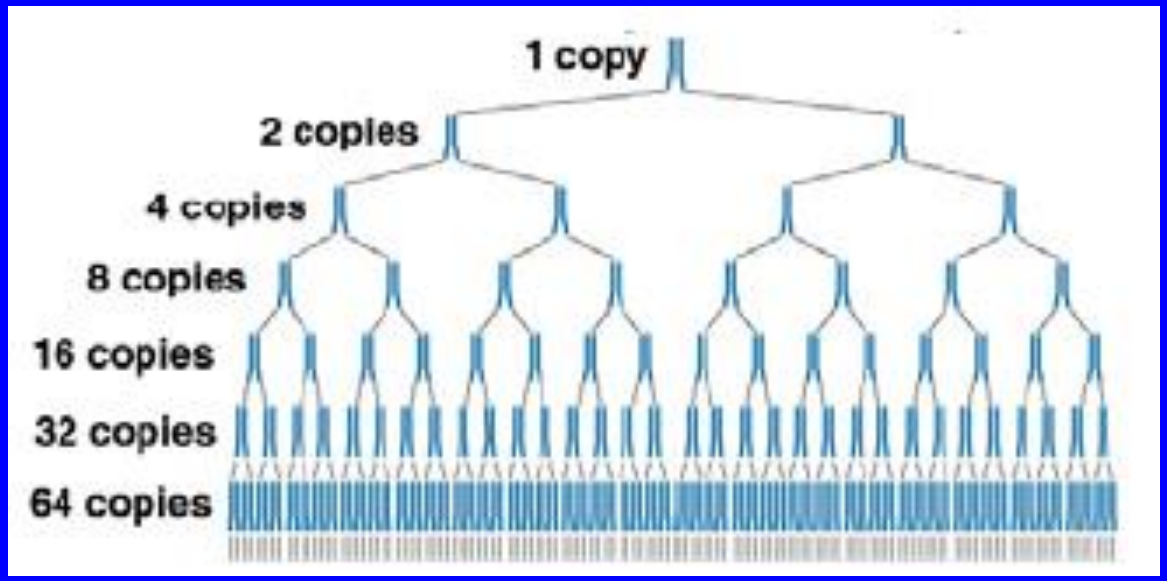
Recombinant DNA Technology

PCR and DNA sequencing

Polymerase Chain Reaction

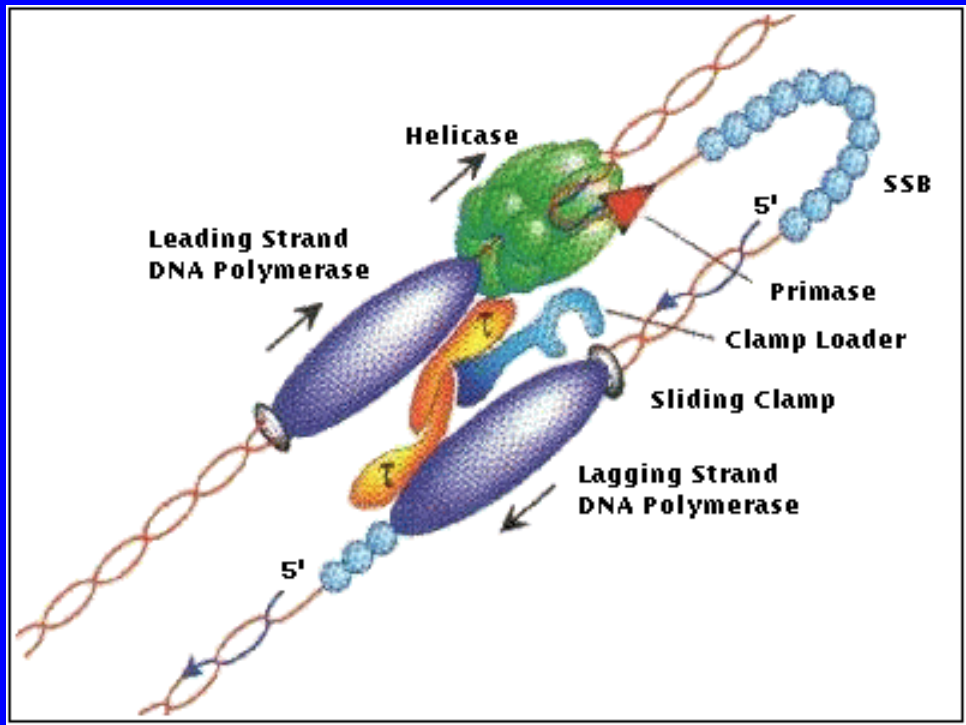
- Powerful technique for amplifying or copying a specific piece of DNA
- Carried out in vitro (in the lab)
- Extremely simple technique



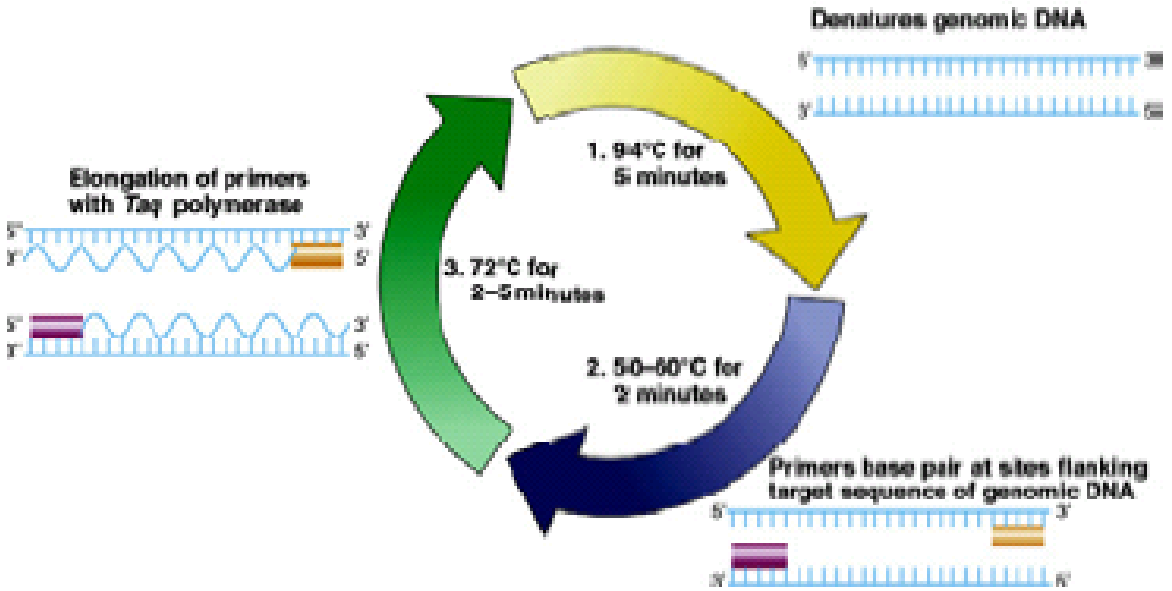
Polymerase Chain Reaction

What's needed -

- two oligonucleotide primers (18-25nt)
  - complementary to opposite strands
  - oriented with 3' OH toward each other
  - less than 2kbp apart
- thermostable DNA Polymerase
  - Taq DNA Pol
- all 4 dNTPs
- template DNA

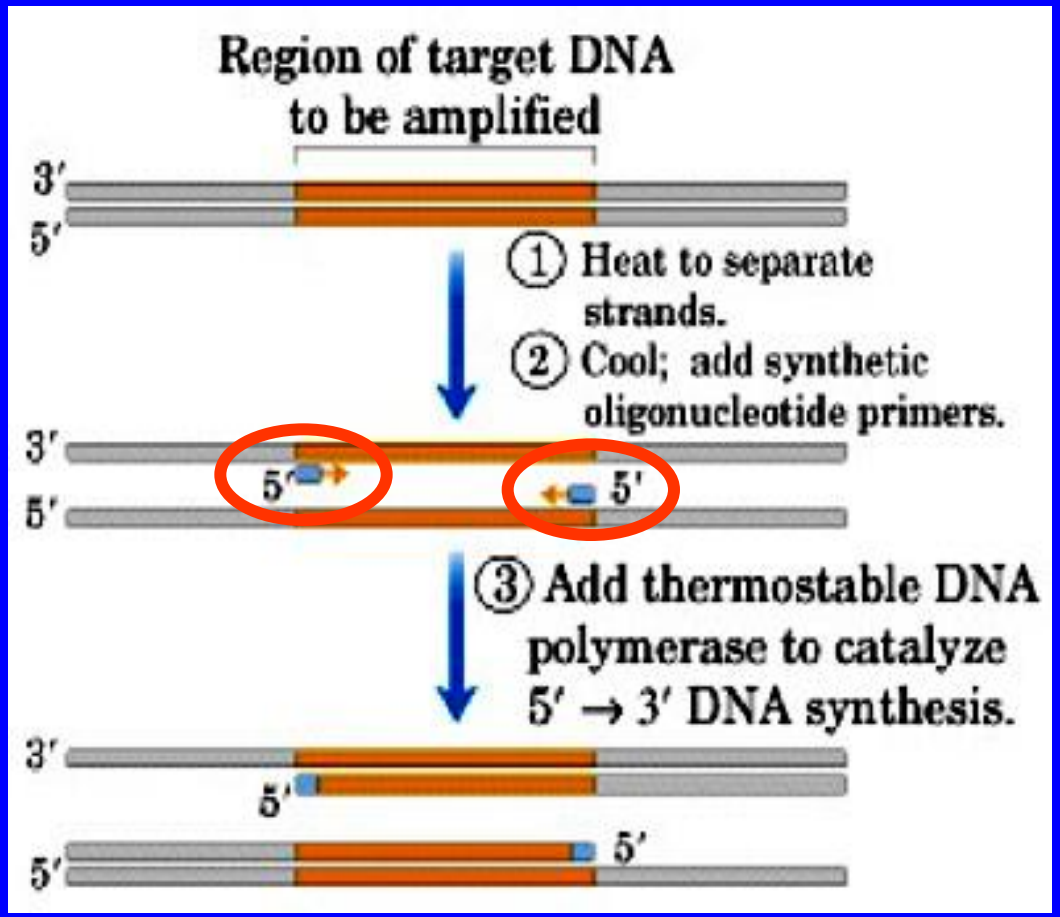


THERMOCYCLER: Rapidly cycles
Denaturation, Annealing, Elongation steps



Polymerase Chain Reaction: principio

CICLI TERMICI:



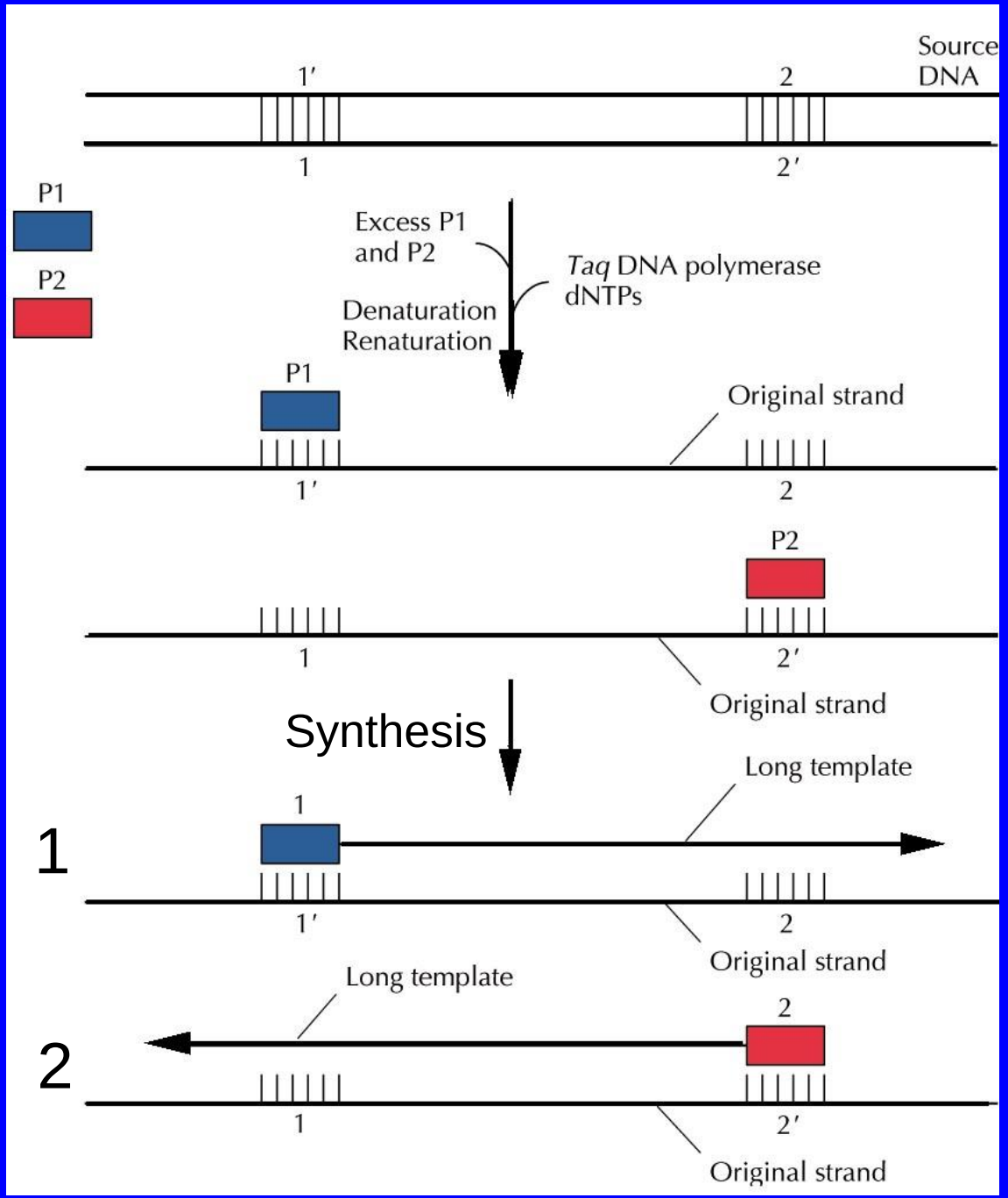
DENATURAZIONE (94-96°C)

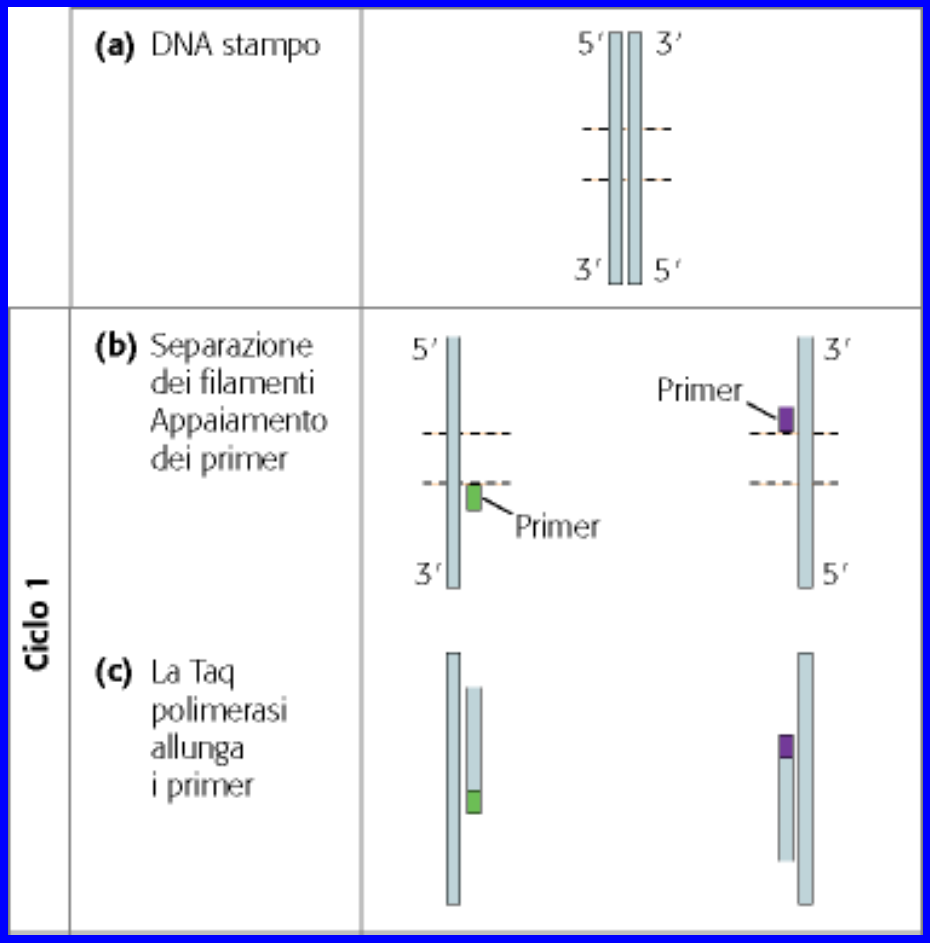
ANNEALING (50-65°C)

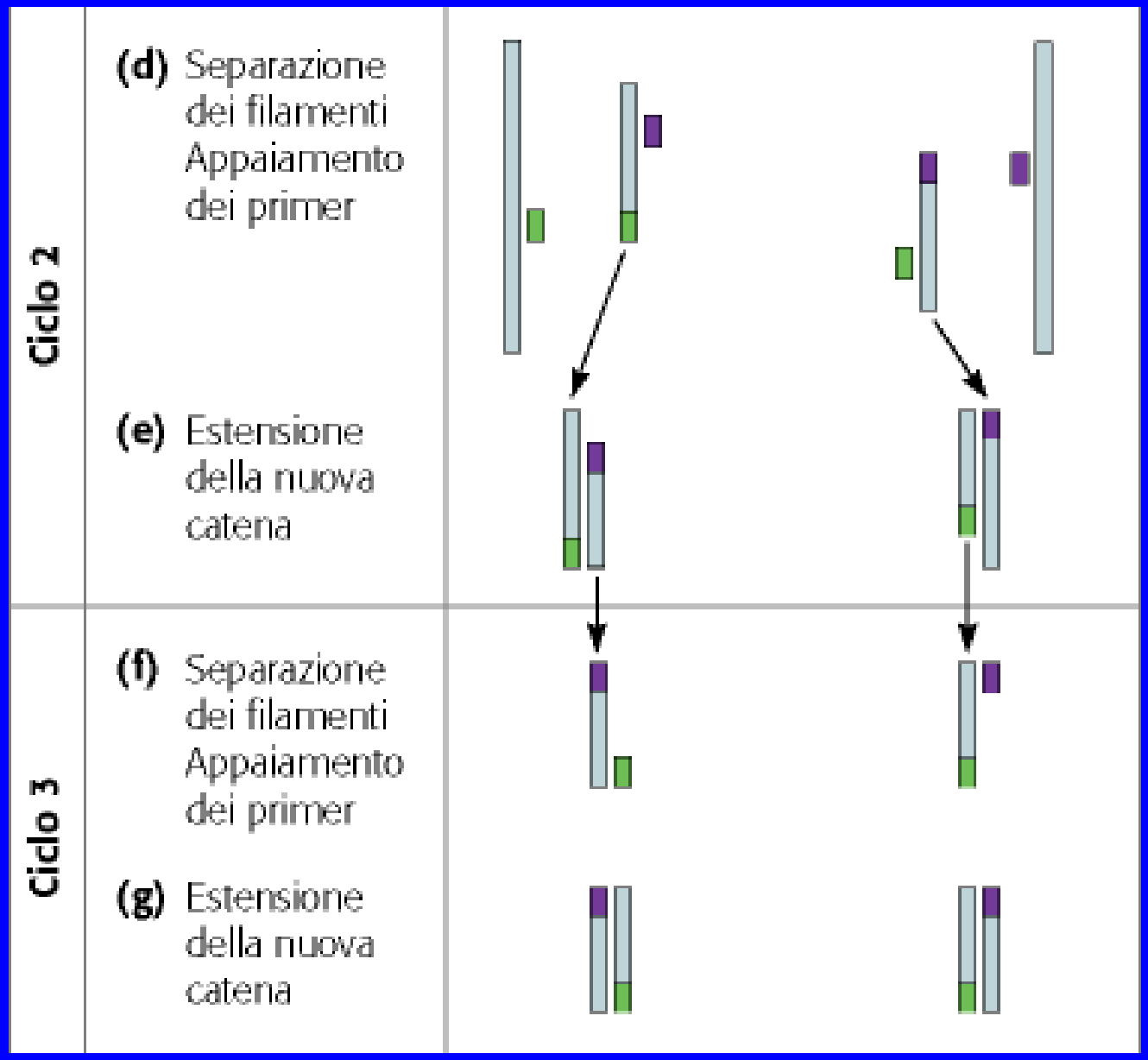
EXTENSION (72°C)

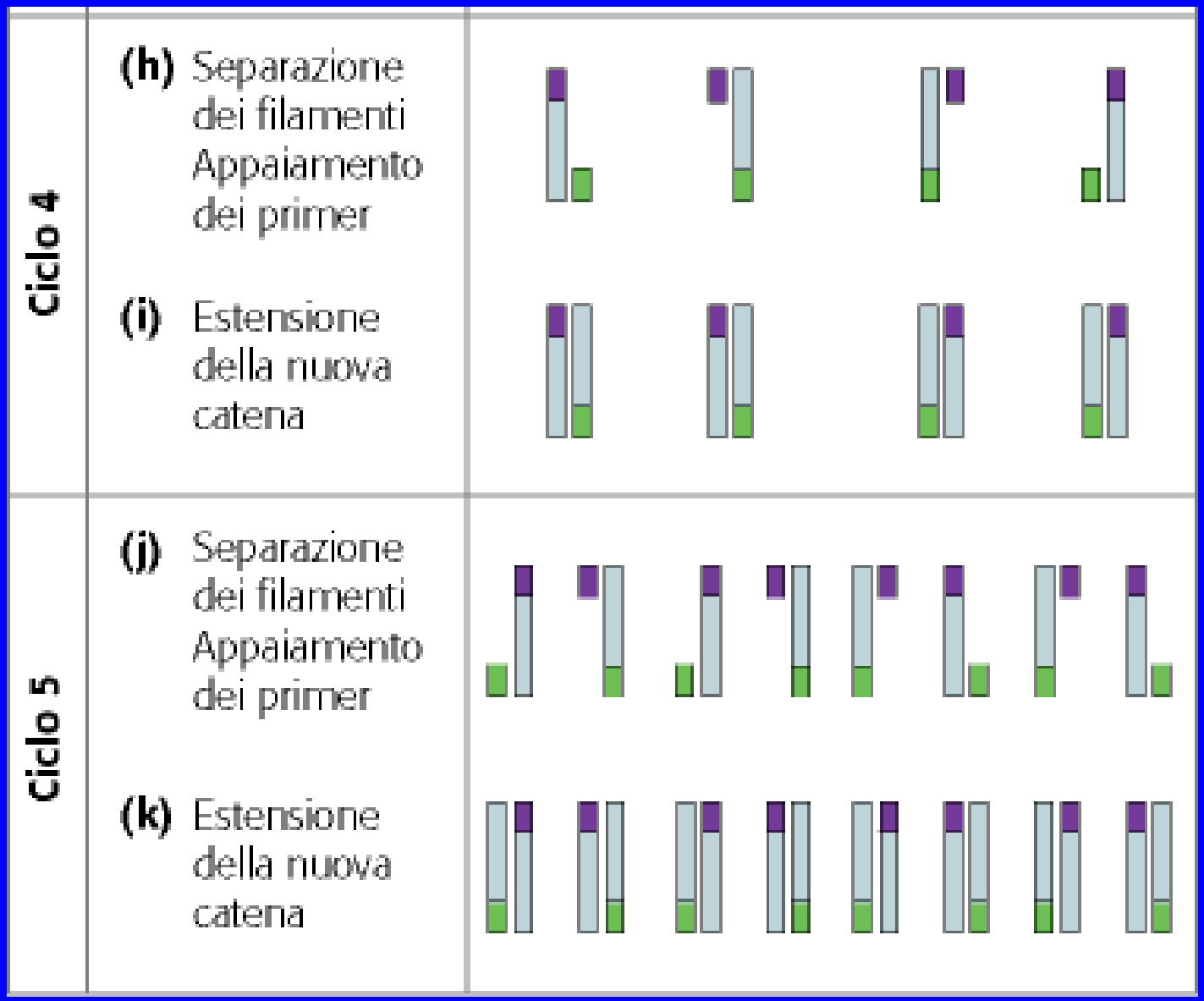
PCR:
1st Cycle

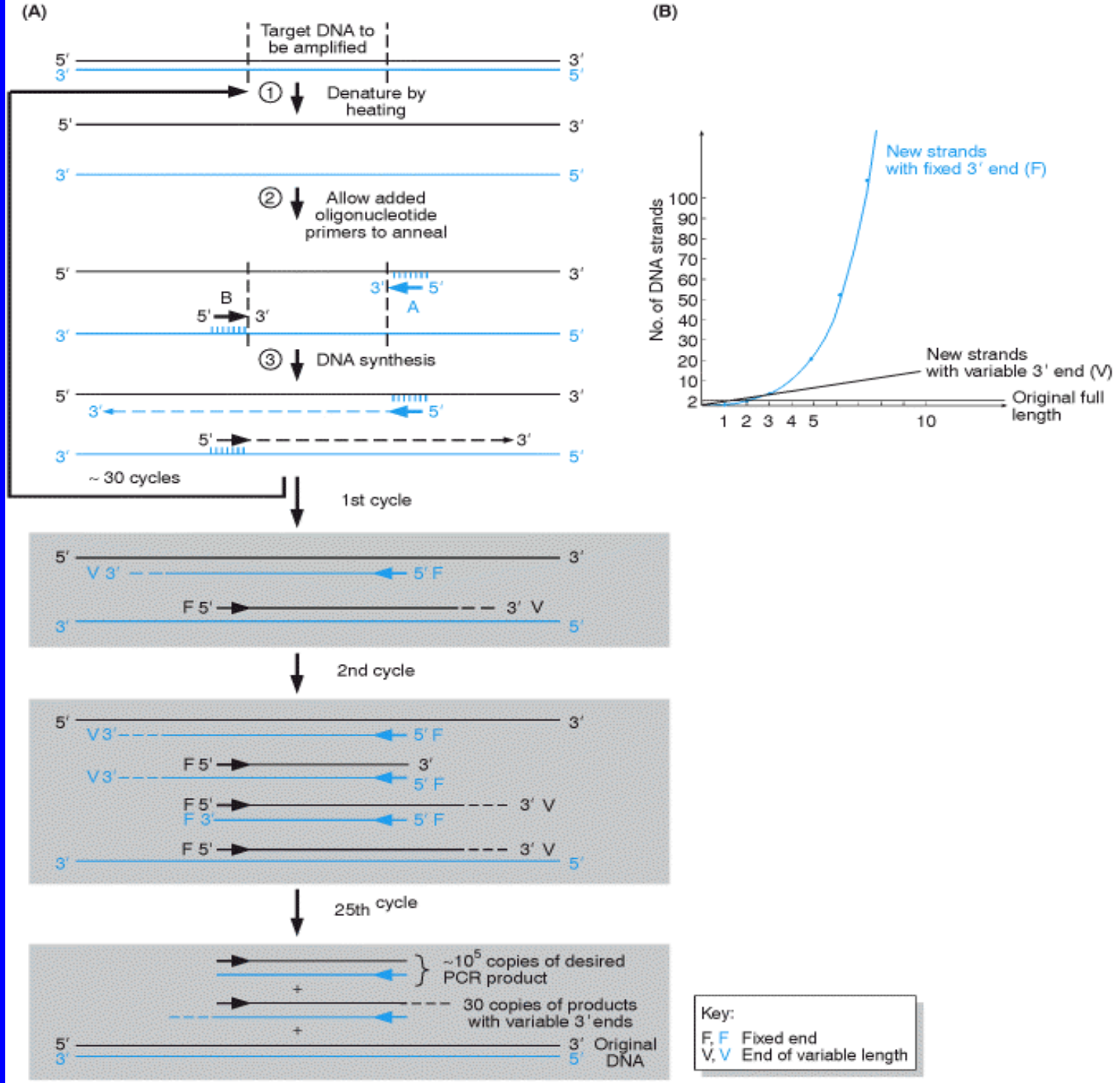
amount of DNA has doubled




(a) DNA stampo
5′
3′
3′
5′
Ciclo 1
(b) Separazione dei filamenti Appaiamento dei primer
5′
Primer
3′
Primer
3′
5′
(c) La Taq polimerasi allunga i primer


Ciclo 2
(d) Separazione dei filamenti Appaiamento dei primer
(e) Estensione della nuova catena
Ciclo 3
(f) Separazione dei filamenti Appaiamento dei primer
(g) Estensione della nuova catena


Ciclo 4
(h) Separazione dei filamenti Appaiamento dei primer
(i) Estensione della nuova catena
Ciclo 5
(j) Separazione dei filamenti Appaiamento dei primer
(k) Estensione della nuova catena


(A)
Target DNA to be amplified
5′
3′
3′
5′
① Denature by heating
② Allow added oligonucleotide primers to anneal
B
A
③ DNA synthesis
~ 30 cycles
1st cycle
V 3′
5′ F
F 5′
3′ V
2nd cycle
25th cycle
~10^5 copies of desired PCR product
30 copies of products with variable 3′ ends
Original DNA
(B)
No. of DNA strands
New strands with fixed 3′ end (F)
New strands with variable 3′ end (V)
Original full length
Key:
F, F Fixed end
V, V End of variable length

PCR: Additional Cycles

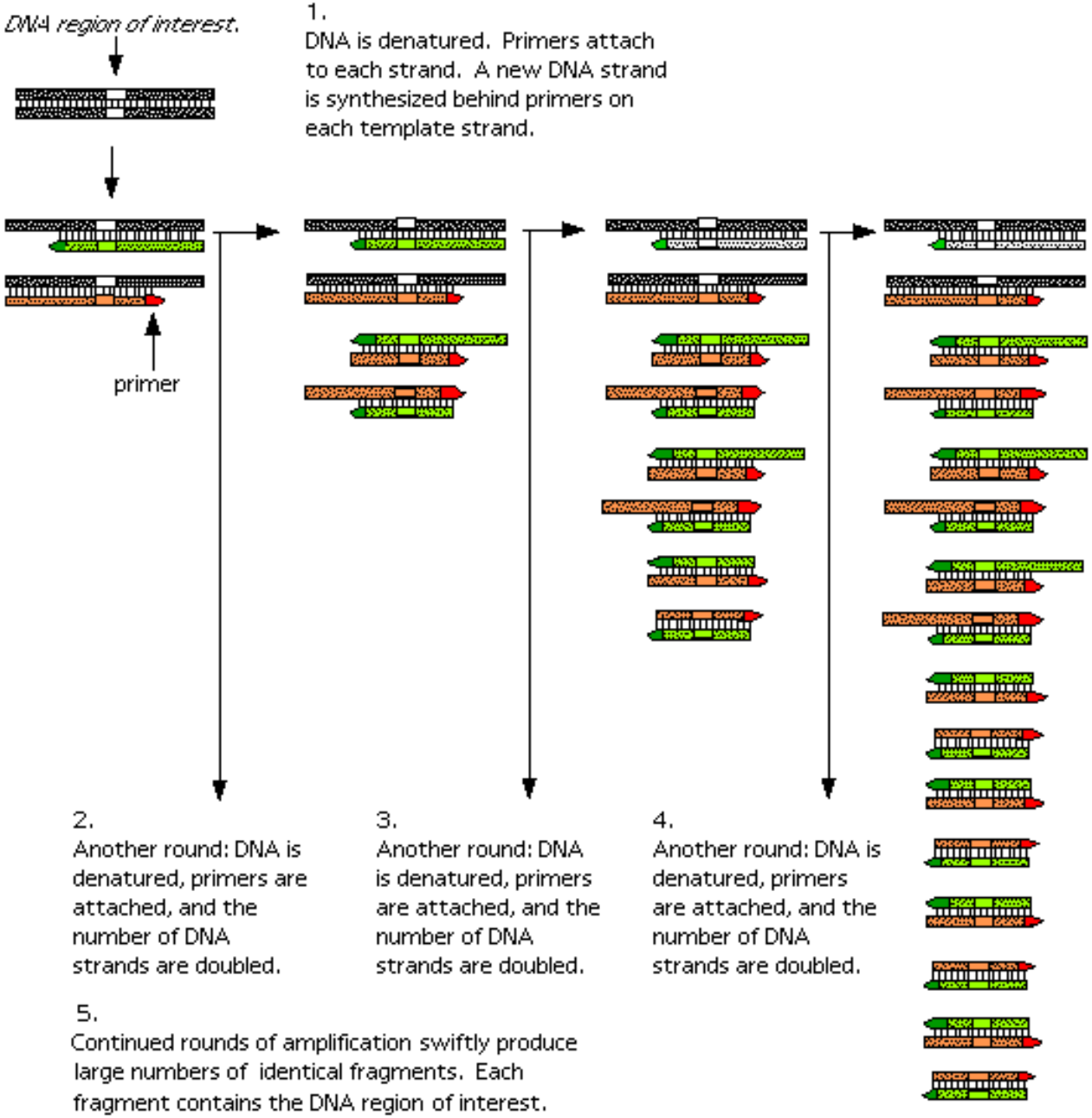
- 2nd Cycle
- again double the number of strands ($4x = 2^2x$)
- 3rd Cycle
- again double the number of strands ($8x = 2^3x$)
- 30th Cycle
- again doubles the number of strands ($2^{30}x$)

PCR is an in vitro method for amplifying DNA sequences using defined oligonucleotide primers.

Oligonucleotide primers A and B are complementary to DNA sequences located on opposite DNA strands and flanking the region to be amplified. Annealed primers are incorporated into the newly synthesized DNA strands.

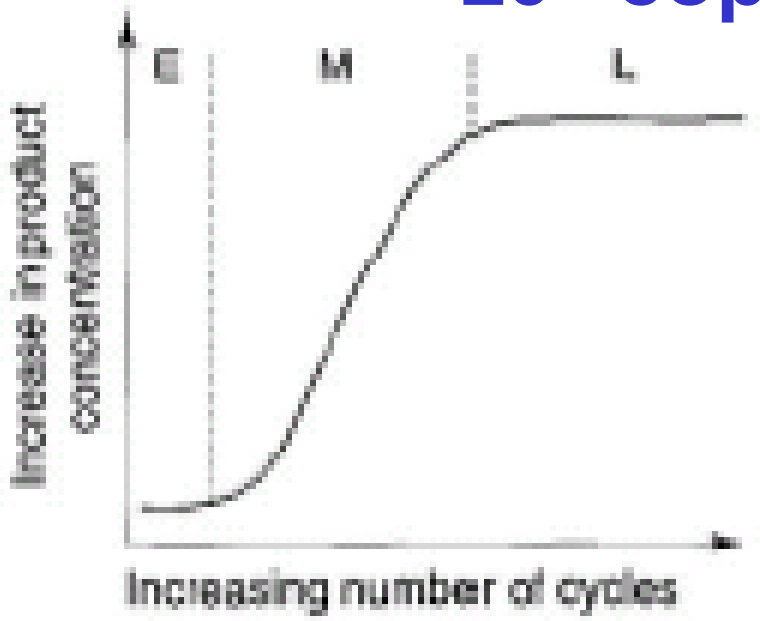
The first cycle will result in two new DNA strands whose 5 end is fixed by the position of the oligonucleotide primer but whose 3 end is variable ('ragged' 3 ends). The two new strands can serve in turn as templates for synthesis of complementary strands of the desired length (the 5 ends are defined by the primer and the 3 ends are fixed because synthesis cannot proceed past the terminus of the opposing primer). After a few cycles, the desired fixed length product begins to predominate.

POLYMERASE CHAIN REACTION



Cycle	Relative amount
1	2
2	4
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1024
11	2048
12	4096
13	8192
14	16384
15	32768
.....	
25	33 554 432
.....	
30	1 073 741 824

10^{12}copies



$$Y = (1 + x)^n$$

X= mean efficiency of a cycle
N= number of cycles
Y= fold amplification

Per Cycle Efficiency (%)	Extent of amplification (20 cycles)	Relative yield
100	1 048 576	1.00
95	631 964	0.60
90	375 900	0.36
85	220 513	0.21
80	127 452	0.12

What are the factors determining the "Plateau Effect" ????

What is limiting?

Template?
Primers?

dNTPs?
Taq Polymerase?

Polymerase Chain Reaction: plateau

Resa effettiva: effetto plateau

Il processo di duplicazione non procede “all’infinito”, esso è limitato da:

- Quantità dei primers
- Attività della Taq polimerasi
- Reannealing dei filamenti

Raggiunto il plateau non si osserva più un incremento nei prodotti

Length	Usually about 20 nt for target sequences in complex genomic DNA; can be much less if target DNA is less complex
Base composition	Substantial tandem repeats of one or more nucleotides to be avoided. Overall %GC plus length to be chosen so that the T_m of each oligonucleotide (*Table 5.2*) should be equal or nearly identical
Secondary structure	Avoid sequences prone to secondary structure which could form hairpins etc. (see *Figure 1.7A*)
3′ end	Base complementarity of the two bases at the extreme 3′ end of the two primers to be avoided. Otherwise primer dimers can result, reducing amplification efficiency

PCR primer design

Designing PCR Primers

- Primers should be ~20 bases long.
- The G/C content should be 45–55%.
- The annealing temperatures should be within 1°C of one another.
- The 3′-most base should be a G or C.
- The primers must not base pair with each other or with themselves or form hairpins.
- Primers must avoid repetitive DNA regions.

Primers That Form Hairpins

- A primer may be self-complementary and be able to fold into a hairpin:

```
5′-GTTGACTTGATA
        ||||| T
    3′-GAACTCT
```

- The 3′ end of the primer is base-paired, preventing it annealing to the target DNA.

Primers That Form Dimers

- A primer may form a dimer with itself or with the other primer.

```
5′-ACCGTAGCCACGAATTCGT-3′
          ||||||||||
      3′-TGCTTAAGCACCGATGGCCA-5′
```

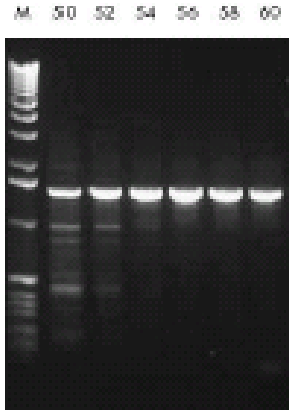
- Primer dimers can be an excellent, but unwanted, substrate for the Taq polymerase.

Help With Primer Design

- Researchers agreed early on that the design of PCR primers was difficult and unreliable.
- Computer programs devised to take all of the design criteria into account.
- Primer3 program at the Whitehead Institute is the most reliable and versatile tool currently available.

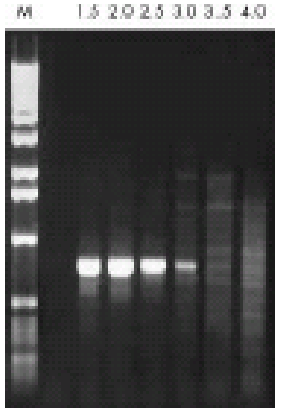
Optimising the Annealing Temperature

- Primers have a calculated annealing temperature (*e.g.* 54°C).
- Temperature must be confirmed practically.
- Temperature steps of 2°C above and below.
- Use gradient cycler.



Optimising the Mg^{2+} Concentration

- The fidelity of the PCR depends on $[Mg^{2+}]$.
- Vary $[Mg^{2+}]$ in steps of 0.5 mM.
- Sometimes a compromise between yield and specificity.



Fidelity of the Reaction

- *Taq* DNA polymerase lacks the 3′→5′ proof-reading activity commonly present in other polymerases.
- *Taq* mis-incorporates 1 base in 10^4.
- A 400 bp target will contain an error in 33% of molecules after 20 cycles.
- Error distribution will be random.

Do Errors Matter?

- Yes, if you want to clone the amplified DNA — an individual molecule may harbour several mutations.
- No, if you want to sequence the amplified DNA or cut it with restriction enzymes.
- Use a proof-reading thermo-stable enzyme rather than *Taq*.

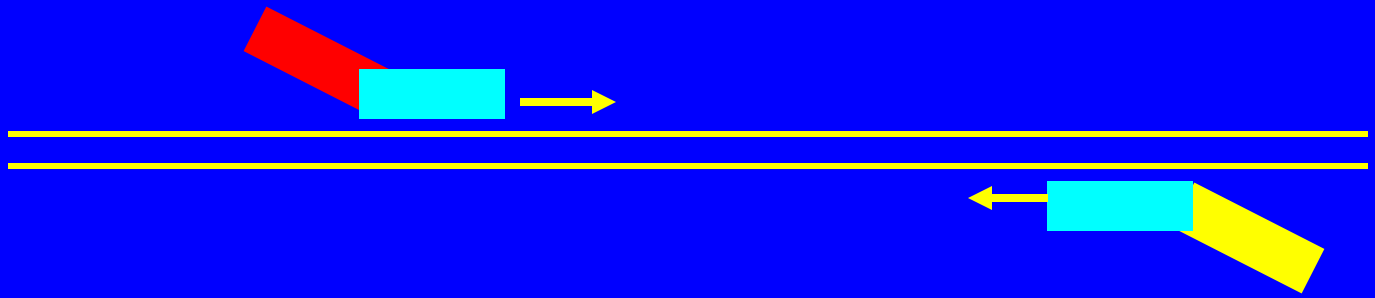
Table 2. Reported Fidelity for Several Different Thermal Stable DNA Polymerases Using the Forward Mutation Assay (1).		
DNA Polymerase	**Error Rate x 10^{-6}**	**Accuracy x 10^{5}**
Pfu	1.3 ± 0.2 S.D.	7.7
Deep $Vent_R$™	2.7 ± 0.2 S.D.	3.7
Tli ($Vent_R$®)	2.8 ± 0.9 S.D.	3.6
Taq	8.0 ± 3.9 S.D.	1.3
UlTma®	55.3 ± 2.0*	0.2
**Range of duplicates.*		

PCR Applications

- All aspects of scientific research
  - cycle sequencing (Fig 5.27)
  - mutation detection
  - gene assembly (Fig 5.26)
  - generation of specific mutations
  - pathogen detection
  - routine molecular biology - cloning
  - etc.

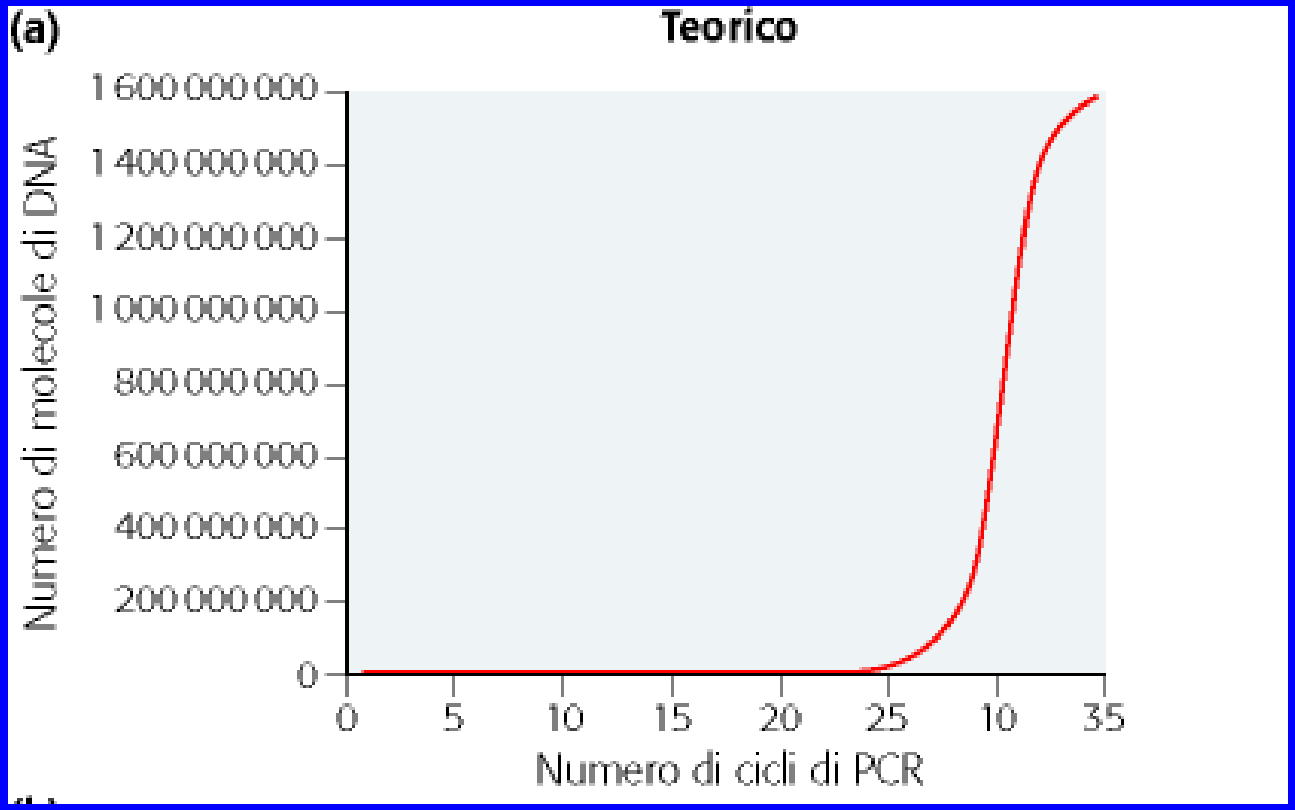
PCR Application

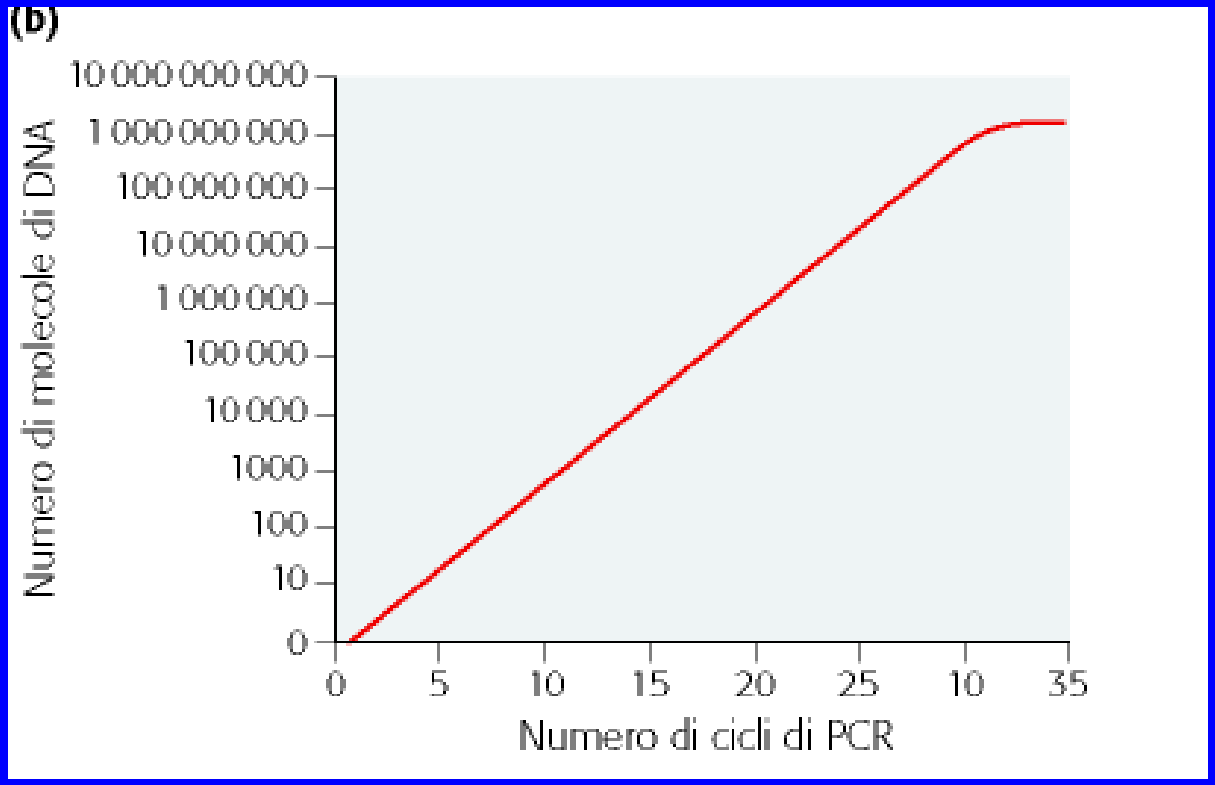
- primers for PCR may have additional sequences at the 5’ end
- not necessary for those bases to pair with template

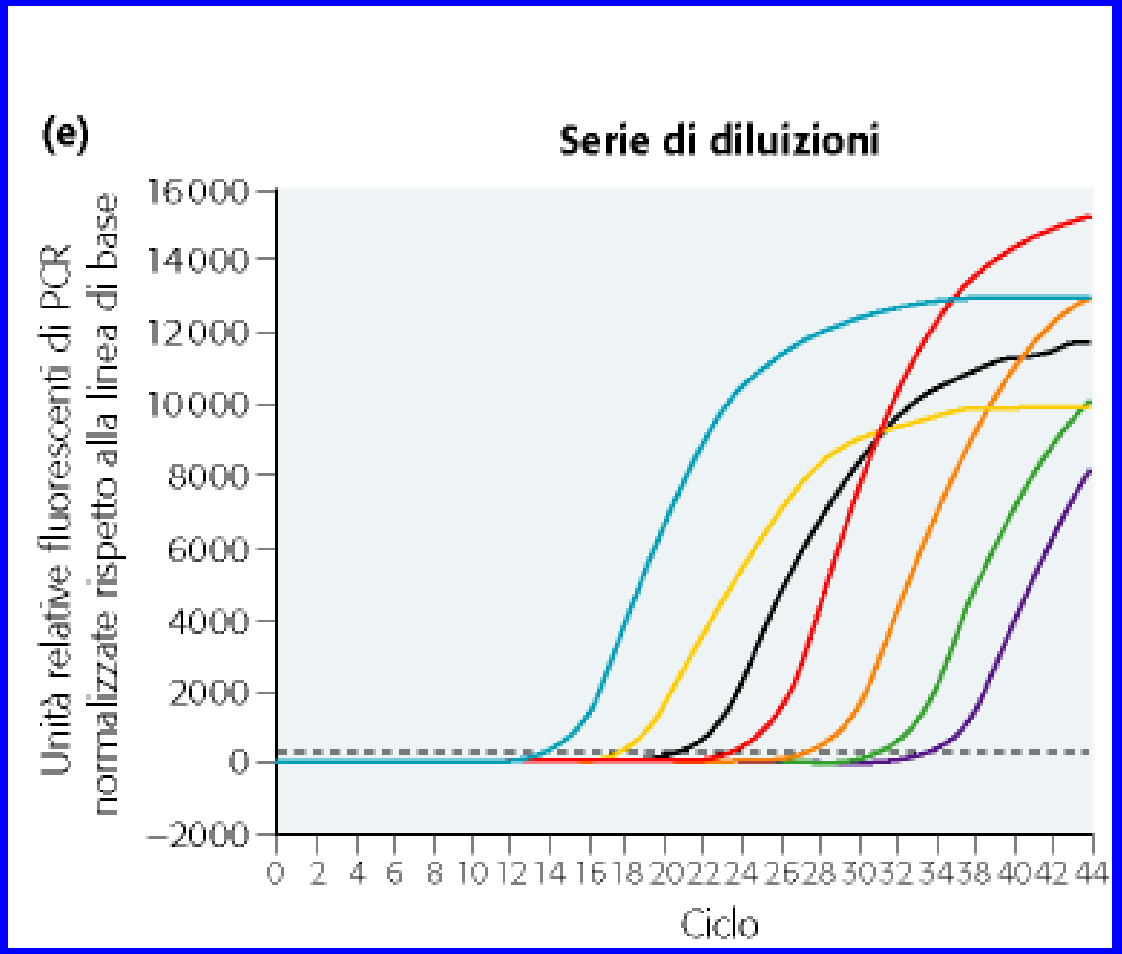


final product

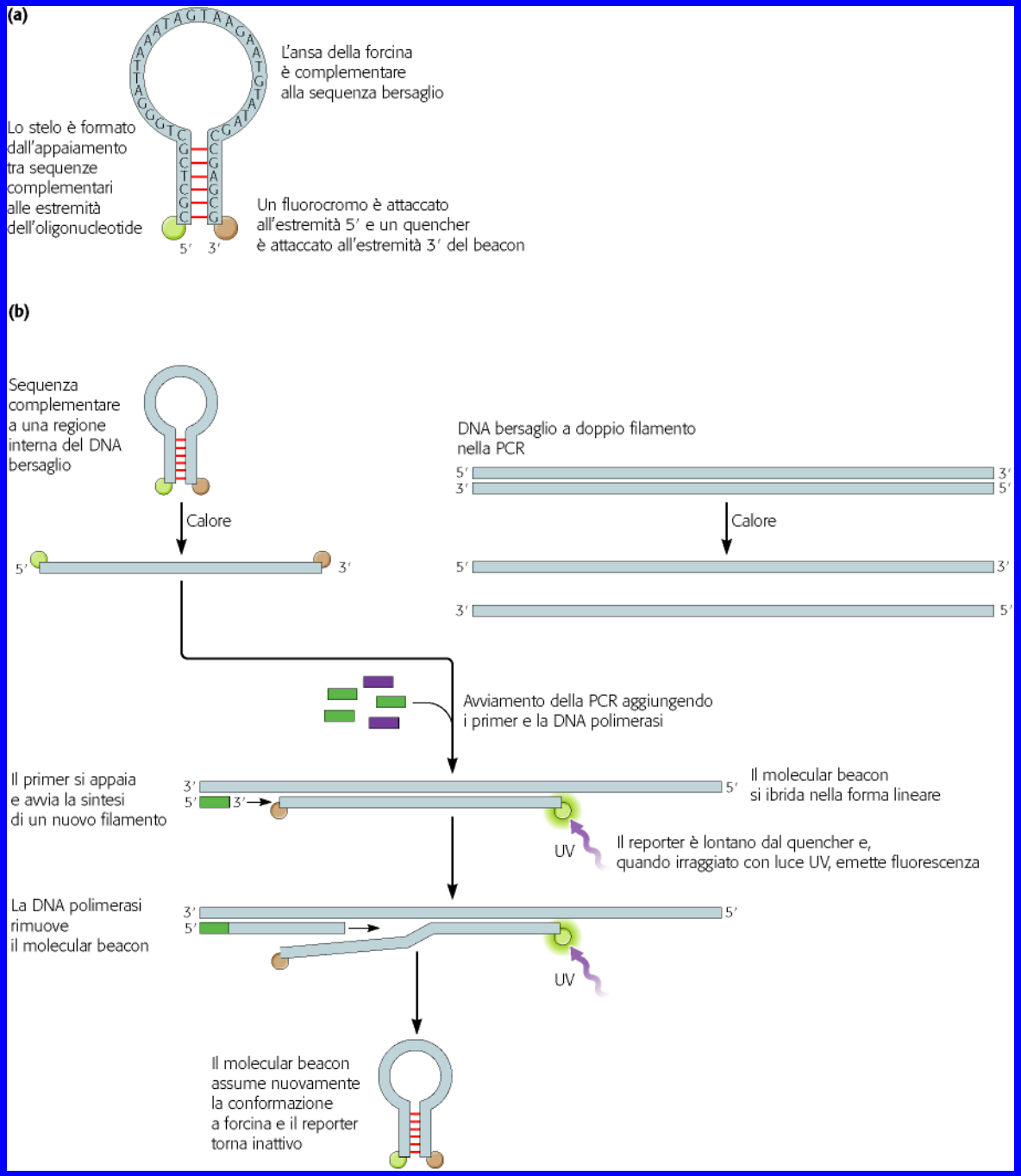
Quantitative aspects of PCR


(a)
Teorico
1 600 000 000
1 400 000 000
1 200 000 000
1 000 000 000
800 000 000
600 000 000
400 000 000
200 000 000
0
Numero di molecole di DNA
0
5
10
15
20
25
10
35
Numero di cicli di PCR

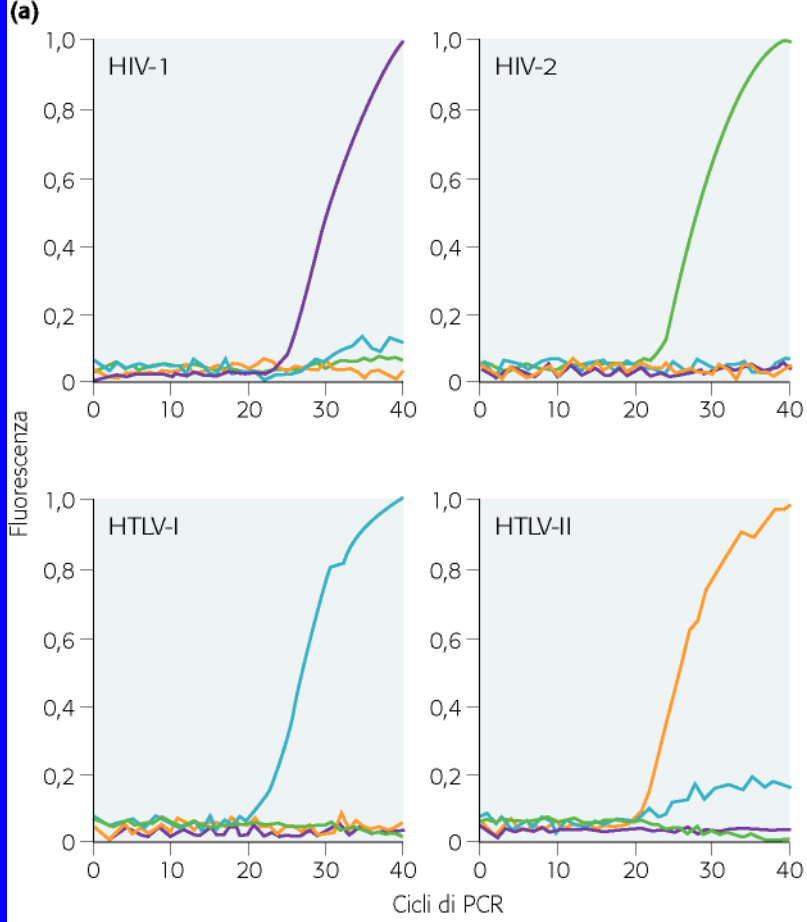

(b)
Numero di molecole di DNA
10 000 000 000
1 000 000 000
100 000 000
10 000 000
1 000 000
100 000
10 000
1000
100
10
0
0
5
10
15
20
25
10
35
Numero di cicli di PCR


(e)
Serie di diluizioni
Unità relative fluorescenti di PCR normalizzate rispetto alla linea di base
16000
14000
12000
10000
8000
6000
4000
2000
0
−2000
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 38 40 42 44
Ciclo

Molecular beacon



Quantification of many genes in a single Real-time PCR (use of specific probes)



Real-Time PCR: applicazioni

Viral Quantitation

Quantitation of Gene Expression

Array Verification

Drug Therapy Efficacy

DNA Damage measurement

Quality Control and Assay Validation

Pathogen detection

Genotyping

Mutagenesis mediated by PCR

Gene Sequencing

DNA Sequencing

- Extremely important technique for understanding organisms
- Can now determine sequence of whole genomes - including human

- Technique is based on enzymatic DNA synthesis using dideoxynucleotides

DNA Sequencing - Sanger Method

- Reaction mix:
  - DNA Polymerase
  - Primer
  - Template
  - 2’ deoxynucleotides (dNTP)
  - 2’,3’ dideoxynucleotides (ddNTP) also used
  - Mix of dNTPs and ddNTP (at lower conc.)

Fig 5.14 Deoxy- and dideoxynucleotides

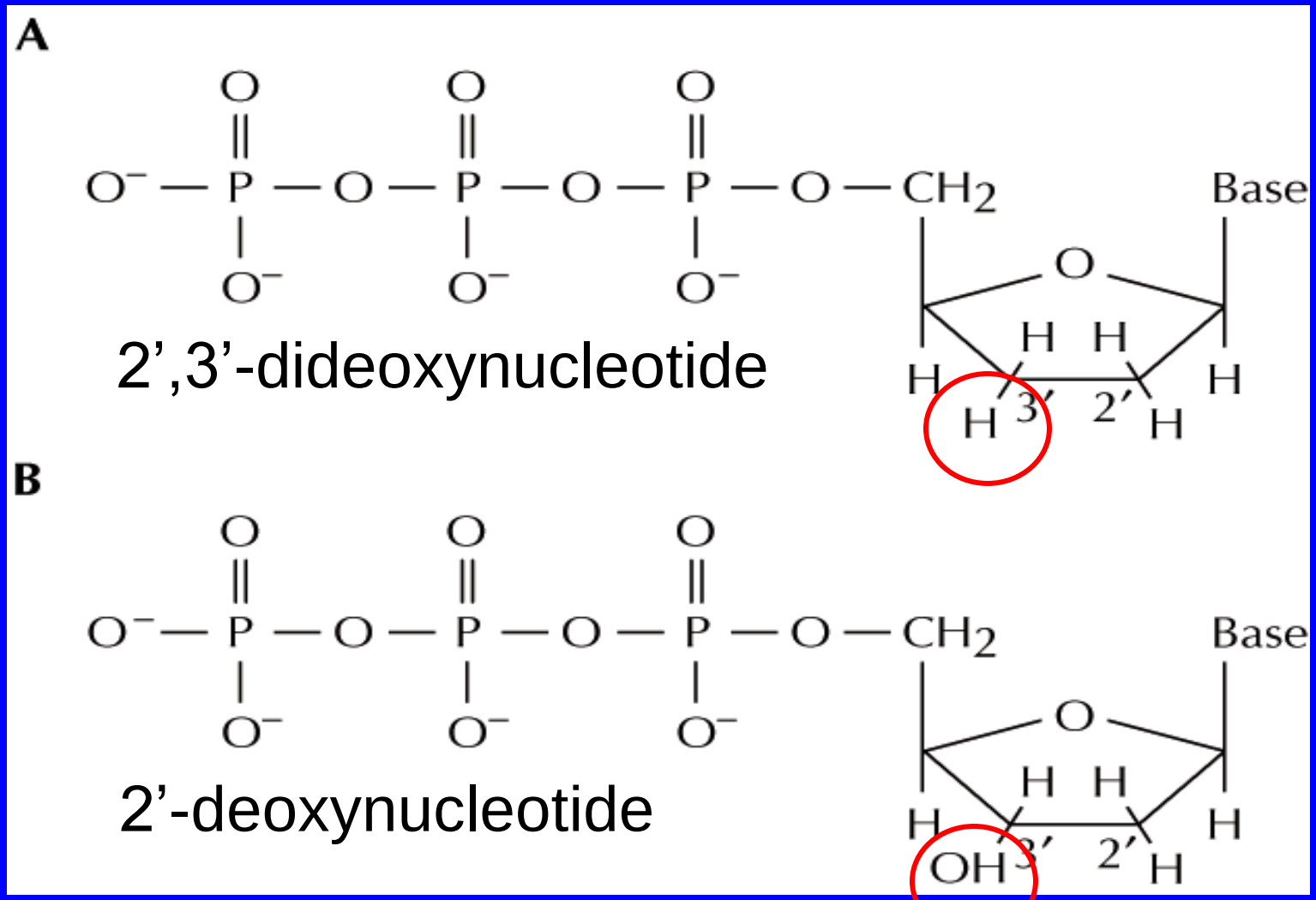


Fig 5.15 Normal DNA Synthesis

Link forms between 3'OH and 5' PO_4

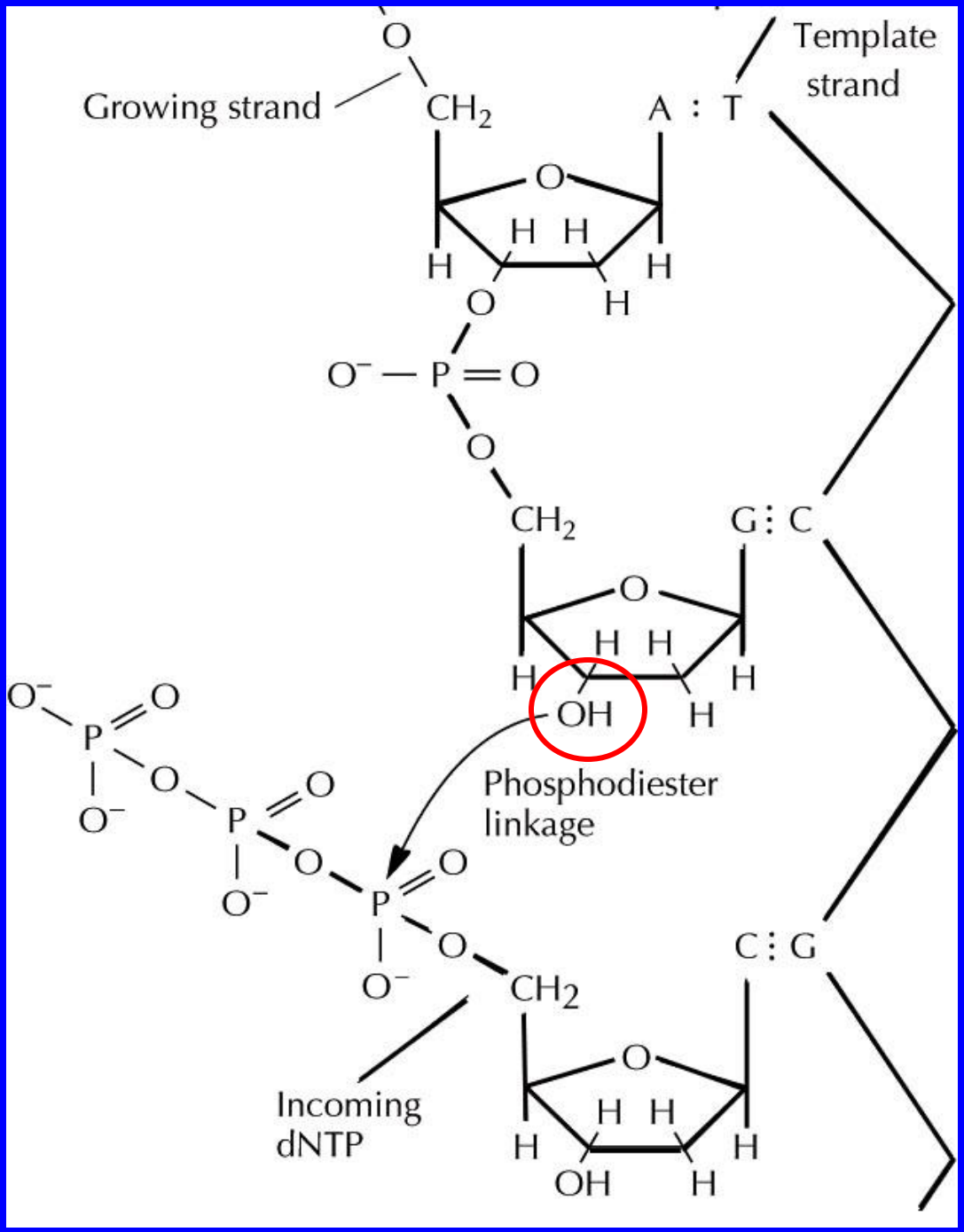
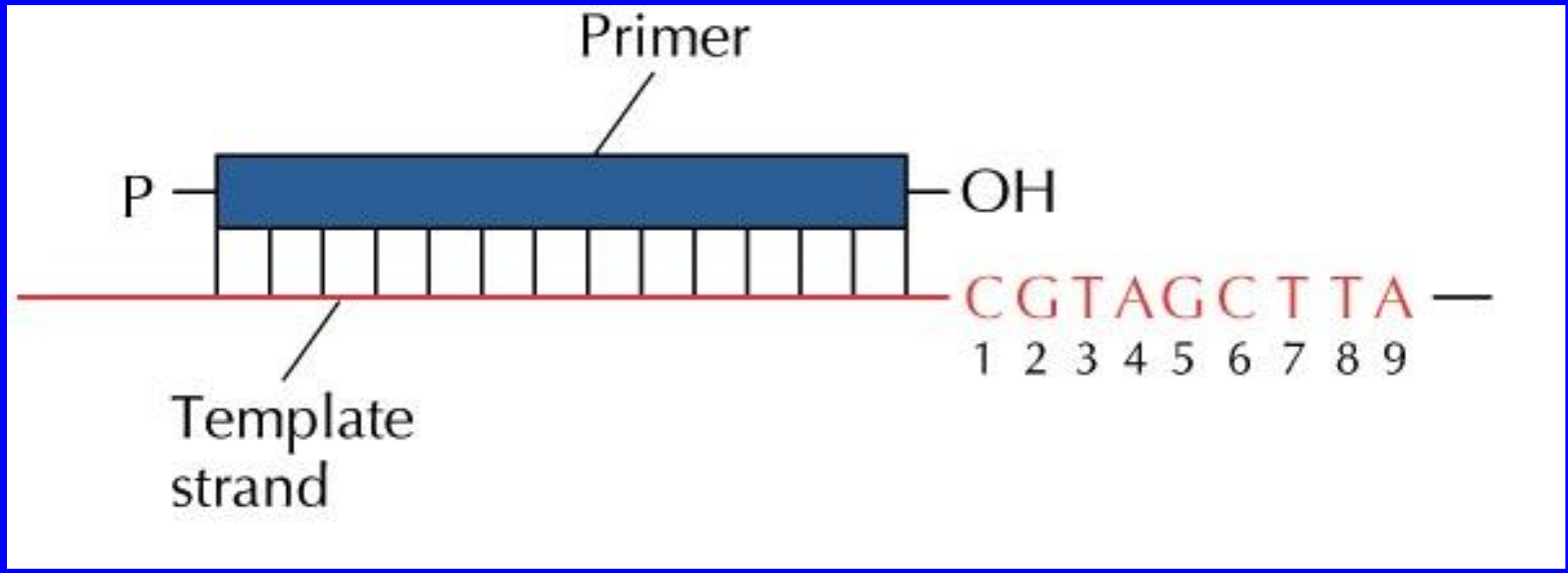


Fig 5.17a Primer Extension during DNA synthesis



ds DNA template is denatured
temperature lowered to allow basepairing of primer

Fig 5.17b Primer extension during DNA synthesis

Contents of reaction tube	Size of primer and extension	Primer and sequence of extension
ddATP + four dNTPs	Primer + 3	Primer – dGdCddA
	Primer + 7	Primer – dGdCdAdTdCdGddA
	Primer + 8	Primer – dGdCdAdTdCdGdAddA
ddCTP + four dNTPs	Primer + 2	Primer – dGddC
	Primer + 5	Primer – dGdCdAdTddC
ddGTP + four dNTPs	Primer + 1	Primer – ddG
	Primer + 6	Primer – dGdCdAdTdCddG
ddTTP + four dNTPs	Primer + 4	Primer – dGdCdAddT
	Primer + 9	Primer – dGdCdAdTdCdGdAdAddT

DNA Sequencing

Primer

GATCACTGTCGACGTAATCGGCATTGCAACGT

Template

Polymerase extends primer until a ddNTP is incorporated

CTAGTGACAGCTGCA
GATCACTGTCGACGTAATCGGCATTGCAACGT

CTAGT
GATCACTGTCGACGTAATCGGCATTGCAACGT

CTAGTGACAGCTGCATTAGCCGTAAGCTTG
GATCACTGTCGACGTAATCGGCATTGCAACGT

CTAGTGACTGCAGCATTAGCC
GATCACTGTCGACGTAATCGGCATTGCAACGT

DNA Sequencing

- Separate fragments
  - Denaturing polyacrylamide gel
  - Higher resolution than agarose
- Detect fragments
  - Fluorescent dye on ddNTP
  - radioactive label incorporated

Fig 5.18
Simulated autoradiograph of DNA sequencing gel

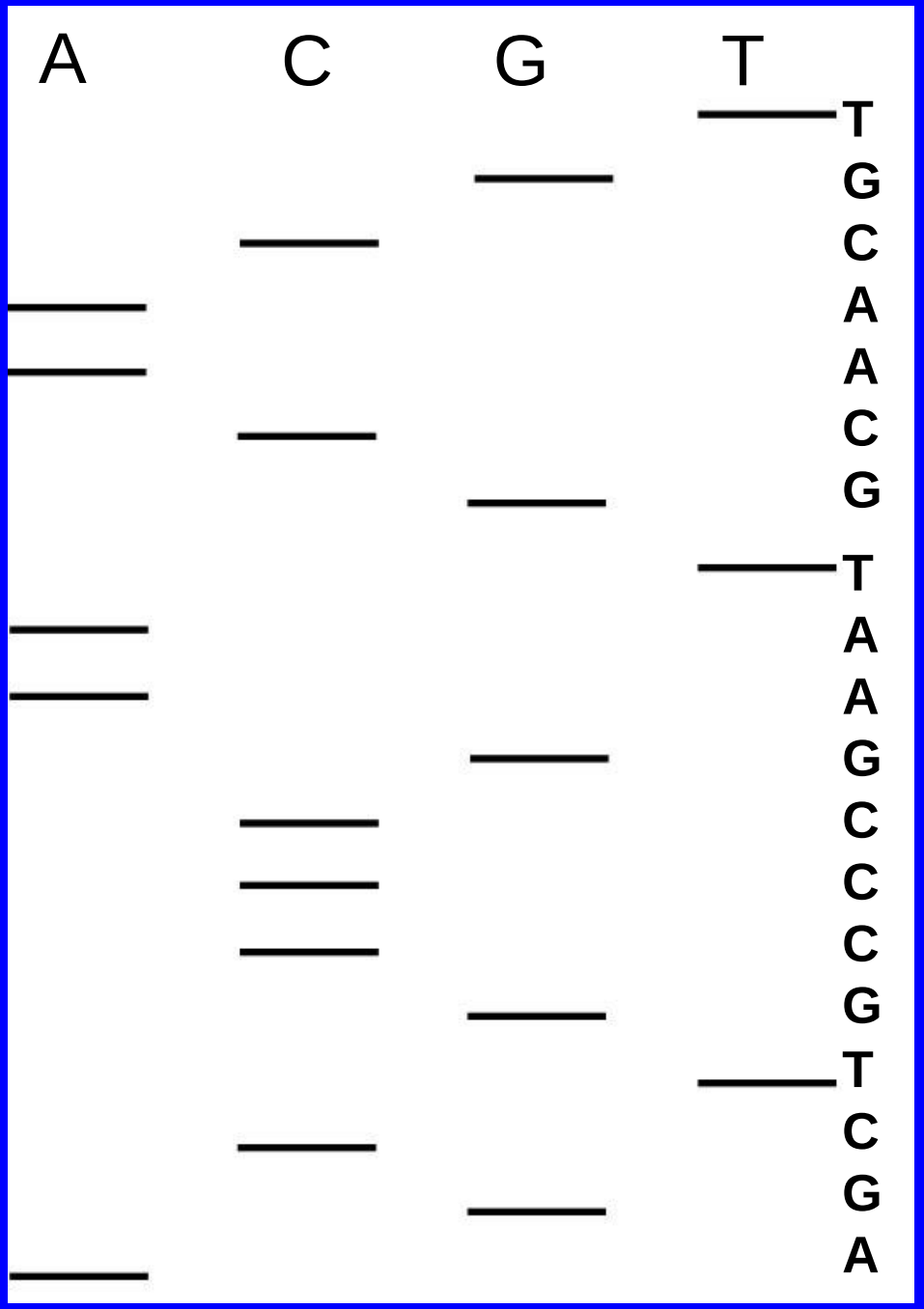
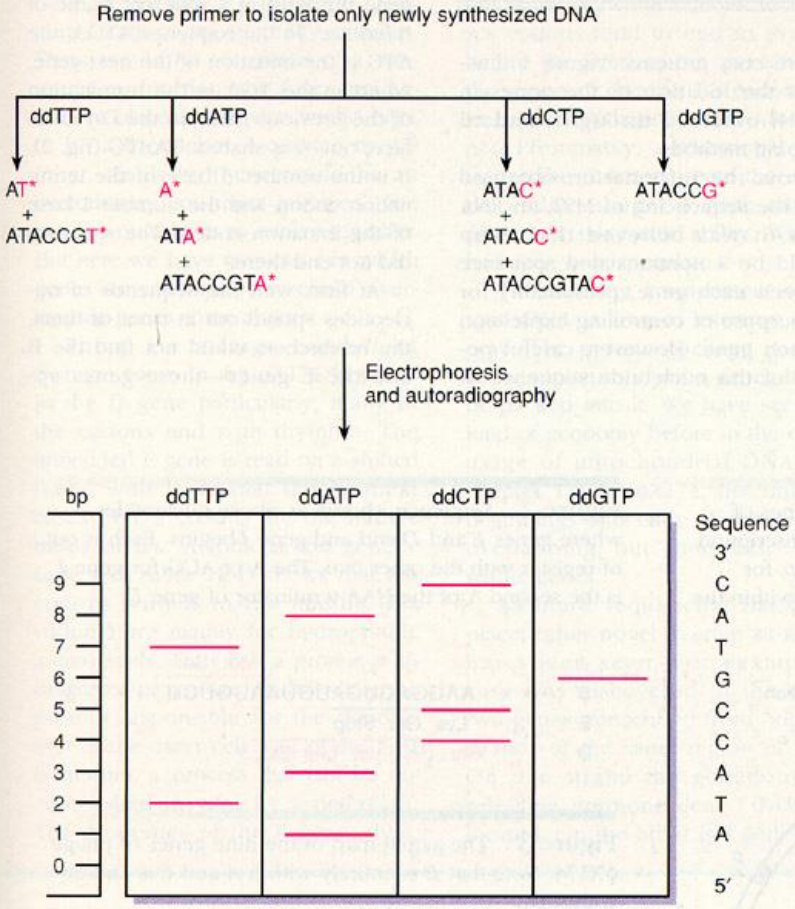


Figure 12.36 Electrophoresis of segments produced by the dideoxy method of DNA sequencing that allows direct reading of the sequence. The *asterisks* indicate the dideoxynucleotides. The newly synthesized reaction products seen in figure 12.35 are isolated by removal of the primer and template. Each reaction mixture (e.g., ddTTP is the mixture containing dideoxythymines) produces specific products of specific lengths that can be determined by electrophoresis. In the case of the ddTTP mixture, two fragments ending in thymine are possible; one is two bases long, the other seven bases long. Thus, the complement of thymine, adenine, appears in positions 2 and 7 of the original piece of DNA. However, either the original strand or its complement (new synthesis) gives the original sequence since DNA is a double helix in which the sequence in one strand is defined by the complementary sequence in the other strand.



Automated Fluorescence Sequencing (See Fig 5.19)