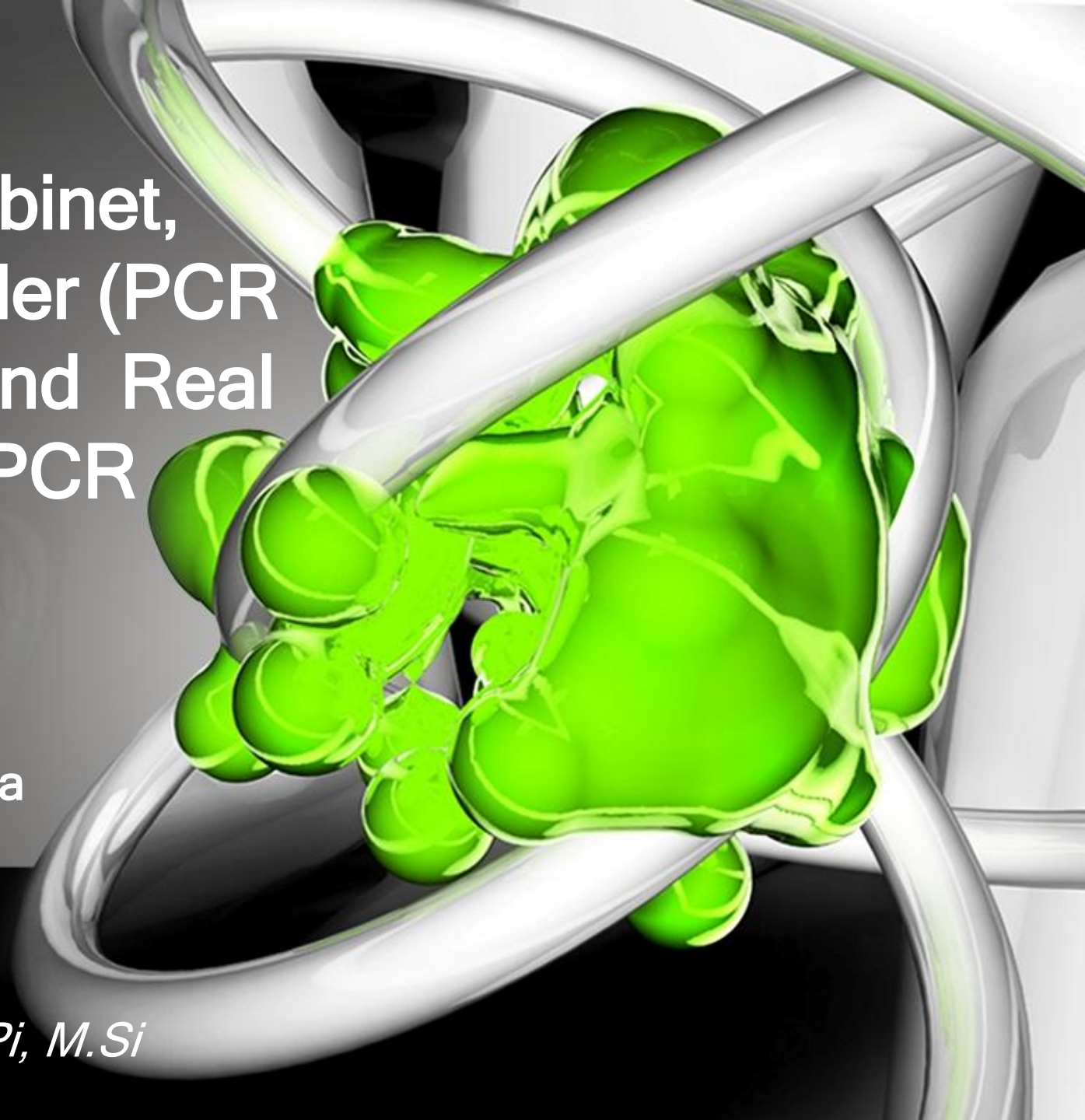
PCR Cabinet, Thermocycler (PCR Mechine) and Real Time -PCR

Meet 6,
Instrumentasi
Bioteknologi
Universitas Esa
Unggul



By: Seprianto, S.Pi, M.Si

Structure of DNA

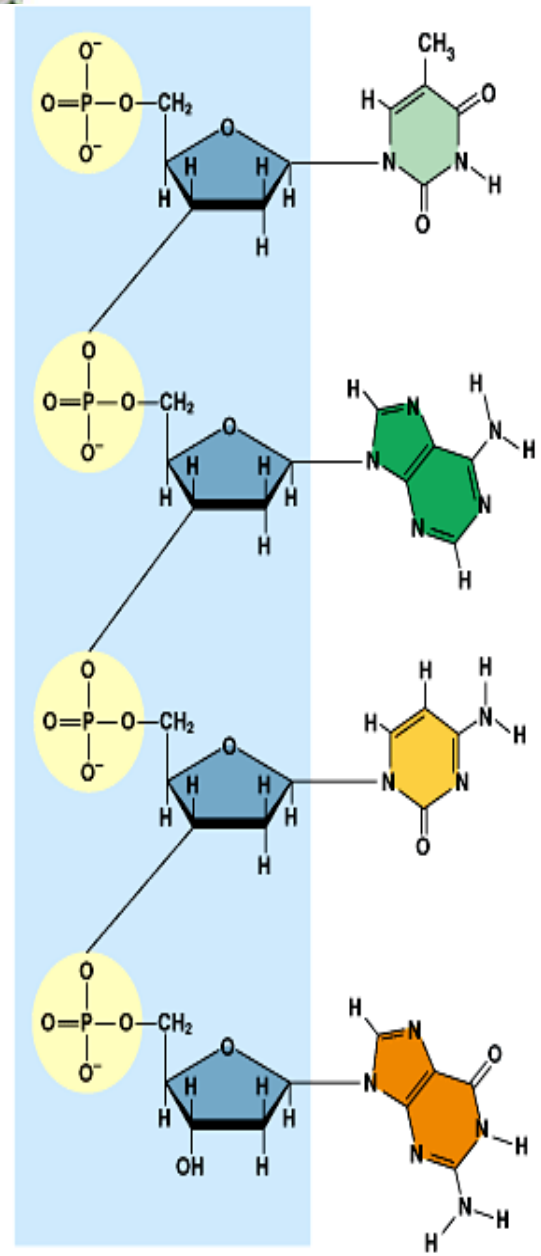
- Double Stranded Helix
- Strands run Anti-Parallel
- Strands Held Together by H-Bonds
- Bases Read 5′ to 3′
- Strands have Complementary Sequence

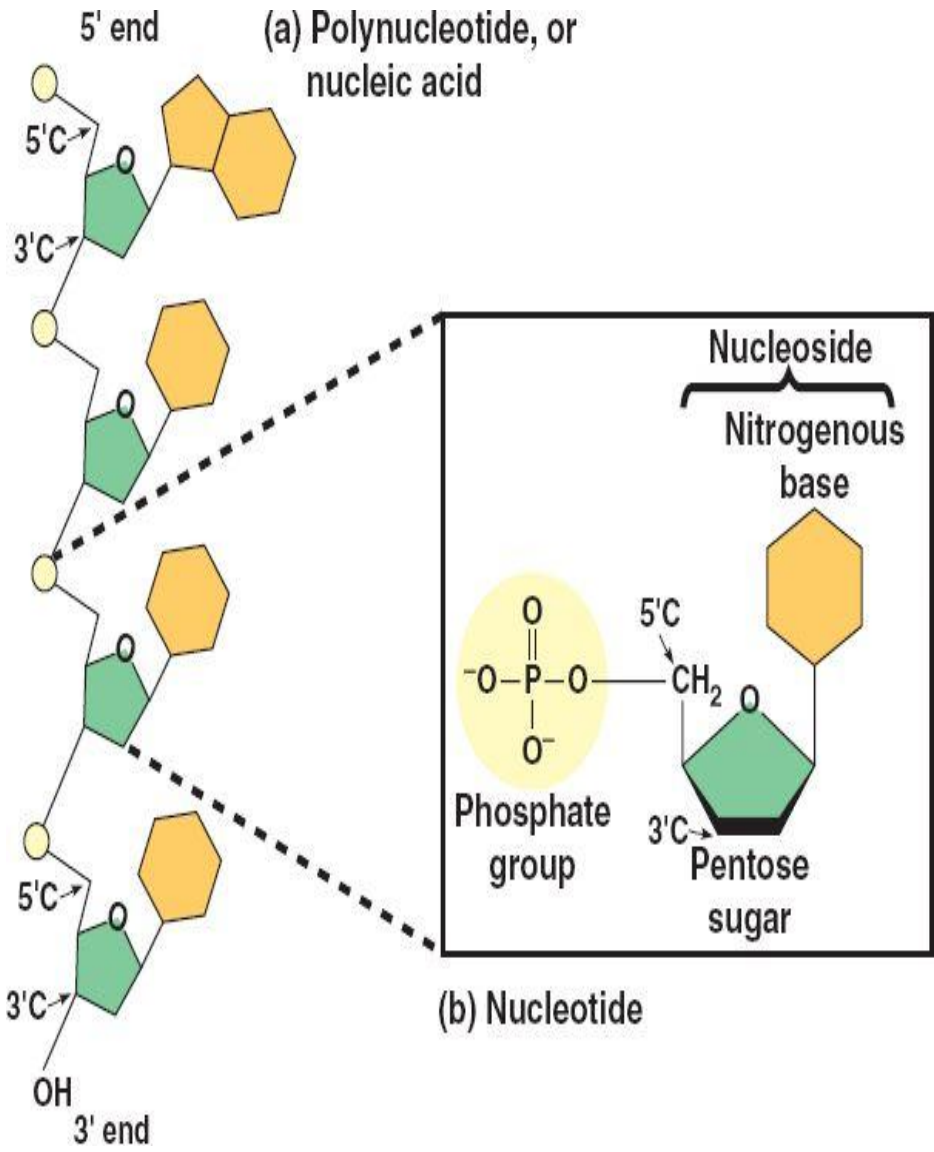
Composition of DNA

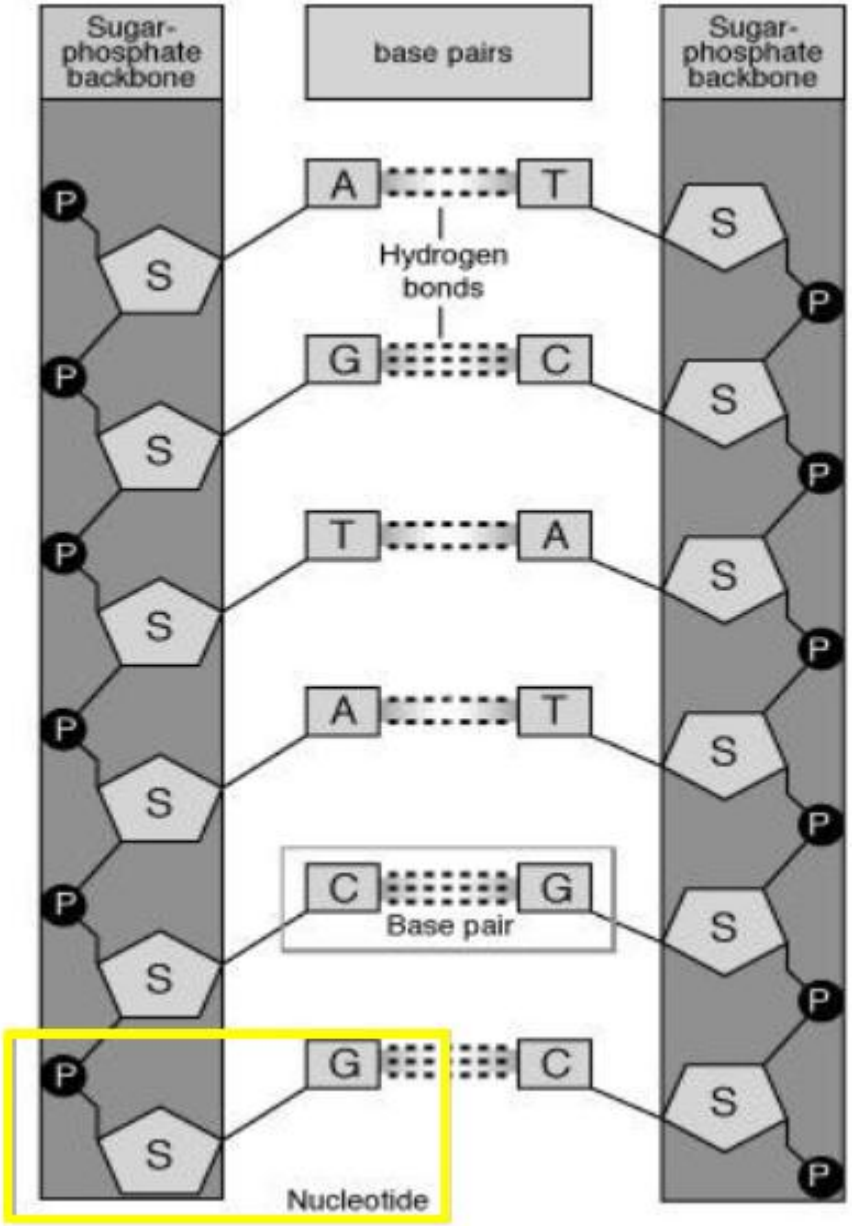
- Consists of 4 Nucleotides
- Each Nucleotide Contains a Phosphate, a sugar and a nitrogenous base.
- The sugar is deoxyribose (ribose in RNA)
- The 4 bases are **A**denine, **C**ytosine, **G**uanine and **T**hymine (**U**racil in RNA)

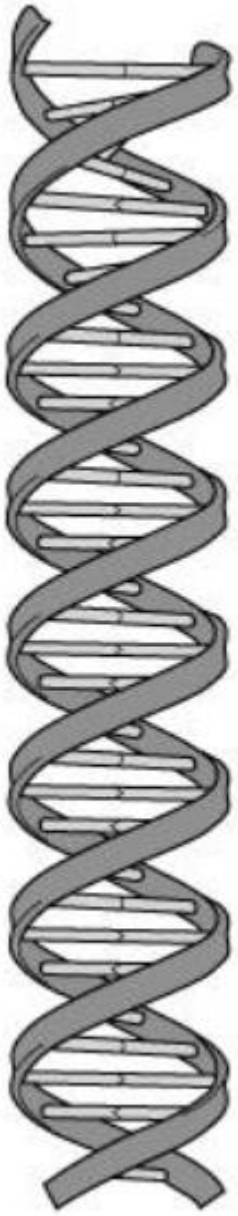
Composition of DNA

- A & G are purines
- C & T are pyrimidines
- [purines] = [pyrimidines]
- [A] = [T] ; [C] = [G]
- A/T base pairs have 2 H-bonds
- C/G base pairs have 3 H-bonds




5' end
(a) Polynucleotide, or nucleic acid
5'C
3'C
Nucleoside
Nitrogenous base
Phosphate group
Pentose sugar
(b) Nucleotide
OH
3' end


Sugar-phosphate backbone
base pairs
Sugar-phosphate backbone
A
T
Hydrogen bonds
G
C
T
A
A
T
C
G
Base pair
G
C
P
S
Nucleotide



Physical Characteristics of DNA

- DNA is water soluble
- DNA precipitates in alcohols
- DNA absorbs UV light at 260 nm
- DNA subject to shear forces
- DNA has characteristic melting & annealing temperatures
- DNA carries a net negative charge

Melting point

- Tm = T at which strands separate
- All DNA has a characteristic Tm
- ↑ duplex length ↑ Tm
- ↑ GC content ↑ Tm
- Salt concentration affects Tm
- Tm can be calculated from sequence

Importance of Tm

- Information on DNA
- Primer Design
- Hybridization Temperature
- PCR application

Making DNA without a cell

Techniques of DNA Amplification (PCR)

- **PCR**: Polymerase Chain Reaction
- **Purpose of PCR**: to make large number of copies of a piece of DNA (amplification = exponential)
- The PCR process was invented by Dr. Kary Mullis, while working at Cetus in 1983.
- Dr. Mullis was given the Nobel Prize in Chemistry in 1993 for his contribution to science.

Dr. Kary Mullis

Making DNA without a cell

We need all the things a cell has:

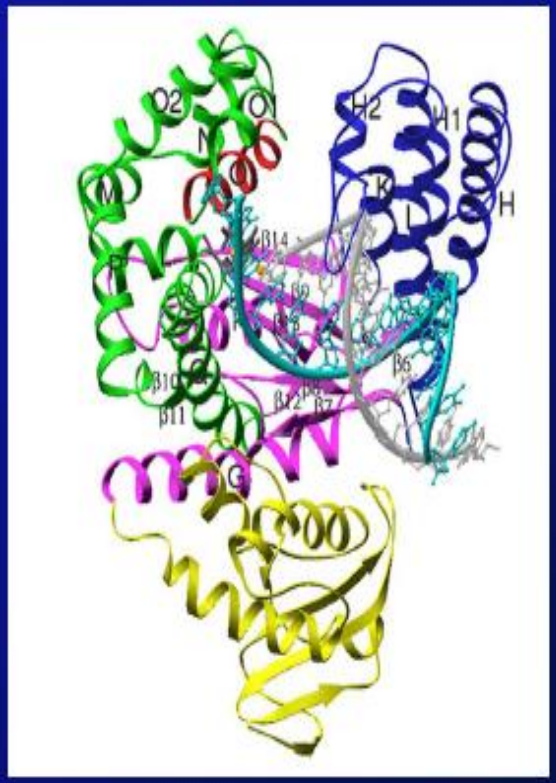
- template DNA
- deoxynucleotide triphosphates (dATP, dTTP, dCTP, dGTP)
- DNA polymerase
- ATP (energy)

And some things not needed by cells

- primers - small single stranded pieces of DNA

Taq polymerase

- The key to PCR is a unique thermo-stable DNA polymerase
- *Taq* DNA polymerase was derived from a thermophilic ("heat loving") bacteria called *Thermus aquaticus*.
- Polymerizes DNA at 72°C
- Remains functional after short incubations at 95°C.



Thermocycler (Mesin PCR)

- *Thermocyclers*, or thermal cyclers, are instruments used to amplify DNA and RNA samples by the polymerase chain reaction
- The thermocycler raises and lowers the temperature of the samples in a holding block in discrete, pre-programmed steps, allowing for denaturation and reannealing of samples with various reagents

Thermocycler (Mesin PCR)

Amplified genetic material can be used

- ✓ Cloning
- ✓ Sequencing
- ✓ Expression analysis
- ✓ Food Phatogen detection,
- ✓ And genotyping

Thermocycler (Mesin PCR)

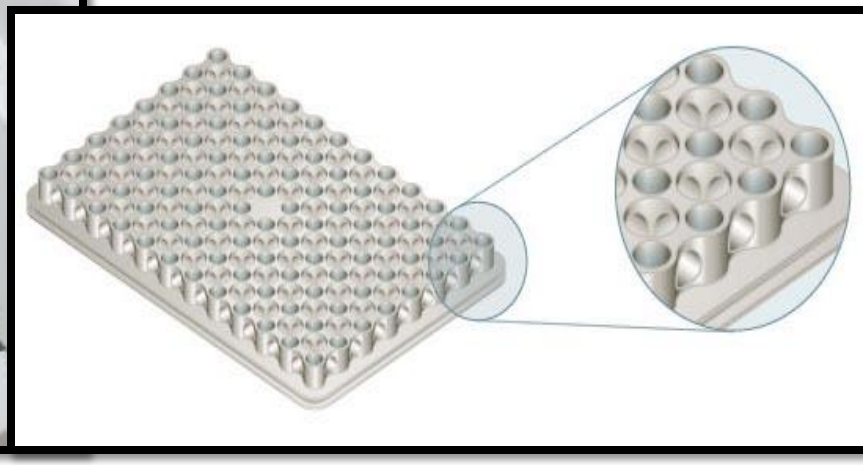
Component the Thermocyler

- ✓ Heater lid (pintu penutup)
- ✓ Well Block (holding block)
- ✓ Power Supply
- ✓ Control Program

Thermocycler (Mesin PCR)

Well Block

48 and 96 well block
Material : Peltier



Thermocycler (Mesin PCR)

PCR reaction tubes

- Material Plastic polypropelene
- Volume : 200 ul
- DNAse and RNAse free
- Disposible

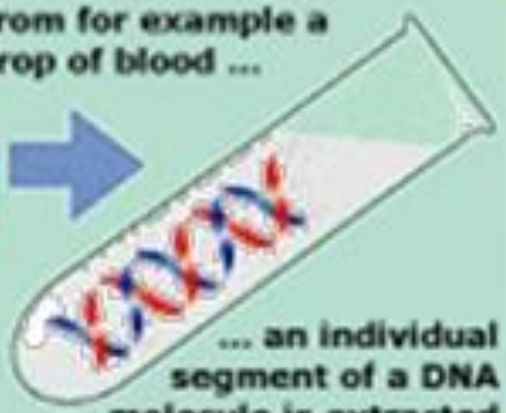
Mesin Thermal Cycler

Mesin Thermal Cycler Generasi I

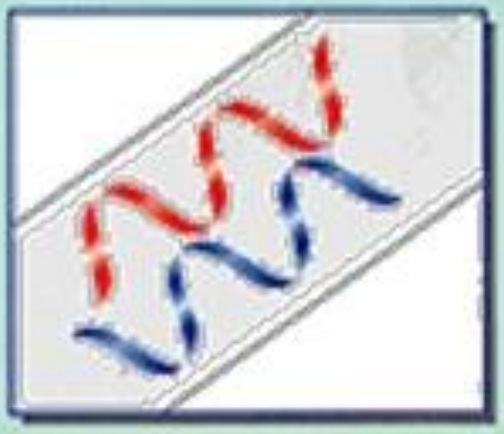
Mesin Thermal Cycler Generasi Terbaru

Mesin Real Time PCR

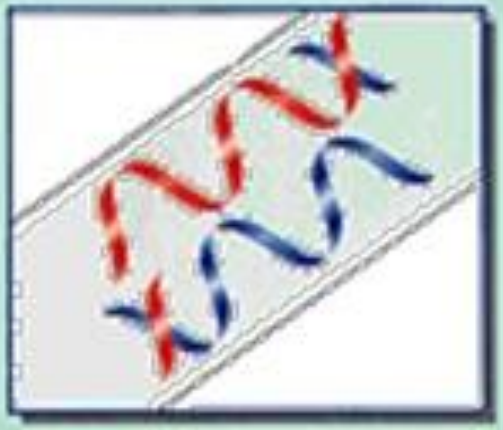
From for example a drop of blood ...



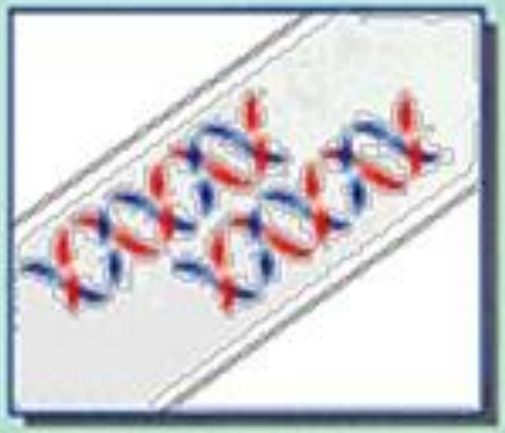
... an individual segment of a DNA molecule is extracted



By raising the temperature to about 90°C the strands are separated.



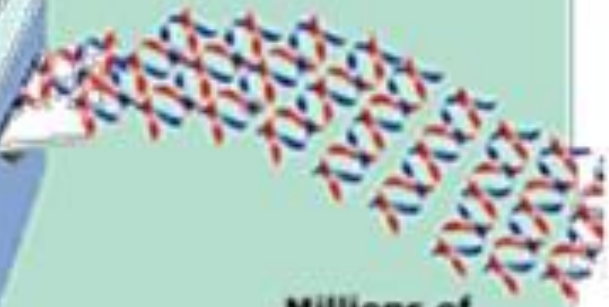
The temperature is lowered about 55°C and synthetic DNA framgents are added. These bind to the strands at the correct positions.



The temperature is now raised to about 70°C and the enzyme DNA polymerase which is added builds up two new complete copies of the DNA strands.

By cycling through the three temperatures the strands are separated and built up again.

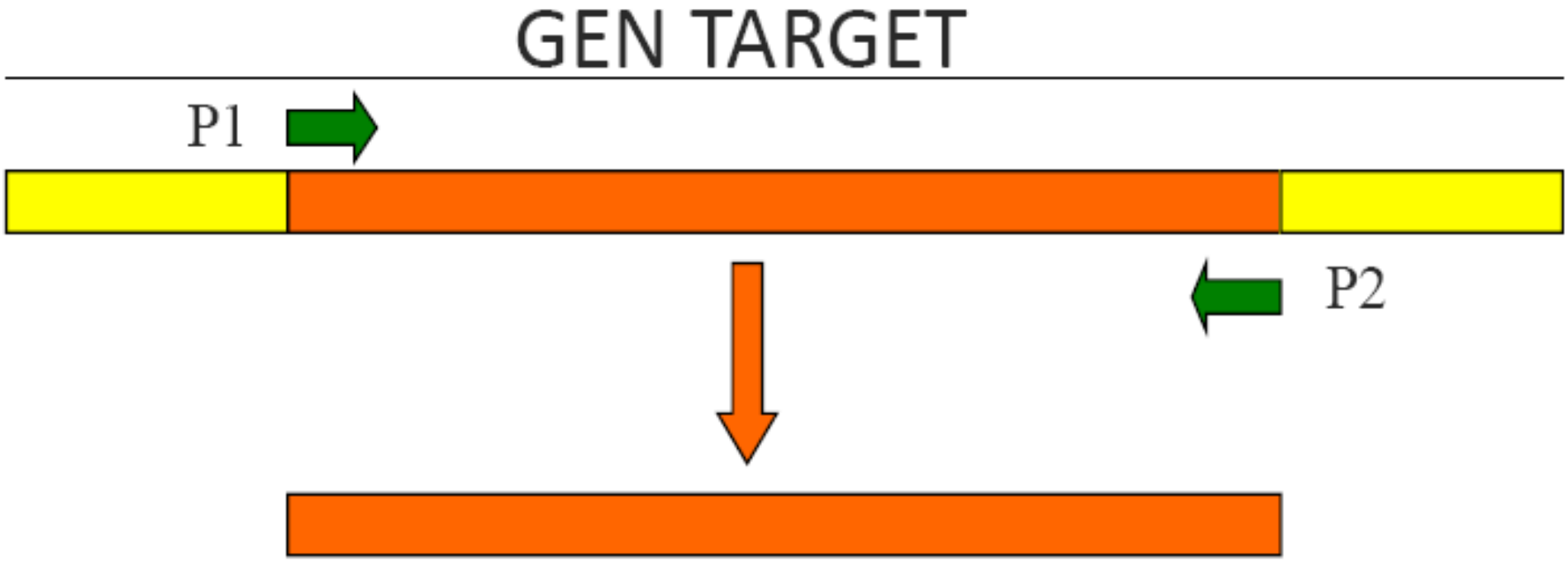
The whole process works like a copying machine.



Millions of copies an hour ...

Source:.gif.pcr/illpres/1993/laureates/chemistry/nobel_prizes/org.nobelprize//:Source: http

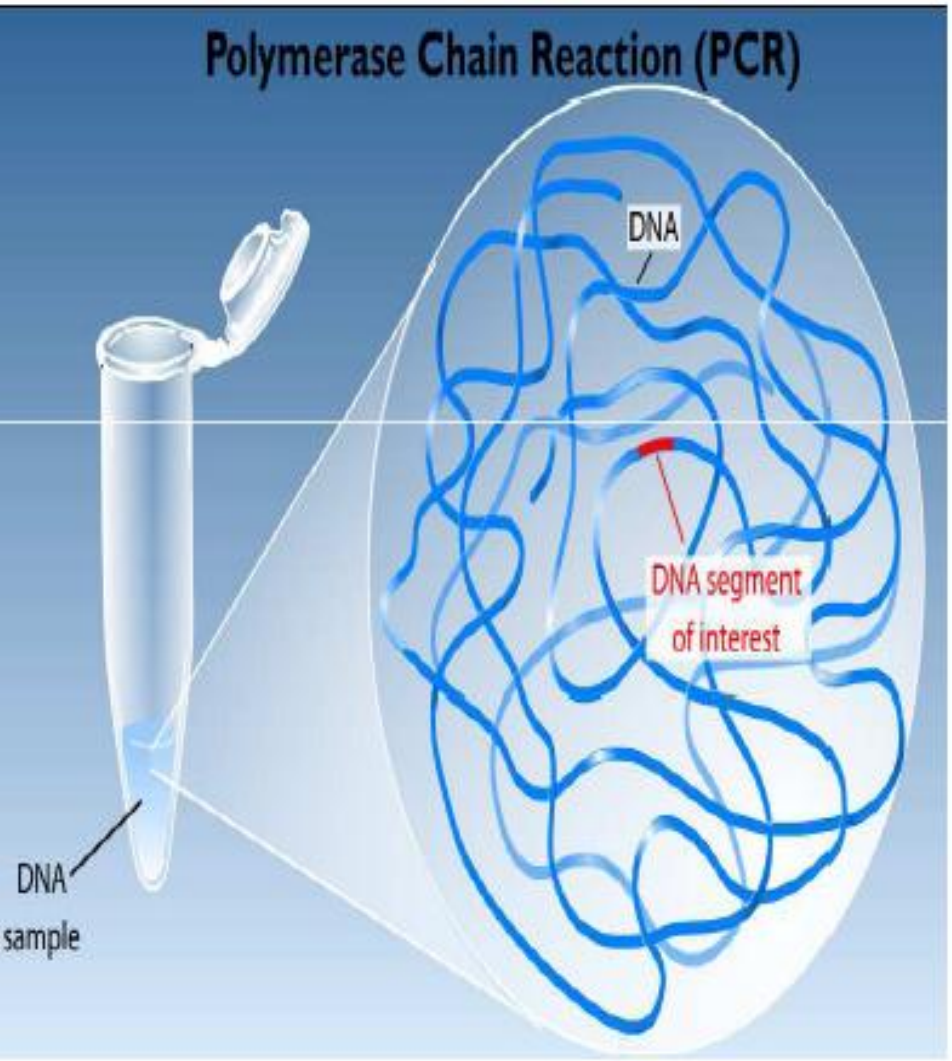
PCR

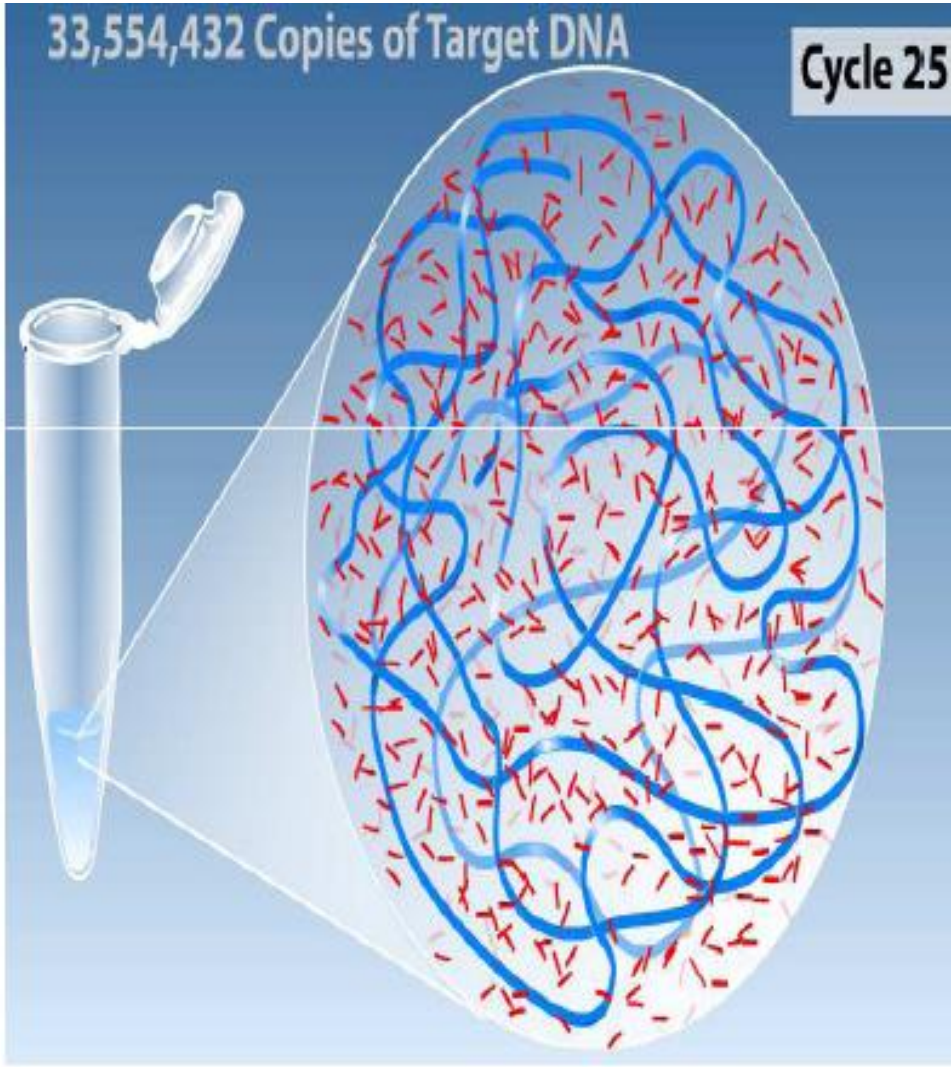


PRODUK PCR: 490 pb

TARGET HARUS SPESIFIK PATOGEN

Amplifikasi DNA Target dalam Genom Organisme





Prinsip PCR

- Merupakan metode untuk amplifikasi potongan DNA secara in vitro pada daerah spesifik yang dibatasi oleh dua buah primer oligonukleotida.
- Mampu memperbanyak sebuah urutan 105-106 kali lipat dari jumlah nanogram dari DNA template.
- Proses ini mirip dengan proses replikasi DNA secara in vivo yang bersifat semi konservatif.

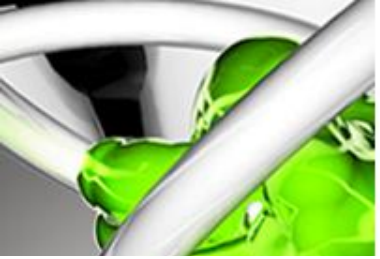
Prinsip PCR

- Pada setiap n siklus PCR sempurna akan diperoleh 2^n DNA target.

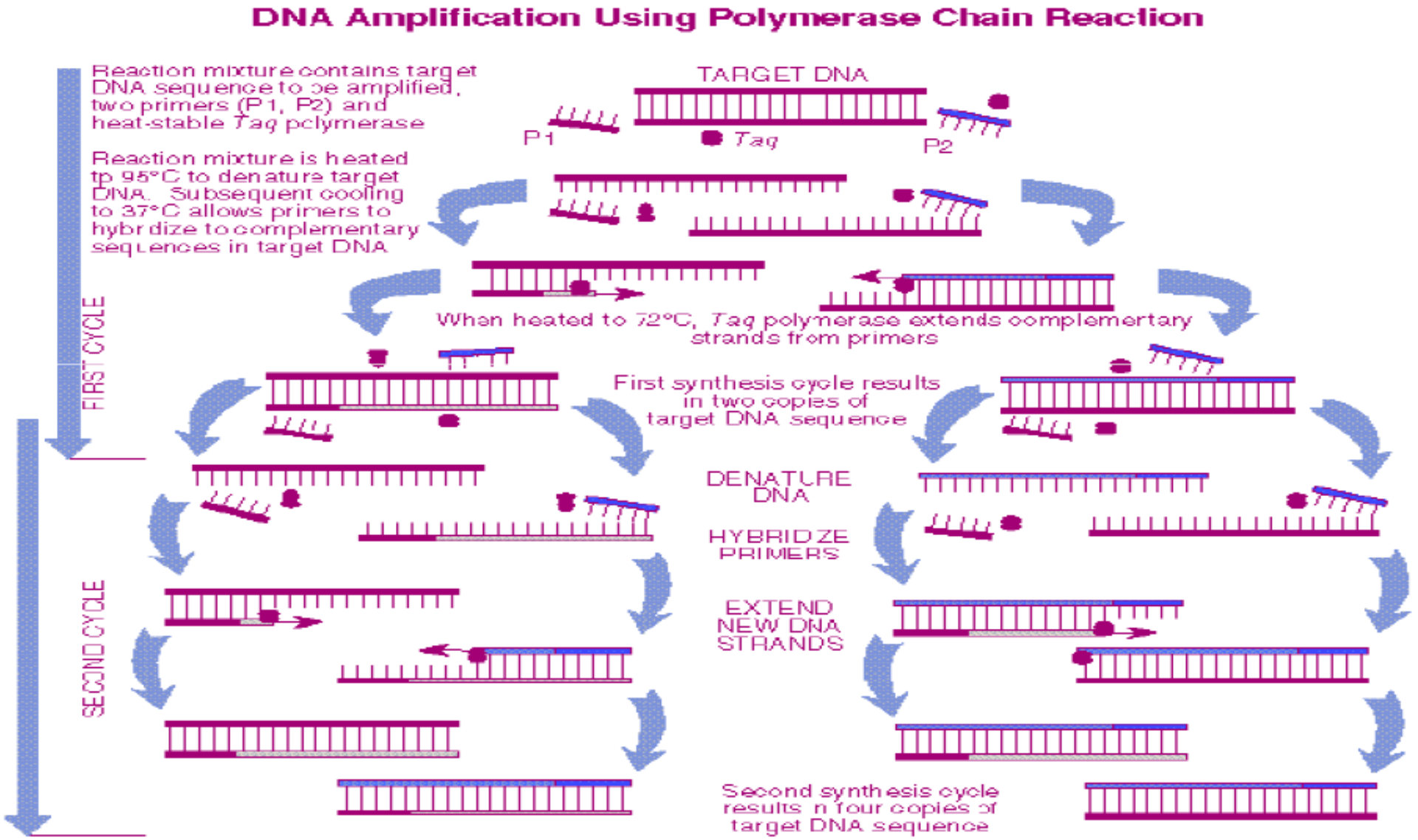
  Misal: DNA target (template) awal = 1 molekul, maka setelah 30 siklus, DNA target akan berjumlah 2^{30} molekul = 1.073.741.824 molekul.

- Pada umumnya terdiri atas 3 tahap yaitu:

  1. Denaturasi DNA
  2. Penempelan primer
  3. Reaksi polimerisasi



Prinsip PCR

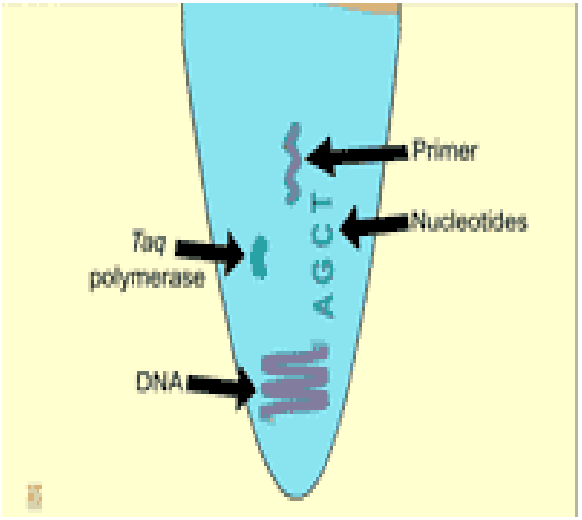

DNA Amplification Using Polymerase Chain Reaction
Reaction mixture contains target DNA sequence to be amplified, two primers (P1, P2) and heat-stable Taq polymerase
Reaction mixture is heated to 95°C to denature target DNA. Subsequent cooling to 37°C allows primers to hybridize to complementary sequences in target DNA
TARGET DNA
P1
Taq
P2
When heated to 72°C, Taq polymerase extends complementary strands from primers
First synthesis cycle results in two copies of target DNA sequence
FIRST CYCLE
DENATURE DNA
HYBRIDIZE PRIMERS
EXTEND NEW DNA STRANDS
SECOND CYCLE
Second synthesis cycle results in four copies of target DNA sequence

Tujuan PCR

- Amplifikasi fragmen DNA
- Isolasi fragmen DNA / gen
- Deteksi organisme/fragmen DNA/gen
- Kuantifikasi jumlah DNA/RNA
- Studi level ekspresi gen (Real time-PCR)

Komponen-Komponen PCR

- DNA Template
- Primer
- DNA Polimerase (Taq Polimerase)
- Deoxynucleoside triphosphates (dNTPs)
- Larutan buffer
- Kation divalen (misal: Mg^{2+})



Komponen-Komponen Penting dalam Replikasi DNA

a. **DNA cetakan :** molekul DNA yang akan direplikasi

b. **Molekul deoksiribonukleotida :** dATP, dTTP, dCTP, dan dGTP

c. **DNA Polimerase :** enzim yang mengkatalisis proses polimerisasi nukleotida menjadi untaian DNA

d. **DNA primase :** enzim yang mengkatalisis sintesis primer untuk memulai replikasi DNA

e. **DNA helikase dan DNA girase :** enzim yang membuka ikatan untaian DNA induk & berperan dalam mencegah aktivitas supercoiling.

f. ***Single Strand Binding Protein*** **(SSB) :** molekul protein yang menstabilkan untaian DNA yang sudah terbuka

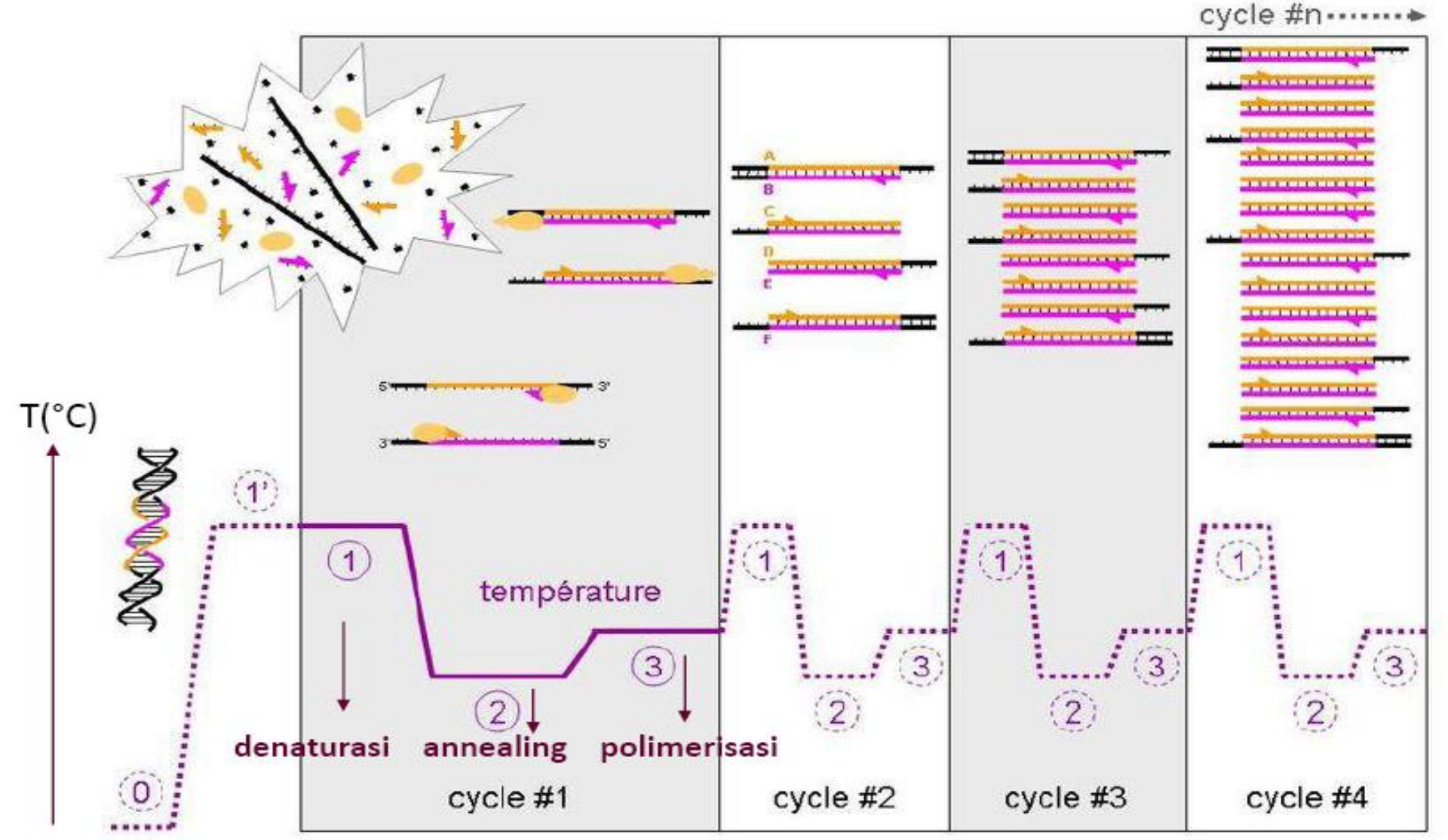
g. **DNA ligase :** enzim yang menggabungkan fragmen-fragmen DNA (fragmen okazaki)

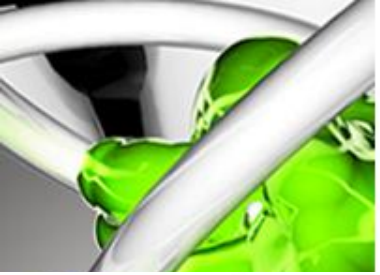
Komponen-Komponen Penting dalam Replikasi DNA

Suggested Variable Values for Standard PCR

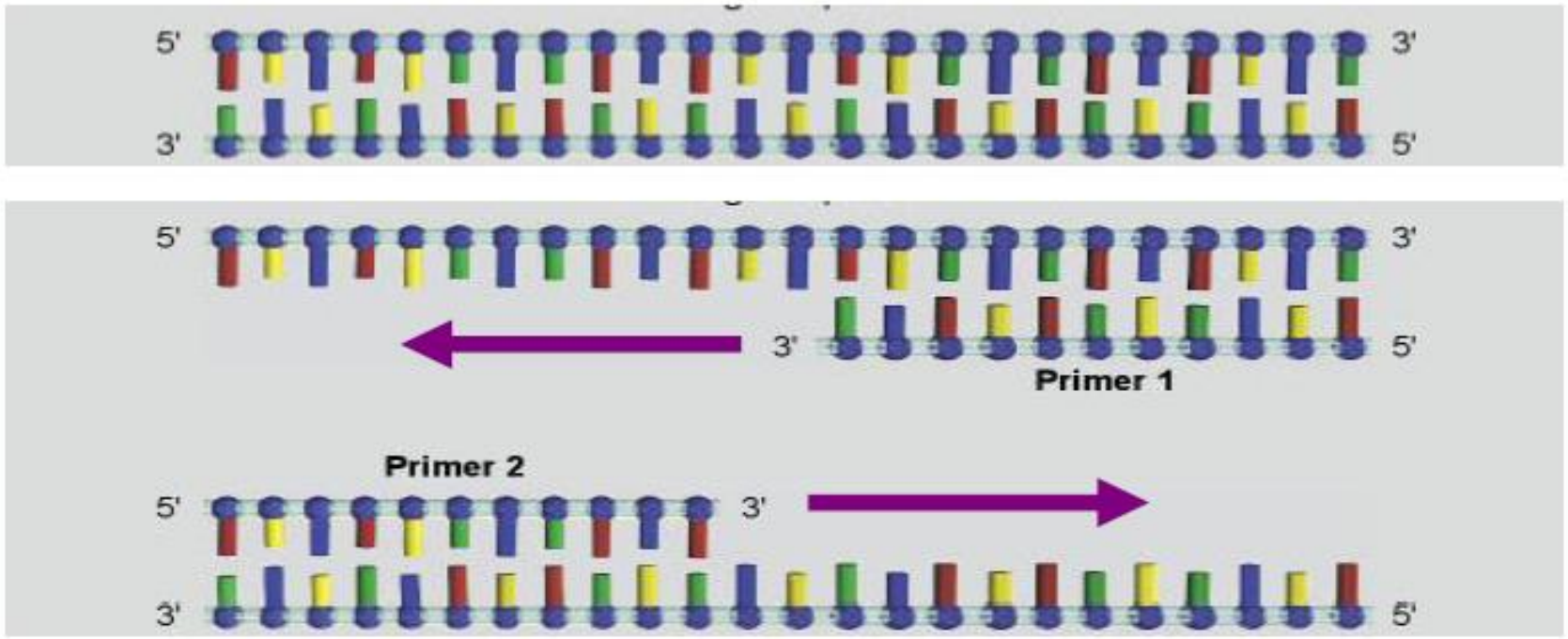
Reaction Volume	100 µL
Taq Polymerase	2- 2.5 U
Primers	0.1 - 1.0 µM each
dNTPs	0.2 mM each
Salt	6 - 50 mM KCl or (NH4)2SO4
Mg++	1.5 - 5.0 mM MgCl2 or MgSO4
Buffer	Tris-HCl 10 - 50 mM, pH 7.5 - 9.0
Template	102 - 105 copies
Source	"PCR Primer: A Laboratory Manual" 1995

Siklus PCR


cycle #n
T(°C)
1'
1
0
2
3
température
denaturasi
annealing
polimerisasi
cycle #1
cycle #2
cycle #3
cycle #4
A
B
C
D
E
F
5'
3'
3'
5'



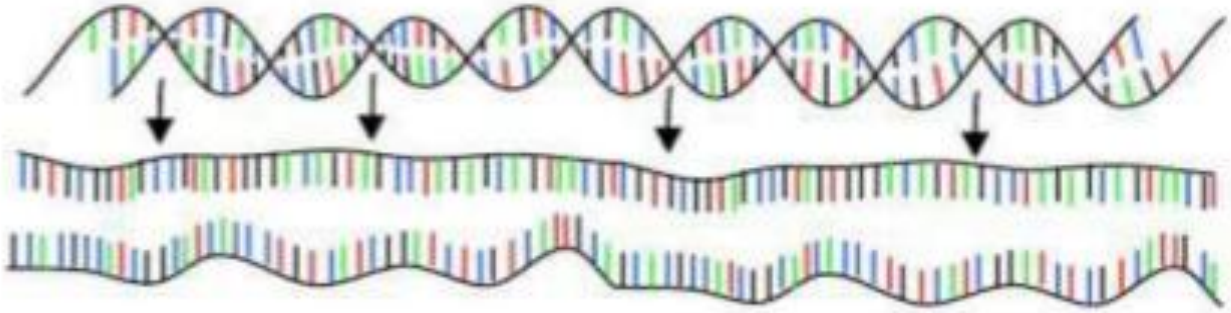
Primer Untuk PCR



5′-TACTTTGAGGTTATCGATTTAGCAAGCGATGCAACCATTACTGATCGA-3′

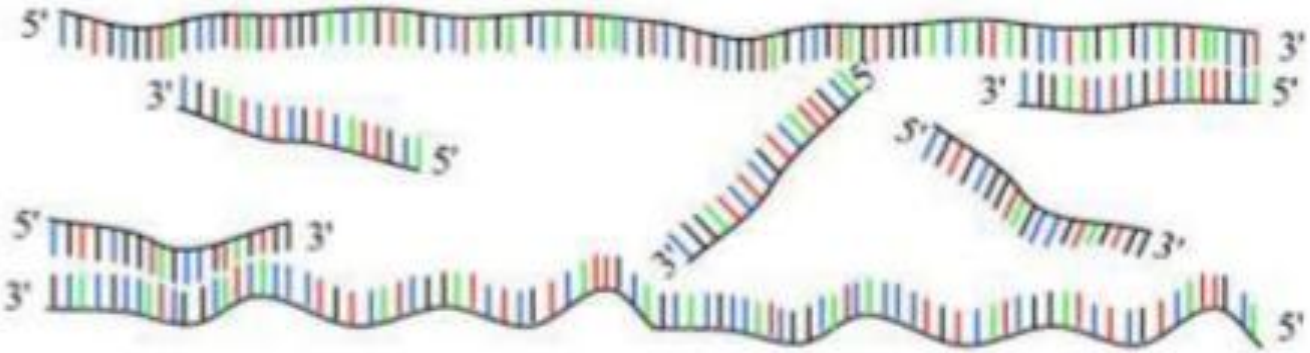
3′-ATGAAACTCCAATAGCTAAATCGTTCGCTACGTTGGTAATGACTAGCT-5′

PRIMER ?



Step 1 : denaturation

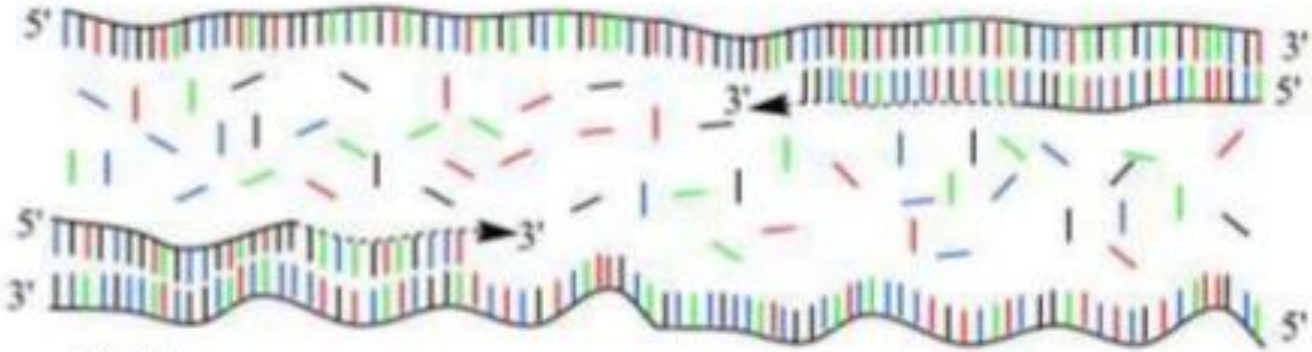
1 minut 94 °C



Step 2 : annealing

45 seconds 54 °C

forward and reverse primers !!!



Step 3 : extension

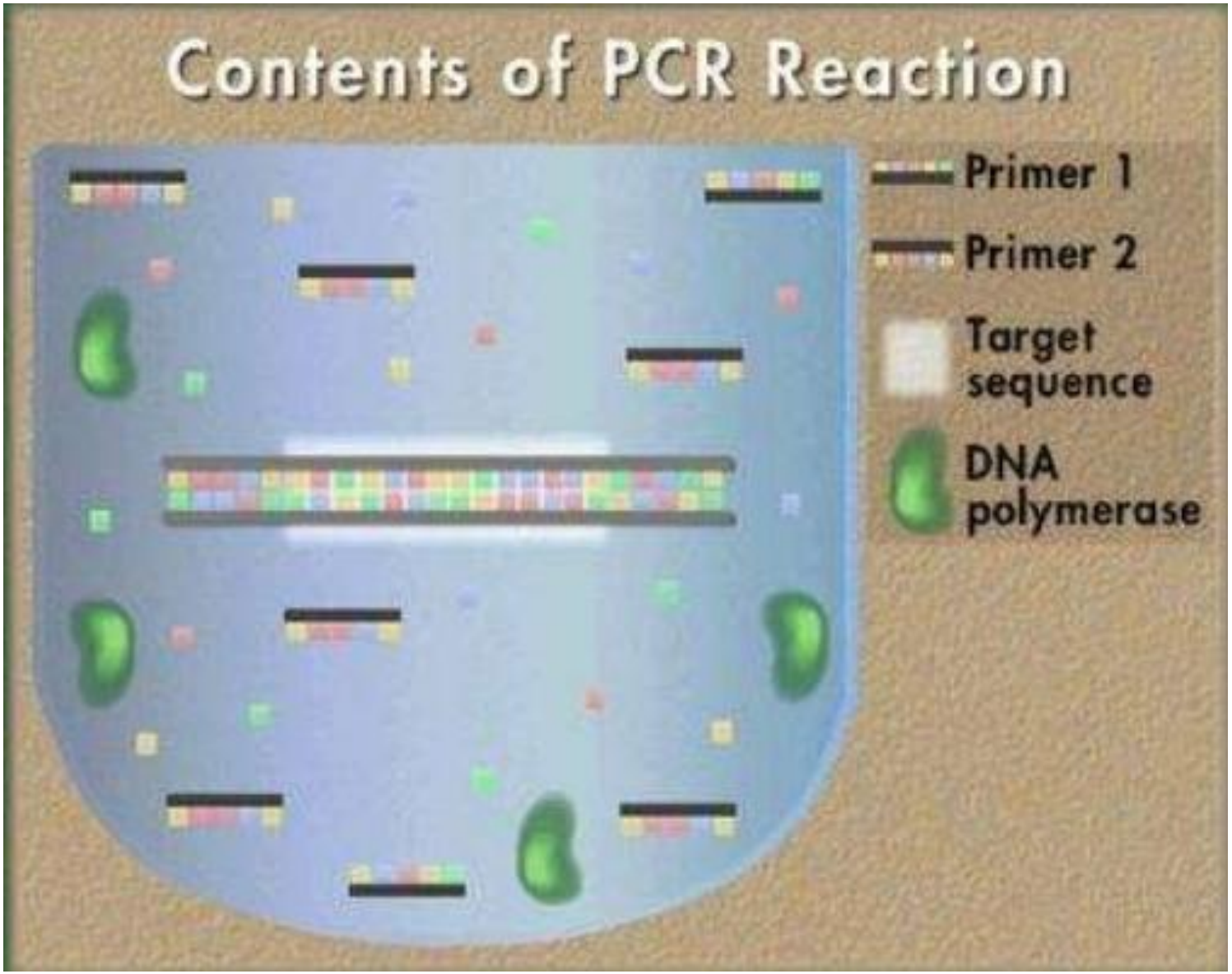
2 minutes 72 °C

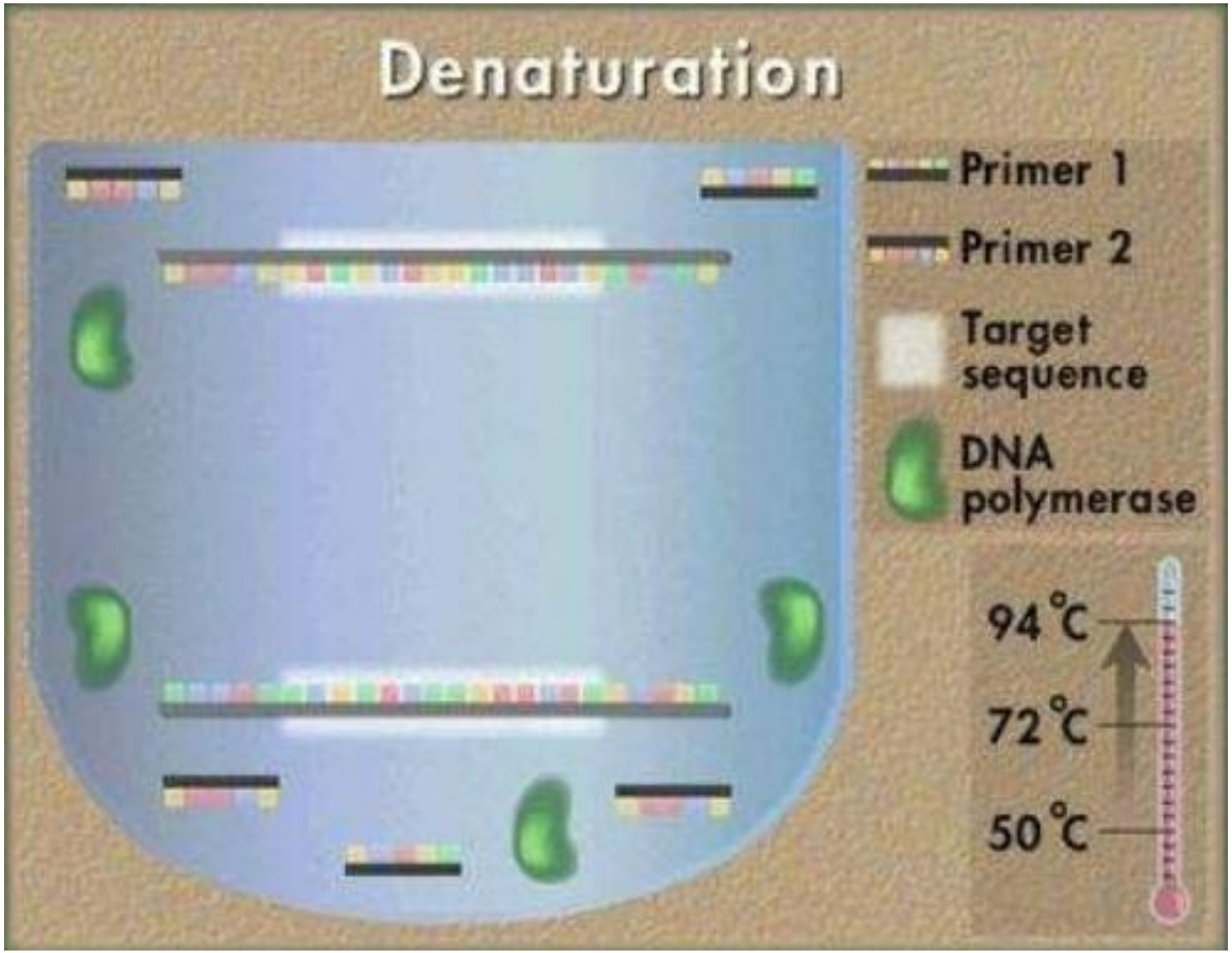
only dNTP's

(Andy Vierstraete 1999)

1. Denaturasi DNA

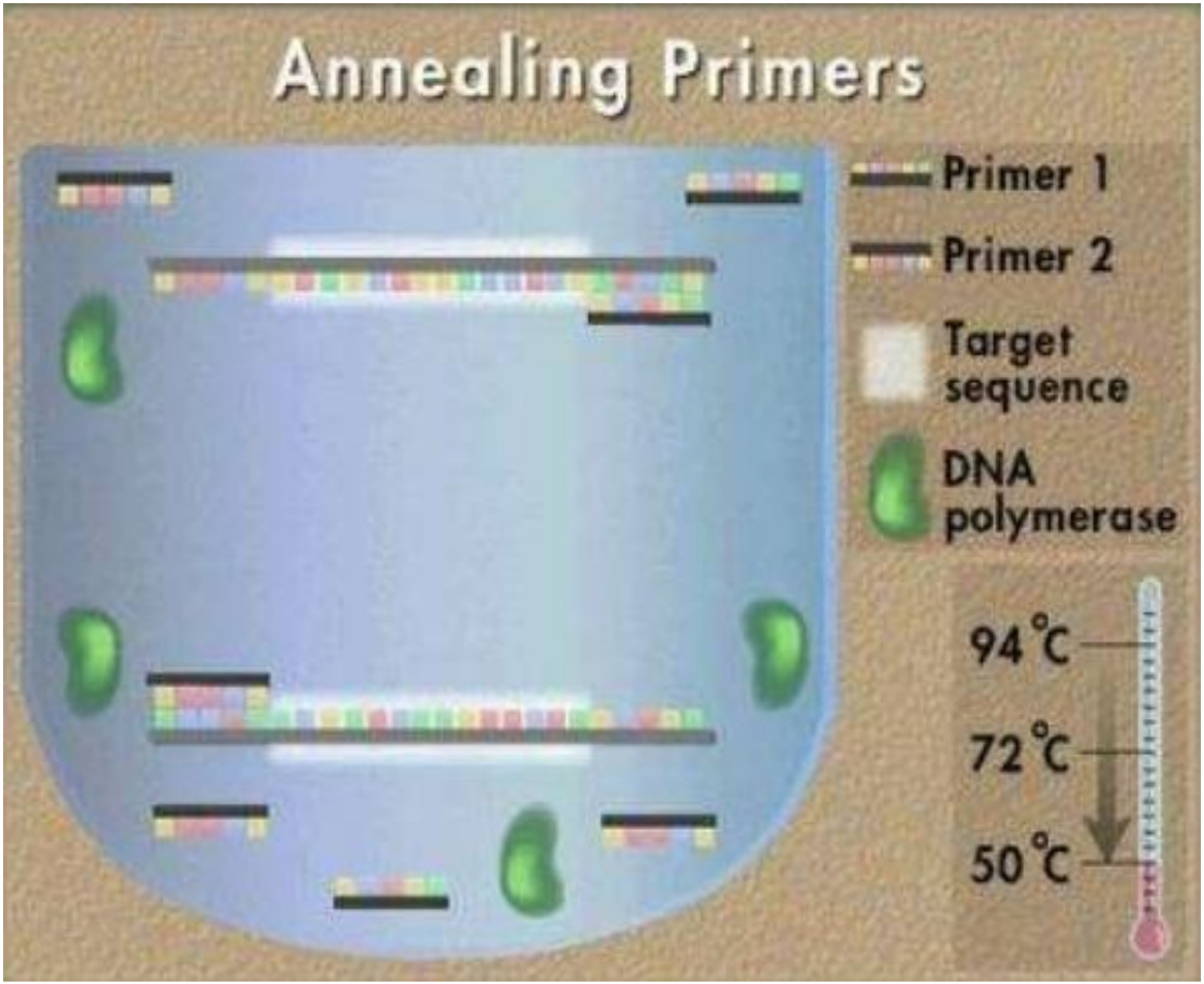
- Selama proses denaturasi, DNA untai ganda akan membuka menjadi DNA untai tunggal.
- Suhu tinggi menyebabkan putusnya ikatan hidrogen diantara 2 basa nitrogen yang komplemen.
- Tahap ini berlangsung sekitar 1-2 menit.
- Denaturasi biasanya dilakukan antara suhu 90-98 °C.


Contents of PCR Reaction
Primer 1
Primer 2
Target sequence
DNA polymerase


Denaturation
Primer 1
Primer 2
Target sequence
DNA polymerase
94°C
72°C
50°C

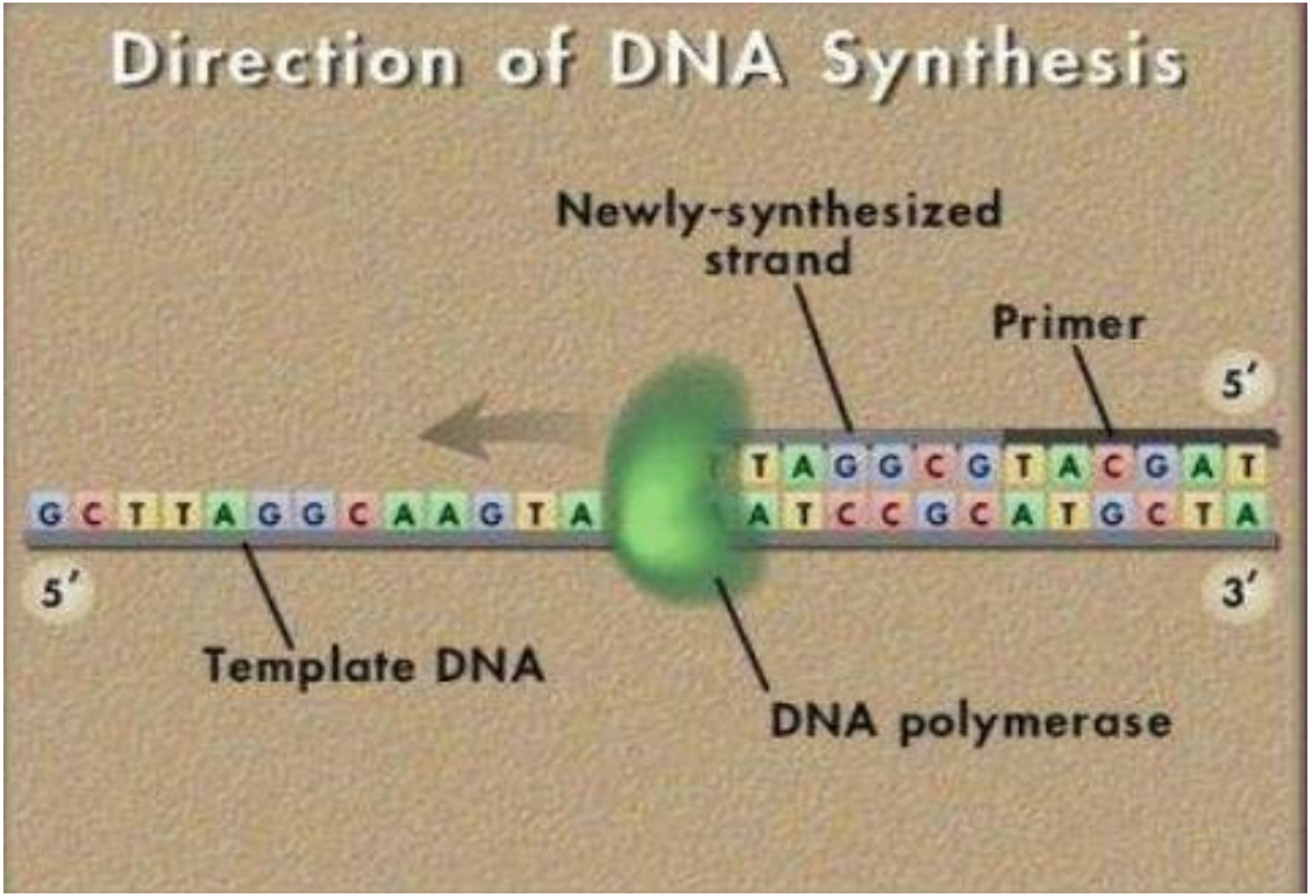
2. Penempelan Primer (*Annealing*)

- Pada tahap *annealing*, primer akan menuju daerah spesifik yang komplemen dengan urutan primer.
- Ikatan hidrogen akan terbentuk antara primer dengan urutan komplemen pada template selama 1-2 menit.
- Proses ini biasanya dilakukan pada suhu 45-60 °C.
- Selanjutnya, DNA polimerase akan berikatan sehingga ikatan hidrogen tersebut akan menjadi sangat kuat dan tidak akan putus kembali apabila dilakukan reaksi polimerisasi selanjutnya, misalnya pada suhu 72 °C.

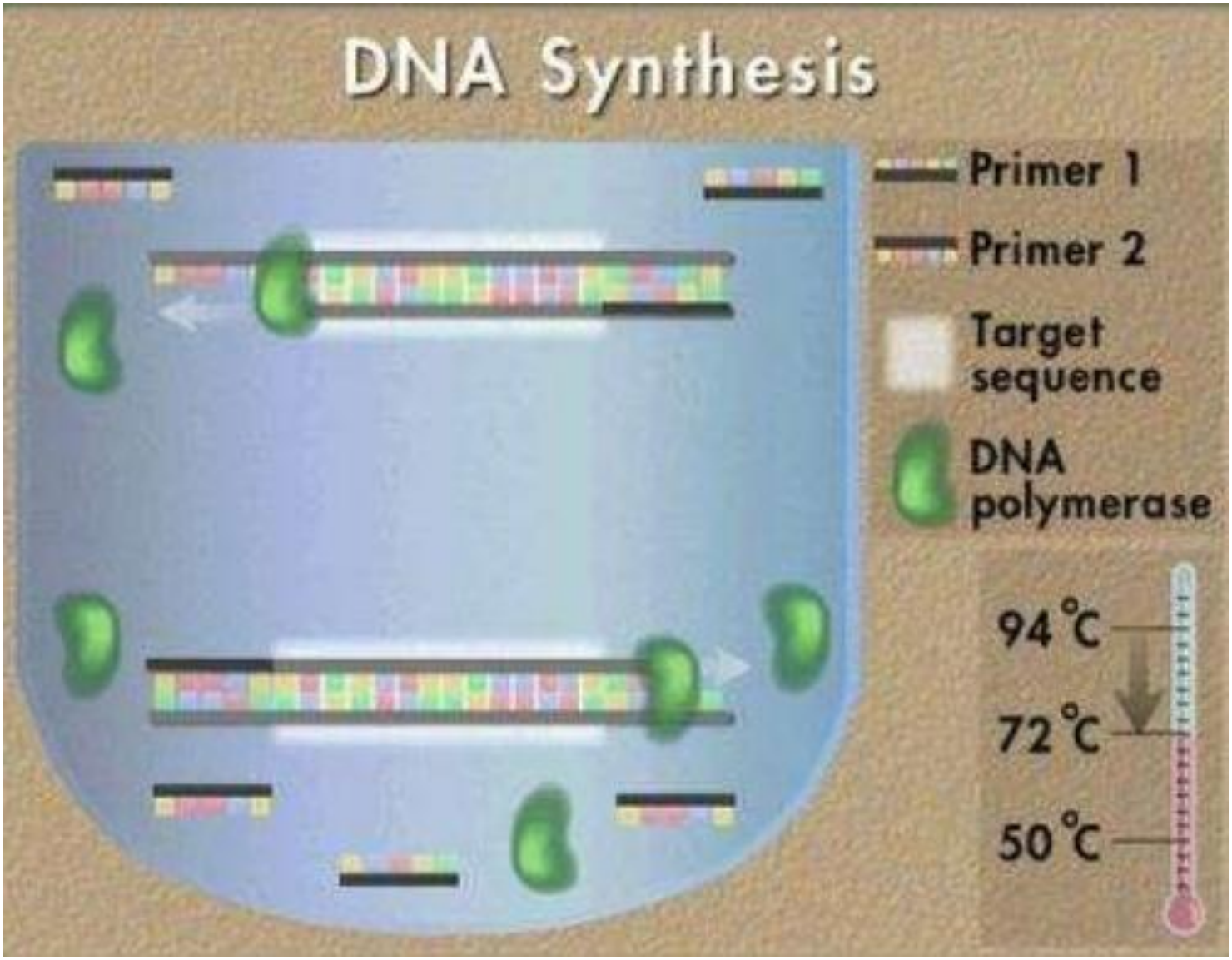

Annealing Primers
Primer 1
Primer 2
Target sequence
DNA polymerase
94°C
72°C
50°C

3. Reaksi Polimerisasi (*Elongation*)

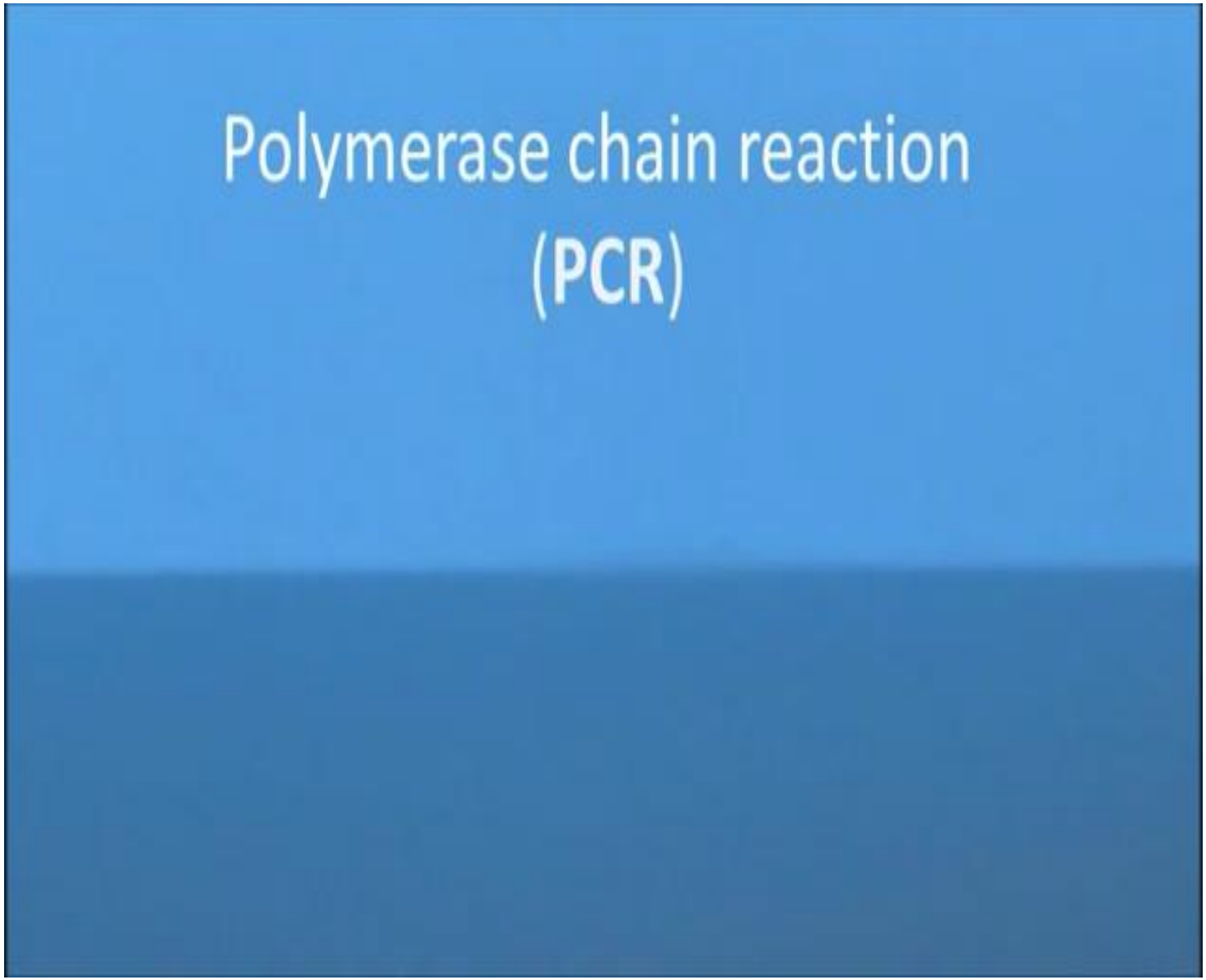
- Reaksi polimerisasi terjadi pada suhu 72 °C.
- Primer yang telah menempel tadi akan mengalami perpanjangan pada sisi 3'nya dengan penambahan dNTP yang komplemen dengan template oleh DNA polimerase.
- Durasi tahap ini biasanya 1 menit.


Direction of DNA Synthesis
Newly-synthesized strand
Primer
5′
T A G G C G T A C G A T
G C T T A G G C A A G T A
A T C C G C A T G C T A
5′
3′
Template DNA
DNA polymerase

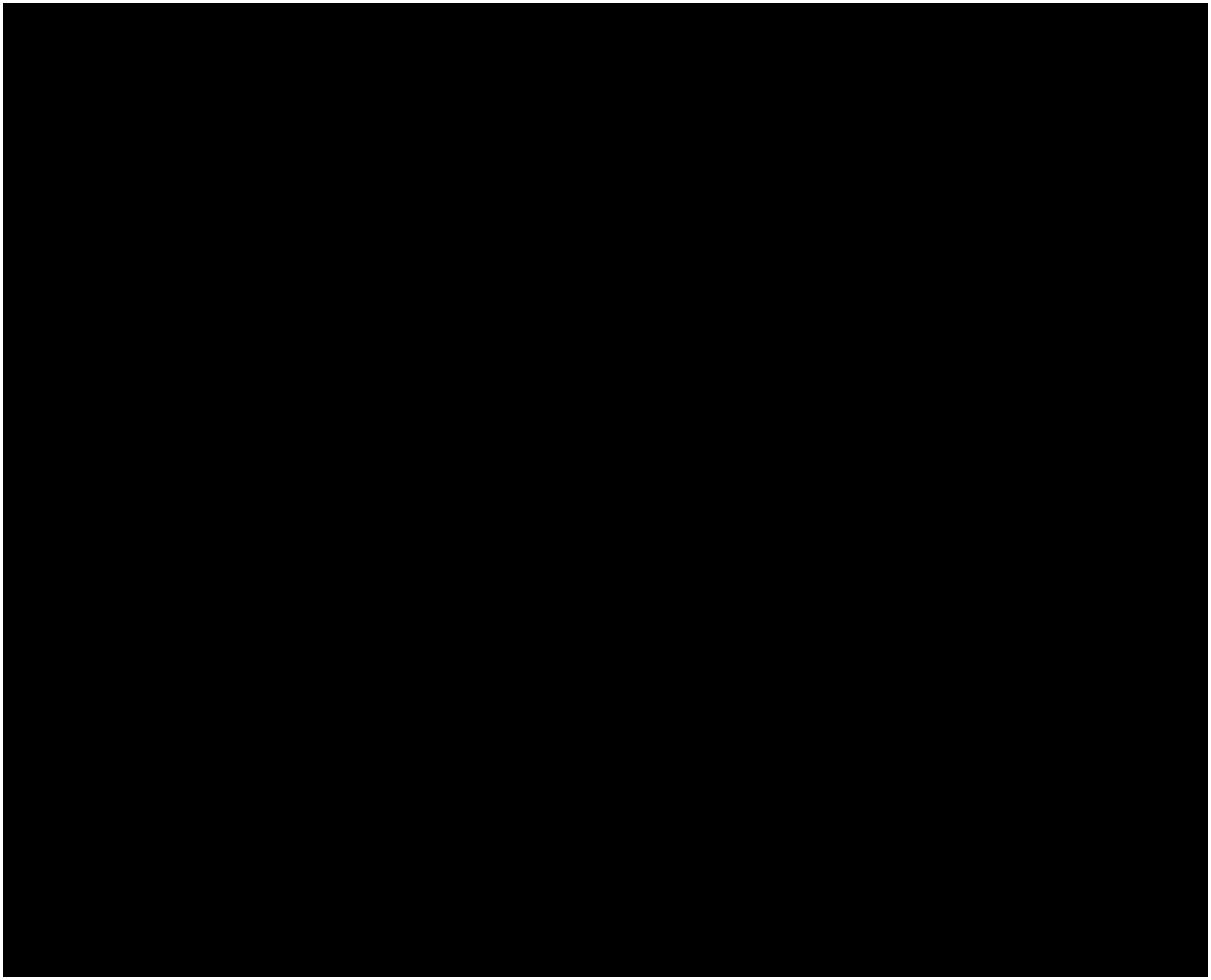
PCR CYCLE


DNA Synthesis
Primer 1
Primer 2
Target sequence
DNA polymerase
94 °C
72 °C
50 °C

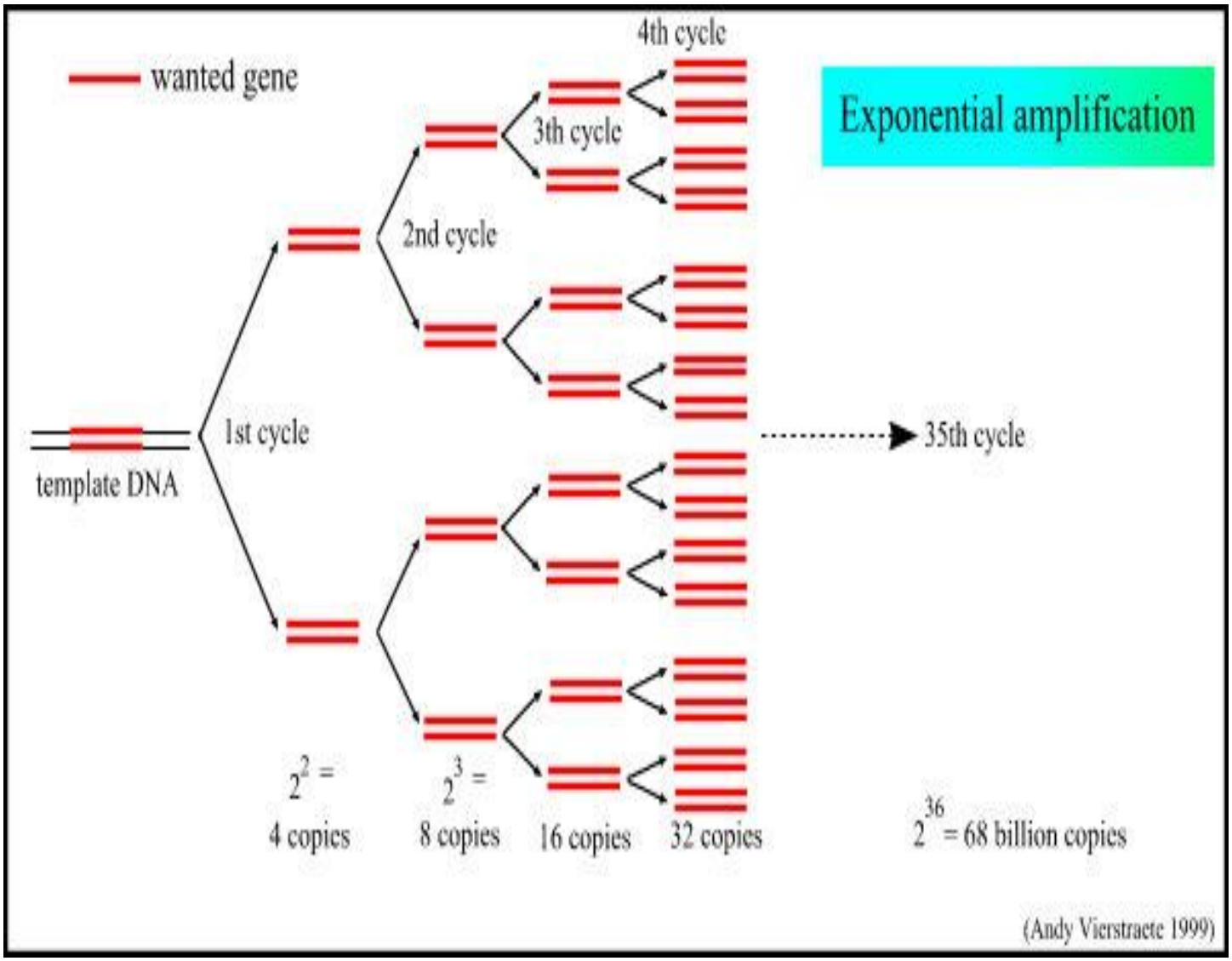
PCR CYCLE



PCR CYCLE



Polymerase Chain Reaction



PCR Cabinet

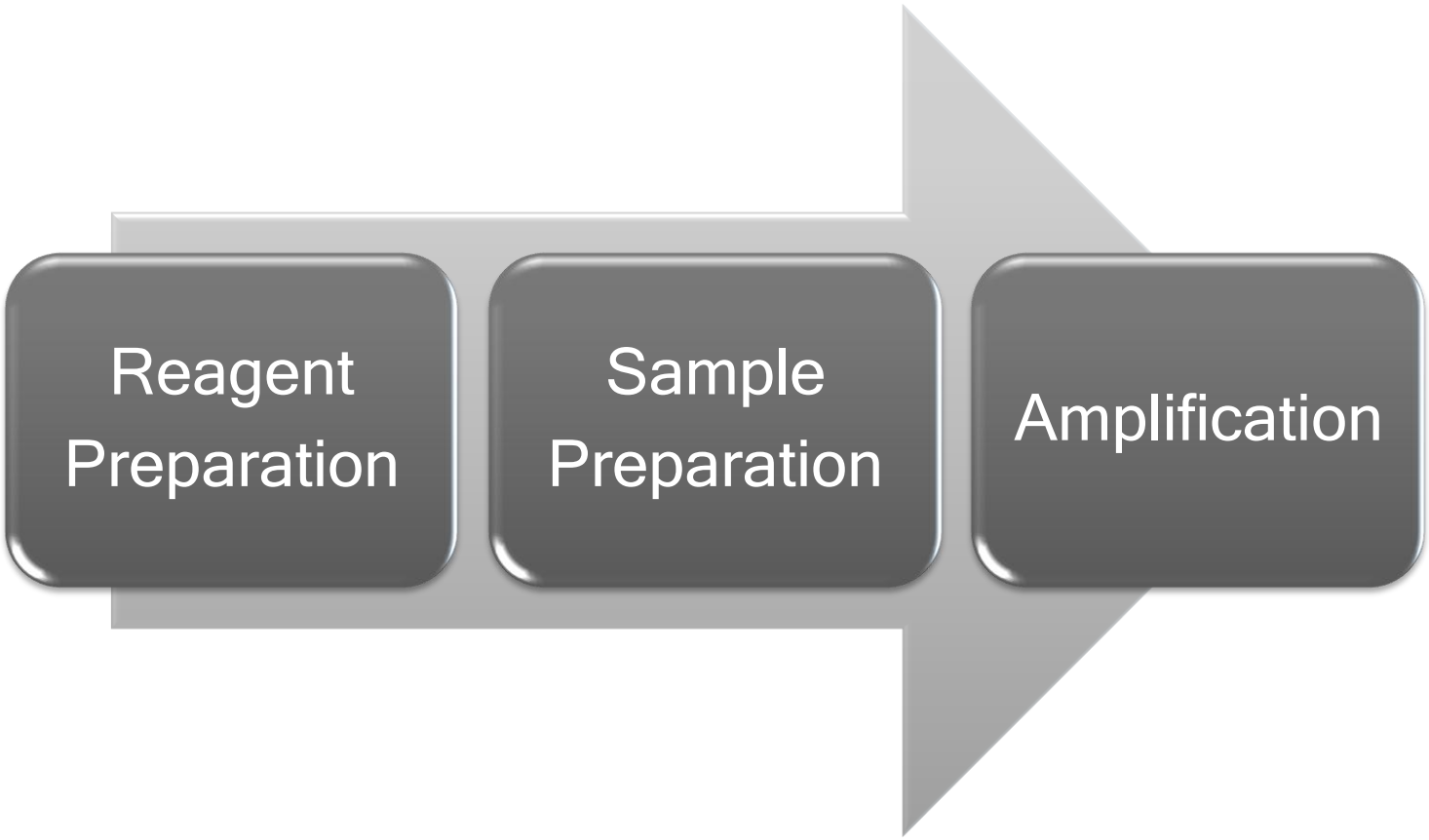
Main Features

- Greater protection against contamination from the ambient environment and cross-contamination within the main chamber.
- High quality polyester pre-filter and main HEPA filter with a typical efficiency of >99.99% at 0.3 microns.
- Built-in UV lamp with timer to facilitate decontamination between PCR cycles

Why PCR Cabinet

- it is essential to prevent possible contamination of the PCR reaction.
- Reagents should be prepared in the reagent preparation area and transferred to the sample preparation area through a pass box or inside closed containers
- The tubes should be transferred to the amplification area, again through a pass box or in a closed container
- Reagent temperature maintained in cold conditions

Why PCR Cabinet

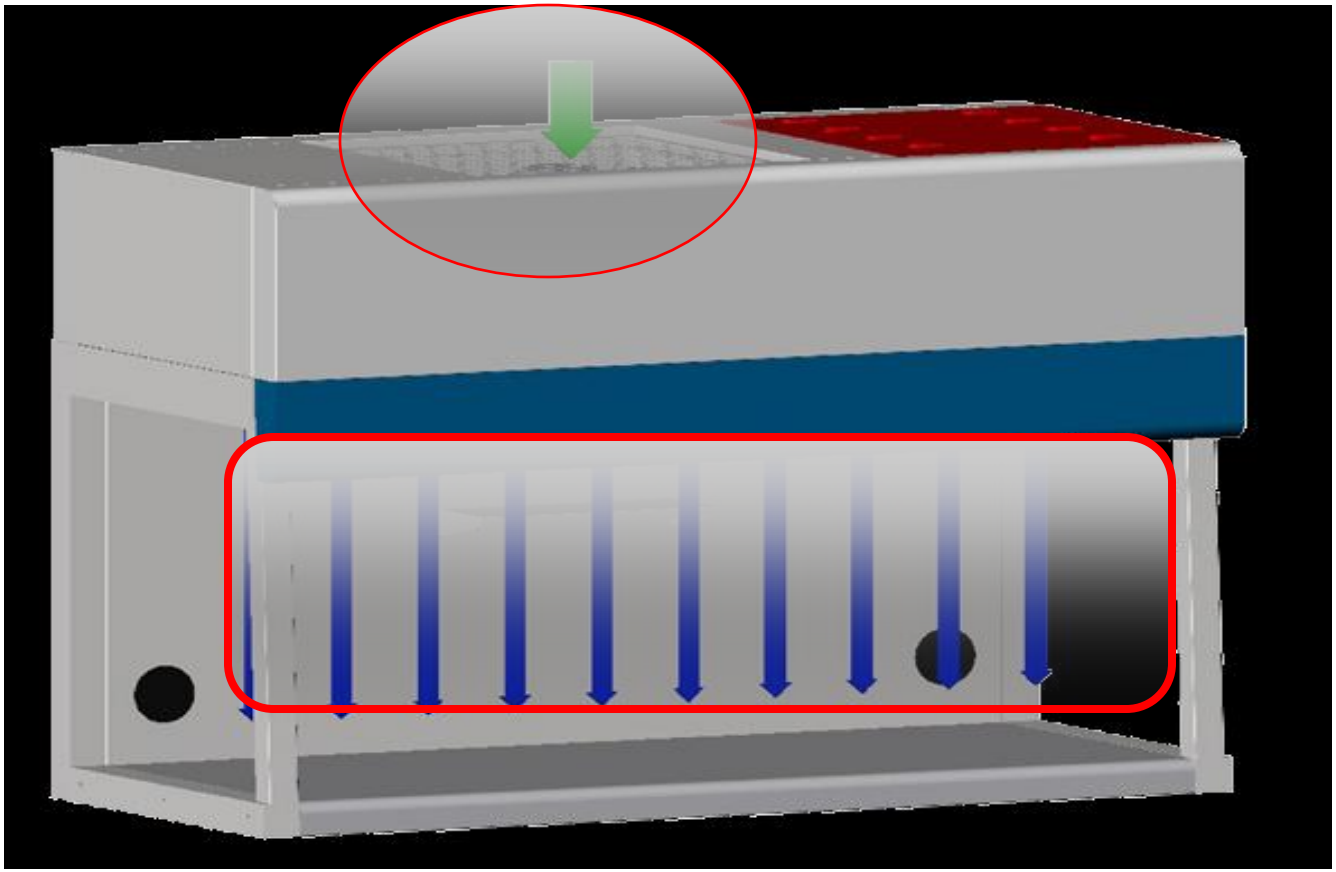


Component of PCR cabinet

- **UV Decontamination Technology**

Component of PCR cabinet

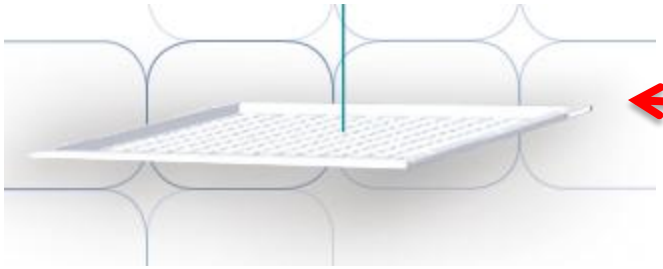
- **HEPA-Filtered Laminar Airflow**



Component of PCR cabinet

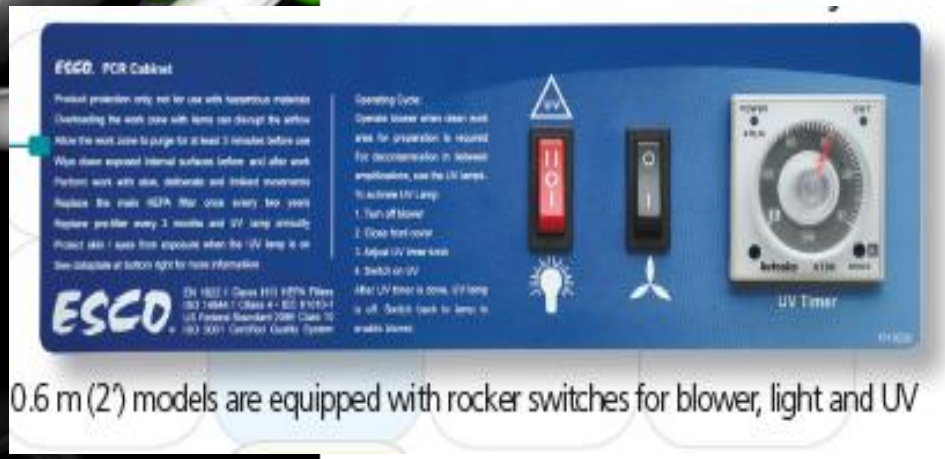
- **Pre-Filters**

An additional disposable pre-filter traps large particles in the inflow air prior to reaching the main filter, protecting against damage and prolonging filter life.



Component of PCR cabinet

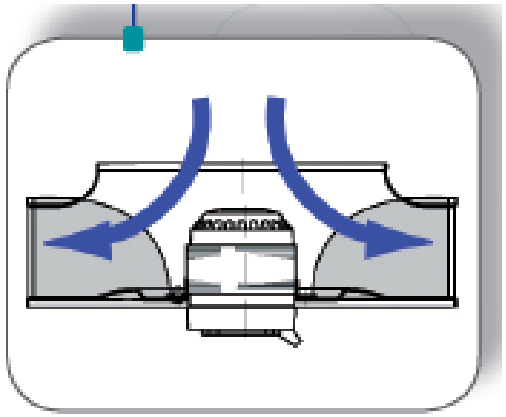
- **Control System**

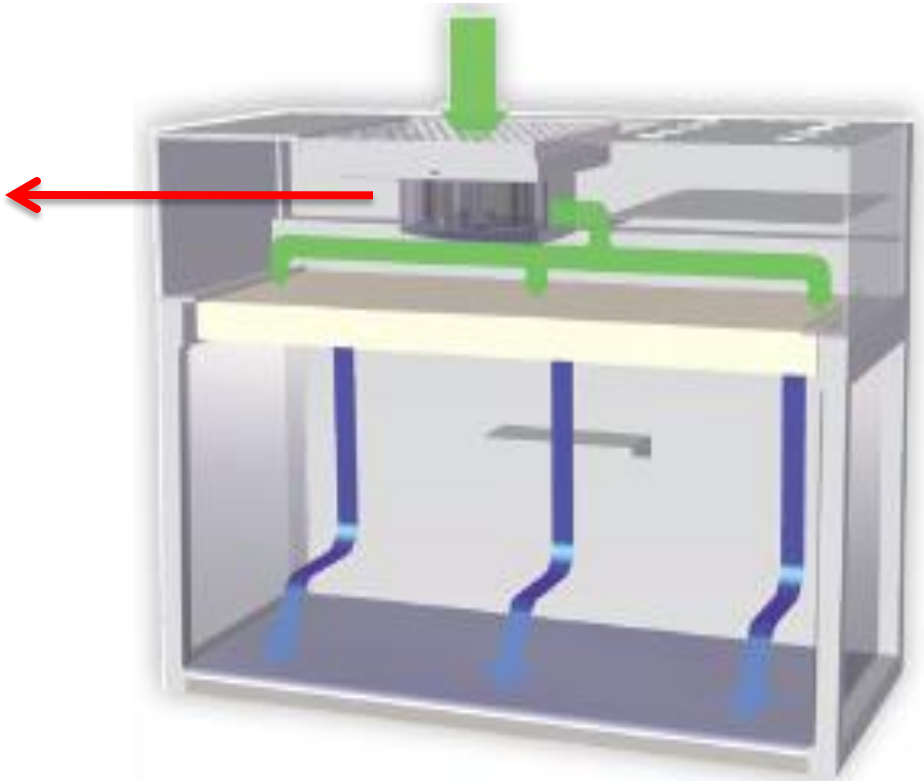


Component of PCR cabinet

- **Blower**

To provide and accommodate a large flow of air or gas to various processes





Macam-Macam PCR

1. Real Time PCR
2. RT-PCR
3. Nested PCR
4. Multiplex-PCR
5. dan lain-lain

Real Time PCR (qPCR)

- Teknik ini digunakan untuk mengamplifikasi sekaligus kuantifikasi jumlah target molekul DNA hasil amplifikasi tersebut.
- Cara kerja : DNA yang telah diamplifikasi dihitung setelah diakumulasikan dalam reaksi secara real time sesudah setiap siklus amplifikasi selesai. Dengan ada probe yang berflorescense yang di tangkap oleh DNA – binding dye (SYBR green) ada suhu annealing.

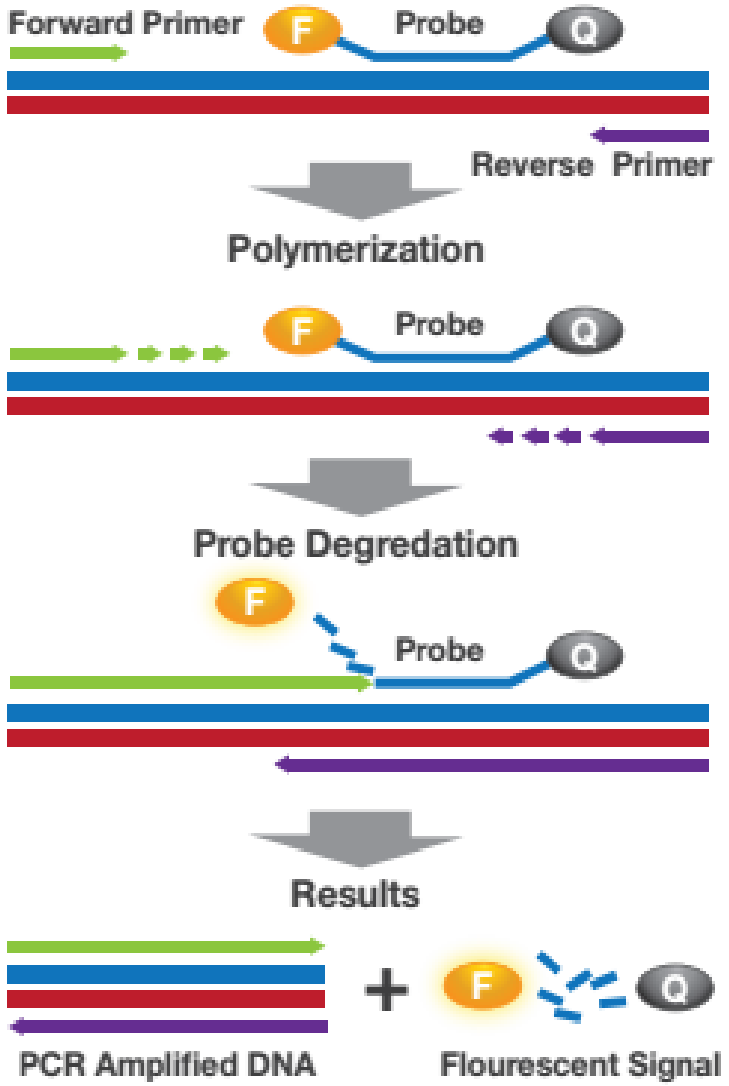
Real Time PCR (qPCR)

Step 1:
Primers and probe bind to target DNA.

Step 2:
PCR occurs, primers are extended on forward and reverse DNA strands.

Step 3:
Probe is degraded as a result of polymerization and flourescent signal is generated.

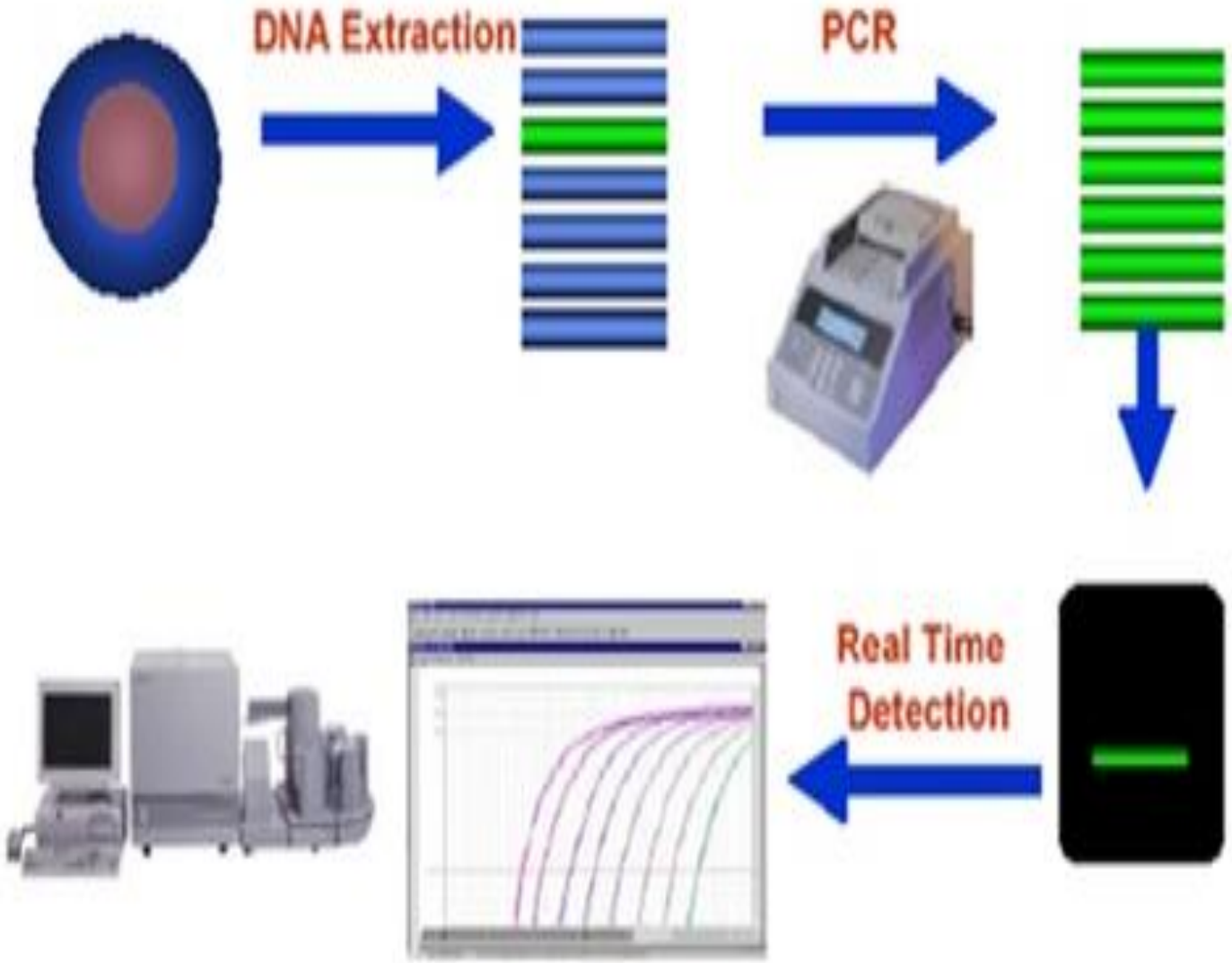
Step 4:
Taregt DNA is amplified and flourescent signal can be measured and quantified.



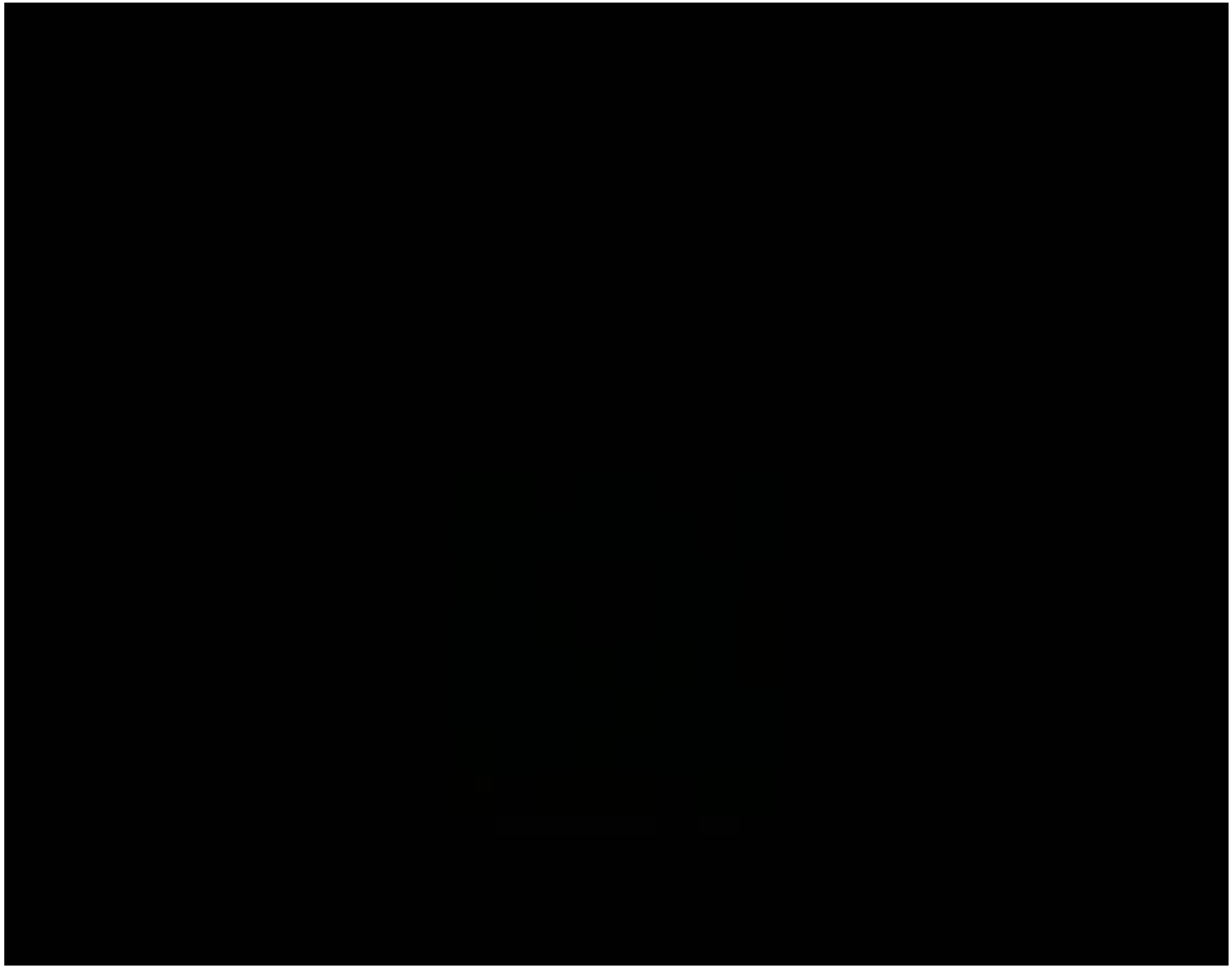
Real Time PCR

PCR Konvensional	Real Time PCR
Sensitivitas rendah	Sensitivitas tinggi
Presisi rendah	Presisi tinggi
Tidak otomatis	Otomatis
Hasil tidak dalam bentuk angka	Data dikumpulkan dalam fase pertumbuhan eksponensial PCR
Ada tahapan setelah PCR	Tidak ada tahapan setelah PCR
Deteksi keberadaan DNA dilakukan pada akhir reaksi	Pengamatan dapat dilakukan saat reaksi berlangsung
Pengamatan keberadaan DNA hasil amplifikasi dilakukan di gel agarosa setelah dilakukan elektroforesis	Keberadaan DNA hasil amplifikasi dapat diamati pada grafik yang muncul sebagai hasil akumulasi fluoresensi dari probe (penanda)

Real Time PCR



Real Time PCR vs PCR konvensinal



RT-PCR (Reverse Transcriptase-PCR)

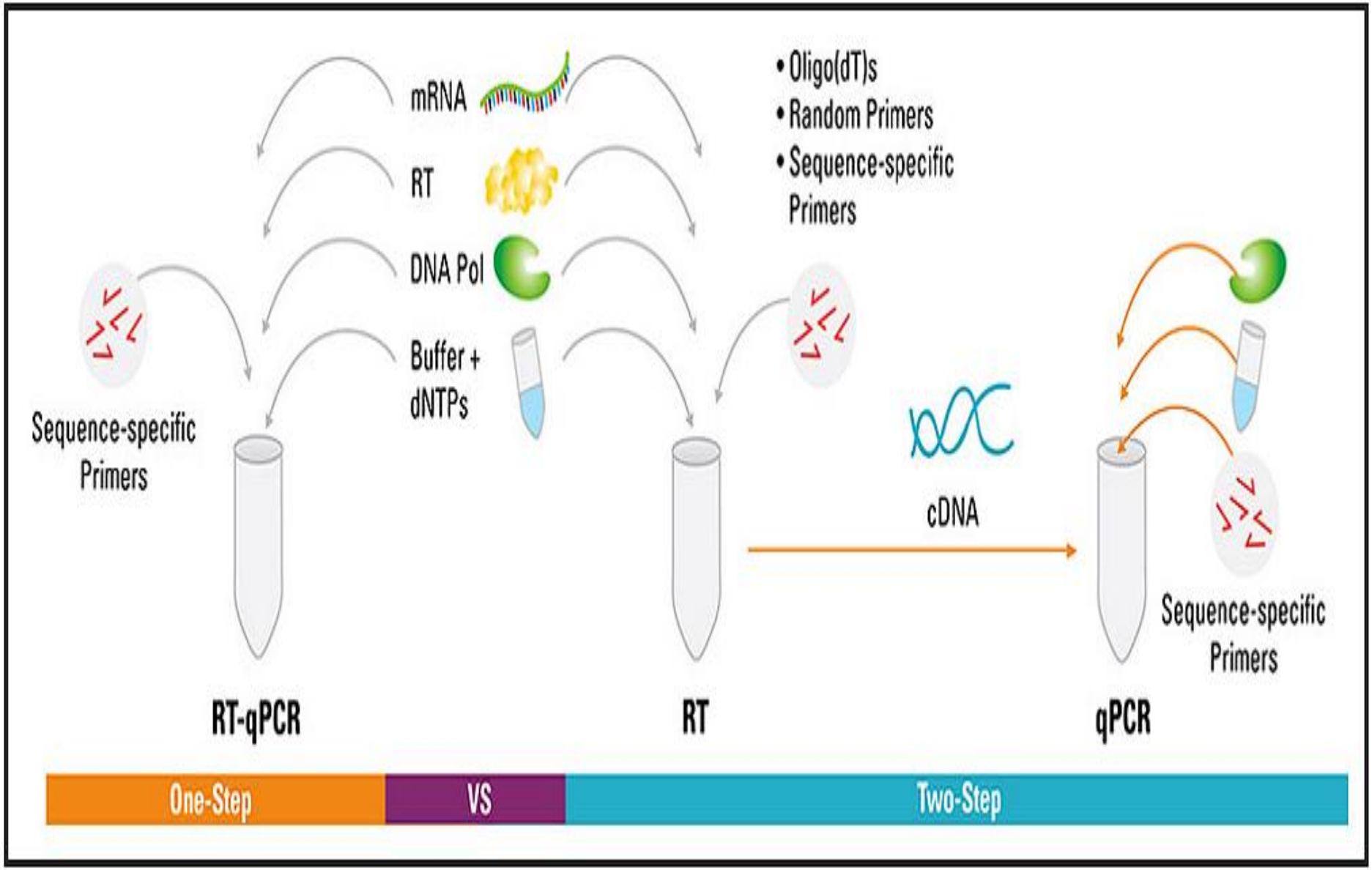
- Merupakan modifikasi dari PCR, dimana yang diamplifikasi berupa mRNA.
- Sampel yang digunakan bukan DNA melainkan RNA.
- Proses RT-PCR dibantu oleh enzim Reverse Transcriptase (dapat mensintesis DNA dengan cetakan RNA).
- RT-PCR penting digunakan sebagai alat diagnostik untuk mendeteksi dan menentukan serotipe virus, sebagai informasi untuk studi epidemiologi.

RT-PCR (Reverse Transcriptase-PCR)

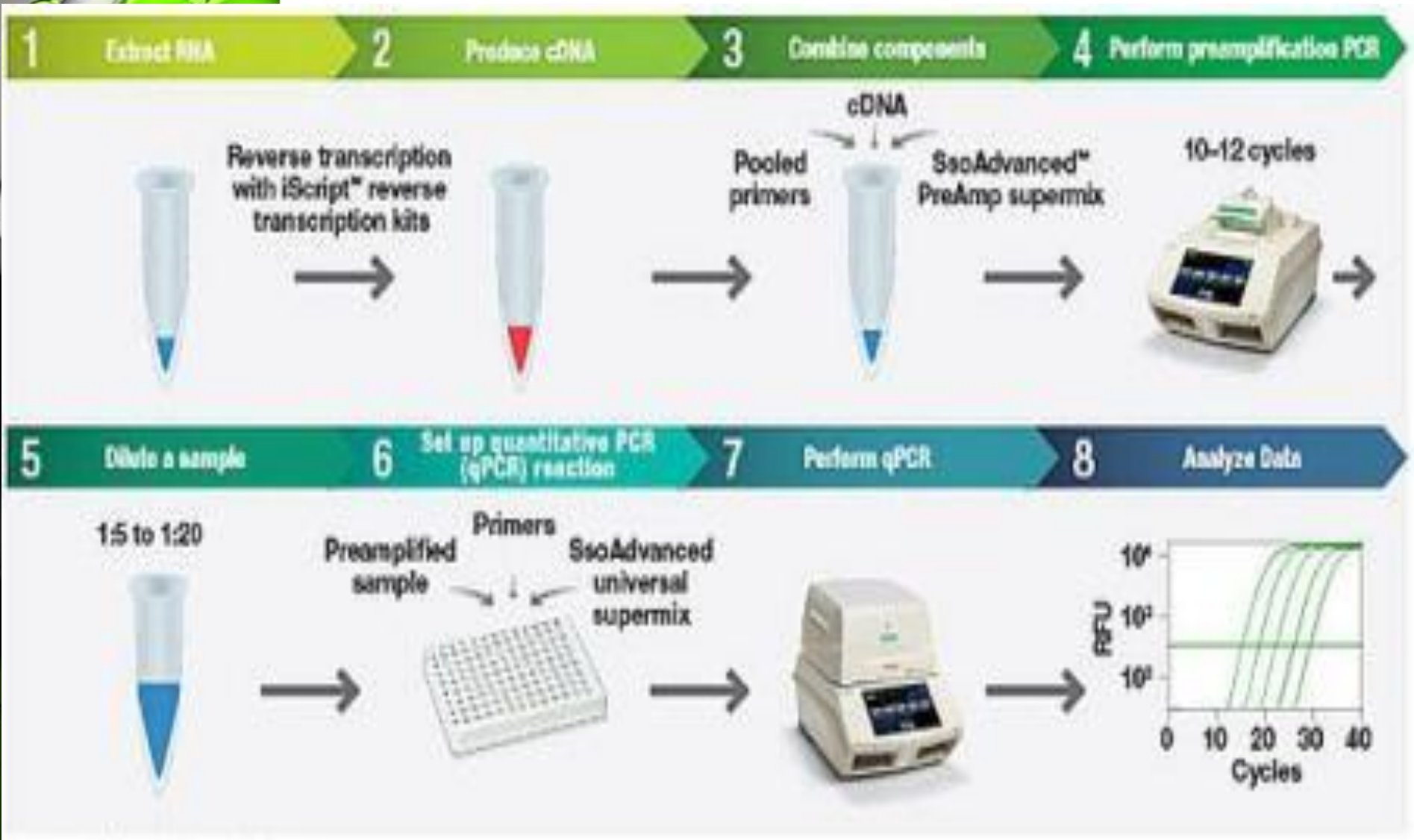
Tahapan:

- RNA diubah dulu jadi DNA oleh enzim reverse transcriptase, yg disebut komplemen DNA (cDNA).
- Sintesis cDNA dari perpasangan antar gugus basa U dan A, serta G dan C.
- Dari cDNA dilipatgandakan segmen DNA yang mirip urutan basa nukleotidanya dengan RNA, hanya U diganti kembali ke T.
- Terjadi proses annealing untuk memasangkan primer untuk memperpanjang segmen cDNA.
- Setelah terbentuk segmen cDNA, baru kemudian masuk ke proses PCR biasa.

RT-PCR (Reverse Transcriptase-PCR)



RT-PCR (Reverse Transcriptase-PCR)



Nested PCR

- Suatu teknik perbanyakan (replikasi) sampel DNA menggunakan enzim DNA polimerase yang menggunakan 2 pasang primer untuk mengamplifikasi fragmen.
- Jika ada fragmen yang salah diamplifikasi, maka kemungkinan bagian tersebut diamplifikasi untuk kedua kalinya oleh primer yang kedua → sangat spesifik dalam melakukan amplifikasi.

Nested PCR

PCR Konvensional	Nested PCR
Digunakan 1 pasang primer	Digunakan 2 pasang primer, untuk meminimalkan kesalahan amplifikasi gen
Hasil fragmen DNA lebih panjang	Hasil fragmen DNA lebih spesifik (lebih pendek)
Waktu reaksi lebih sebentar, karena hanya dilakukan 1 kali reaksi PCR	Waktu reaksi lebih lama, karena dilakukan 2 kali reaksi PCR

Multiplex-PCR

- Merupakan beberapa set primer dalam campuran PCR tunggal untuk menghasilkan amplikon dari berbagai ukuran yang spesifik untuk sekuens DNA yang berbeda.
- Dengan penargetan gen sekaligus, informasi dapat diperoleh dari reaksi tunggal yang tidak membutuhkan banyak reagen dan waktu.
- Temperatur annealing untuk masing-masing set primer harus dioptimalkan untuk bekerja dengan benar dalam reaksi tunggal, dan ukuran amplikon.
- Panjangnya pasangan basa harus cukup berbeda untuk membentuk pita yang berbeda ketika divisualisasikan dengan elektroforesis gel.

Kontrol Kualitas PCR

- Kontrol positif : cetakan atau sel yang pasti memberikan hasil positif (tabung berbeda)
- Kontrol negatif : tanpa cetakan atau sel (hasil harus negatif)
- Kontrol internal : DNA yang pasti memberikan hasil positif, dilakukan pada tabung sampel

Aplikasi PCR

Applications of PCR

Molecular Identification

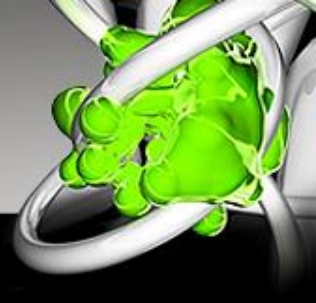
- Molecular Archaeology
- Molecular Epidemiology
- Molecular Ecology
- DNA fingerprinting
- Classification of organisms
- Genotyping
- Pre-natal diagnosis
- Mutation screening
- Drug discovery
- Genetic matching
- Detection of pathogens

Sequencing

- Bioinformatics
- Genomic cloning
- Human Genome Project

Genetic Engineering

- Site-directed mutagenesis
- Gene expression studies



TERIMA KASIH