Introduction to hybridisation and related techniques

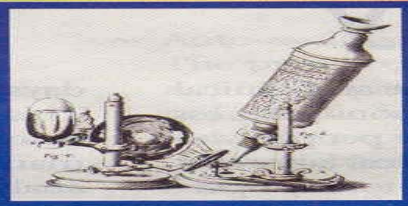
M. RAMAEL
DEPT. PATHOLOGY
St. ELISABETH HOSPITAL HERENTALS

The History of Laboratory Medicine (up to the end of the 19th century)

700 B.C. – 1600 A.D.

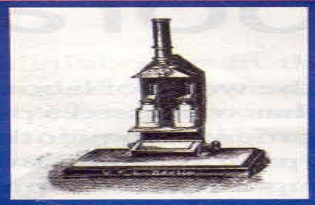
Theory of the four humours
(Blood. phlegm, yellow bile and black bile)
Humoral pathology
Visual aspects of blood and urine

– 1800



Corpuscular theory,
Theory of blood "sickness"
Invention of the microscope,
Discovery of blood cells and bacteria,
Qualitative chemical blood analysis

–1900



Cell pathology
Fixing and staining
Differentiation of cells and bacteria
Bacterial cultures
Quantitative chemical blood analysis

The History of Laboratory Medicine (20th Century)

– 1920

Colorimetry
Photometry
Counting Chambers

Quantification of metabolites, electrolytes, metal ions and blood cells.

– 1960

Enzymology, Blood groups, Separation techniques:

Quantification of enzyme activity and metabolism. Transfusion medicine.

– 1980

Immunoassays, HPLC Automation:

Quantification of plasma proteins, hormones. Serology of infectious disease

– 2000

PCR, FACS, Sequencers Mass spectrometry, Data Processing:
Genotyping
Virus load determination
Immunohaematology
Drug monitoring
Online analysis

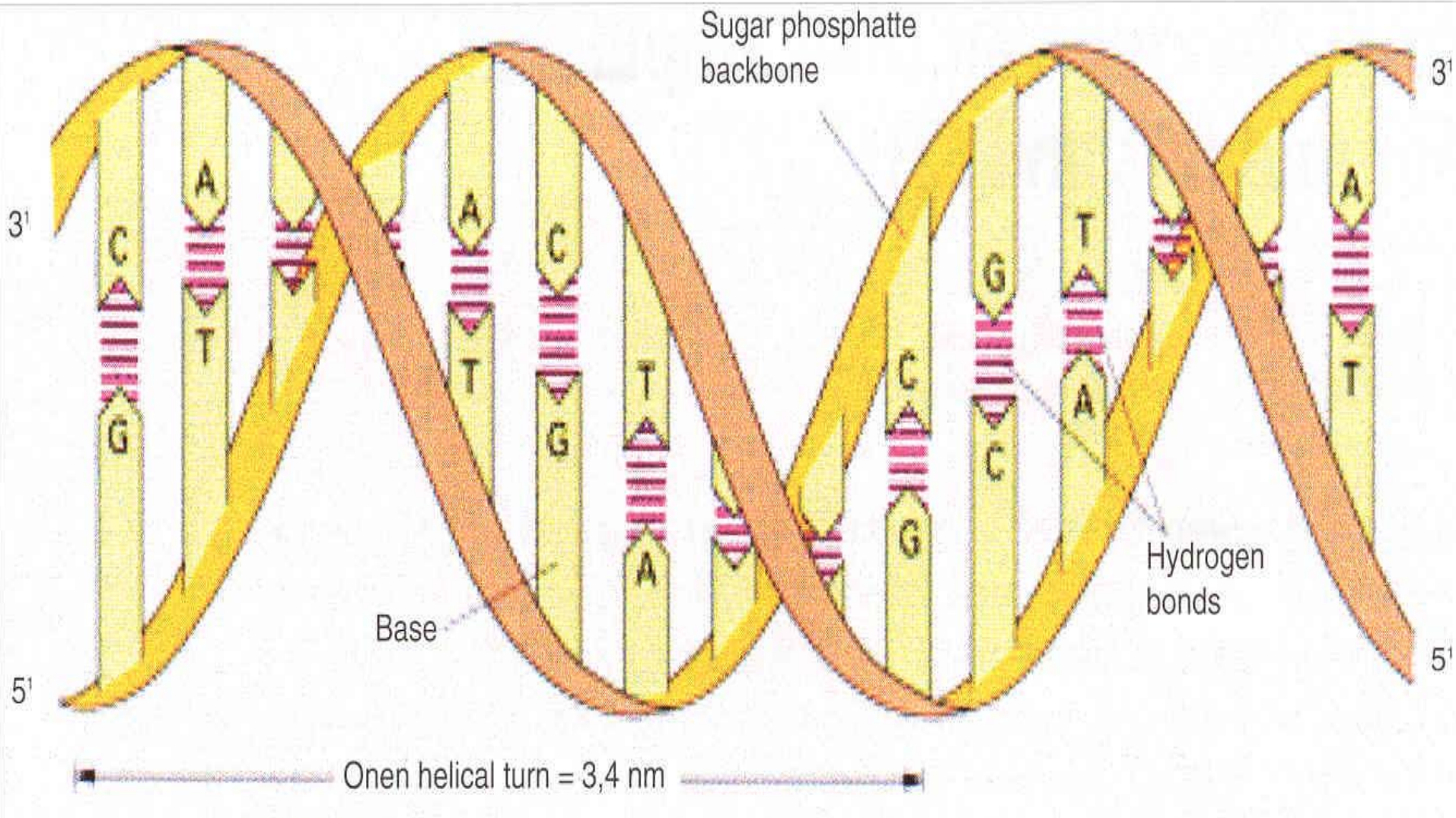
The Future of Laboratory Medicine (21st Century)

'Molecular Medicine'

Sequencing of the human genome
(Bio-) Informatics, Robotics,
Semi-conductor technologies (Chips)
Tandem-Mass spectrometry

Genomics
Proteomics
Lab-on-a-chip

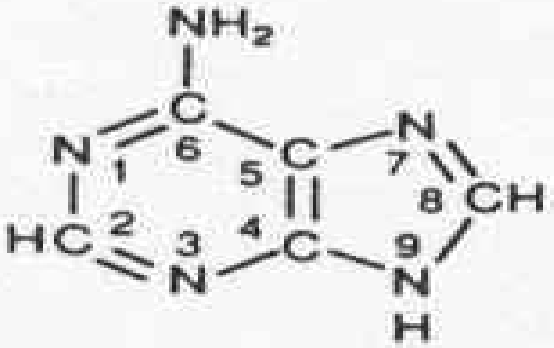
DNA STRUCTURE



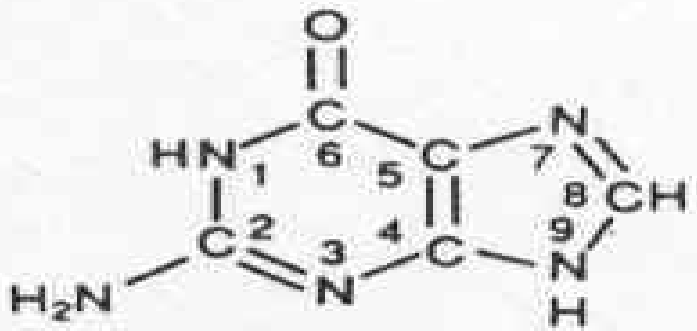
Components of DNA

- Deoxyribose
- Bases:
  - adenine
  - guanine
  - cytosine
  - thymidine

PURINES

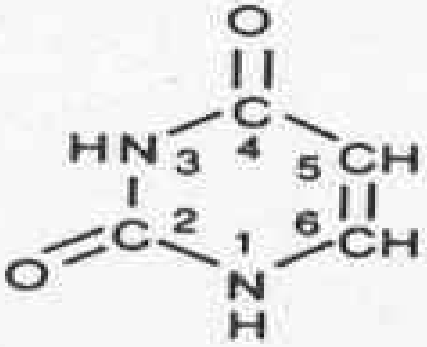


Adenine (A)

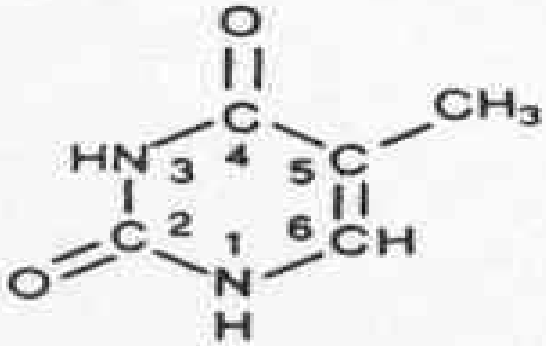


Guanine (G)

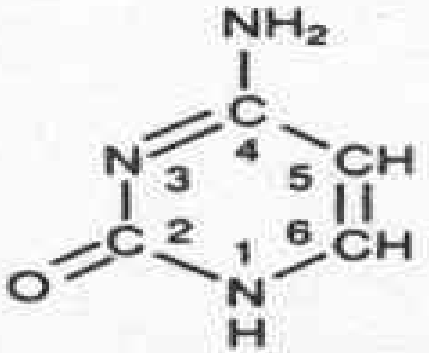
PYRIMIDINES



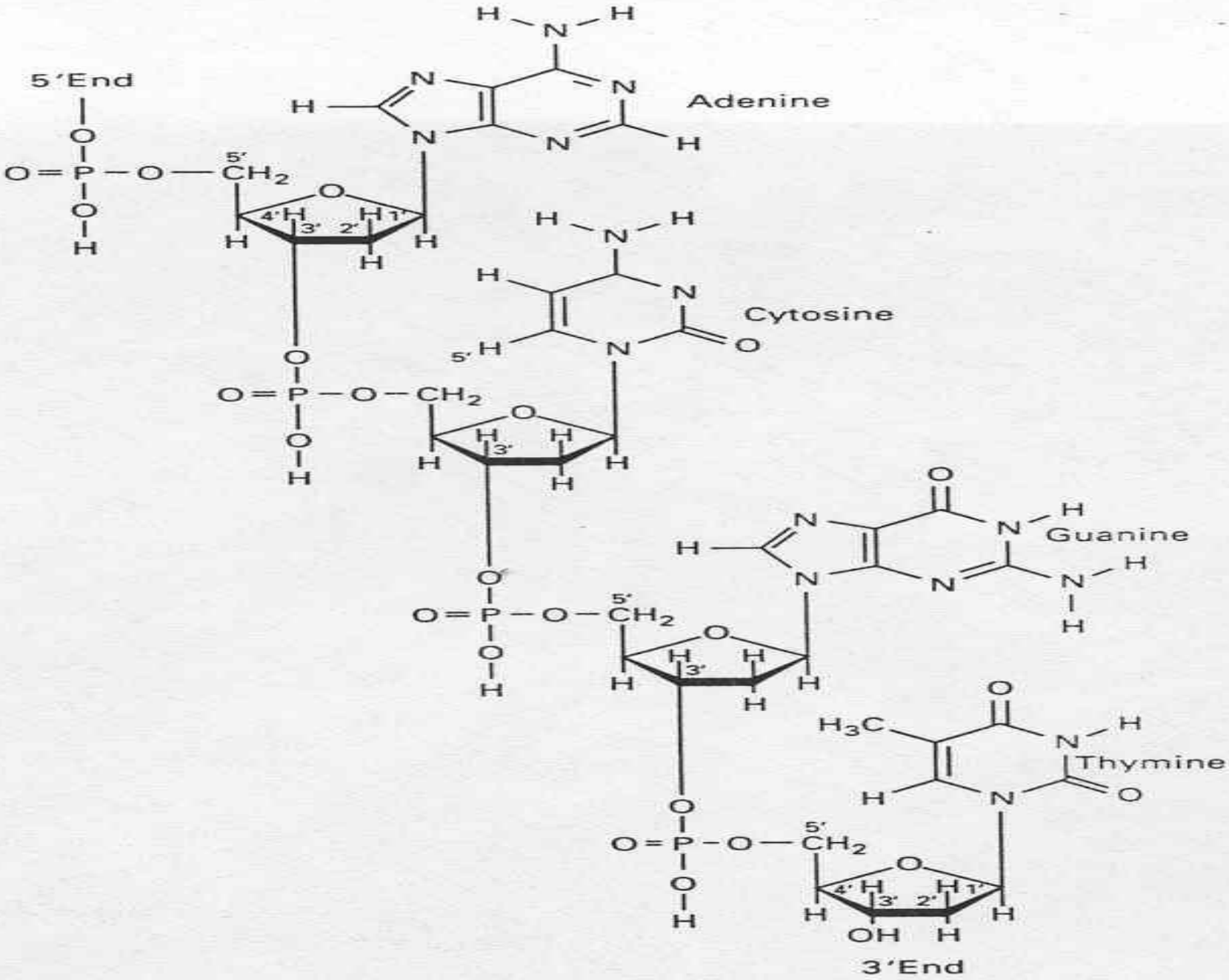
Uracil (U)



Thymine (T)



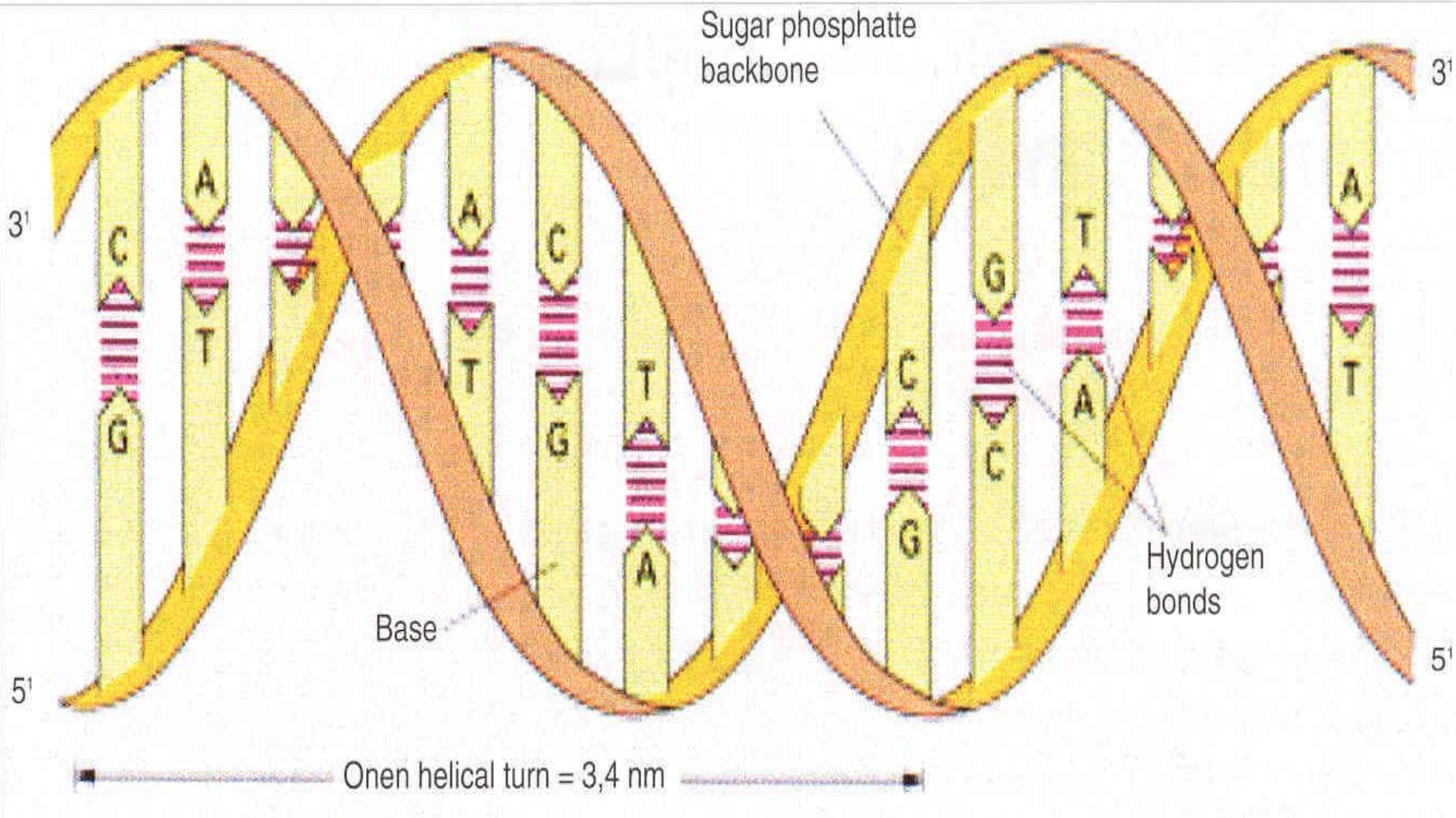
Cytosine (C)


5′End
Adenine
Cytosine
Guanine
Thymine
3′End

Double Helix DNA

- Back-bone= DEOXYRIBOSE SUGAR
- Base pairing
- Adenine - Thymidine
- Guanine-Cytosine

DNA STRUCTURE



BASICS

- DNA = AMPHOPHILIC MOLECULE
- HYDROPHILIC : SUGAR BACKBONE
- HYDROPHOBIC: BASES

Components of RNA

- Ribose
- Bases:
  - adenine
  - guanine
  - cytosine
  - uracil

CLASSICAL TECHNIQUES

- SOUTHERN BLOTTING

- NORTHERN BLOTTING
- DOT BLOT TECHNIQUE

Hybridisation

- Basic principle
- Complementarity of both strands
- based on hydrogen bounds between bases

- 5 AATGGCC**CAAAATGCATT**AGCT 3
- 3’ **GTTTTACGTAA** 5’

GoldenOFormula

- Tm= 81.5 + 16.6 logM + 0.41(%G+C)- - 500/ L - 0.62 (% formamide)

Southern Blotting

- GENOMIC DNA
- RESTRICTION ENZYMES
- ELECTROPHORESIS
- BLOTTING NYLON MEMBRANE
- HYBRIDIZATION RADIOACTIVE PROBE

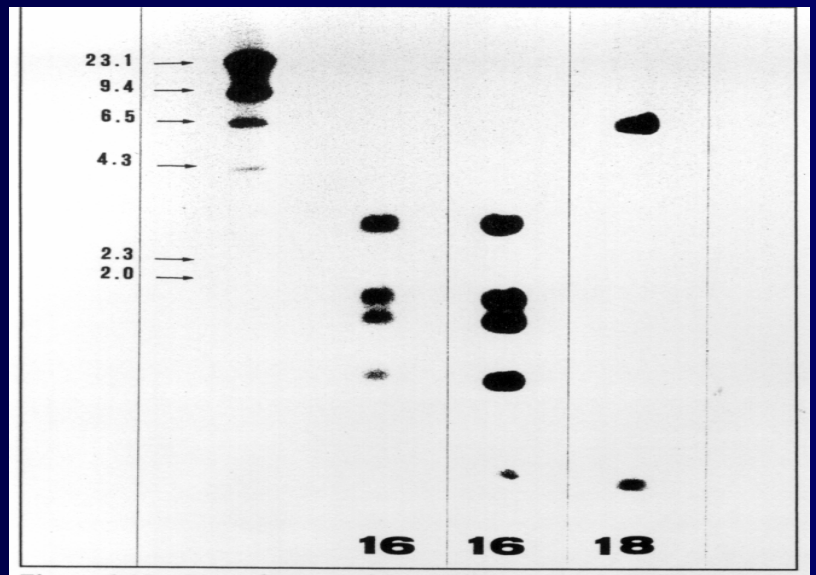
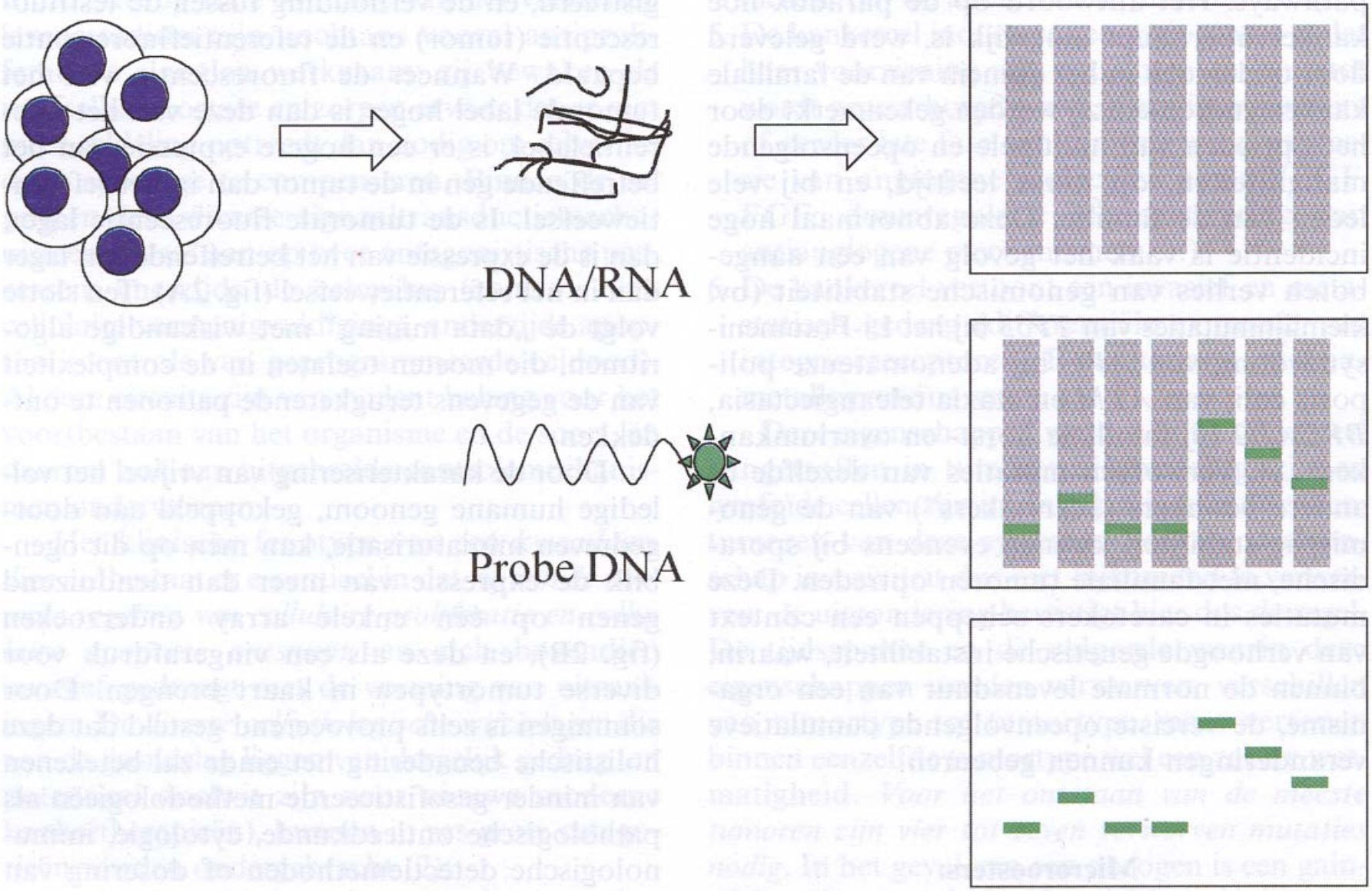


Figure 6.15 Autoradiogram of three DNA samples studied by Southern blot. On the left, a size marker (λ-Hind III) ranging from 2.0 to 23.1 kilobases is indicated. Three DNAs extracted from HGSIL are also illustrated: two of them were proved to contain HPV–16 and the other HPV–18 as revealed by their Pst I pattern and high–stringency conditions of hybridization.

Northern Blotting

- PURIFIED RNA
- ELECTROPHORESIS
- BLOTTING NYLON MEMBRANE
- HYBRIDIZATION RADIOACTIVE PROBE

Northern/Southern blotting



Dot-Blot Technique

- GENOMIC DNA
- NYLON MEMBRANE
- DNA PROBES

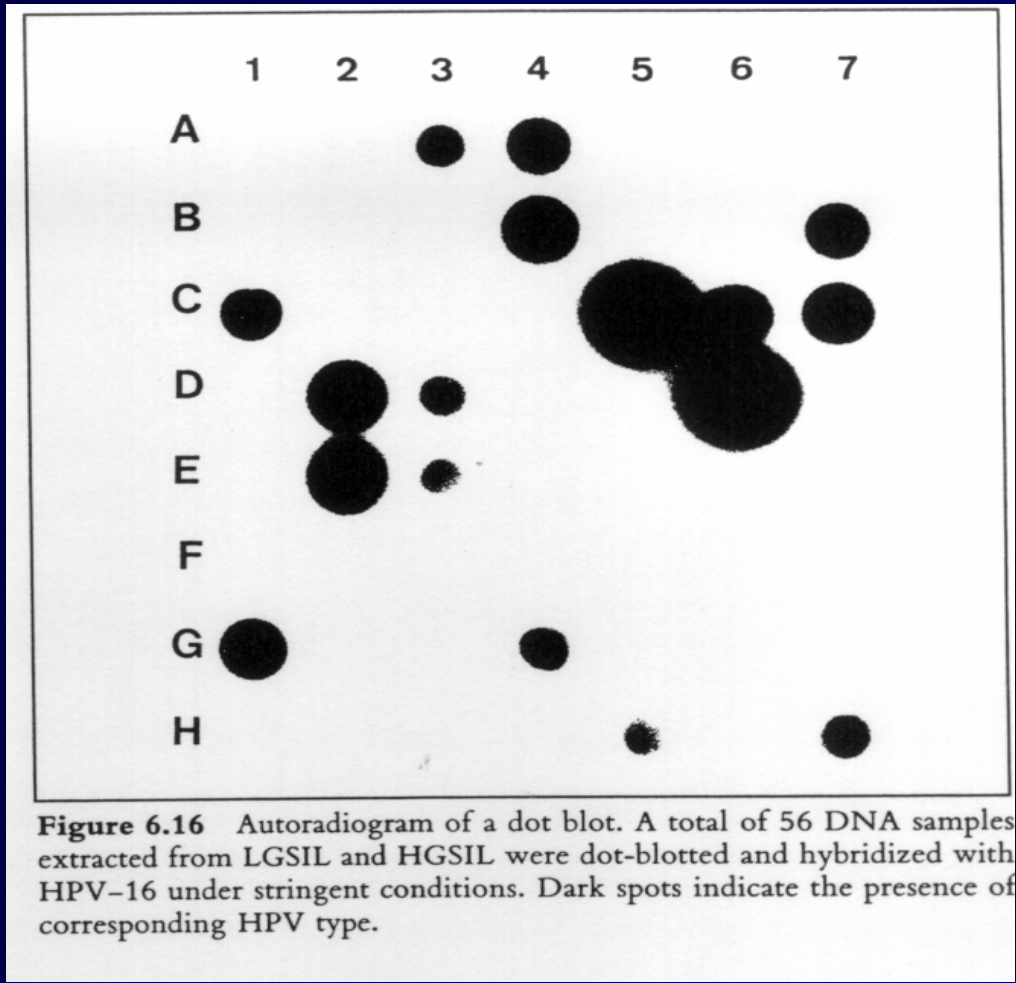
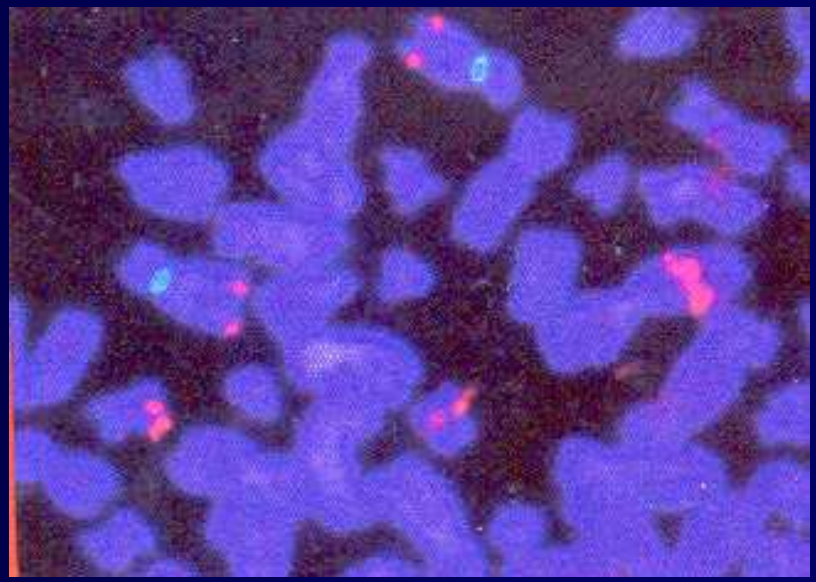


Figure 6.16 Autoradiogram of a dot blot. A total of 56 DNA samples extracted from LGSIL and HGSIL were dot-blotted and hybridized with HPV–16 under stringent conditions. Dark spots indicate the presence of corresponding HPV type.

IN SITU

- Morphological technique
- Chromosomes
- Whole cells
- Tissue: frozen, PET



Applications in pathologyO

- Detection of viruses
- Detection of chromosomal aberrations
- Detection of gene transcripts (mRNA)

Principles of hybridization

- PRETREATMENT
- HYBRIDIZATION
- POST-HYBRIDIZATION WASHING
- VISUALIZATION

PRETREATMENT

- Paraffin embedded tissue
- Frozen tissue sections
- Coated slides: silanized
- Proteolytic enzymes

Hybridization

- **S**odium **S**aline **C**itrate = SSC
- FORMAMIDE
- PROBE
- DEXTRANSULPHATE

Probes

- RNA probes: detection of both RNA/DNA
- DNA probes: idem

DNA Probes

- WHOLE GENOMIC PROBES
- OLIGONUCLEOTIDE PROBES
- Cosmid probes
- PNA PROBES

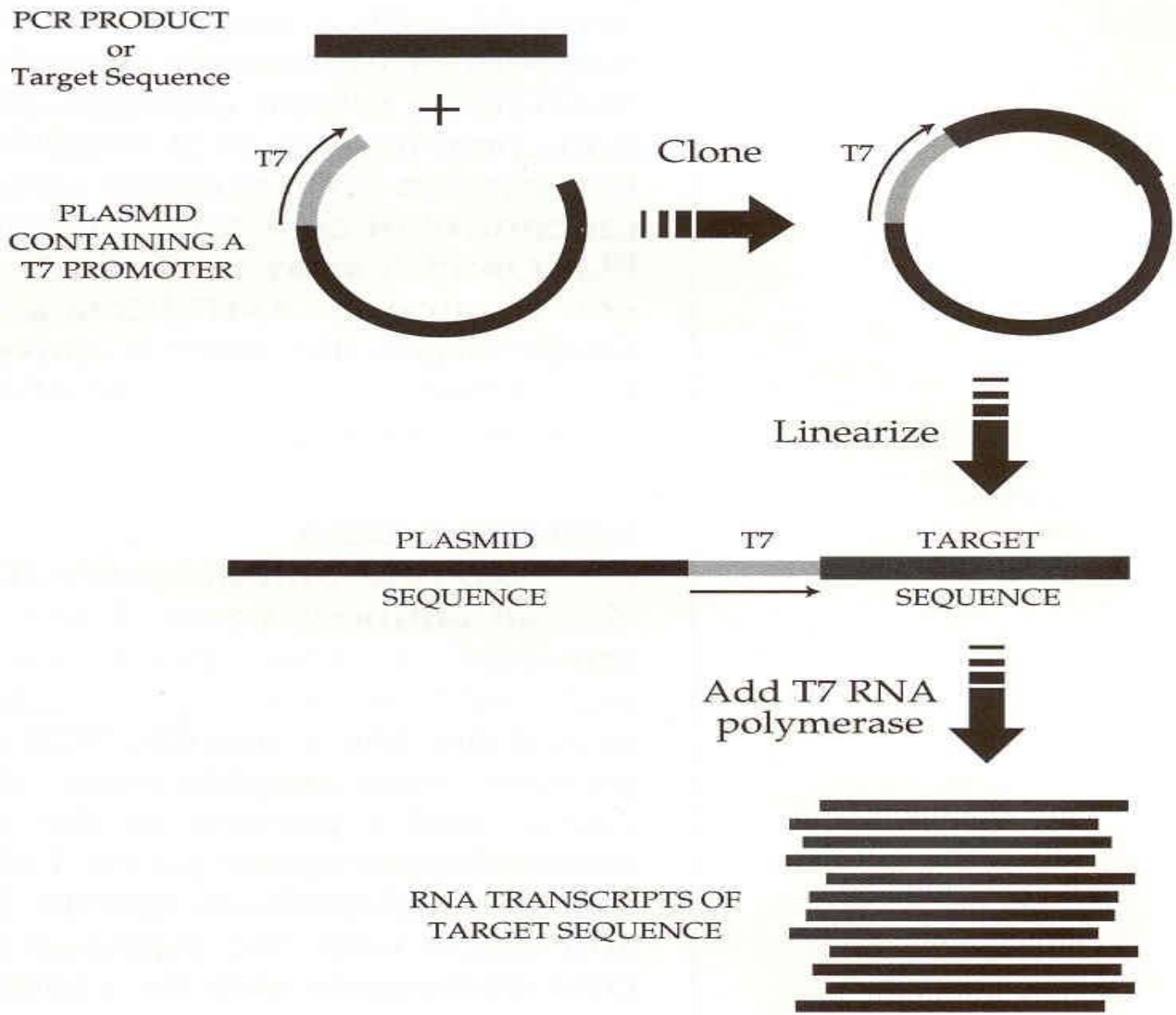


FIGURE 7 Preparation of RNA probes from PCR products or other DNA fragments. PCR products or other DNA fragments are cloned into a plasmid containing a T7 RNA polymerase promoter. After propagation and purification of the plasmid DNA, the DNA is linearized and RNA transcripts are produced.

Post-Hybridisation washing

- Low stringency: high salt-low formamide
- High stringency: low salt-high formamide

Visualisation

- Radioactive
- Fluorescence: FITC, TRITC, …
- Immunohistochemistry: biotine, digoxygenine

PCR Reaction Mixture

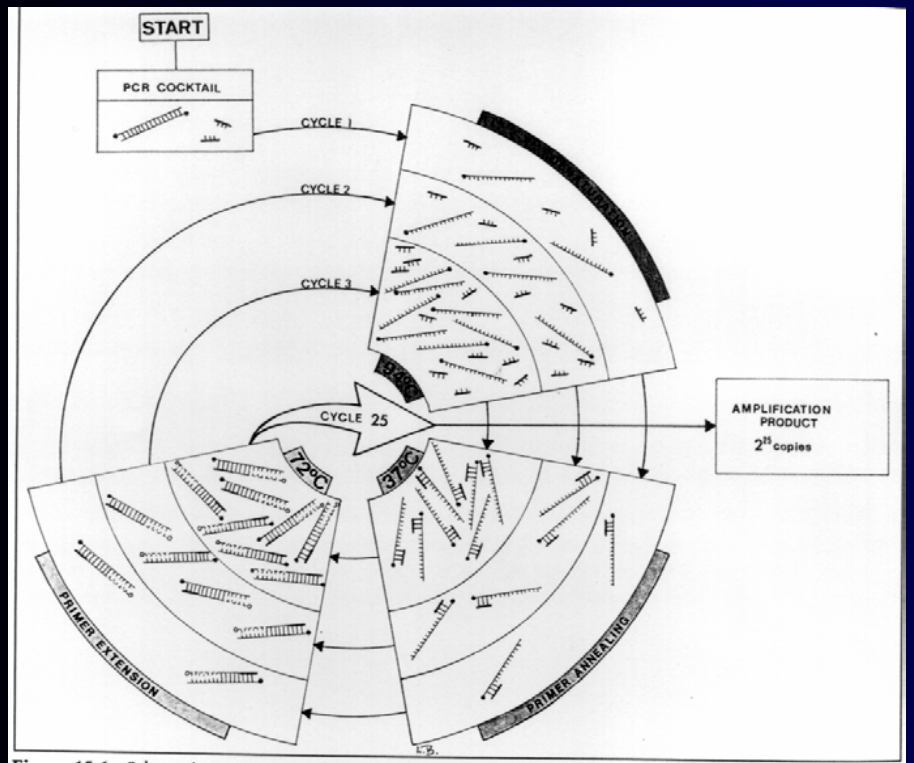
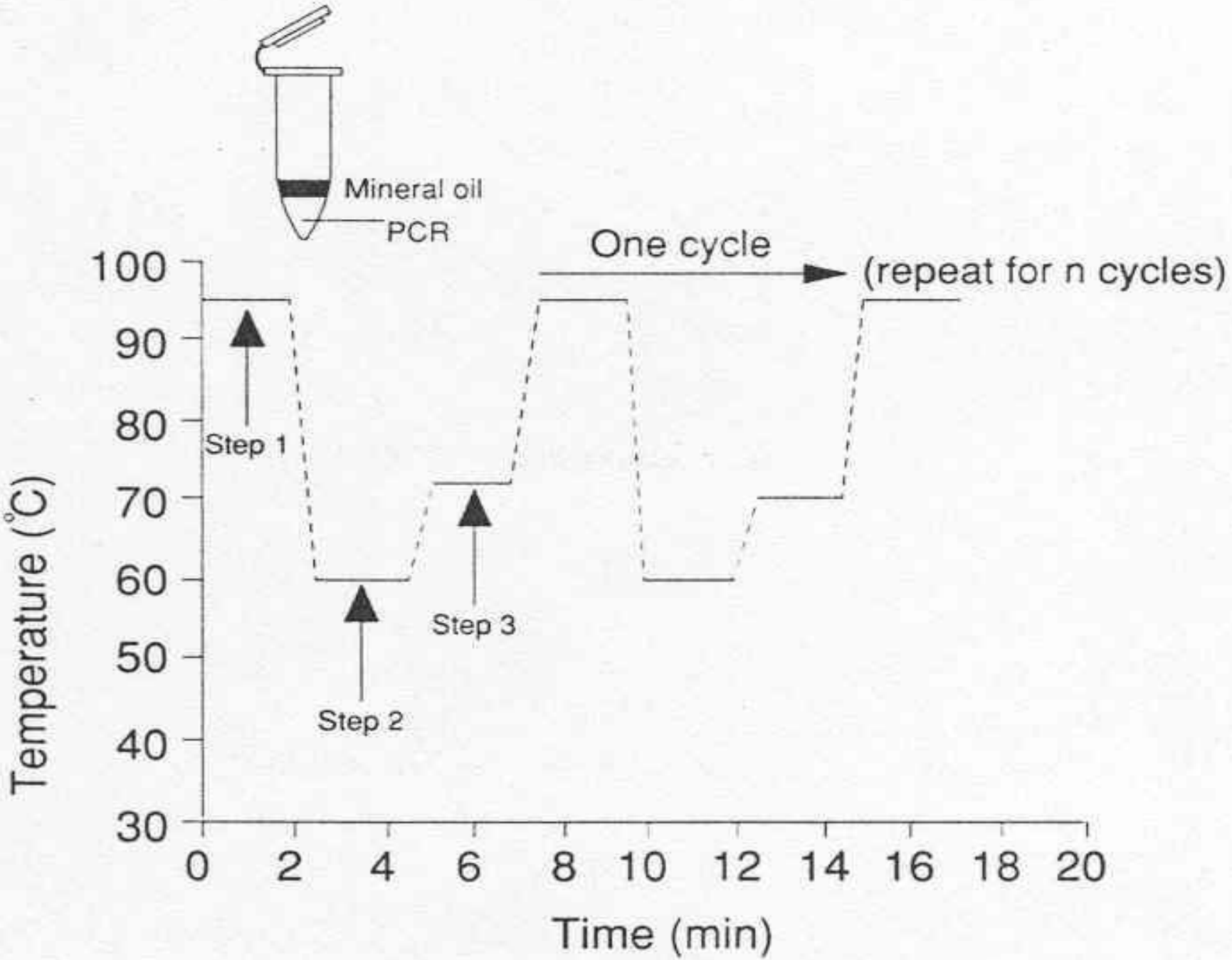


Figure 15.6 Schematic presentation of the polymerase chain reaction. Denaturation (94°C); annealing of primers (37°C); and primer extension (72°C) are illustrated with corresponding amplification of DNA molecules.

- DNA or cDNA
- Taq-polymerase
- $MgCl_2$
- Primer

PCR-PRIMERS

- Synthetic oligonucleotides
- 20- 30 bp
- %GC


Mineral oil
PCR
One cycle
(repeat for n cycles)
Step 1
Step 2
Step 3
Temperature (°C)
100
90
80
70
60
50
40
30
0
2
4
6
8
10
12
14
16
18
20
Time (min)

PCR Reaction cycle

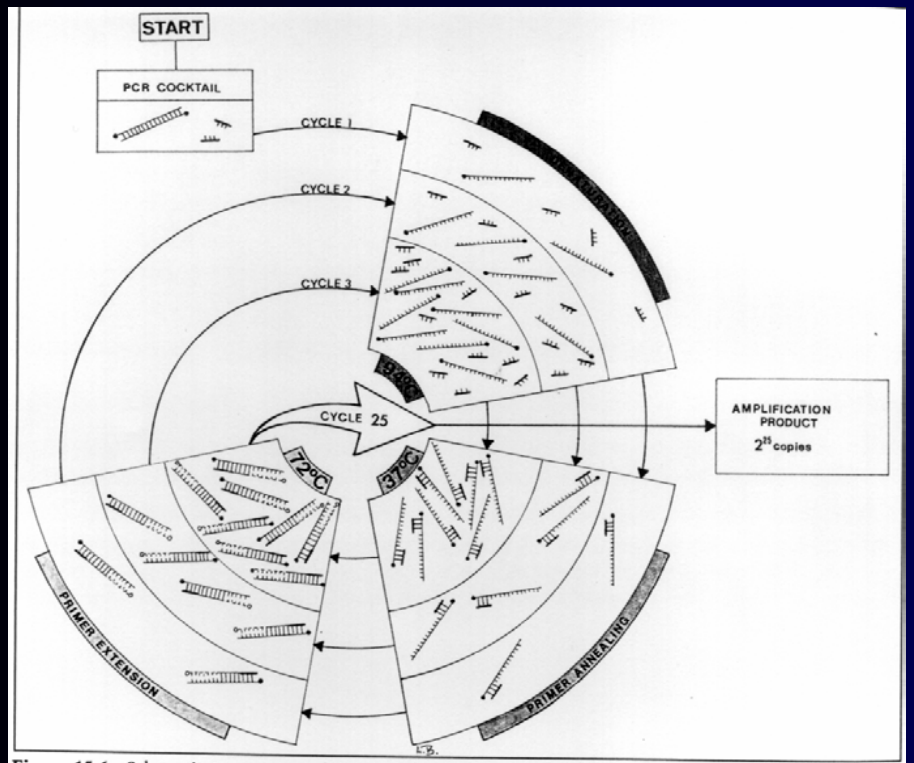
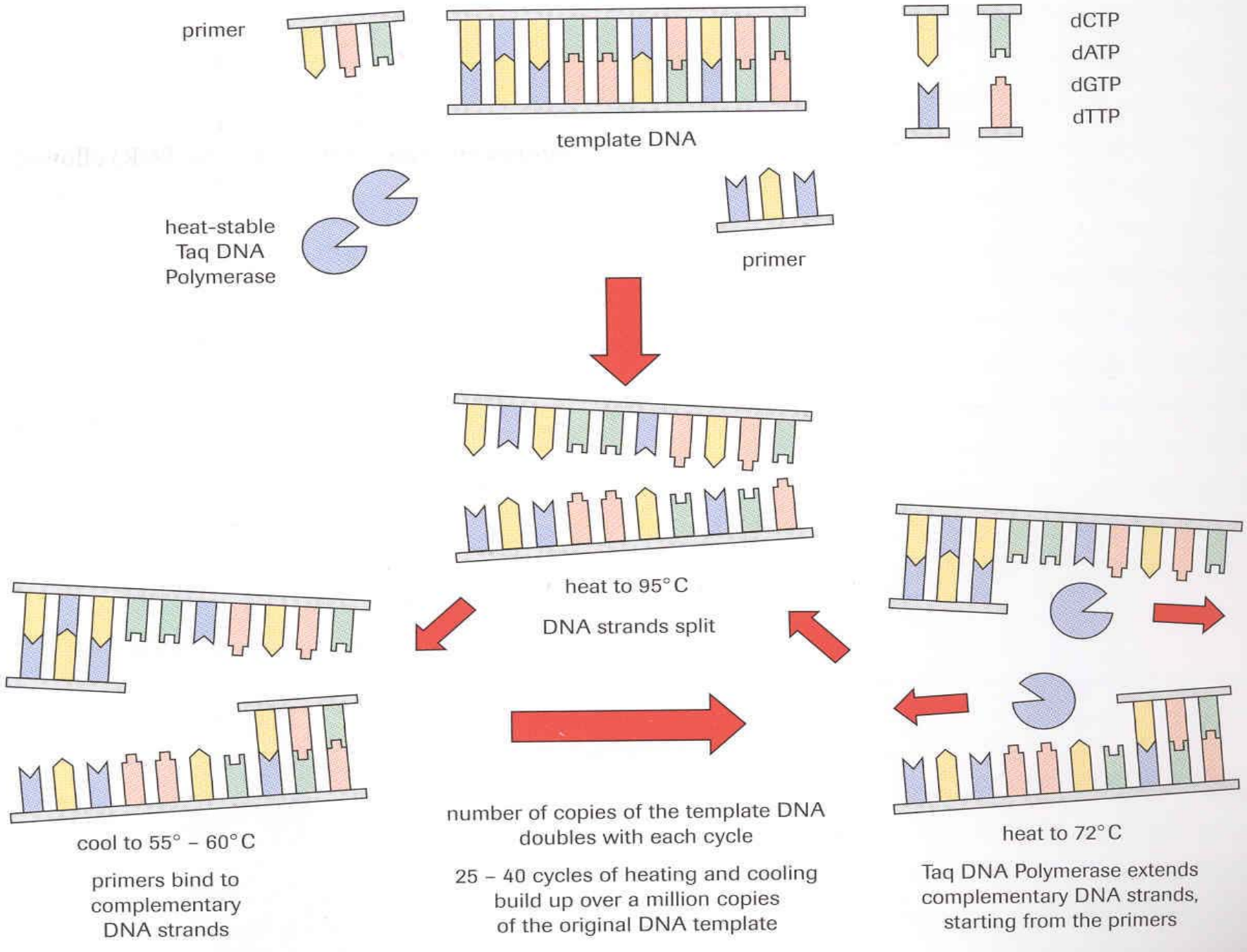
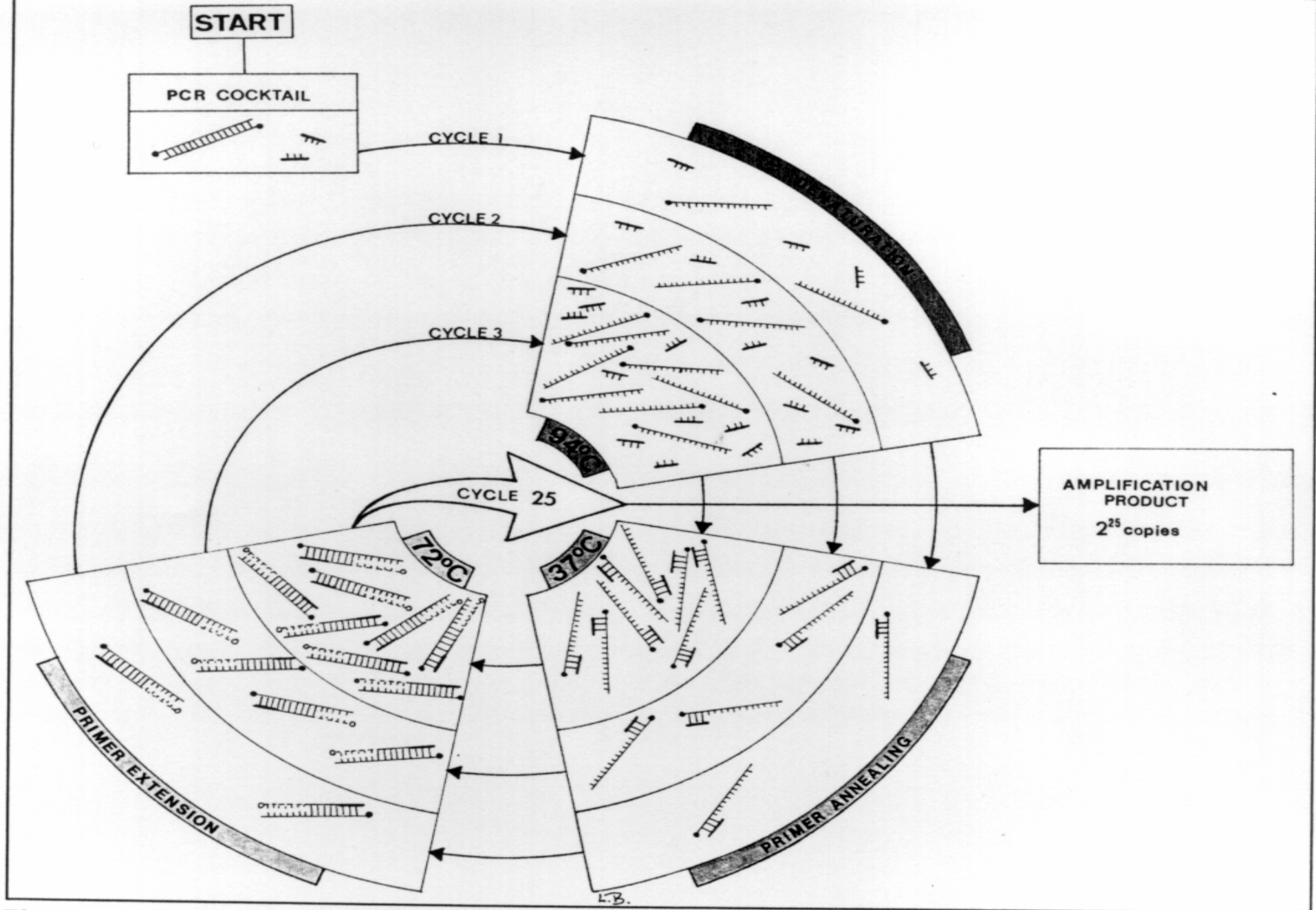


Figure 15.6 Schematic presentation of the polymerase chain reaction. Denaturation (94°C); annealing of primers (37°C); and primer extension (72°C) are illustrated with corresponding amplification of DNA molecules.

- Denaturation: 95°C
- Annealing: 50-60°C
- Elongation: 72°C


primer
template DNA
dCTP
dATP
dGTP
dTTP
heat-stable
Taq DNA
Polymerase
primer
heat to 95° C
DNA strands split
cool to 55° – 60° C
primers bind to
complementary
DNA strands
heat to 72° C
Taq DNA Polymerase extends
complementary DNA strands,
starting from the primers
number of copies of the template DNA
doubles with each cycle
25 – 40 cycles of heating and cooling
build up over a million copies
of the original DNA template



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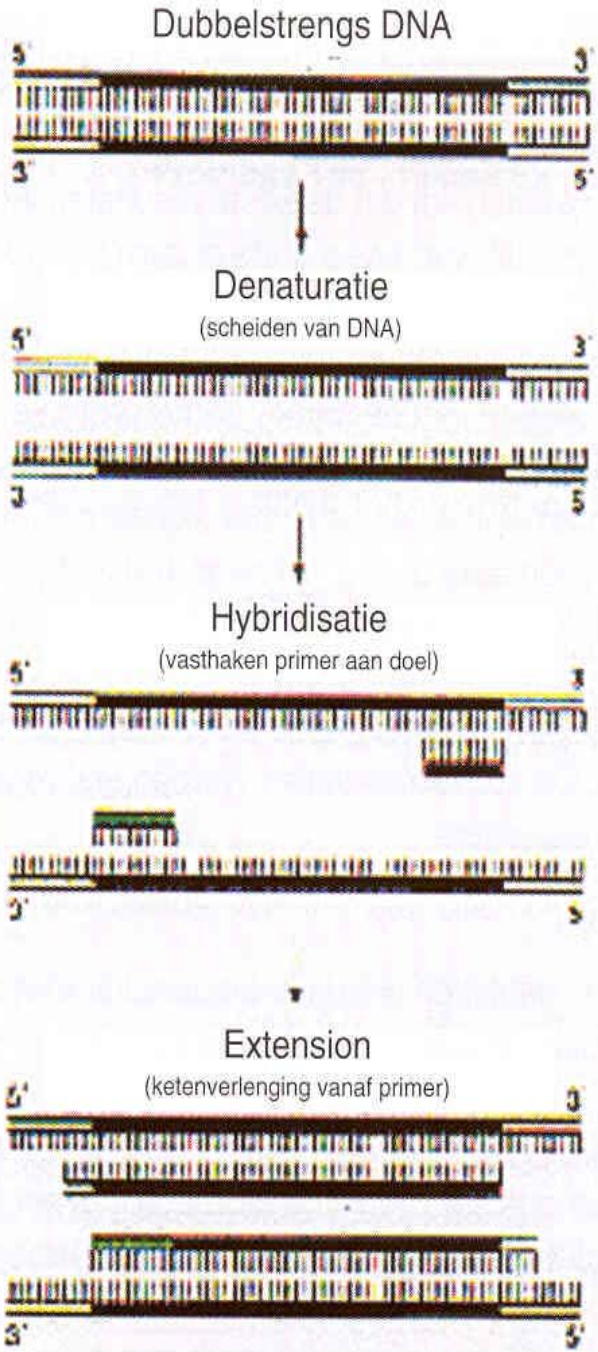

Dubbelstrengs DNA
5'
3'
3'
5'
Denaturatie
(scheiden van DNA)
5'
3'
3'
5'
Hybridisatie
(vasthaken primer aan doel)
5'
3'
3'
5'
Extension
(ketenverlenging vanaf primer)
5'
3'
3'
5'

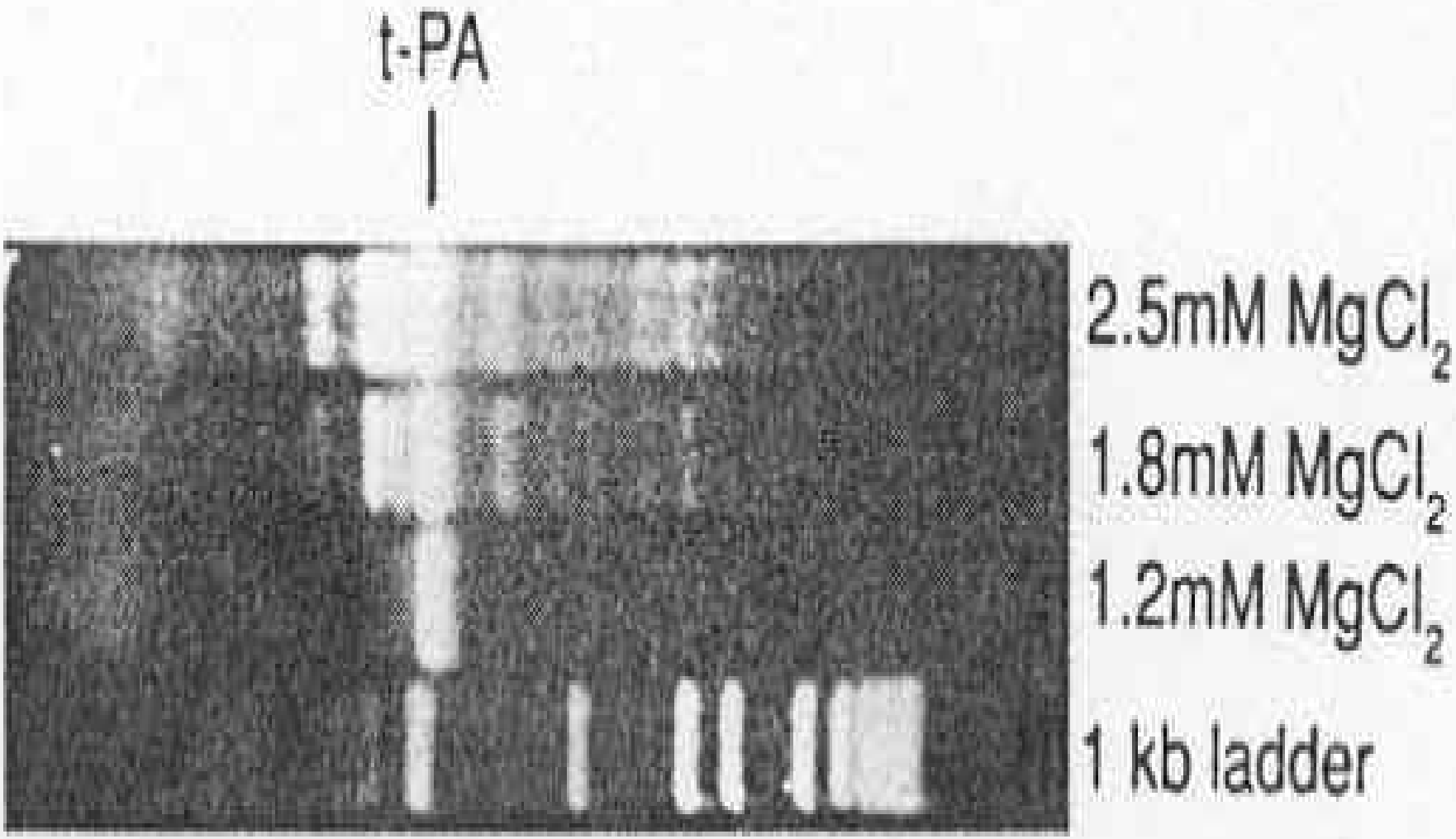

t-PA
2.5mM $MgCl_2$
1.8mM $MgCl_2$
1.2mM $MgCl_2$
1 kb ladder

TABLE 2.4: *Enhancers of PCR*

Substance	Concentration
Formamide	5%
Dimethyl sulfoxide (DMSO)	<10%
Tetramethylammonium chloride (TMAC)	10–100 μM
Polyethylene glycol 6000 (PEG)	5–15%
Glycerol	10 –15 %
Tween® 20	0.1 –2.5%
Gene 32 protein (Pharmacia)	1 nM
7 deaza-dGTP	Replace 75% of dGTP with deaza-dGTP
Perfect Match® (Stratagene)	1 unit
Taq Extender™ PCR additive (Stratagene)	1 unit
E. coli single-strand DNA binding protein (ssb)	5 μg ml^{-1}

At higher concentrations these 'enhancers' are known to, or are likely to, inhibit *Taq* DNA polymerase, and so determination of the optimum concentration for enhancement has to be determined empirically.

TABLE 1.1: *Quantitative analysis of a PCR before and after 25 cycles (amplification efficiency = ~70%)*

	Before PCR				After PCR			
	Weight	Moles	Molarity	Molecules	Weight	Moles	Molarity	Molecules
Template[a]	1 ng	3.10×10^{-17}	3.10×10^{-13}	1.86×10^{7}	1 ng	3.00×10^{-17}	3.00×10^{-13}	1.81×10^{7}
Target[b]	10 pg	3.00×10^{-17}	3.00×10^{-13}	1.81×10^{7}	1 µg	3.00×10^{-12}	3.00×10^{-8}	1.81×10^{12}
Primers[c]	1623 ng	2.00×10^{-10}	2.00×10^{-6}	1.20×10^{14}	1574 ng	1.94×10^{-10}	1.94×10^{-6}	1.17×10^{14}
dNTPs[d]	39 µg	8.00×10^{-8}	8.00×10^{-4}	4.82×10^{16}	37 µg	7.70×10^{-8}	7.70×10^{-4}	4.64×10^{16}
Magnesium ion[e]	3.6 µg	1.50×10^{-7}	1.50×10^{-3}	9.03×10^{16}	3.6 µg	1.50×10^{-7}	1.50×10^{-3}	9.03×10^{16}
Taq DNA polymerase[f]	12.5 µg	1.33×10^{-13}	1.33×10^{-9}	8.01×10^{10}	12.5 µg	1.33×10^{-13}	1.33×10^{-9}	8.01×10^{10}

[a] Bacteriophage lambda (template dsDNA = 48 500 bp).

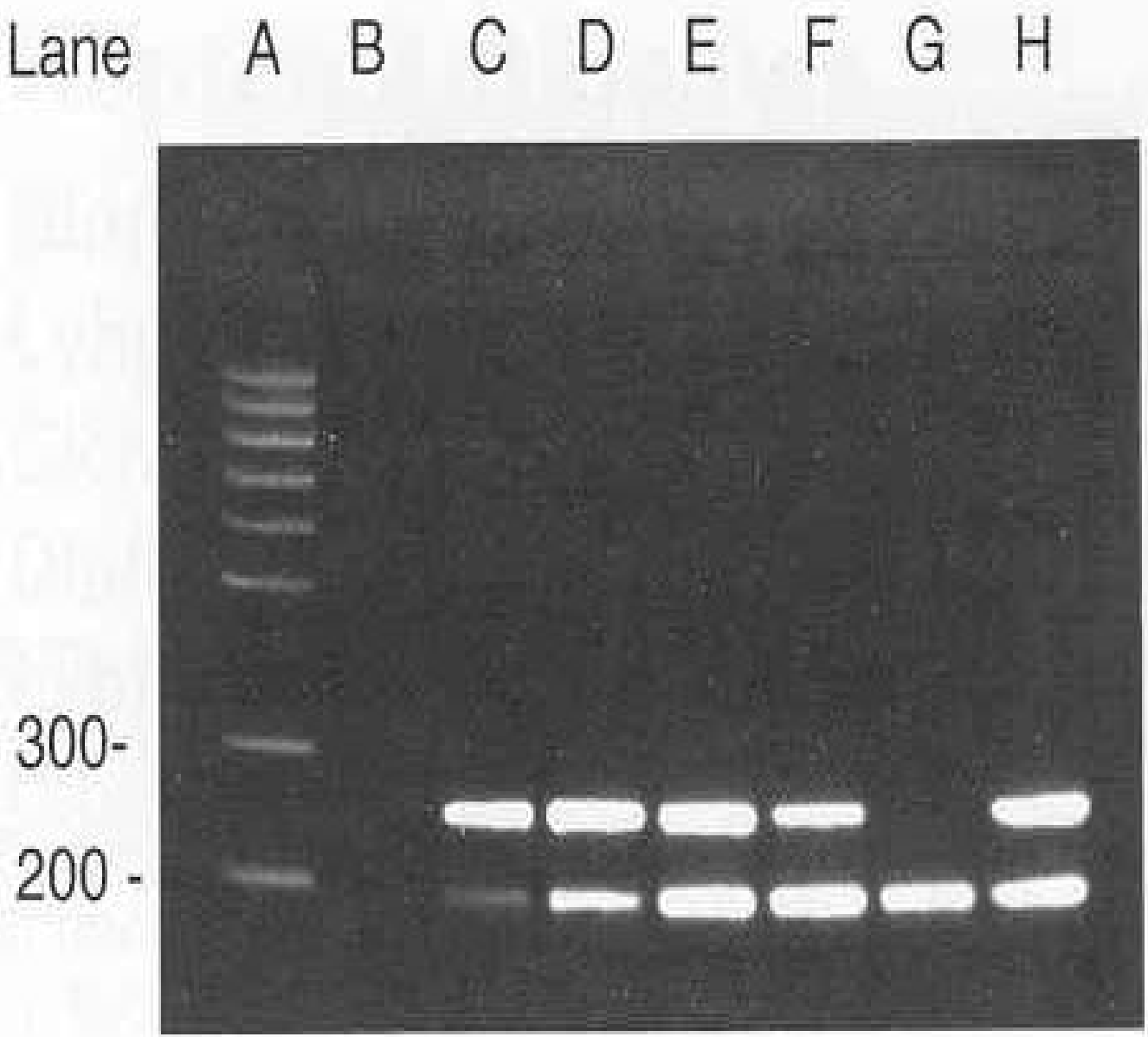
[b] Target is 500 bp.

[c] 1 µM (each) primers, 25-mers.

[d] 200 µM (each) dNTPs; total [dNTPs] = 0.8 mM (average molecular weight of a dNTP is 487 Da; average molecular weight of a dNMP is 325 Da).

[e] Total [$MgCl_2$] = 1.5 mM; free [$MgCl_2$] = 0.7 mM.

[f] 2.5 Units *Taq* DNA polymerase per 100 µl; polymerase activity = 250 000 units mg^{-1}; enzyme half-life not considered.


Lane
A
B
C
D
E
F
G
H
300-
200 -
- C. diphtheriae Ban
- Control Band

PCR-Controls

- Known positive control
- Known negative control with DNA
- negative control without DNA
- quality control of DNA: genomic sequence

Paraffin embedded tissue

- Formalin fixation: breaks in DNA strand
- DNA degeneration
- Maximal amplification: 150-200 bp
- DNA inhibitors
- DNA extraction

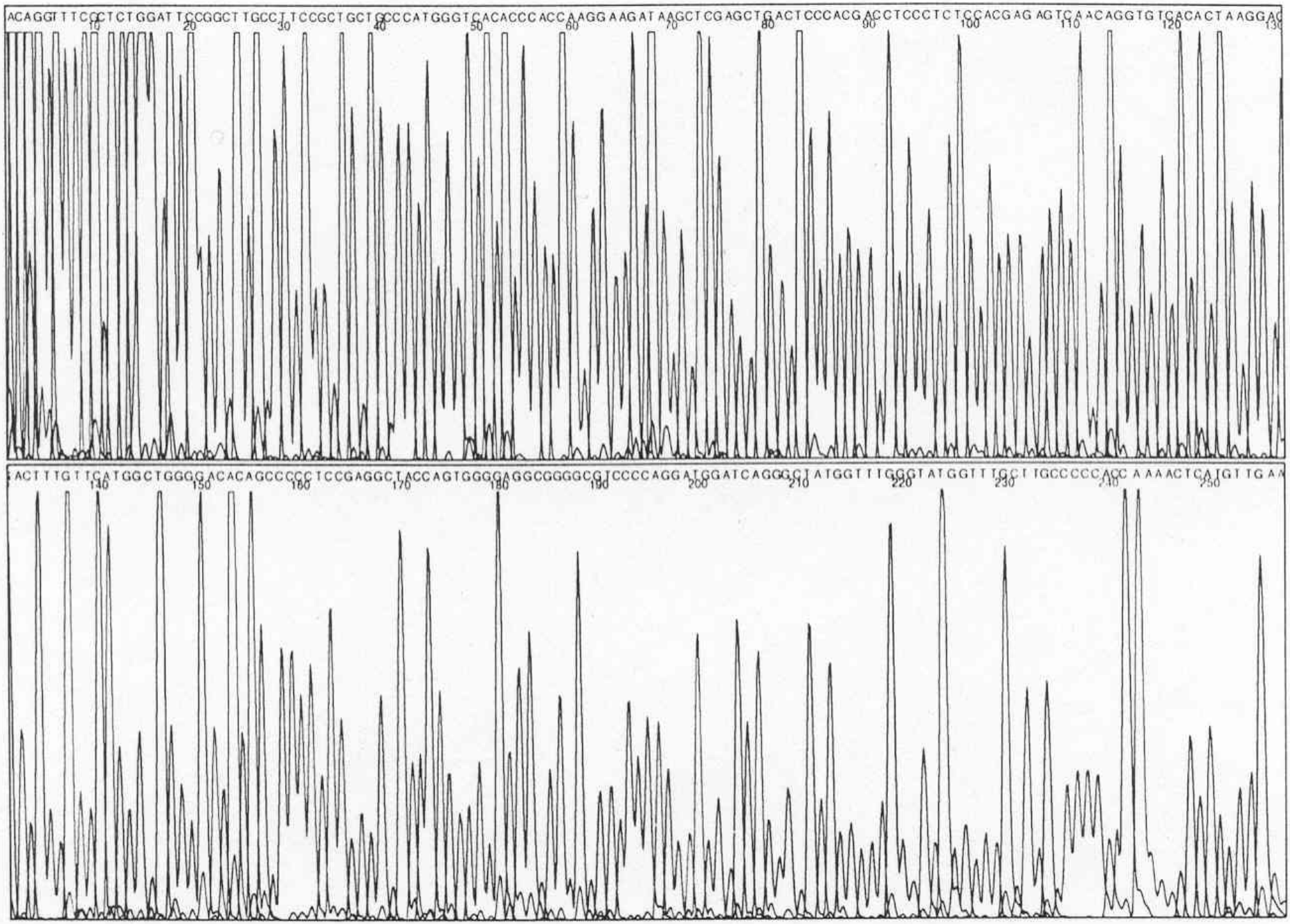
PCR TECHNOLOGY

- Classical gelelectrophoresis
- Sequencing of PCR product
- Enzyme-immunoassay (EIA)
- Linearized Probe assay (LIPA)
- Real time PCR with quantification

TABLE 3.2: *Detection, identification and quantification of PCR products*

Detection	Visualization
Agarose gel and/or polyacrylamide gel electrophoresis	EtBr staining (UV transilluminator, image analyzer)
	Southern blotting (hybridization with labeled probe)
	Incorporation of label into amplicon
	Addition of capture tag followed by detection
	Silver staining
Restriction endonuclease digestion	Agarose or polyacrylamide gel, HPLC
Dot blots	Hybridization with labeled probe (e.g. ASOs)
High-pressure liquid chromatography	UV detection
EtBr incorporation during PCR	UV transilluminator
Electrochemiluminescence	Voltage-initiated chemical reaction/photon detection
Scintillation proximity assay (SPA)	Scintillation counting of captured PCR product
Direct sequencing	Radioactive or fluorescent-based DNA sequencing

Specific methods for the detection of mutations are not shown, although modifications of those listed may be applied to mutation analysis.
Abbreviations: ASOs, allele-specific oligonucleotides; EtBr, ethidium bromide; HPLC, high-pressure liquid chromatography; UV, ultraviolet.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ACTTTGTTCATGGCTGGGGACACAGCCCCCTCCGAGGCTACCAGTGGGGAGGCGGGGCGTCCCCAGGATGGATCAGGGCTATGGTTTGGGTATGGTTTGCTTGCCCCCACCAAAACTCATGTTGAA