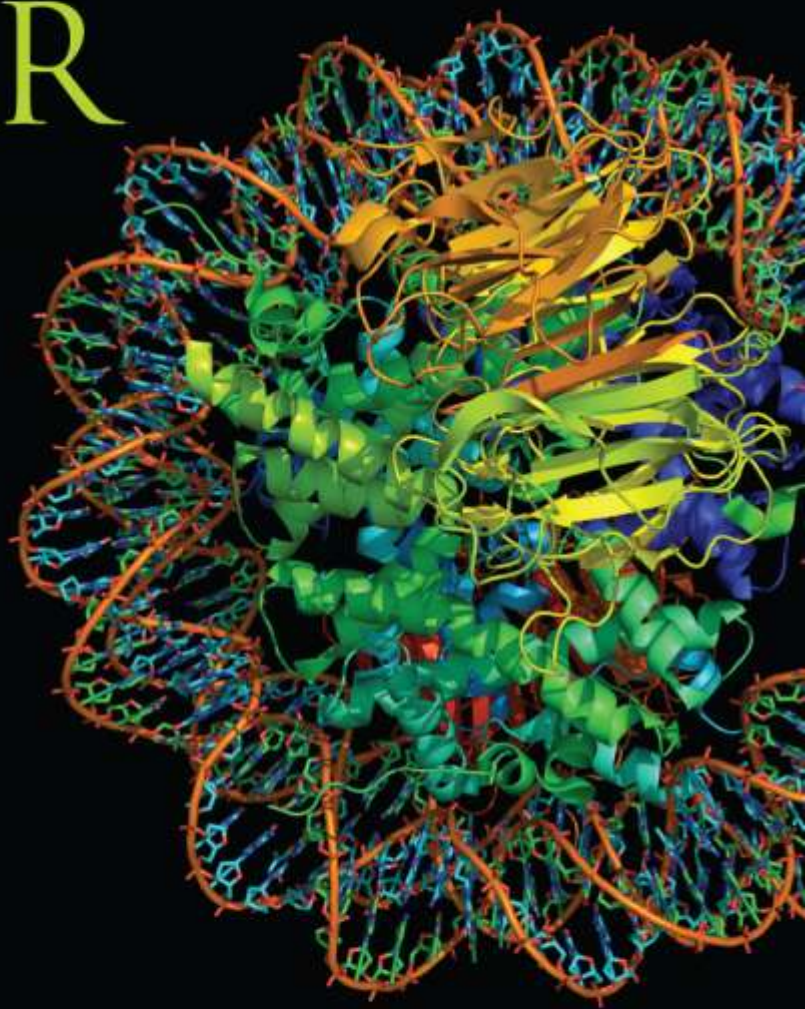
Principles of

MOLECULAR BIOLOGY

BURTON E. TROPP



Chapter 4
Molecular Biology Technology

TECHNIQUES IN MOLECULAR BIOLOGY

DR. NABIL BASHIR

Nucleic Acid Isolation

- Technique depends on the particular source
  - Viral DNA
    - Phenol extraction (removes protein)
  - Bacterial DNA
    - Lysozyme to digest cell envelope
    - Detergent (SDS) disrupts cell membrane
    - After alcohol precipitation chromosomal DNA can be spooled out

Nucleic Acid Isolation

- Plasmid DNA isolation
  - Lysozyme then SDS treatment along with NaOH (hydrolyzes RNA)
    - Neutralize with acid
    - Plasmid remains in solution while chromosomal DNA is in an insoluble aggregate
- Yeast and fungal DNA
  - Need to remove polysaccharide wall – then similar to bacteria

Nucleic Acid Isolation

- Great care required to protect RNA from degradation by RNases during RNA isolation
  - Freeze tissue in liquid nitrogen then grind the frozen cells
  - Resuspend in acidic phenol/chloroform/sodium acetate/guanidinium thiocyanate
  - RNA present in aqueous phase is precipitated with ethanol or isopropanol

Physical Techniques Used to Study Macromolecules

Centrifugation can separate macromolecules based on density

- Modern ultracentrifuges can generate 800,000 x gravity
- Sedimentation rate depends on molecular size and shape

Sucrose gradient centrifugation

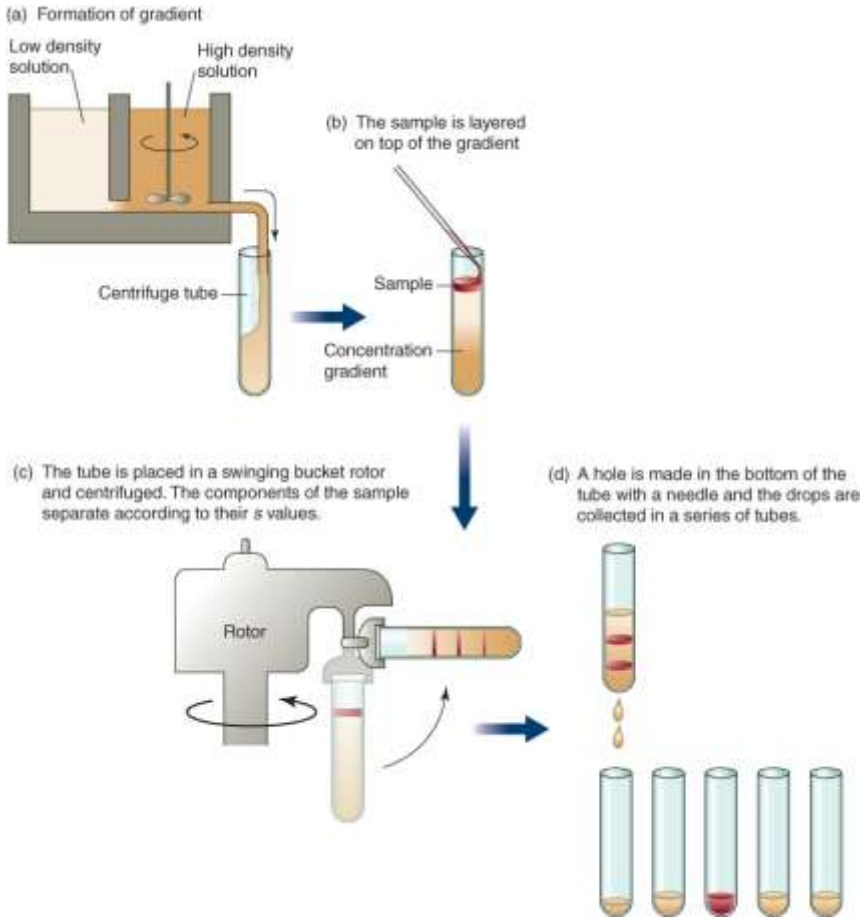


Figure 04.05: Sucrose gradient centrifugation.

Physical Techniques Used to Study Macromolecules

Equilibrium gradient centrifugation

- Nucleic acids are suspended in a CsCl solution
- High speed centrifugation generates a near linear concentration gradient
- Macromolecules seek their neutral density

Physical Techniques Used to Study Macromolecules

- Gel electrophoresis separates macromolecules based on their migration rate in an electric field
  - Nucleic acids typically separated on agarose or polyacrylamide gels
  - Nucleic acid gels are often stained with ethidium bromide

Physical Techniques Used to Study Macromolecules

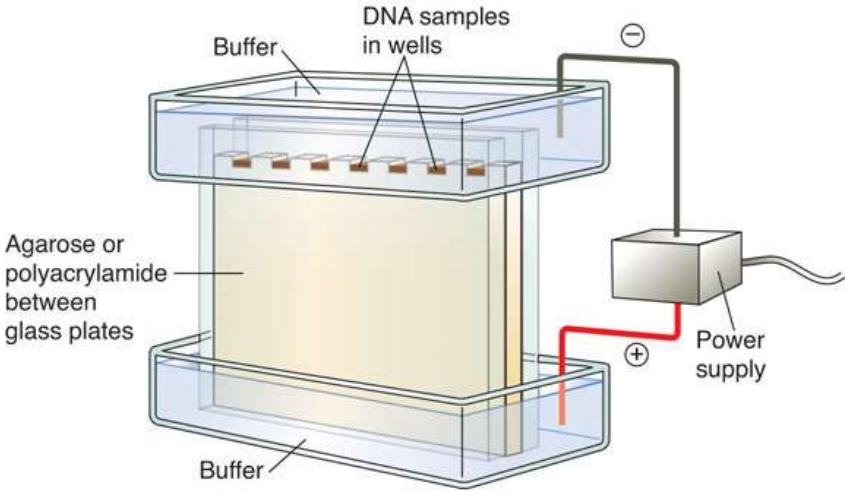


Figure 04.07A: Gel electrophoresis. (a) Apparatus for gel electrophoresis capable of handling several samples simultaneously.

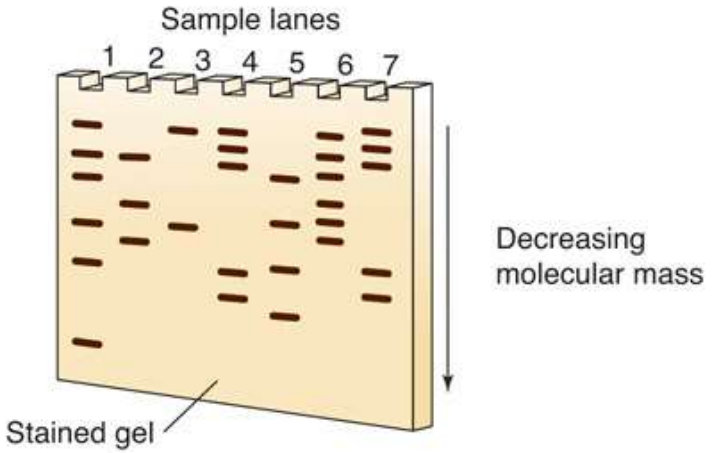


Figure 04.07B: Gel electrophoresis. (b) The separated components of a sample appear as bands, which may either be colored or fluoresce.

Physical Techniques Used to Study Macromolecules

- Electrophoresis can be used to determine a polypeptide's molecular mass however:
  - Unlike DNA, native proteins do not have a uniform charge or shape
  - Requires a specialized electrophoretic technique
- SDS-PAGE
  - Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis

Enzymatic Techniques Used to Modify DNA

- Nucleases
  - Enzymes that digest polynucleotides by cleaving the 5’→3’ phosphodiester bond
    - DNases
      - Some are specific for double stranded DNA others act only on single stranded DNA, some both
    - RNases
    - General nucleases

Enzymatic Techniques Used to Modify DNA

- Endonucleases
  - Nucleases that act within a strand
    - Some are base sequence specific
- Exonucleases
  - Nucleases that act only at the end of a strand
    - 5’ →3’ or 3’ →5’

Enzymatic Techniques Used to Modify DNA

- Restriction endonucleases
  - A class of nucleases that cleave (restrict) viral DNA that enters a cell
    - Cleave within a specific nucleotide sequence (restriction site)
      - Host DNA is protected through methylation of restriction site

Enzymatic Techniques Used to Modify DNA

- Type II restriction enzyme recognition sites are palindromic
- Some generate flush or blunt ends
- Others generate cohesive or sticky ends

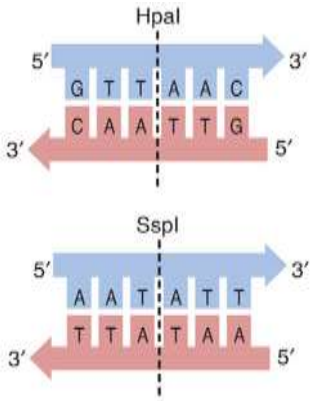


Figure 04.10A: Restriction endonuclease generated ends. Some restriction endonucleases such as HpaI and SspI cut on the line of symmetry to produce blunt ends.

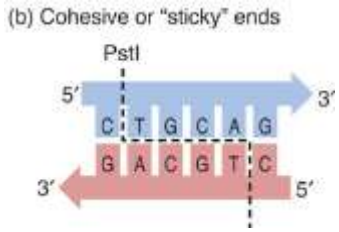


Figure 04.10B: Restriction endonuclease generated ends. Other restriction endonucleases (PstI) cut on either side of the line of symmetry to produce cohesive ends.

(Adapted from an illustration by Maria Price Raposa, Carolina Biological Supply Company.)

Southern Blotting is used to detect specific DNA fragments :and can be used to detect both partial and complete deletions of a gene

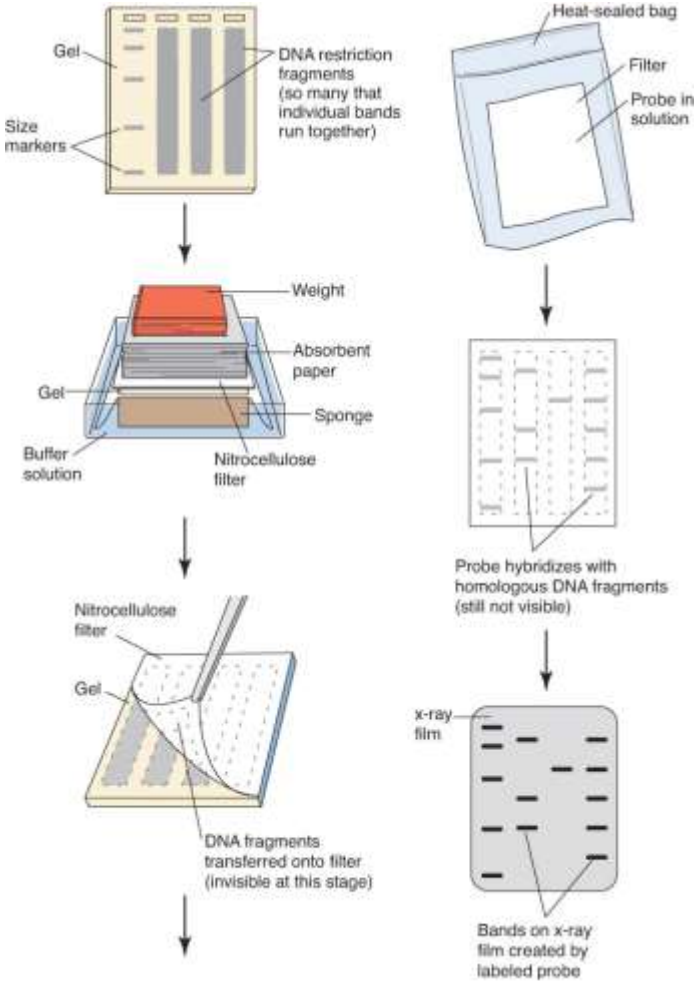
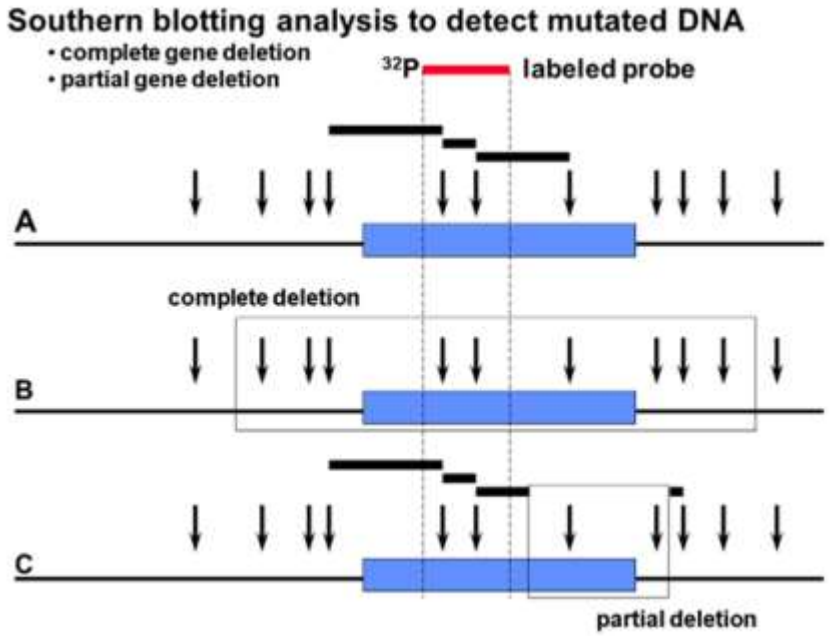
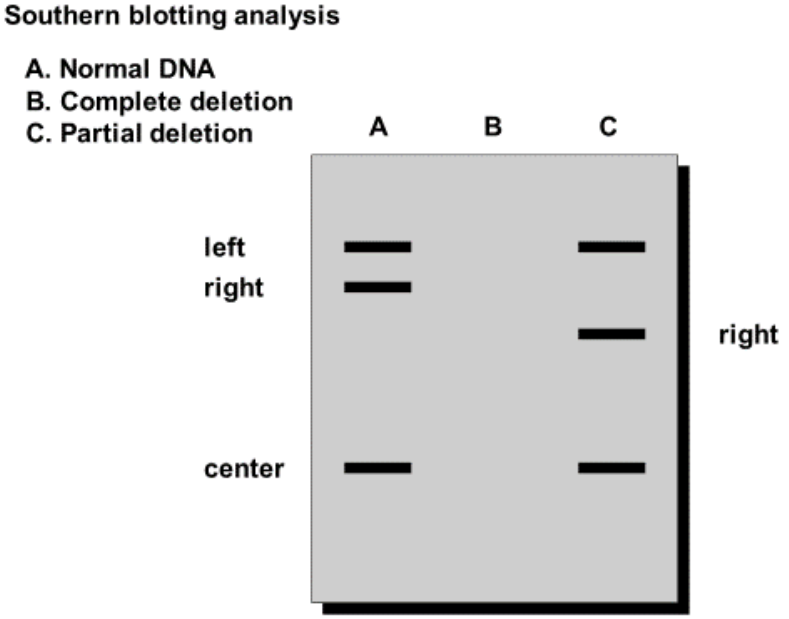


Figure 04.14: Southern blot analysis: an experimental method for identifying a specific DNA fragment in a gel.





Enzymatic Techniques Used to Modify DNA

- Northern Blot:
  - RNA gel blot
- Western Blot
  - Protein gel blot
    - Bound protein is detected using antibody probes

Enzymatic Techniques Used to Modify DNA

- DNA polymerases require a template and primer
  - Primer strand: site of attachment for nucleotides
  - Template strand: determines the order of attachment according to base pairing
  - Grows DNA sequence in 5’→3’ direction
- Several different DNA polymerases are used in the Molecular Biology lab

Enzymatic Techniques Used to Modify DNA

DNA polymerase I (Pol I) function

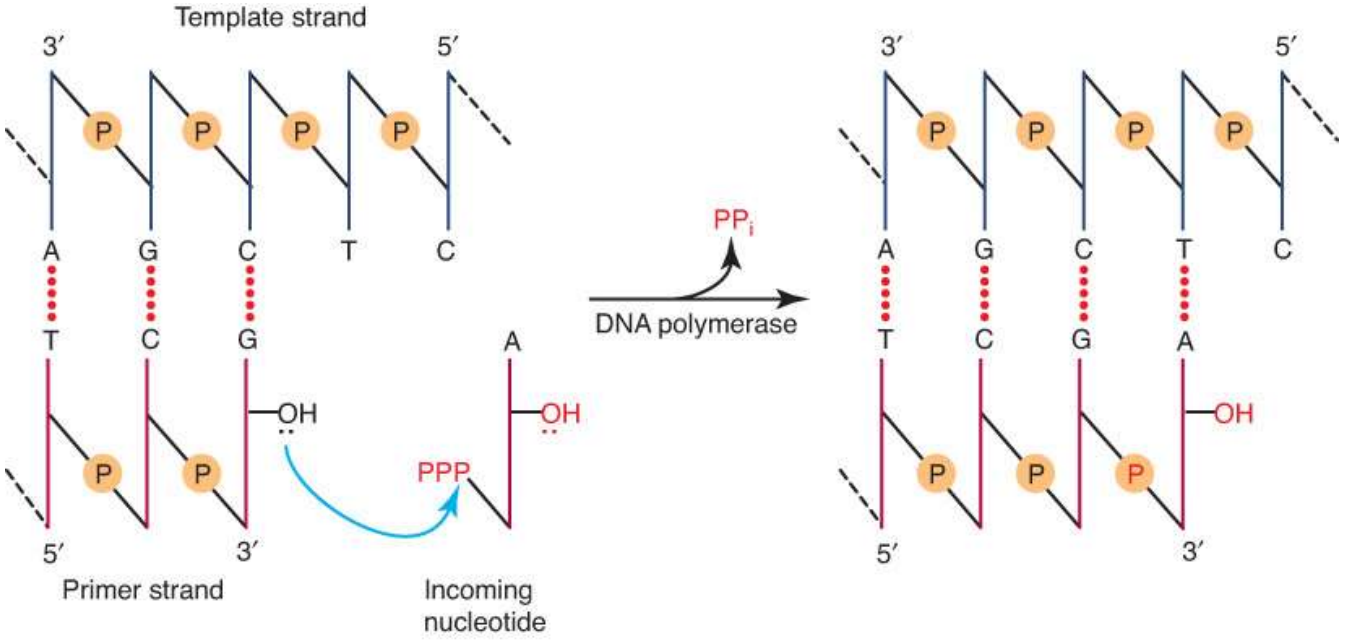


Figure 04.16: DNA polymerase I: polymerase function. DNA polymerase I uses the template strand to determine the next nucleotide that it adds to the primer strand.

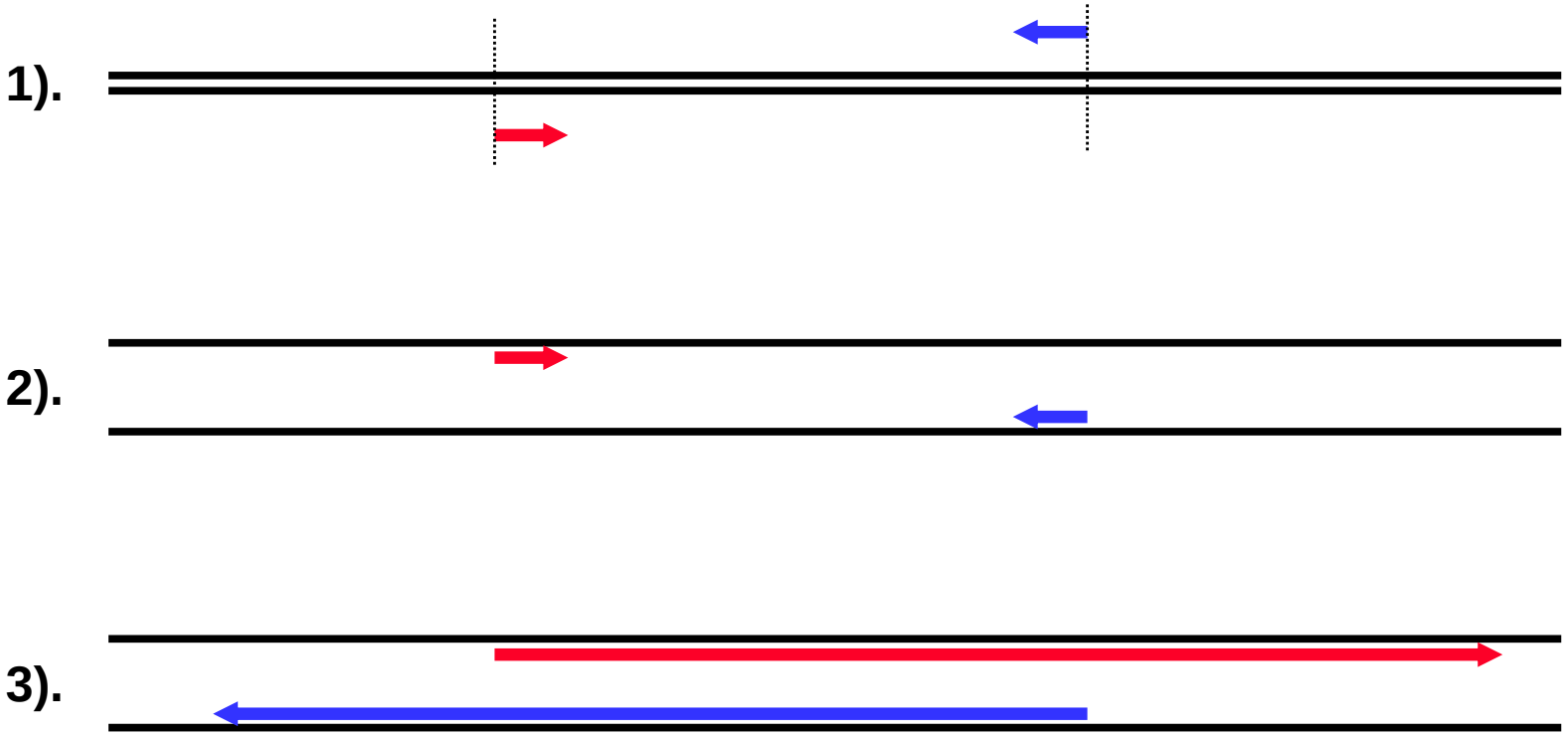
Enzymatic Techniques Used to Modify DNA

DNA polymerase I has both 3’ →5’ and 5’ →3’ exonuclease activity

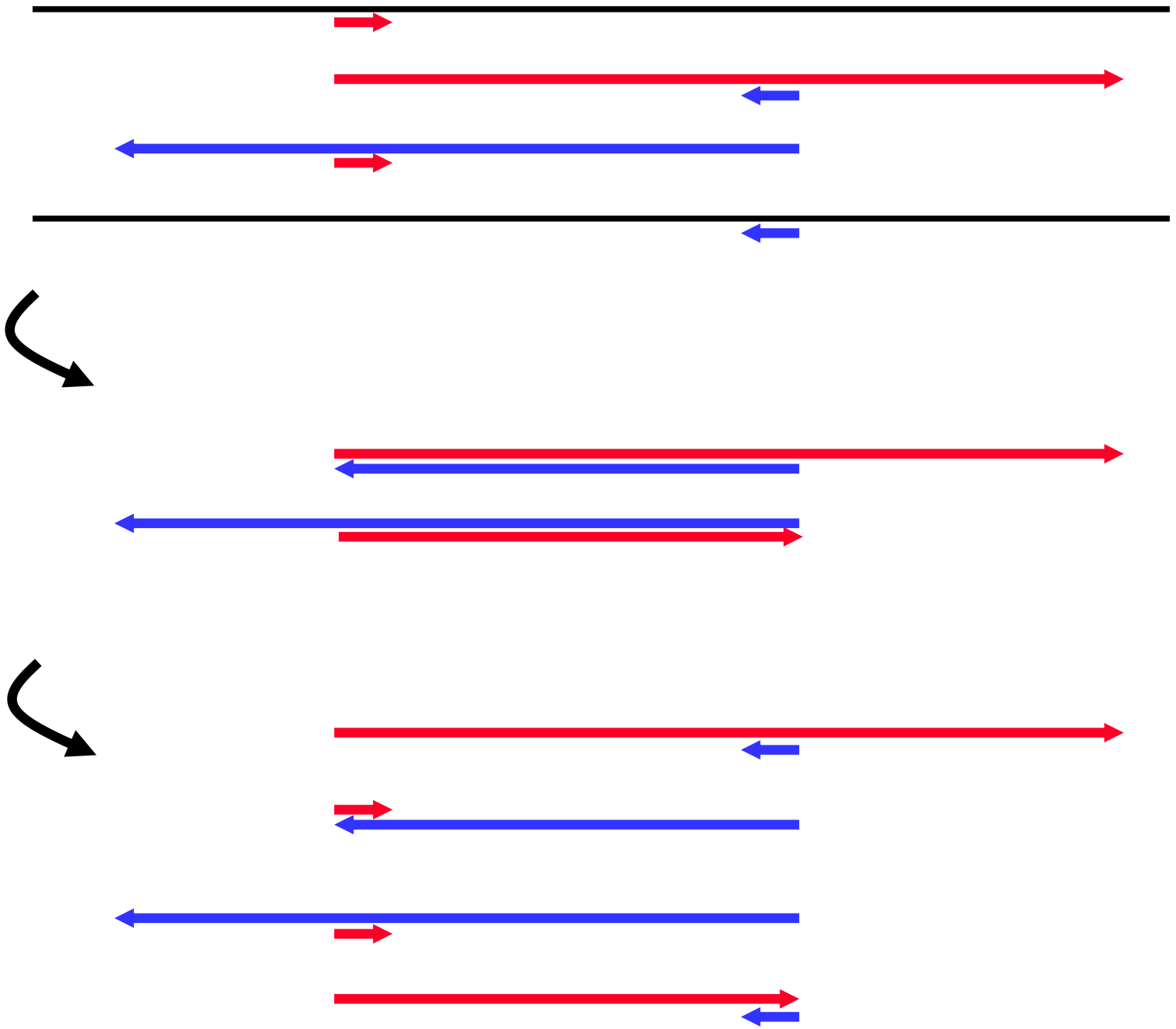
- Highly purified DNA Pol I from *E. coli* consists of a single 928 amino acid polypeptide
- 3’ →5’ exonuclease – proofreading function
- 5’ →3’ exonuclease – editing function

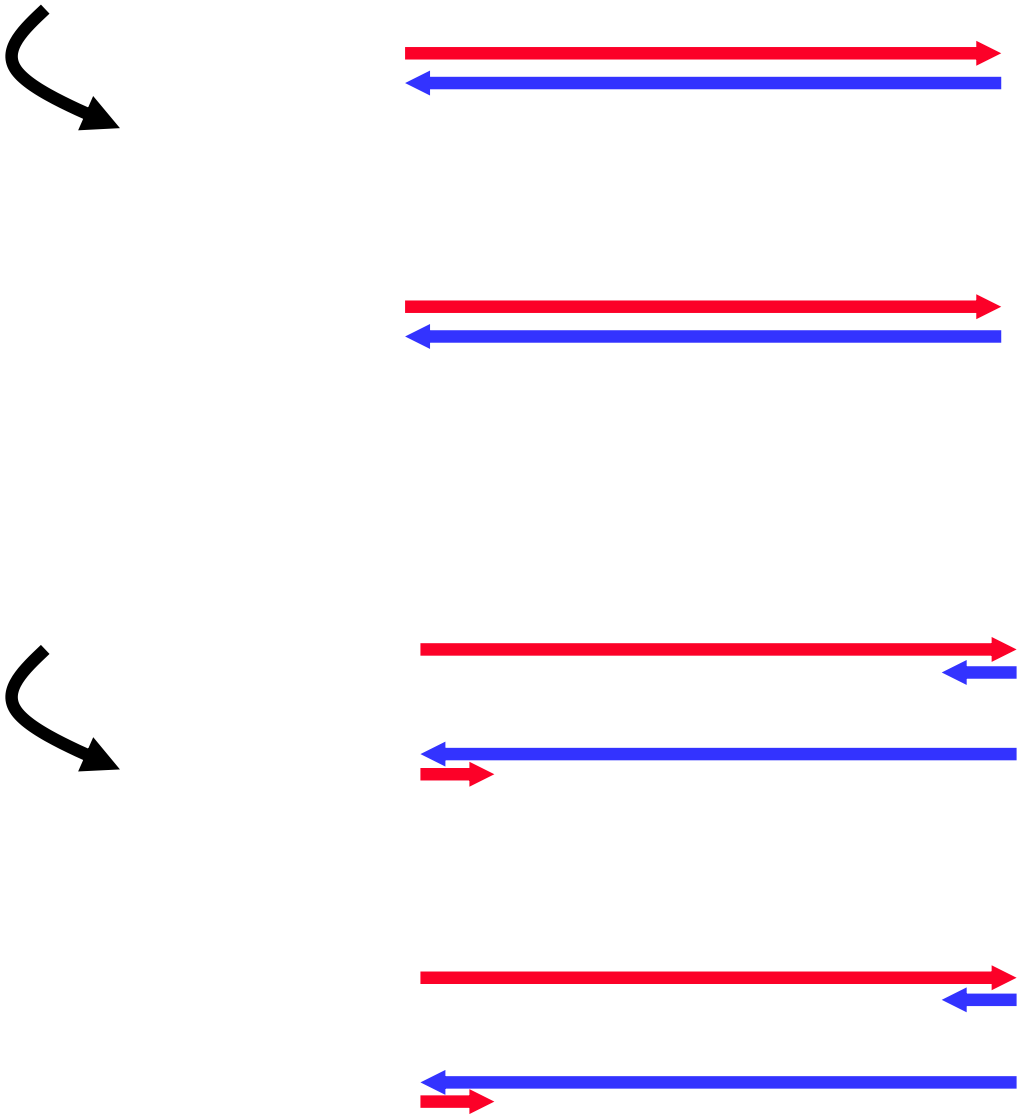
Polymerase chain reaction (PCR) analysis

1). primers are designed to flank the region to be amplified in target DNA
2). primers are annealed to denatured DNA
3). DNA is synthesized using Taq polymerase (from Thermus aquaticus)
4). primers are annealed again and the process is repeated through 20-30 cycles, geometrically amplifying the target sequence
5). DNA is analyzed by gel electrophoresis



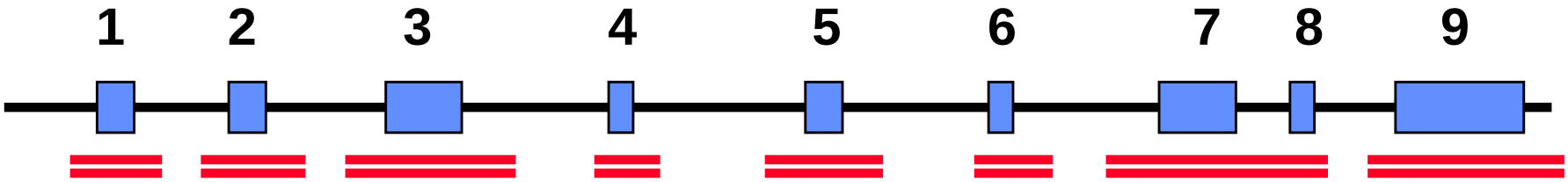
4).





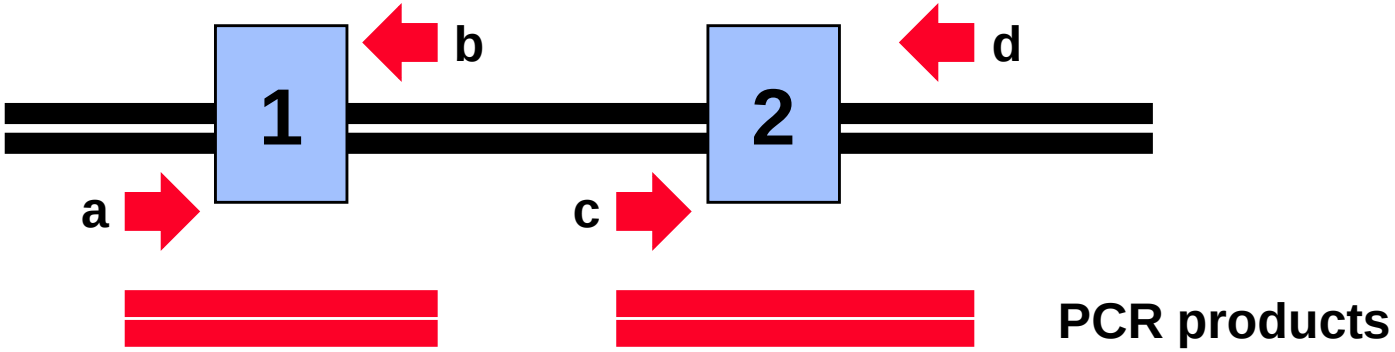
Detection of Lesch-Nyhan deletions by multiplex PCR

- exons in the HGPRT (HPRT) gene

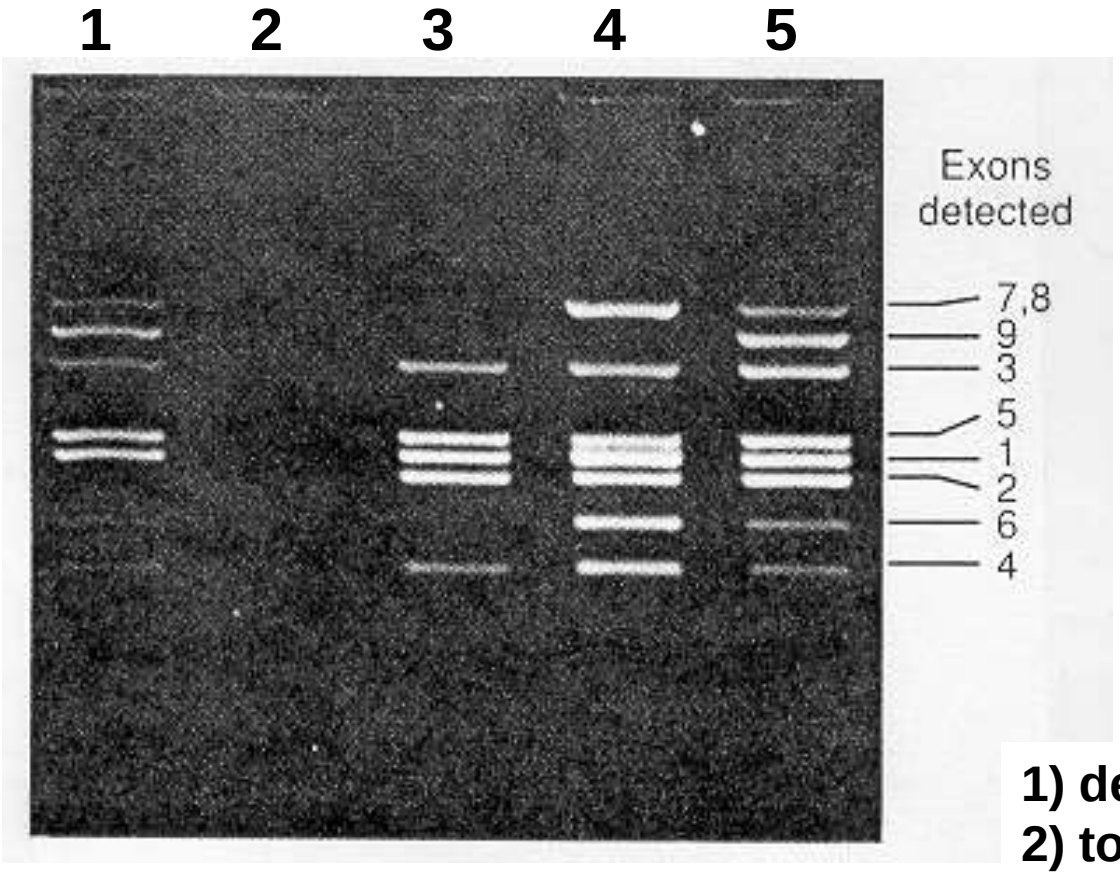


- PCR products resulting from the use of multiplex primers -- each product is flanked by its own set of leftward and rightward primers

- for example, for exons 1 and 2:



Detection of Lesch-Nyhan deletions by Multiplex PCR



1) deletion of exon 2
2) total gene deletion
3) deletion of exons 6-9
4) deletion of exon 9
5) normal

Enzymatic Techniques Used to Modify DNA

- The Polymerase Chain Reaction (PCR) is used to amplify DNA
  - The PCR cycle
    1. Denaturing linear duplex DNA
    2. Annealing an oligonucleotide primer
    3. Extending the DNA primer with heat stable DNA polymerase

The Polymerase Chain Reaction (PCR) Cycle

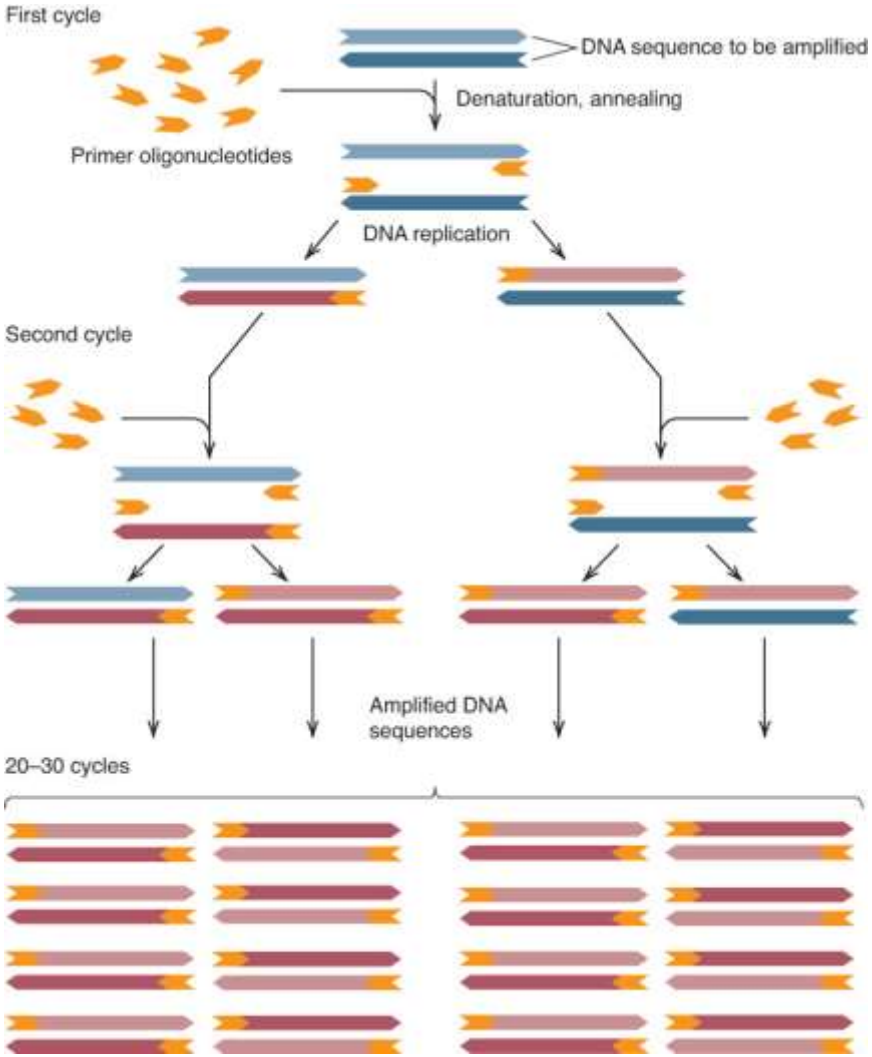


Figure 04.22: Schematic for the polymerase chain reaction (PCR), a method for amplifying a specific target DNA sequence.

Enzymatic Techniques Used to Modify DNA

- PCR can amplify a specific DNA region in only a few hours
  - Amplification of a segment of DNA for genetic engineering
  - Rapid detection of pathogenic bacteria and viruses
  - Detection of inborn errors of metabolism
  - Detection of tumors

DNA SEQUECING

Procedure for the Sanger dideoxy chain termination technique

- **DNA to be sequenced is amplified by PCR**
- **Four reactions are set up -- each containing one of four ddNTPs**
- **DNA is synthesized in the presence of the ddNTPs, giving rise to sets of DNA products representing all of the possible size fragments for the unknown sequence**
- **The fragments are resolved by gel electrophoresis and the sequence is read up from the bottom of the gel by identifying the lane giving the next larger size fragment**

Dideoxynucleotide Sequencing

- The Sanger-Chain Termination Method
- Uses small amounts of dideoxynucleotides that when incorporated cause termination of primer chain extension

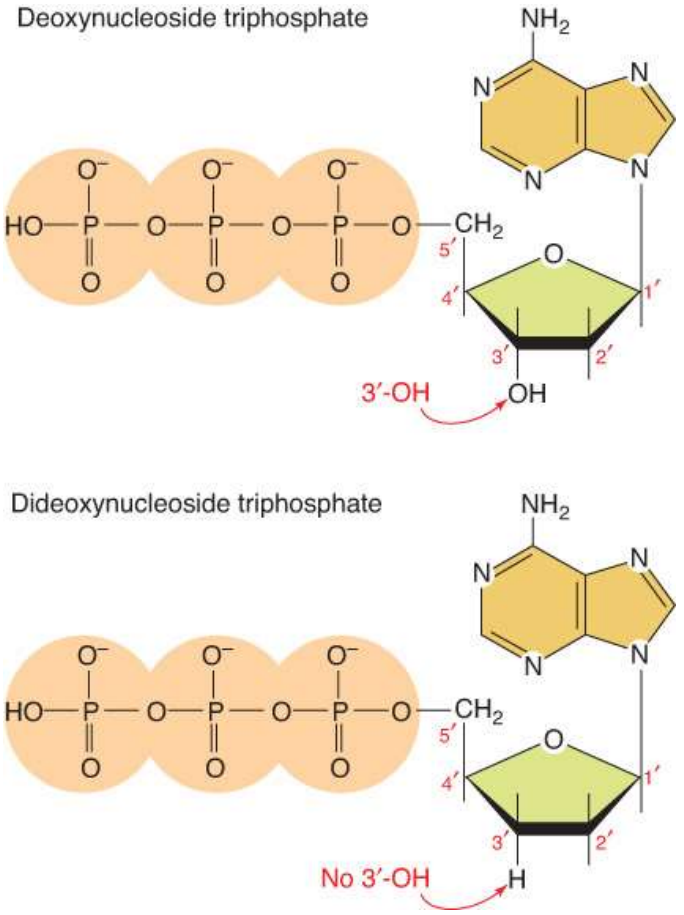
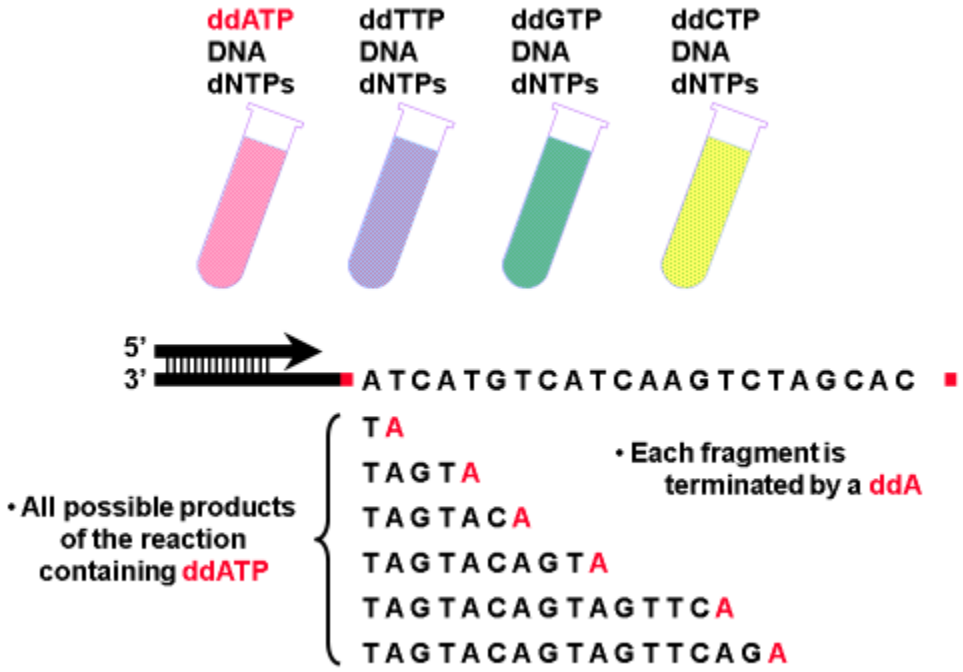
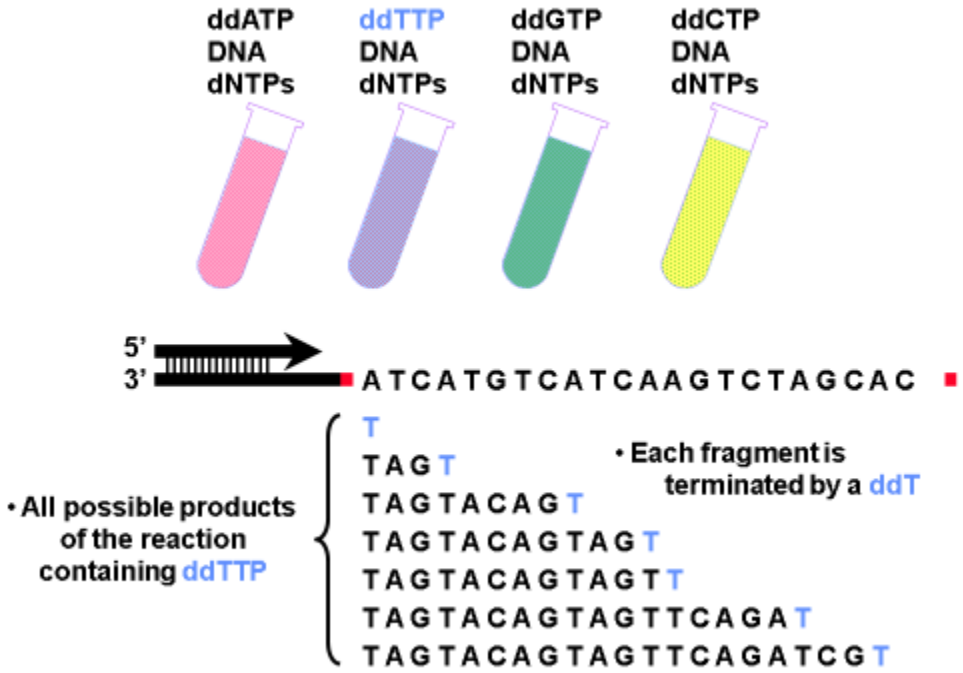
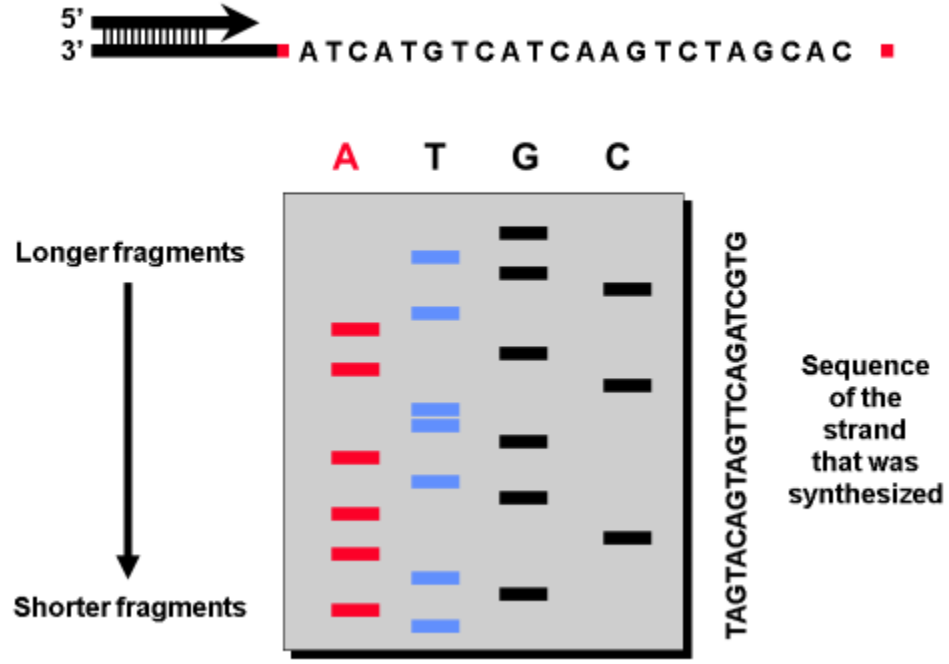


Figure 04.24: Comparison of deoxy- and dideoxynucleoside triphosphate structures.







Dideoxynucleotide Sequencing

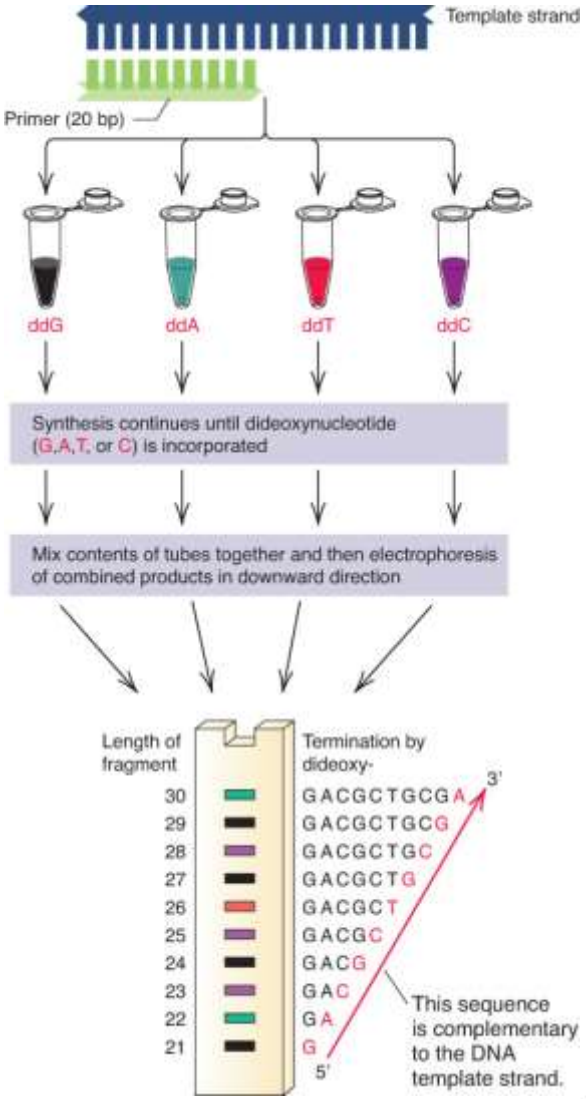


Figure 04.25: Chain termination method for sequencing DNA. Lengths of the terminated DNA fragments are shown at the left of the gel.

Dideoxynucleotide Sequencing

- Dideoxy-Terminator Cycle Sequencing
- 250 bp/day in middle 80,s
  - A major advance in dideoxynucleotide sequencing using a thermal cycler
    - Thermostable DNA polymerase
    - Fragments separated by capillary electrophoresis
  - Developed into a highly automated robotic system
  - 60,000000000 bp/10 min now

The Human Genome Sequence

- The Human Genome Project completed initial sequencing in 2001
  - 2.85 billion of the 3 billion nucleotides have been sequenced
    - Error rate estimated to be less than 1bp in every 10,000bp
    - Work continues to fill in the remaining gaps
  - Contains 20,000-25,000 protein coding genes
  - Almost identical (99.9%) in all people
  - Invaluable reference but requires extensive annotation that continues even today