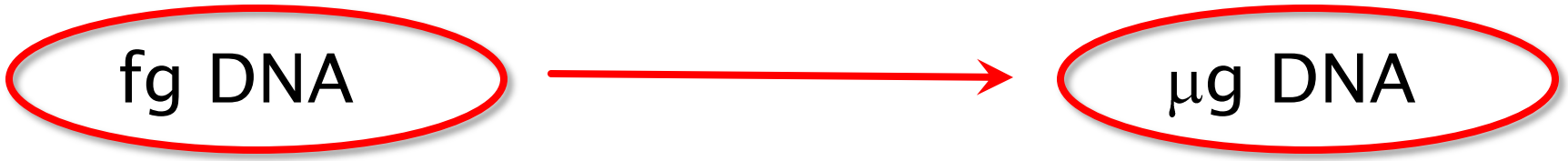
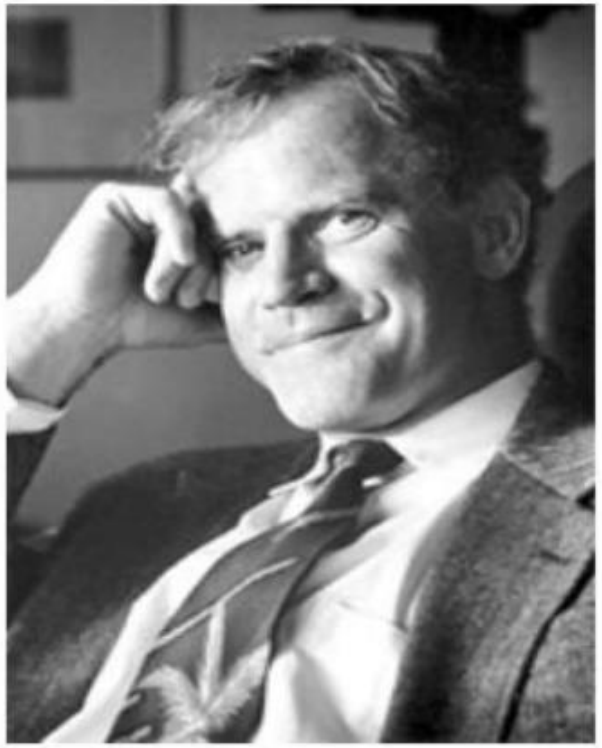
Polymerase Chain Reaction

= multiple rounds of in vitro DNA replication

= a region of DNA lying between two regions of known sequence is amplified hundreds of millions of time within a matter of several hours.



Nobel Prize in chemistry 1993:

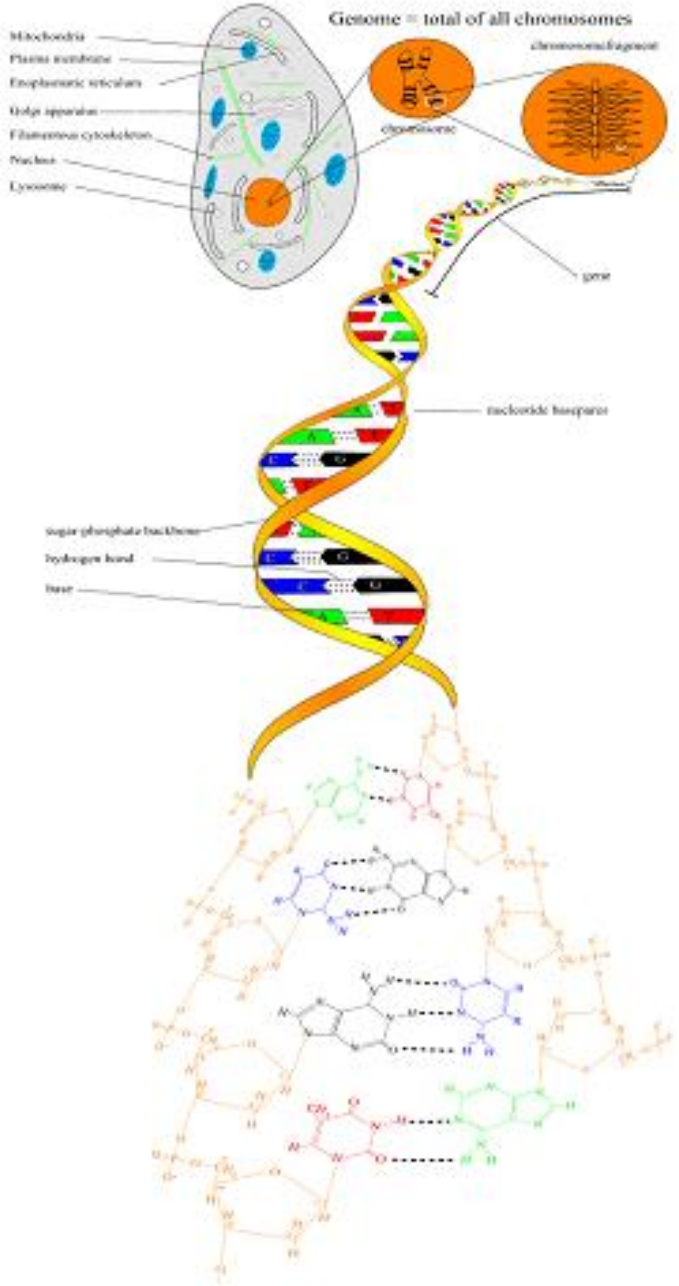


Kary B. Mullis

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"*.

Source: http://www.nobelprize.org/nobel_prizes/chemistry/laureates/1993/



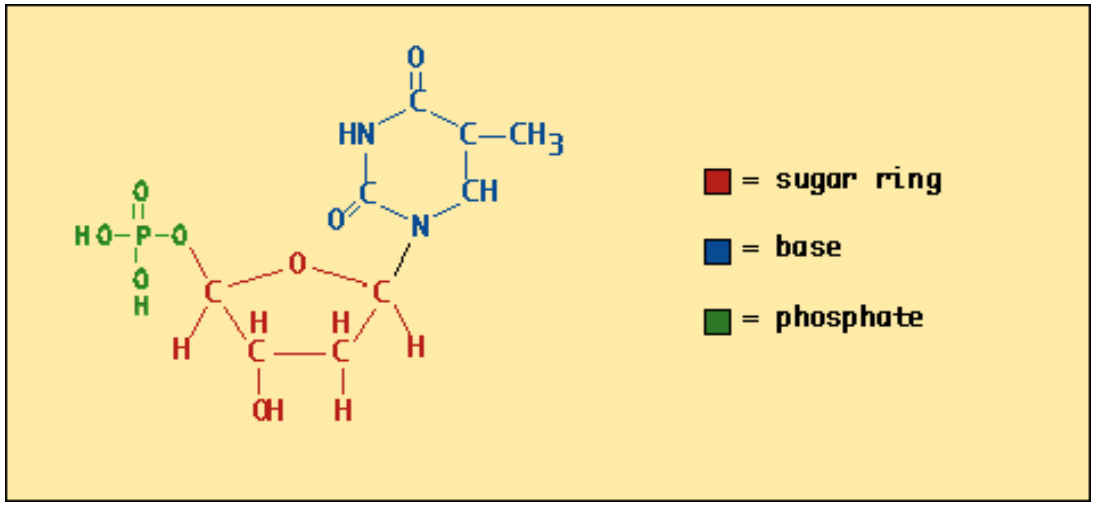
DNA (deoxiribonucleic acid)

- In the nucleus of almost every cell
- Equal amounts in every cell
- Double-stranded: two complementary strands, which form the basis of genetic information coding = genes
- Tightly packed
- During cell replication: condensed into chromosomes

Figure: Andy Vierstraete, 1999

The primary structure of the DNA

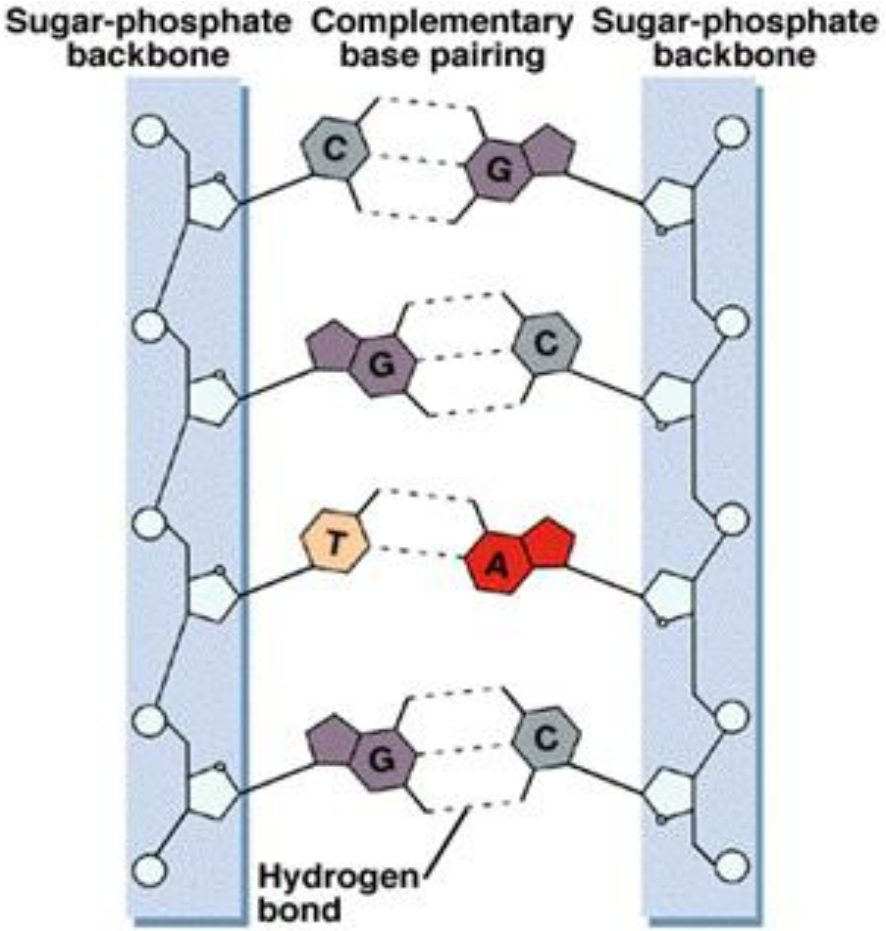
- Nucleotides:



Sugar ring: deoxyribose

Base:

- purine: adenine (A), guanine (G)
- pyrimidine: cytosine (C), thymine (T)

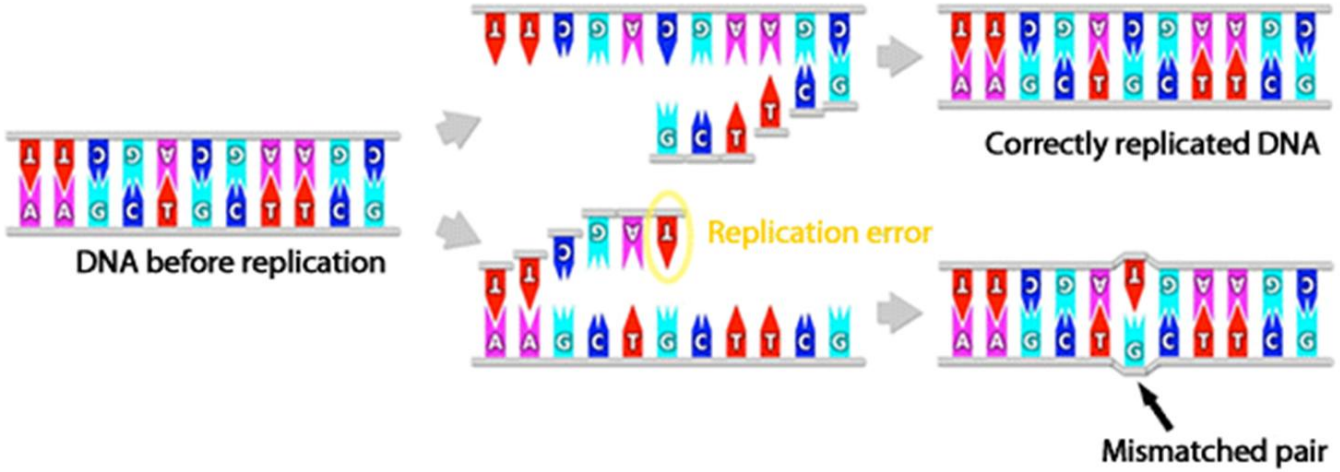


Strict pairing rules:

Purine with pyrimidine:

A = T

G ≡ C



Wrong pairing: mismatch

Transmission of genetic information

3 processes:

PCR

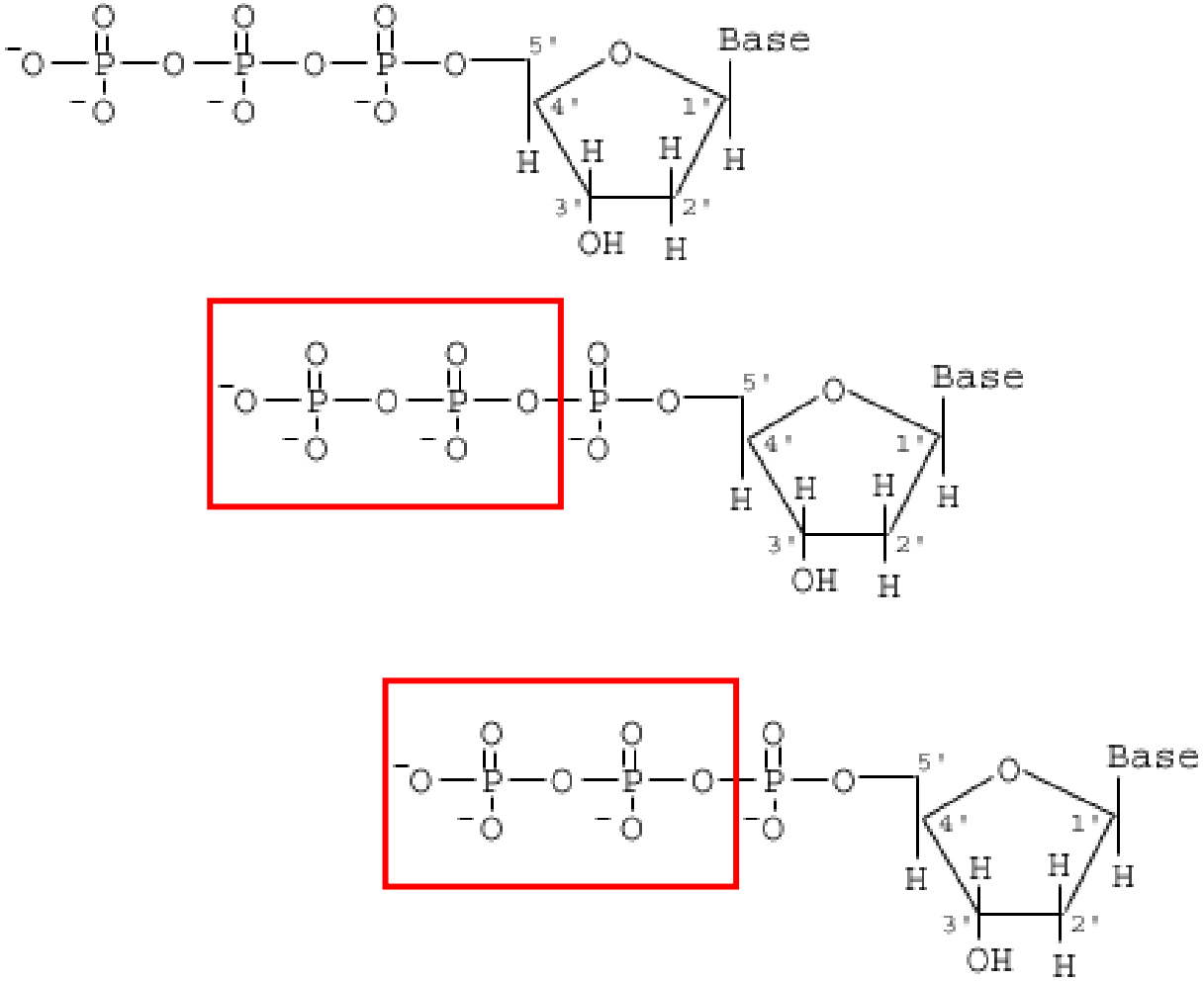
1. replication: dup (whole or part of it)

2. transcription: copy of genetic information contained in a gene into a single stranded RNA

3. translation: copy of information from a particular RNA into proteins

Overview of the DNA replication

Concept of 5’ and 3’ end:



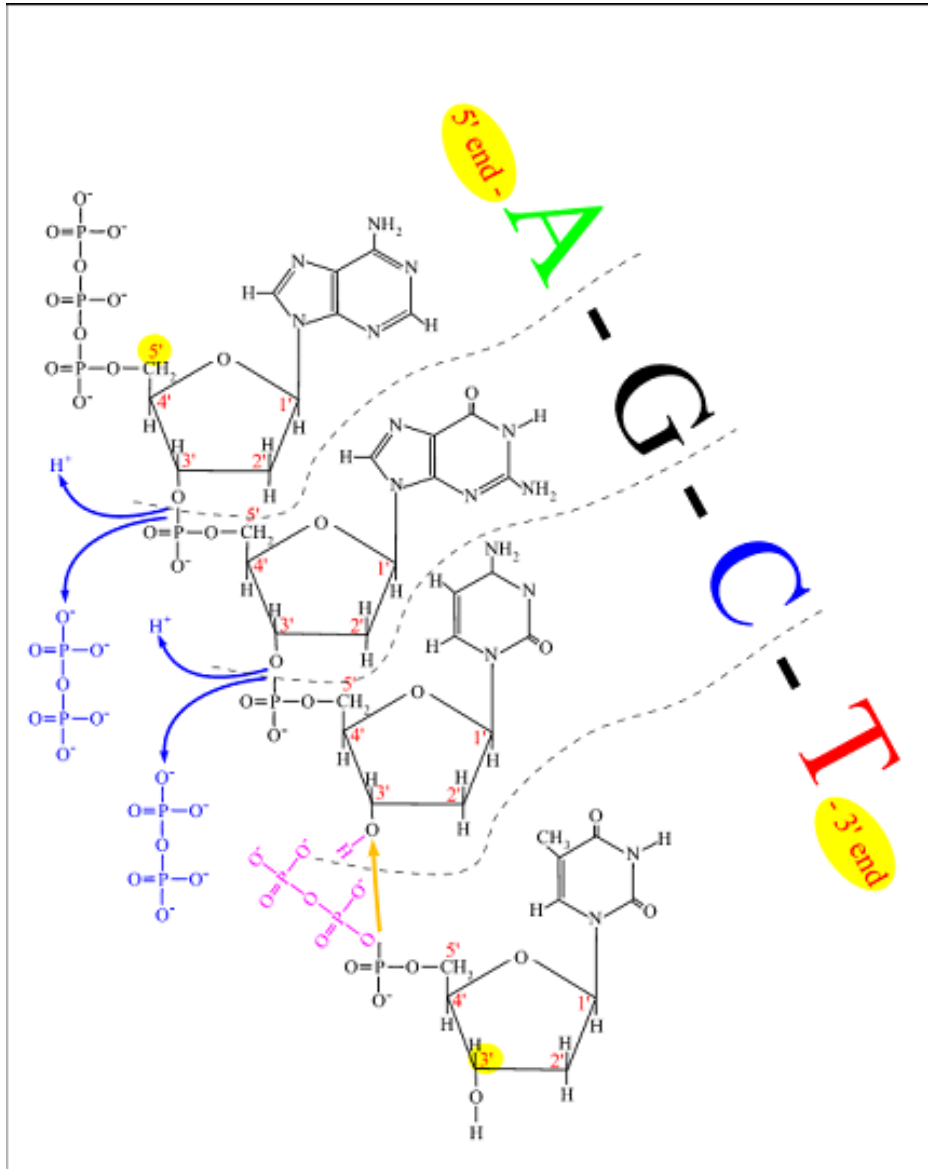
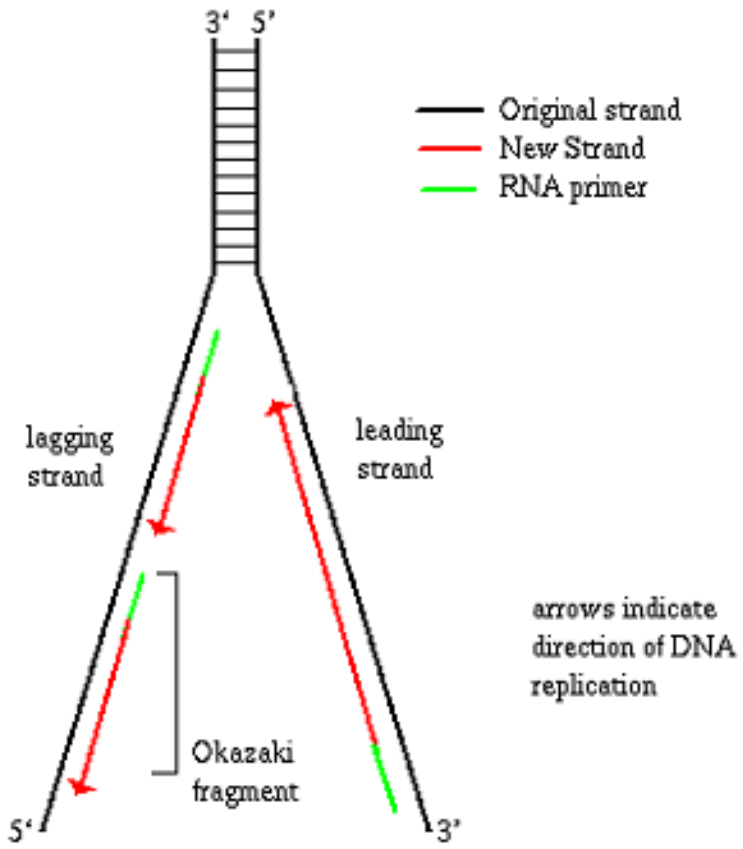


Figure: Andy Vierstraete, 1999

Overview of the DNA replication



1. Unwinding of the double helix and nicking one strand of the DNA
   (topoisomerases and helicases)

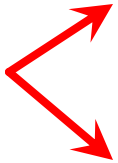
2. Attachment of RNA primer

3. Duplicating the DNA strand nucleotide by nucleotide by the DNA polymerase III

Obligate direction of synthesis: 5' to 3'

Components of a PCR reaction

Target:

DNA 

genomic

cDNA (reverse transcribed from RNA = RT-PCR)

Primers (1 pair: forward and reverse)

Thermostable DNA polymerase

dNTPs (dATP, dCTP, dGTP, dTTT in equal amounts)

Mg^{2+} (cofactor for DNA polymerase)

Main steps of a PCR reaction

1. Denaturation
2. Annealing
3. Extension

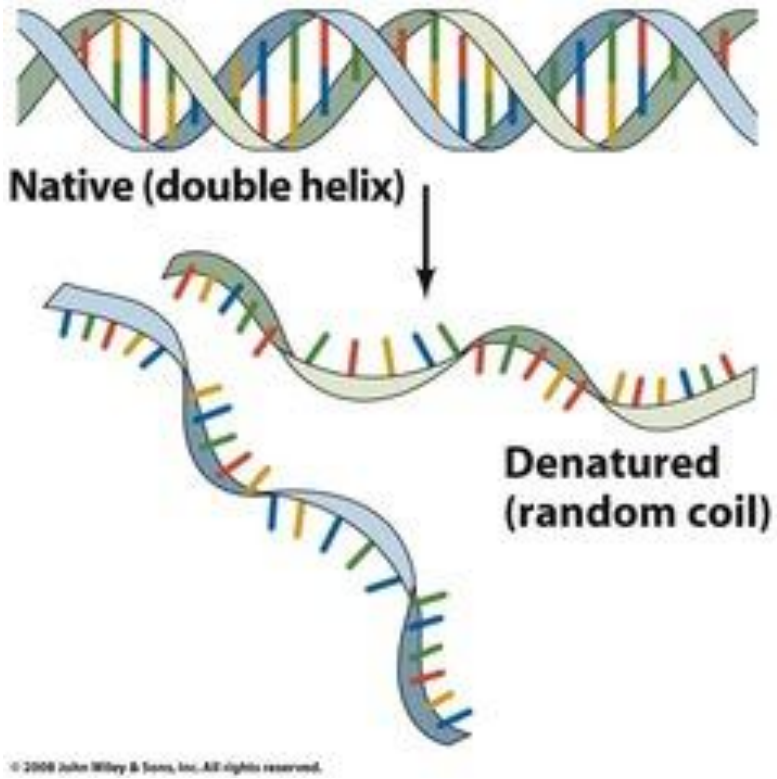
} 1 cycle

1 PCR reaction: multiple repetitive cycles

1. Denaturation

= „melting” of double-stranded DNA at high temperature to convert it into single-stranded DNA

Complete denaturation: at approx. 94 C



T_m = temperature when half of the dsDNA moleules are denatured, ie transformed into ssDNA

Factors influencing T_m:

- G-C content of DNA fragment

The higher the G-C content of a DNA segment, the higher the temperature needed for complete denaturation

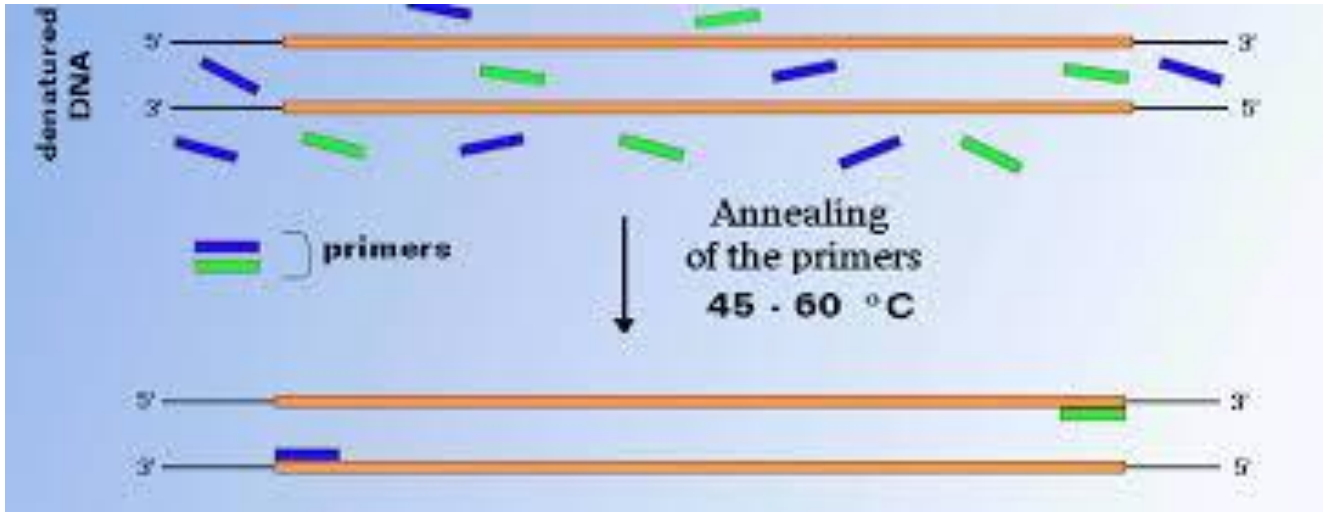
- Type of solvent, salt concentration, pH

Organic solvents (formaldehyde) + low salt concentration + high pH decrease T_m

2. Annealing (= primer annealing)

= hybridisation of the two oligonuleotides, used as primers to the DNA template

Temp: 50-65 C

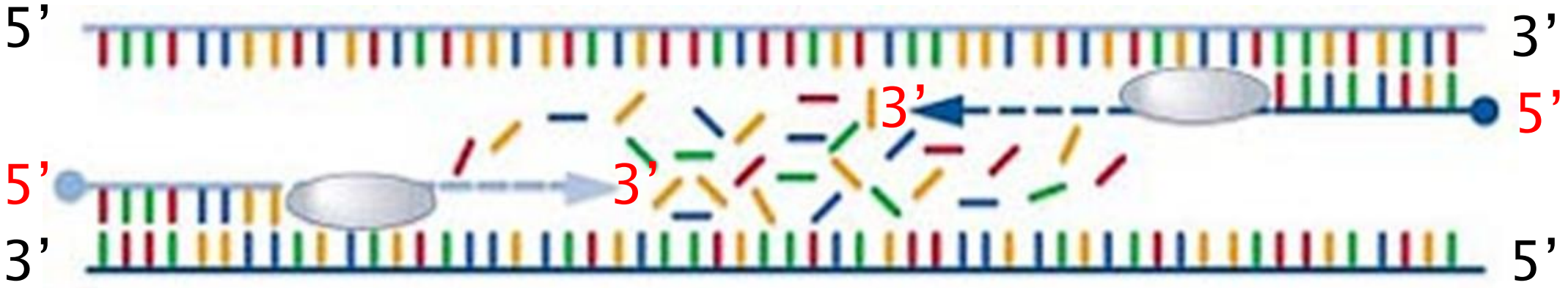


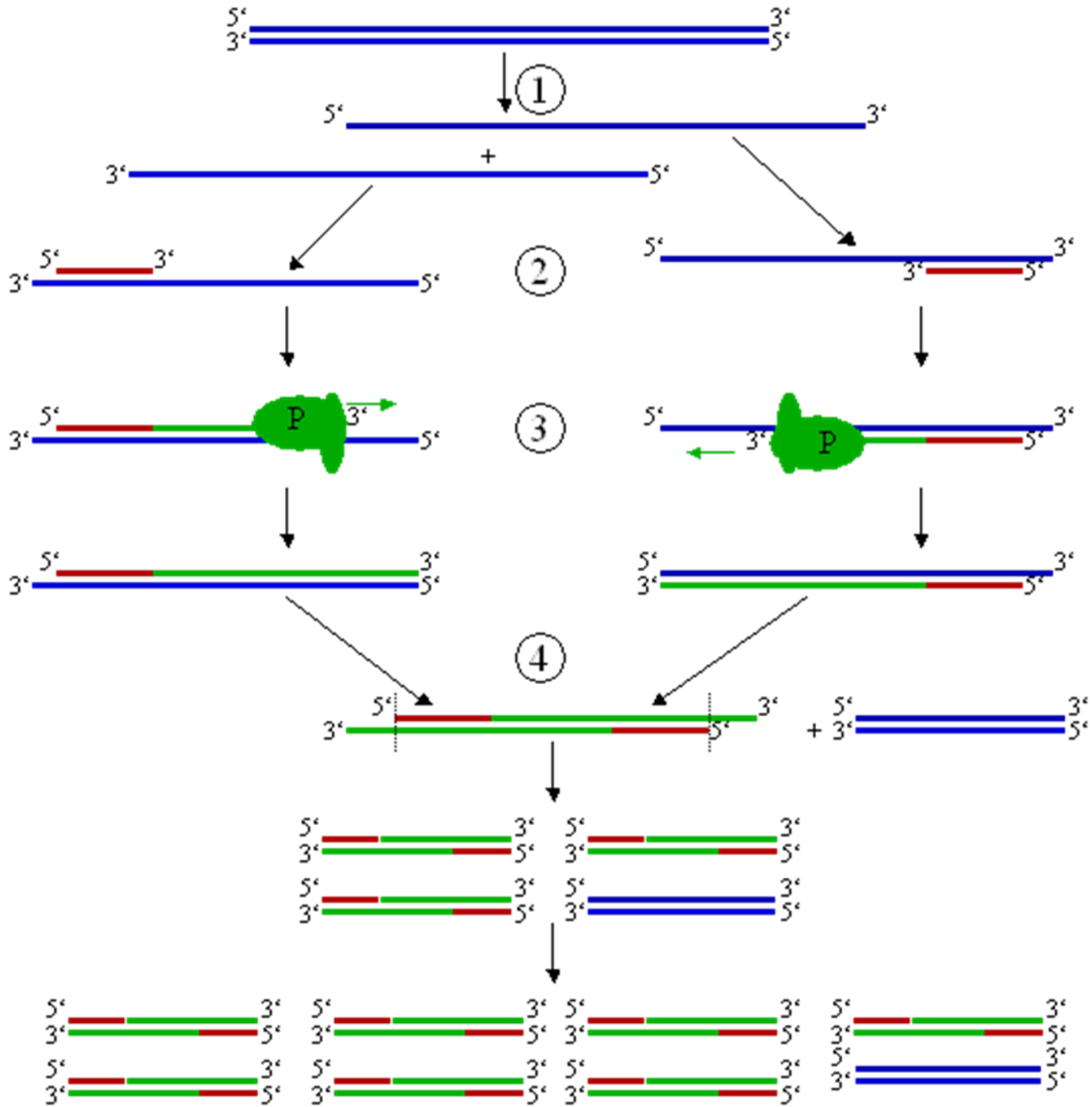
3. Extension (= primer extension)

= extension of the primers across the target DNA sequence by using a heat-stable DNA polymerase in the presence of dNTPs resulting in a duplication of the starting target DNA

Optimal temp: approx. 72 C (for Taq DNA polymerase)

Optimal time: 1 min (depends on the length of DNA target




5'
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2
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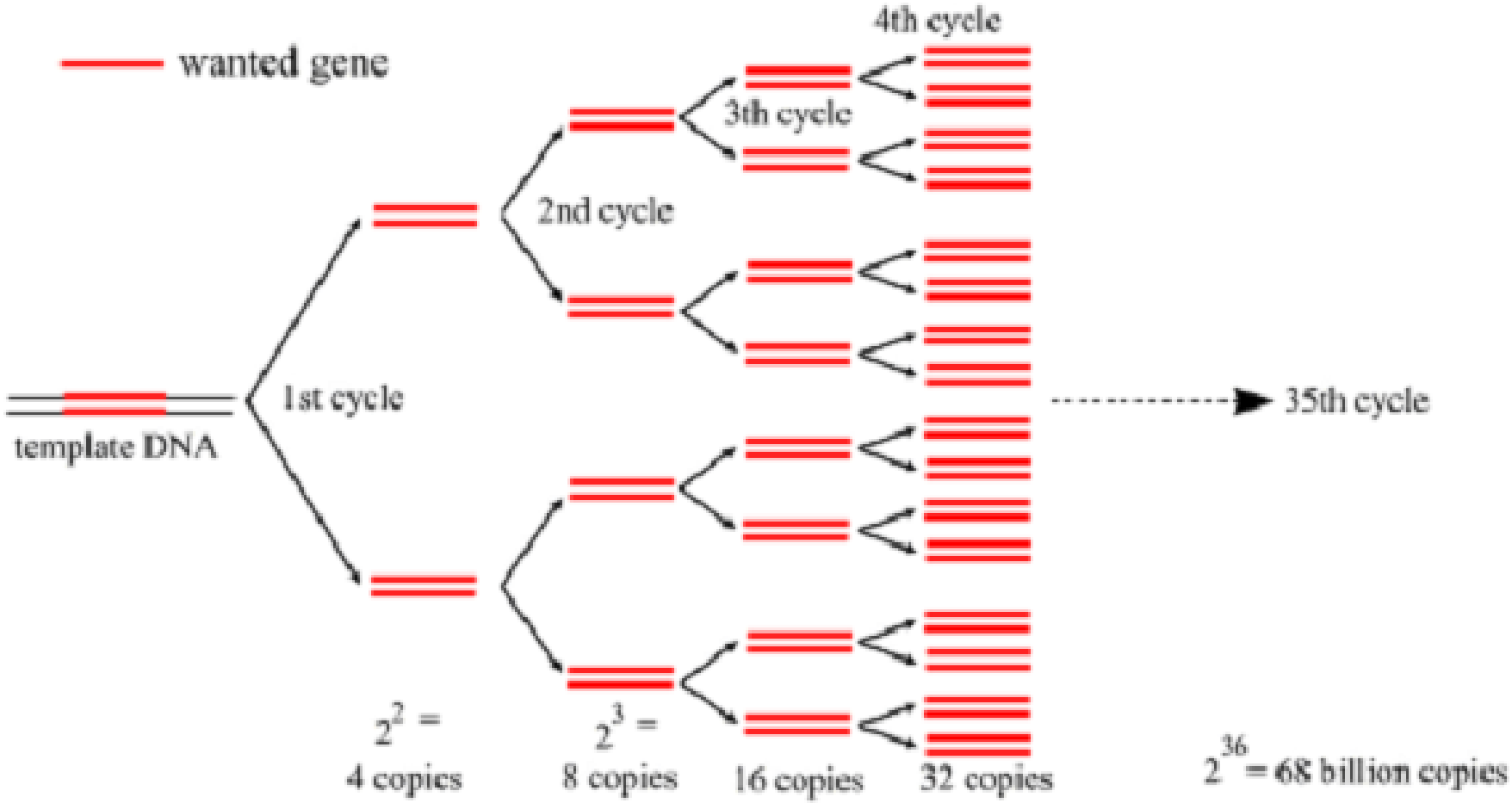
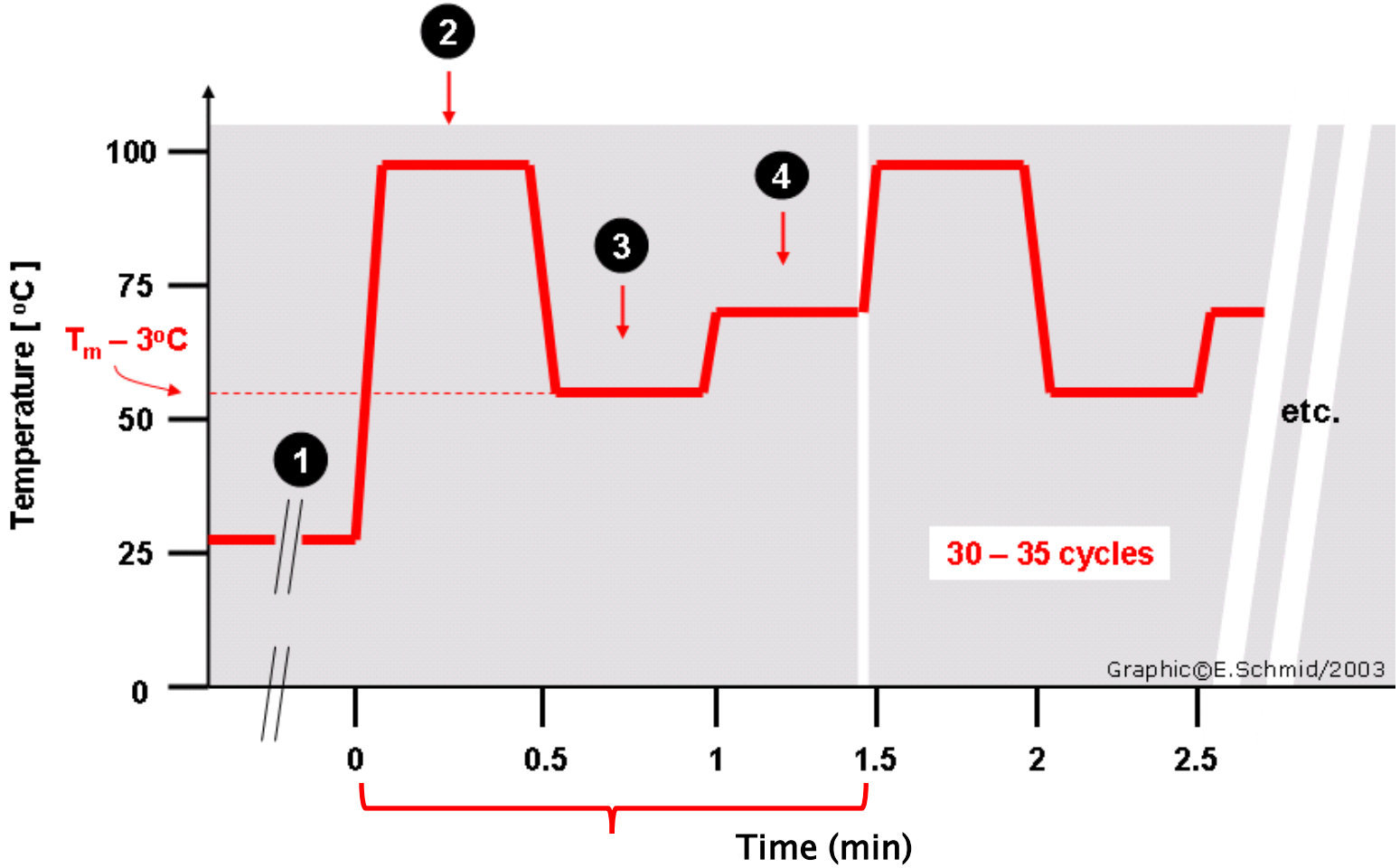


Final number of copies: $(2^n-2n)x$
n: the number of cycles
x: the number of copies of the original template

The Polymerase Chain Reaction (PCR)
M. Somma, M. Querci

Instrumentation:

Thermal cyclers = temperature baths, which could shift their temperatures up and down rapidly and in an automated, programmed manner



Target DNA

Amount:

- min 1 intact copy
- typical: 0.05 – 1 μg

Length:

- Below 0.1 kb up to few kb

Fators inhibiting target amplification:

- Damaged DNA template (nicks)
- Contaminants: detergents mg-chelating agents, heparin, formaline

Primers and primer design:

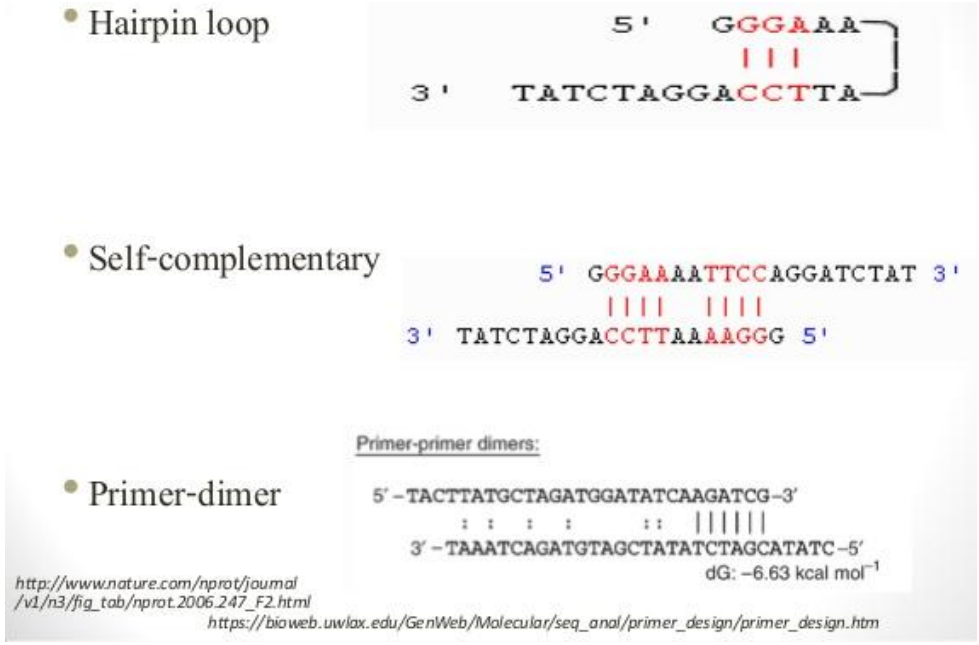
Length:

- 16-30 bp (to allow sufficintly high T_ms)

(Optimal 18.24 bp)

Structure:

- Avoid high G-C content
- Avoid repetitive sequences
- Avoid self comlementarity
- If possible, higher GC content at the 3' end
- Optimal conc: 1 μM (30 cycles)



Primers and primer design:

Optimal annealing temp:

- theoretically: 5 C lower than T_m
- practically: to be determined empirically (!gradient PCR)

T_m:

- Identical for the two primers
- $T_m = 2(A+T) + 4(G+C)$ (3)
  where *A*, *T*, *G*, *C* are the purinic and pyrimidinic bases.
- Several primer designing softwares are available

DNA polymerase:

- Heat-stable!!
- Native or cloned
- Varying half life (40 min to several hours)
- Incorporates nucleotides from the 3' end of a polynucleotice
- 3' – 5' or 5' – 3' exonuclease activity (proofreading!)
- Varying degree of fidelity:
  - Taq DNA pol: 1/10000 error rate
  - Pfu DNA pol: 1/1000000 error rate (high fidelity)
- Varying degree of efficiency (% of conversion of template to product/cycle)

Mg^{2+} and reaction buffer:

- Typical reaction buffer:
  - 10 mM Tris, pH 8.3
  - 50 mM KCl
  - 1.5–2.5 mM **$MgCl_2$** (0.5–5 mM – optimal conc to be empirically determined)

The role of Mg^{2+} is critical

- forms soluble complex with dNTPs (essential for dNTP incorporation)
- stimulates polymerase activity
- increases the T_m of primer/template (stabilize the duplex)

Low Mg: no/low product yield

High Mg: non-specific products (mispriming)

Mg^{2+} and reaction buffer:

Avoid:

- chelating agents (EDTA)
- negative ions (phosphates)

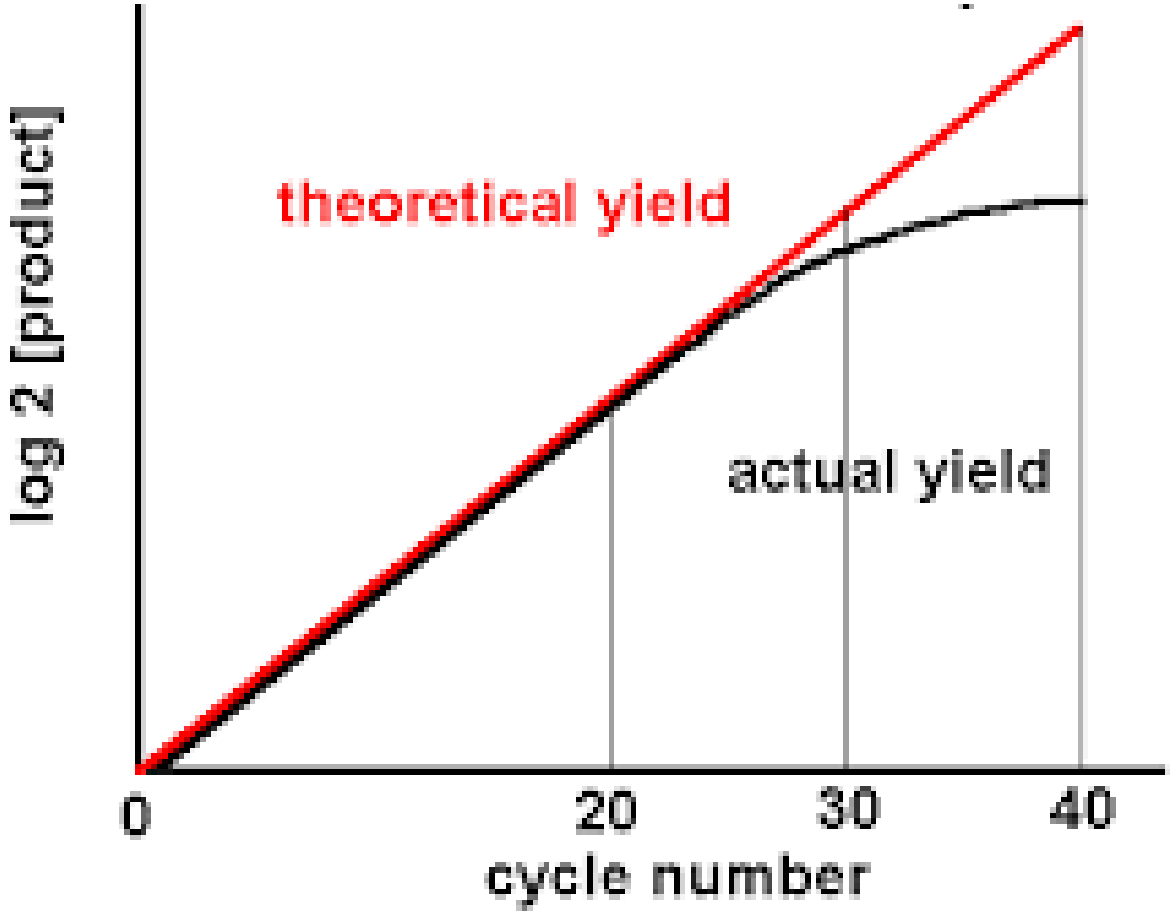
Addition of specific substances to increase specificity and efficiency (for certain polymerases only):

- DMSO
- PEG
- formamide
- glycerol
- detergents, …

dNTPs:

- Typical conc:
  - 2-200 μM for each dNTP
  - used in equivalent conc.
  - usual stock: 1 mM
  - optimal pH: 7-7.5

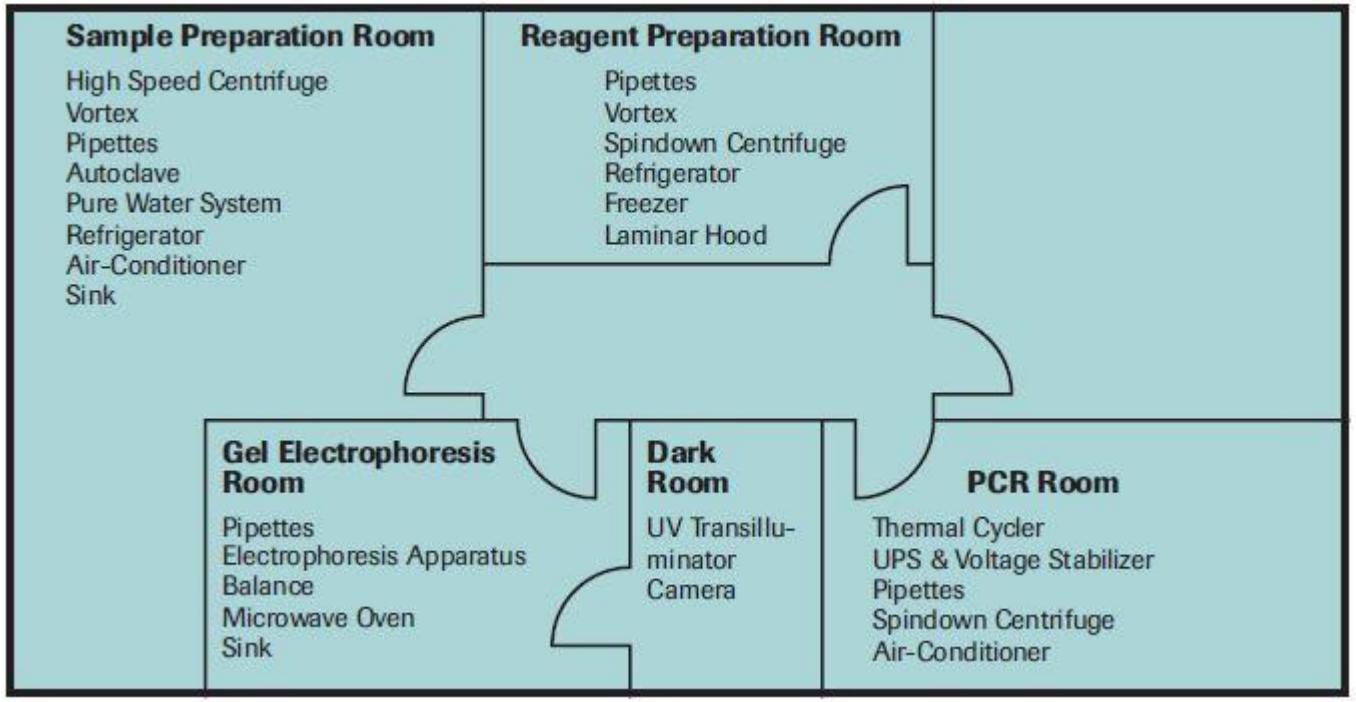
The plateau effect:



Design of a PCR laboratory:

Most critical to perform PCR amplification in a DNA-free environment!!

- Physically separate working areas with dedicated equipment
- Strict compliance with decontamination requirements



The mastermix:

= all reagents (except the template DNA) are mixed in a single tube, in enough volume according to the number of reactions to be performed

This is then aliquotted into tubes and DNA template added.

- Reduces the risk of contamination
- Improves PCR performance

Controls:

Control	Method
Contamination of the reagents with the target DNA	PCR without DNA template (only mastermix) negative control
Specificity of the reaction	Controls to find secondary and non-specific products
Development and sensitivity of the reaction	Positive/negative controls to verify that the desired conditions and yields are fulfilled
Integrity of the PCR mixture	PCR with a DNA positive control

Thank you for your attention!