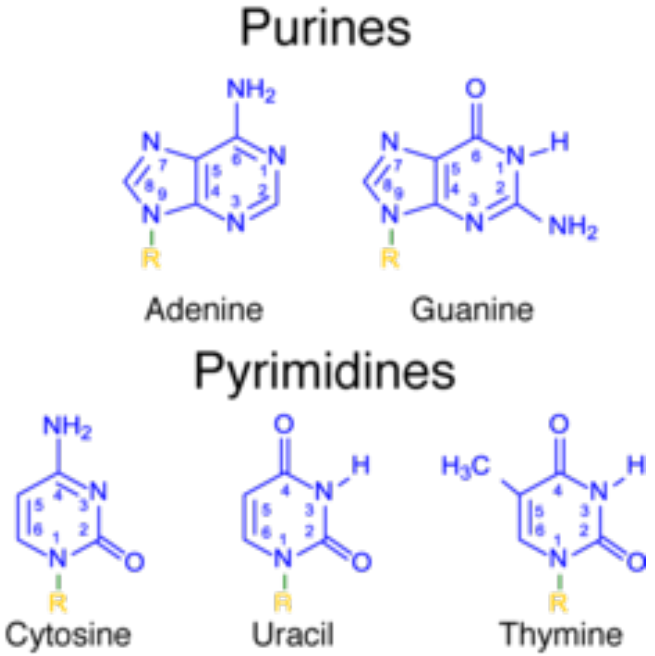
- **Nucleotides (dNTPs)**
  - Added to the growing chain
  - Activated NTP's
  - dATP, dGTP, dCTP, dTTP
  - Stored at 10mM, pH 7.0
  - Add to 20-200 μM in assay



TP2: Polymerase Chain Reaction (PCR)

PCR reaction components



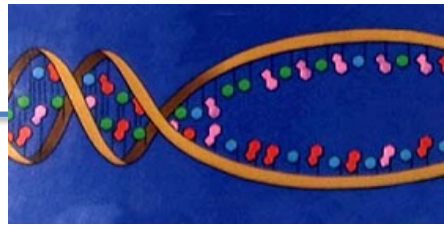


dNTP, deoxyribonucleotide triphosphate. Each dNTP is made up of a phosphate group, a deoxyribose sugar and a nitrogenous base. There are four different dNTPs and can be split into two groups: the purines (**A,G)** and the pyrimidines (**T,C**) that connect through hydrogen bounds.

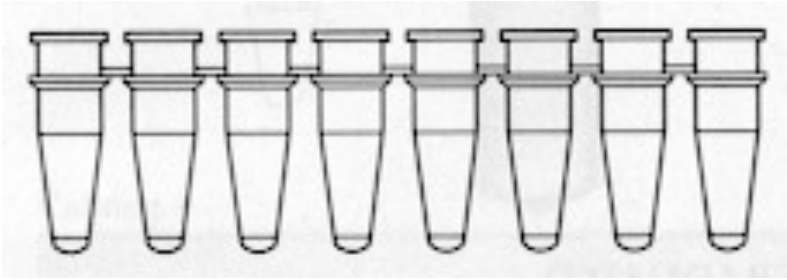
This figure also shows the five common nitrogenous bases found in DNA and RNA on the right.

TP2: Polymerase Chain Reaction (PCR)

PCR reaction components



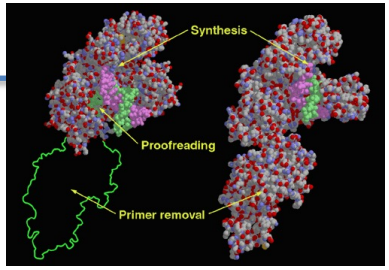
- Water
- Buffer
- DNA template
- Primers
- Nucleotides
- **Mg^{2+} ions**
- DNA Polymerase



- **Mg^{2+} ions**
  - Essential co-factor of DNA polymerase
  - Insufficient concentration reduces yield
  - Stabilizes the DNA double-helix
  - Too much: DNA extra stable, non-specific priming, band smearing
  - Used at 0.5 to 3.5 μM in the assay

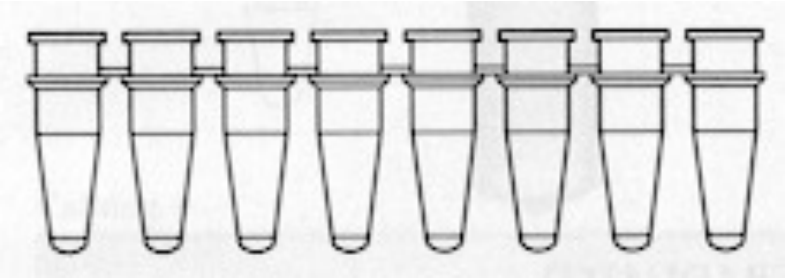
TP2: Polymerase Chain Reaction (PCR)

PCR reaction components



- Water
- Buffer
- DNA template
- Primers
- Nucleotides
- Mg^{2+} ions
- **DNA Polymerase**

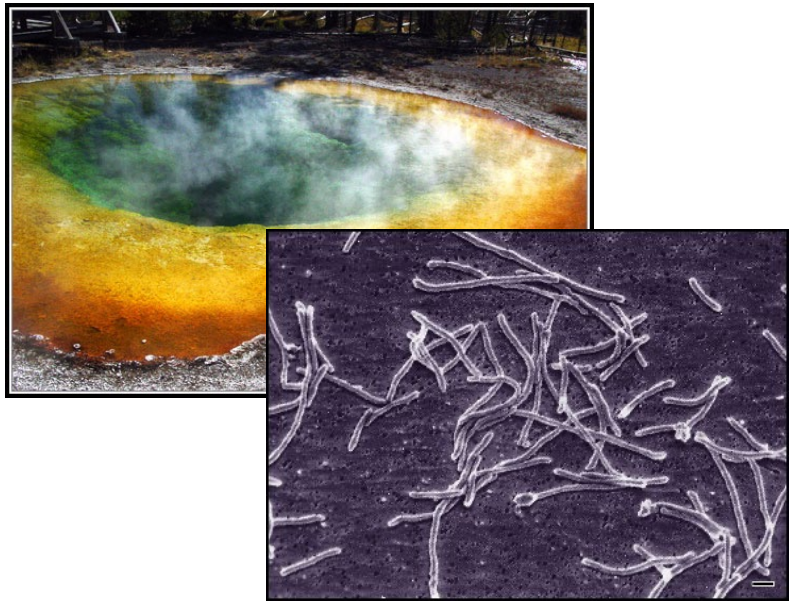
- **DNA Polymerase**
  - The enzyme that does the extension
  - TAQ or similar
  - Heat-stable
  - Approx 1 U / rxn



TP2: Polymerase Chain Reaction (PCR)

Thermostable DNA polymerase (taq DNA polymerase)

When studies of biological organisms in hot springs began in the 1960s, scientists thought that the life of thermopile bacteria could not be sustained in temperatures above about 55 °C.



Thomas D. Brock and Hudson Freeze (Indiana University) reported a new species of thermopile bacterium which they named ***Thermus aquaticus***. The bacterium was first discovered in the Lower Geyser Basin of Yellowstone National Park, near the major geysers and has since been found in similar thermal habitats around the world.

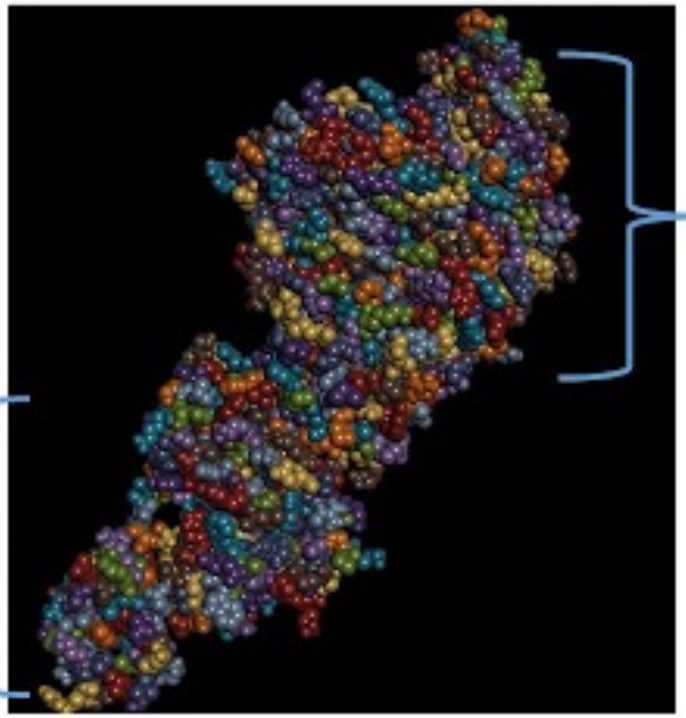
***Taq* DNA polimerase was isolated from *T. aquaticus* in 1969 by Brock & Freeze 1969**

TP2: Polymerase Chain Reaction (PCR)

Structure of DNA Polymerase

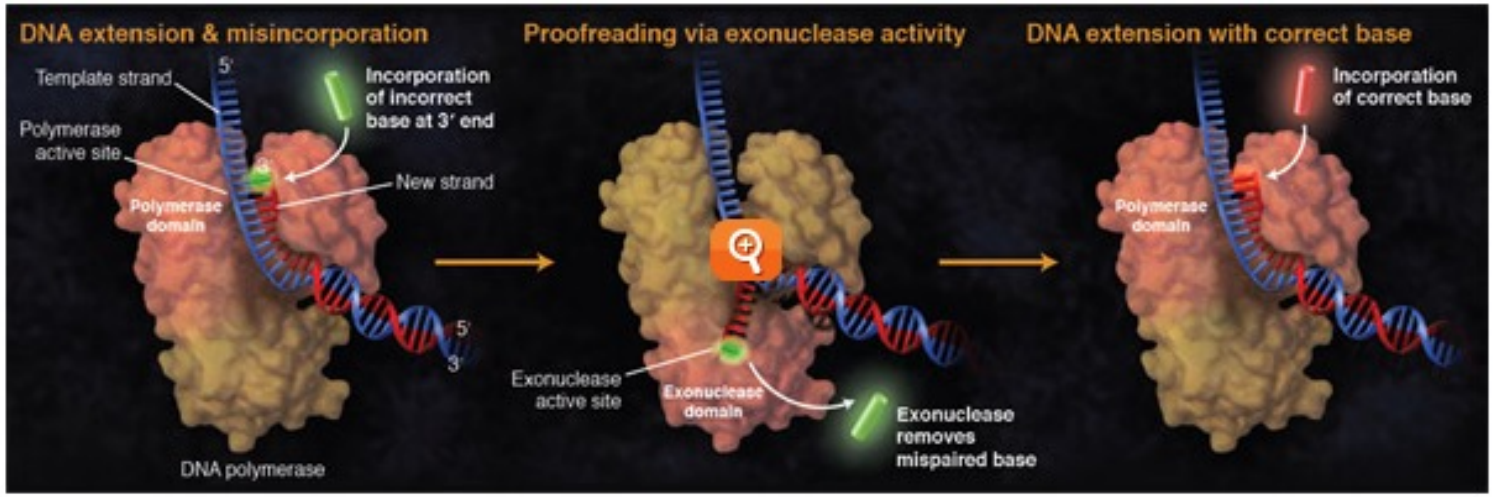
The known DNA polymerases have highly conserved structure, which means that their overall catalytic subunits vary very little from species to species, independent of their domain structures. Conserved structures usually indicate important, irreplaceable functions of the cell, the maintenance of which provides evolutionary advantages. The shape can be described as resembling a right hand with thumb, finger, and palm domains.

DNA polymerase I



TP2: Polymerase Chain Reaction (PCR)

Proofreading



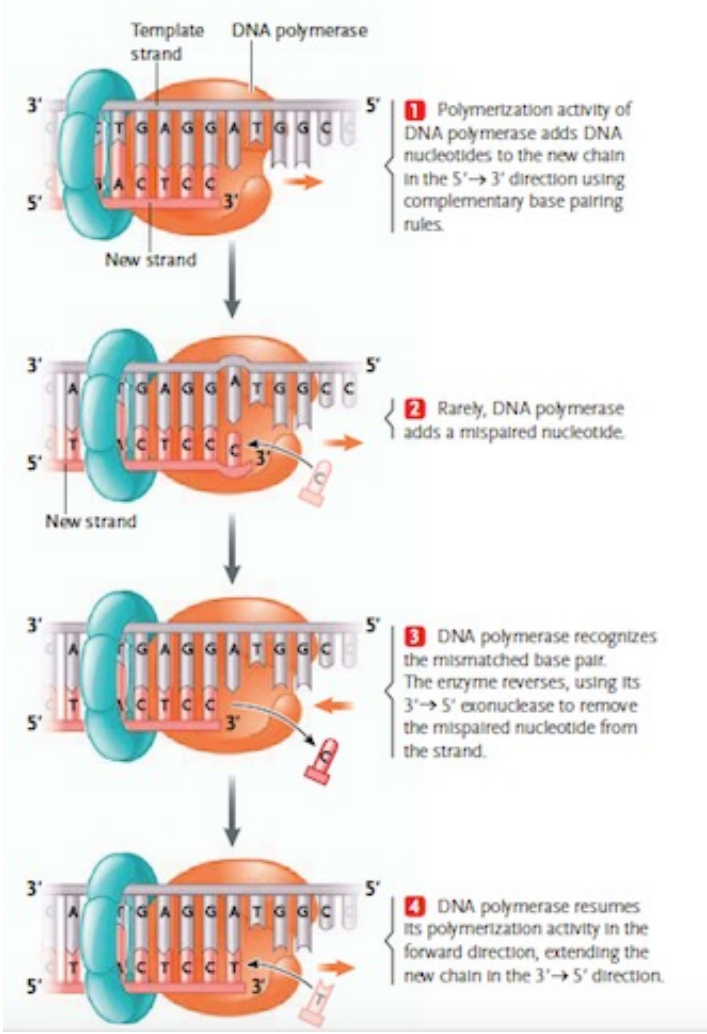
Extension proceeds along the template strand at the 3′ end of the newly synthesized strand. When the polymerase recognizes an error, the mismatched base is transferred to the exonuclease active site and the base is excised. The extended strand returns to the polymerase domain, re-anneals to the template strand, and replication continues.

https://www.youtube.com/watch?v=6O0qD6KCOVE&t=1s

https://international.neb.com/tools-and-resources/feature-articles/polymerase-fidelity-what-is-it-and-what-does-it-mean-for-your-pcr

TP2: Polymerase Chain Reaction (PCR)

Proofreading



	KOD (%)	Phusion HF (%)	Pt *Taq* (%)	Expand HF (%)	FastStart HF (%)	Sequal Prep Long (%)
Overall error rate[a]	0.21	0.11	0.34	0.25	0.23	0.29
Insertions	0.10	0.07	0.14	0.11	0.11	0.11
Deletions	0.06	0.02	0.08	0.07	0.05	0.06
Substitutions	0.01	0.01	0.07	0.04	0.03	0.07
Dots or Dot[b]	0.04	0.01	0.05	0.04	0.04	0.05

https://www.thermofisher.com/pt/en/home/brands/thermo-scientific/molecular-biology/phusion.html

TP2: Polymerase Chain Reaction (PCR)

DNA polymerase properties

1. **Thermal stability.** A denaturation step at approximately 95°C in each PCR cycle separates the two strands of a DNA molecule. DNA polymerase must be robust enough to tolerate high-temperature cycles without compromising activity,

2. **Extension rate.** This refers to the speed at which nucleotides are added, per second, per molecule of DNA polymerase, a factor determined by extension temperature, DNA template sequence and buffer composition. Early polymerases exhibited extension rates of about 1 kb per minute at 72°C,

3. **Fidelity.** Fidelity is an inherent DNA polymerase property defining the frequency of insertion of an incorrect nucleotide per kb of DNA. High-fidelity polymerases are more accurate because of the ability to "proofread" and excise incorrectly incorporated mononucleotides, replacing them with the correct base.

4. **Processivity.** The probability that a polymerase will detach from DNA during extension, indicating the average number of nucleotides the enzyme adds in a single binding event, is known as its processivity. High processivity is important when amplifying long amplicons.

https://www.youtube.com/watch?v=DBiHGc2LCb4

TP2: Polymerase Chain Reaction (PCR)

Example of a PCR reaction

Sterile Water	38.0 µl
10X PCR Buffer	5.0 µl
MgCl2 (50mM)	2.5 µl
dNTP's (10mM each)	1.0 µl
PrimerFWD (25 pmol/ul)	1.0 µl
PrimerREV	1.0 µl
DNA Polymerase	0.5 µl
DNA Template	1.0 µl
Total Volume	50.0 µl

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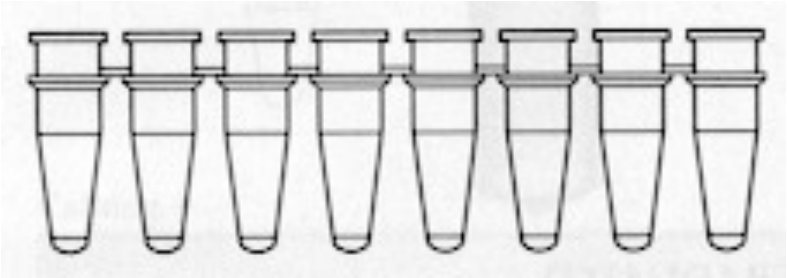
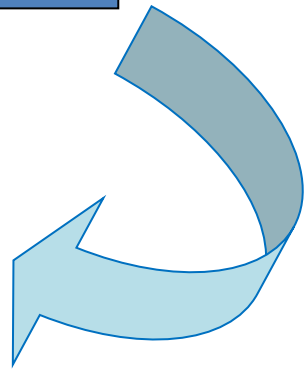
Total Volume	50.0 µl

Mixing Common Reagents Saves Time – **master mix** concept

Component	1X (µ)	20X (µl)
Sterile Water	38.0	760
10X PCR Buffer	5.0	100
MgCl2 (50mM)	2.5	50
dNTP's (10mM each)	1.0	20
PrimerFWD (25 pmol/ul)	1.0	20
PrimerREV	1.0	20
DNA Polymerase	0.5	10
DNA Template	1.0	--
Total Volume	50.0	980



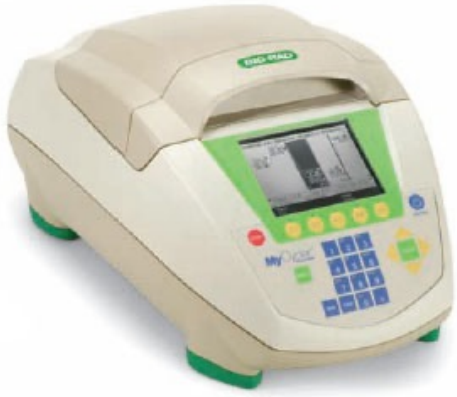
Aliquot
49 ul



TP2: Polymerase Chain Reaction (PCR)

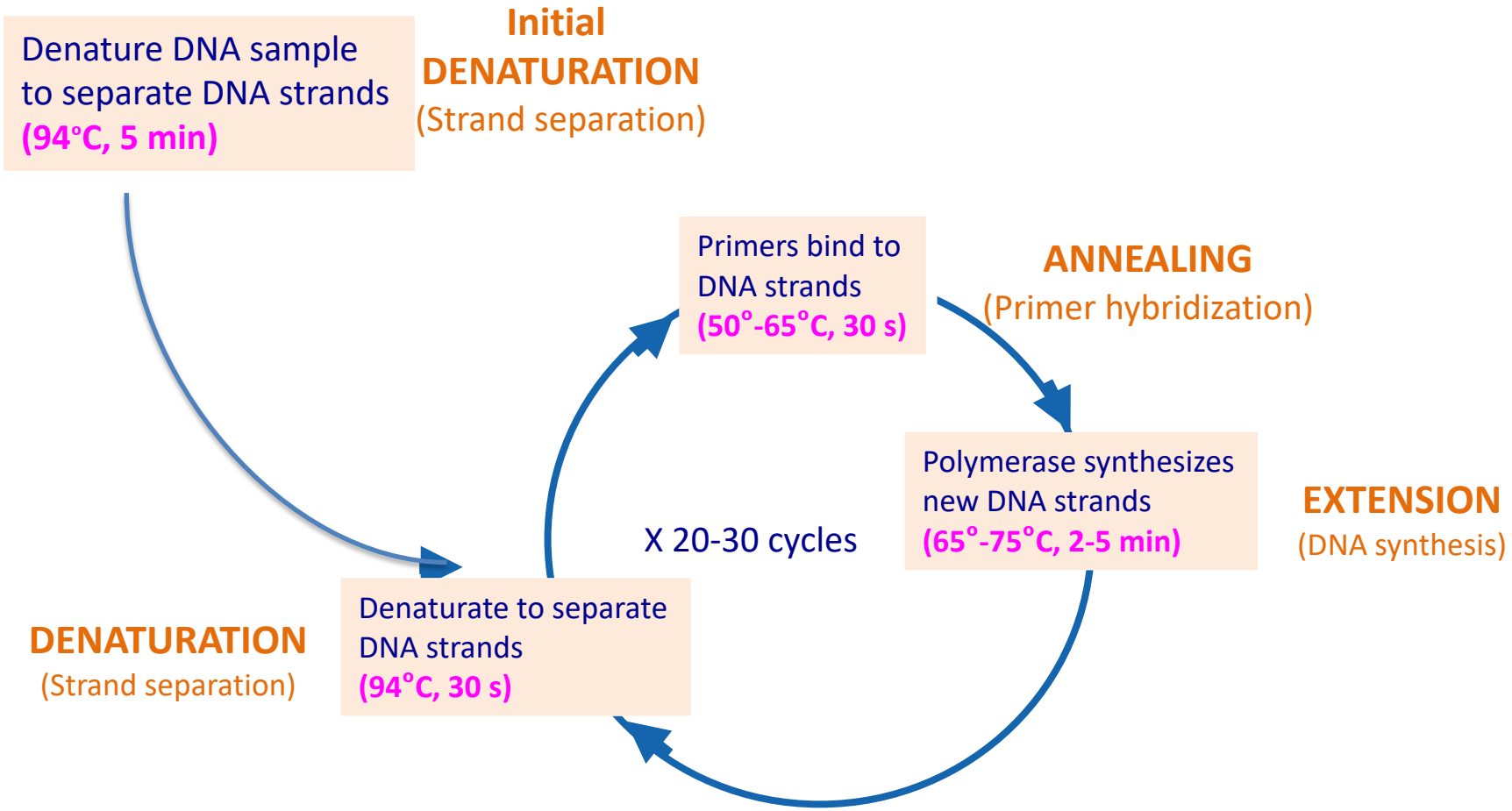
Thermal cycling conditions

Step	Time	Temperature	Comments
1. Denaturation	4 min	94°C	Denaturation of template and primer-dimers
2. Denaturation cycle	0.5 min	94°C	
3. Annealing cycle	0.5 min	55°C	Or 5°C below the lowest primer melting temperature
4. Extension cycle	1 min/kb	72°C	
5. Repeat steps 2–4			Repeat 30 times
6. Final extension	10 min	72°C	
7. End	Infinite	4°C	



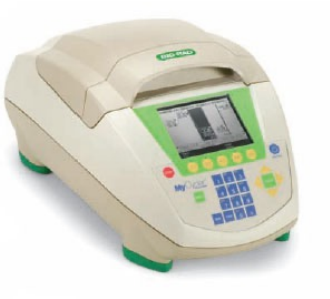
TP2: Polymerase Chain Reaction (PCR)

Thermal cycling steps

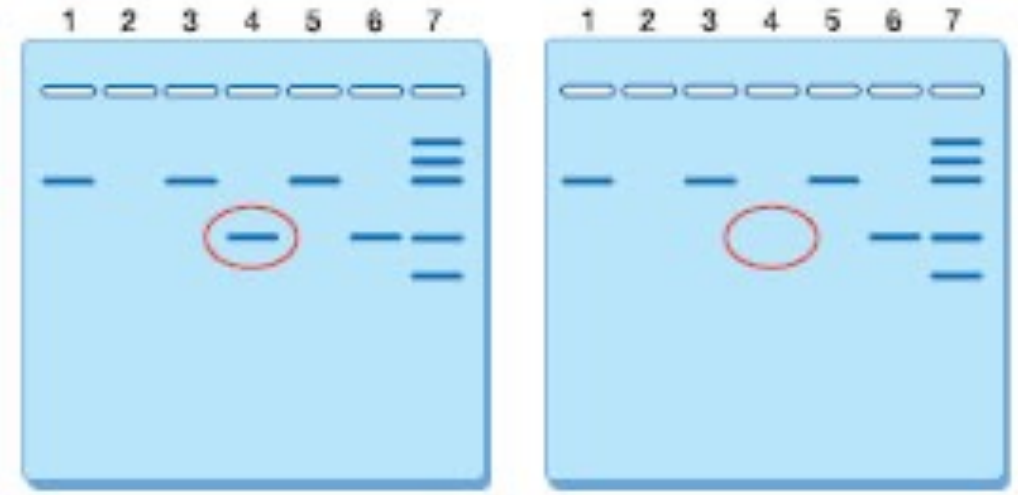


TP2: Polymerase Chain Reaction (PCR)

Visualization of PCR products

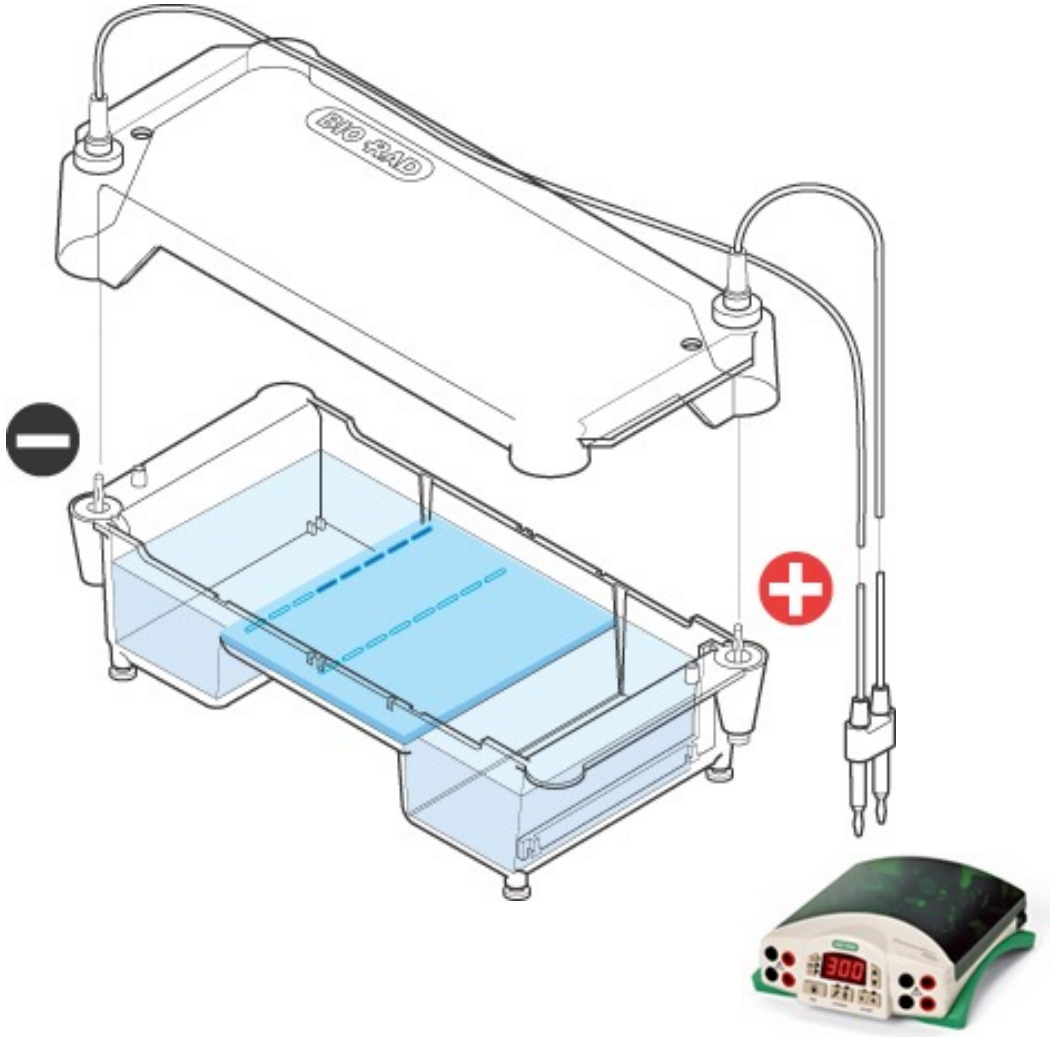


- After thermal cycling, tubes are taken out of the PCR machine.
- Content of tubes is loaded onto an agarose gel.
- DNA is separated by size using an electric field.
- DNA is stained.
- PCR products are visible as different “bands”.



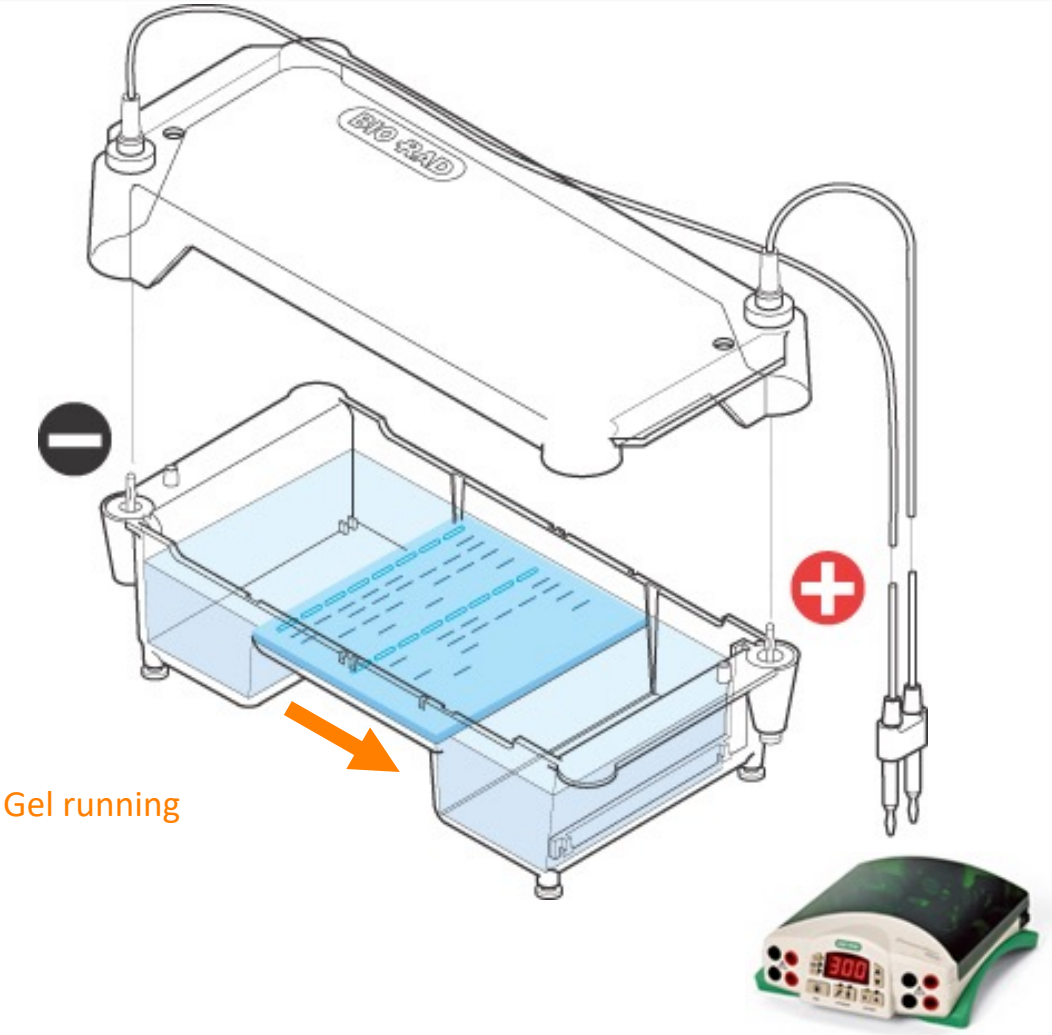
TP2: Polymerase Chain Reaction (PCR)

Visualization of PCR products



TP2: Polymerase Chain Reaction (PCR)

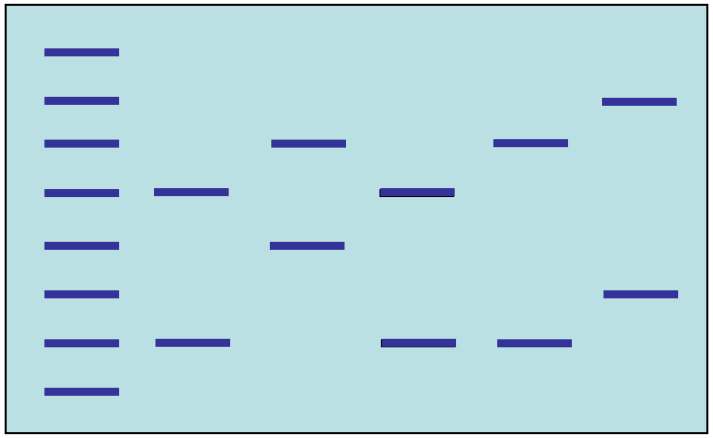
Visualization of PCR products



TP2: Polymerase Chain Reaction (PCR)

Visualization of PCR products

The final result of the traditional PCR procedure is a gel with a series of bands:

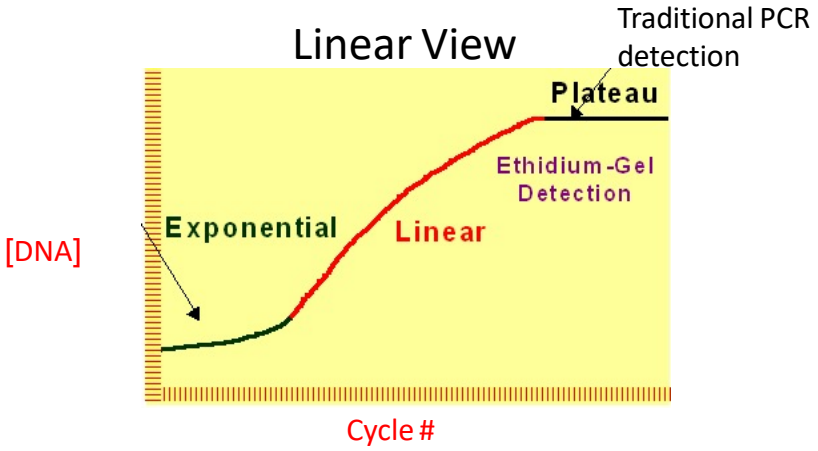


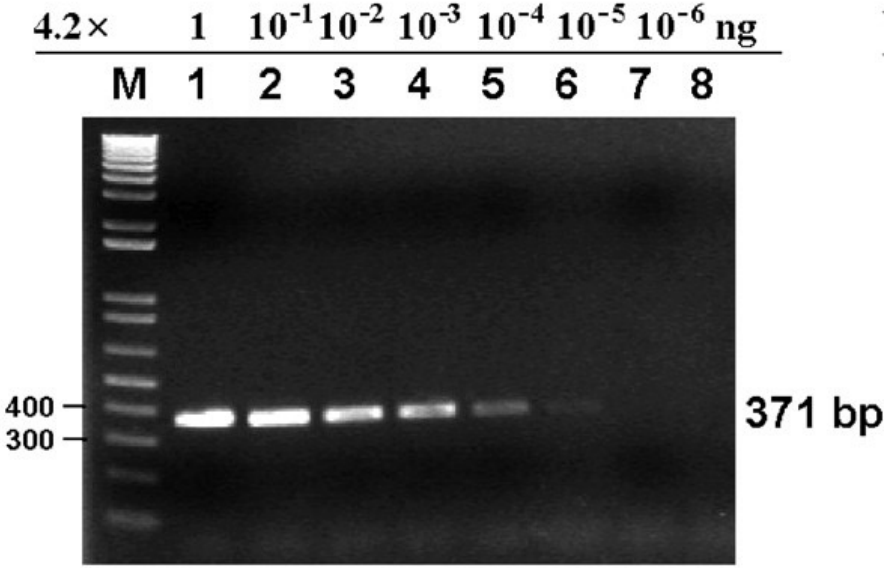
Bands can be compared against each other, and to known size-standards, to determine the presence or absence of a specific amplification product.

TP2: Polymerase Chain Reaction (PCR)

Visualization of PCR products

- End point detection
- Low resolution





Applications

TP2: Polymerase Chain Reaction (PCR)

1. Diagnosis of pathogens	a) PCR b) Nested PCR c) Quantitative PCR d) Multiplex PCR e) Differential on-line and real time PCR
2. Typing genetic markers	a) RFLPs b) AFLPs c) Short tandem repeat polymorphisms
3. DNA template for	a) Genomic mutation screening intron-specific primers flanking exons b) RT-PCR cDNA used as templates for pairs of exon-specific primers to generate overlapping fragments
4. Detecting point mutations	a) Restriction site polymorphisms b) Allele specific amplification
5. cDNA cloning	a) DOP-PCR b) RACE
6. Genomic DNA cloning	New members of a DNA family a) DOP-PCR. Whole genome or subgenomic amplification b) DOP-PCR c) Linker-primed PCR
7. Genome walking	a) Inverse PCR b) Bubble linker (vectored) PCR c) IRE-PCR
8. DNA templates for DNA sequencing	a) ssDNA by asymmetric PCR b) dsDNA for direct sequencing or for cloning followed by sequencing
9. *In vitro* mutagenesis	a) 5' add-on mutagenesis to create a recombinant PCR product b) Mispaired primers to change a single predetermined nucleotide

TP2: Polymerase Chain Reaction (PCR)

- Amplification of specific DNA fragments to clone, or to isolate a specific clone (gene discovery)
- Distinction between alleles
- Detection of polymorphisms, Molecular Typing, Taxonomy, Forensics, Molecular Diagnosis
- Phylogenetic studies
- Direct isolation of a specific segment of DNA of different origins (genomic, plasmidic etc)
- Preparation of probes
- Tagging of genes by insertion mutations, site-directed mutagenesis
- Cloned DNA molecules are sequenced rapidly by new methods based on PCR

TP2: Polymerase Chain Reaction (PCR)