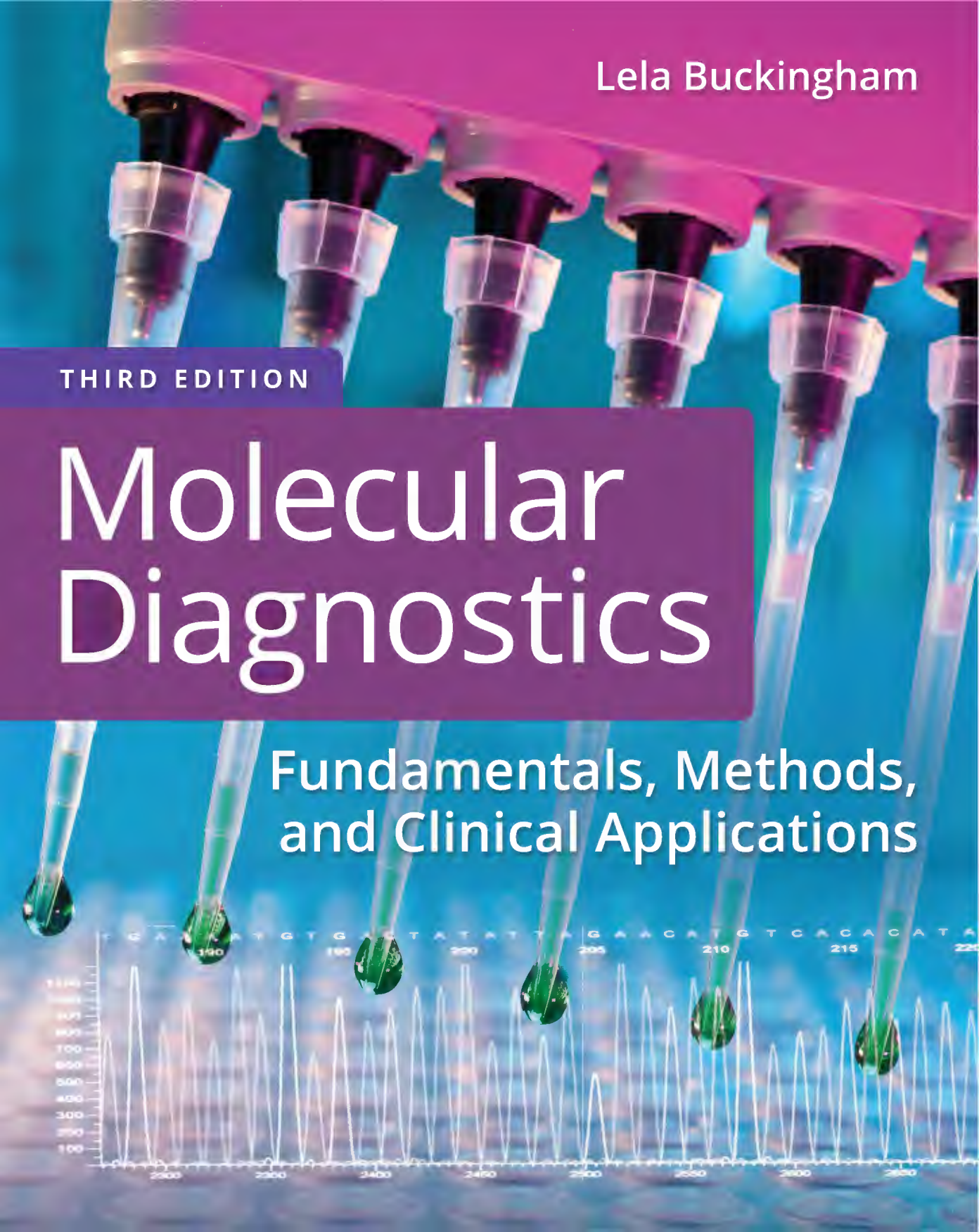
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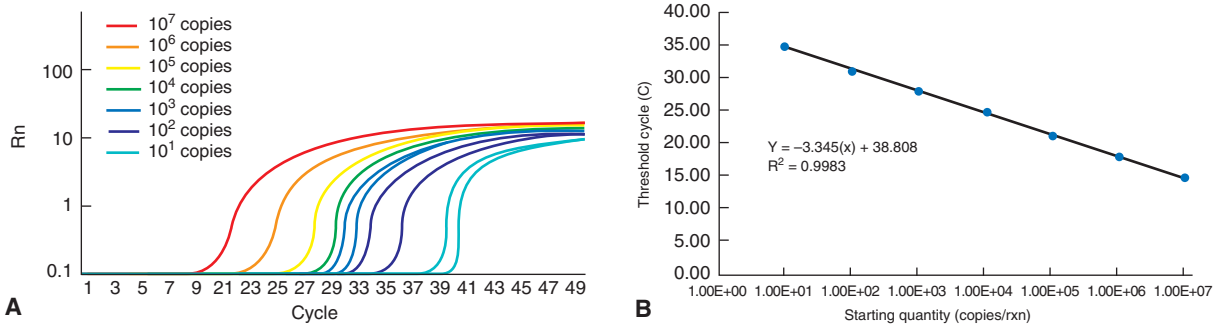
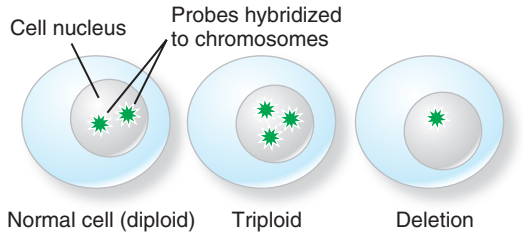
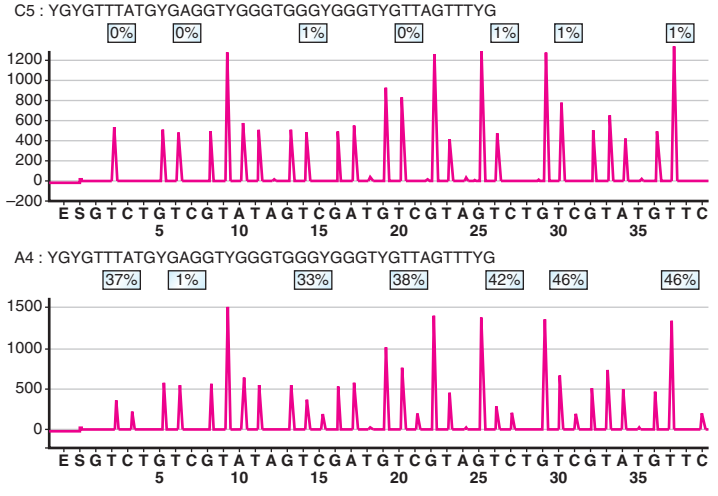


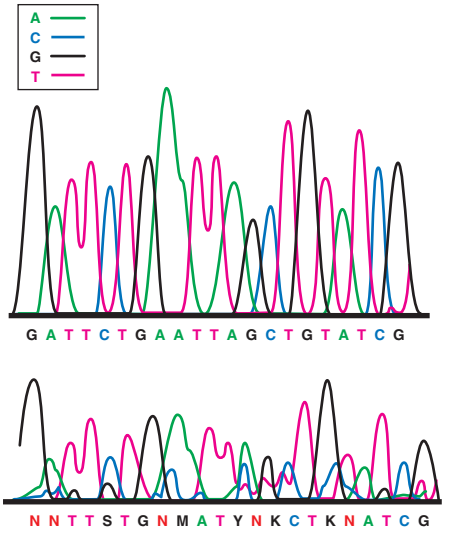
■ Quantitative polymerase chain reaction (PCR)



■ Fluorescence in situ hybridization (FISH) analysis for normal diploid cell (left), triploidy (center), and deletion (right)



■ DNA methylation at cytosine residues detected by pyrosequencing of bisulfite-treated DNA



■ Examples of good sequence quality (top) and poor sequence quality (bottom)

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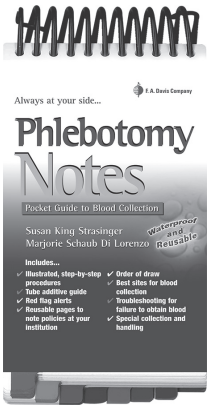

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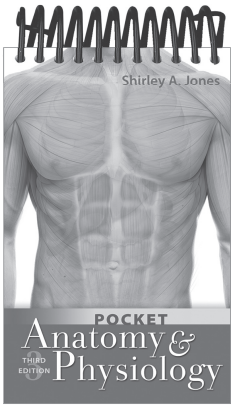

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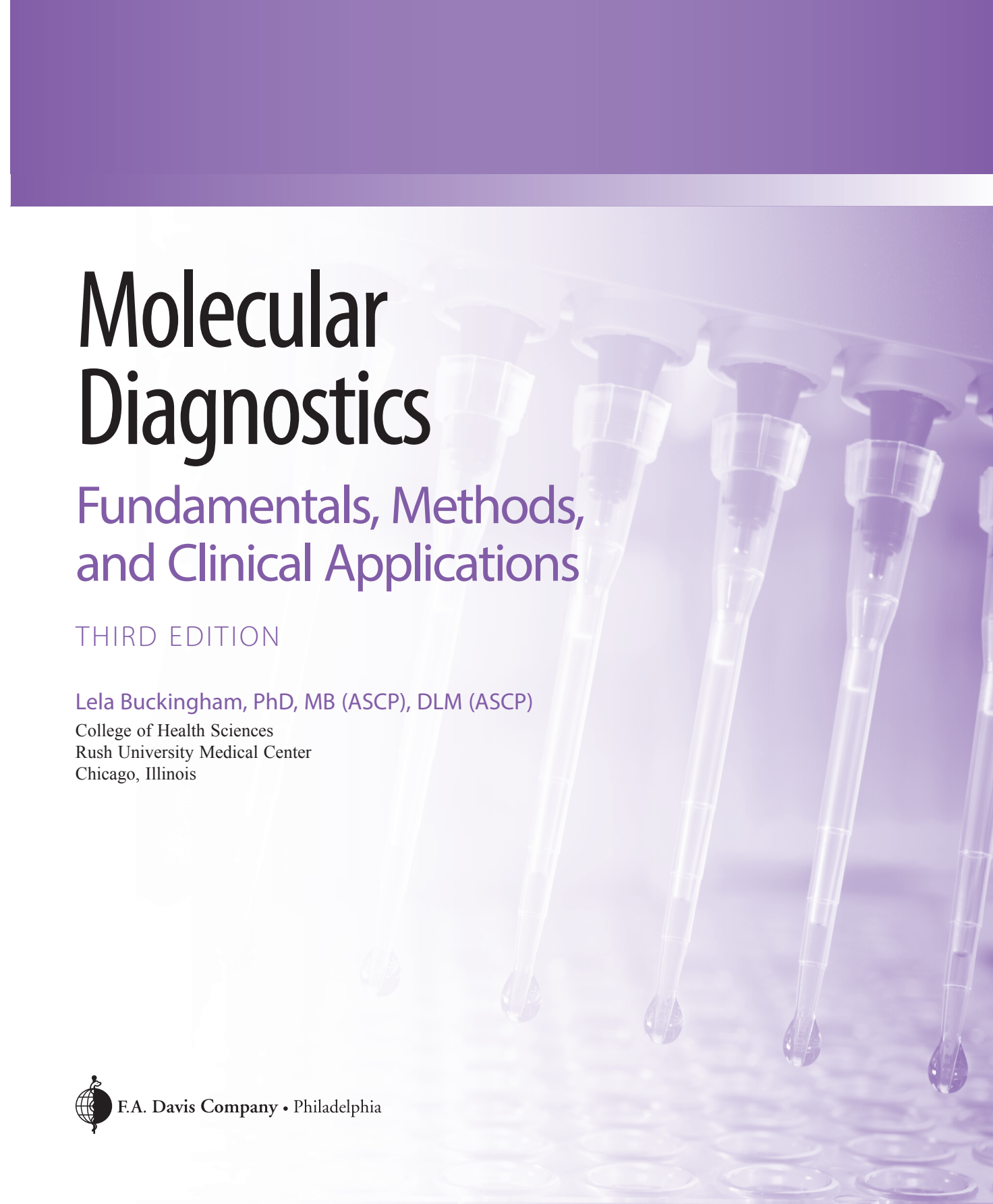

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To our students committed to
service through the practice of Medical Laboratory Science

Preface

Molecular technology continues to grow in importance in the clinical laboratory. Training of health-care professionals routinely includes molecular biology, from laboratory techniques to therapeutic decisions. This textbook was written to provide fundamental knowledge of molecular biology, current methods, and their clinical applications.

The primary audience for this text is students enrolled in Clinical/Medical Laboratory Science programs at all levels. It explains the principles of molecular technology that are used for diagnostic purposes. Examples of applications of molecular-based assays are included in the text, along with case studies that illustrate the use and interpretation of these assays in patient care.

This text is also appropriate for those in other health-related disciplines who need to understand the purpose, principles, and interpretation of the molecular diagnostic tests that they will be ordering and assessing for their patients.

Students who are first learning about molecular-based assays will find the text useful for explaining the fundamental principles. Practitioners who are performing and interpreting these assays can use this text as a resource for reference and troubleshooting and to drive the implementation of additional molecular-based assays in their laboratories.

An Instructor's Resource package has been developed for educators who adopt this text for a course. These resources, including PowerPoint presentations, a test-item bank, and additional case studies, are available on DavisPlus at http://davisplus.fadavis.com.

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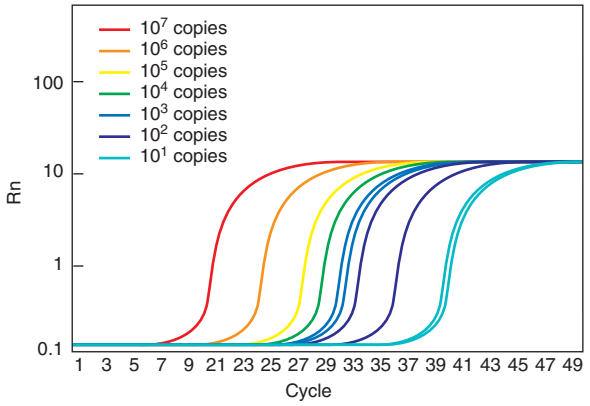
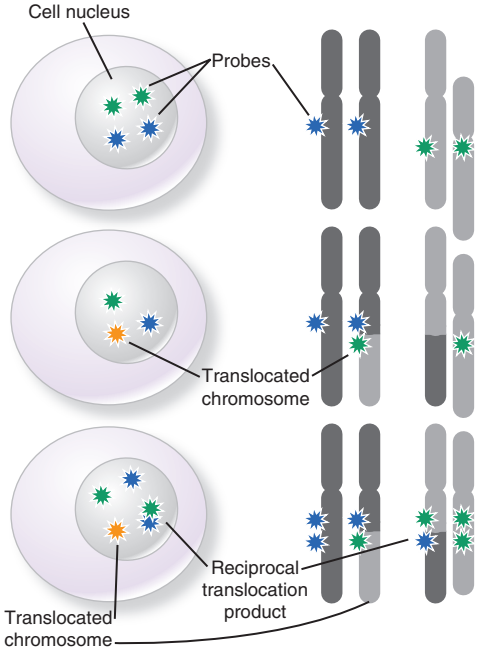
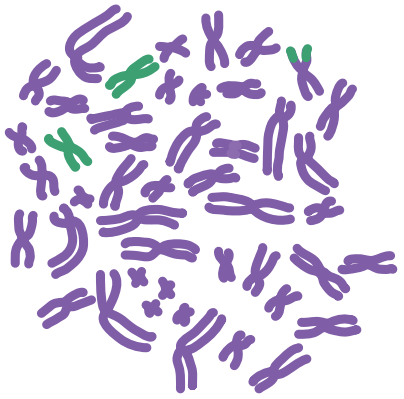


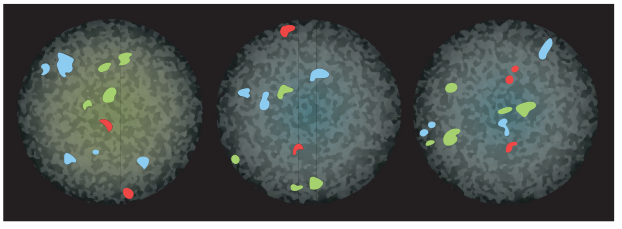
COLOR PLATE 1 A plot of the accumulation of polymerase chain reaction (PCR) product over 50 cycles of PCR. In this sigmoid curve, the generation of fluorescence occurs earlier with more starting template (solid lines) than with less (dotted lines). See Figure 6.13A in the text.



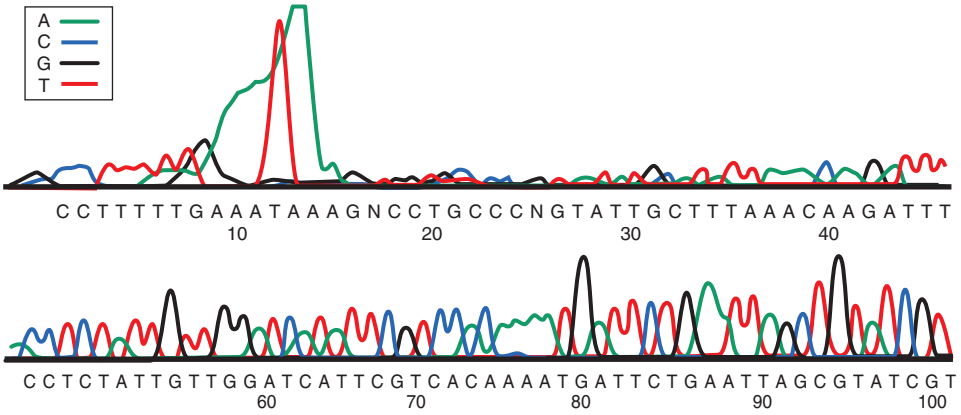
COLOR PLATE 2 Fluorescence in situ hybridization (FISH) analysis using distinct probes to detect a translocation. A normal nucleus has two signals from each probe *(top)*. A translocation involving the two chromosomes combines the two probe colors *(middle)*. Dual-fusion probes confirm the presence of the translocation by also giving a signal from the reciprocal breakpoint *(bottom)*. See Figure 7.16 in the text.



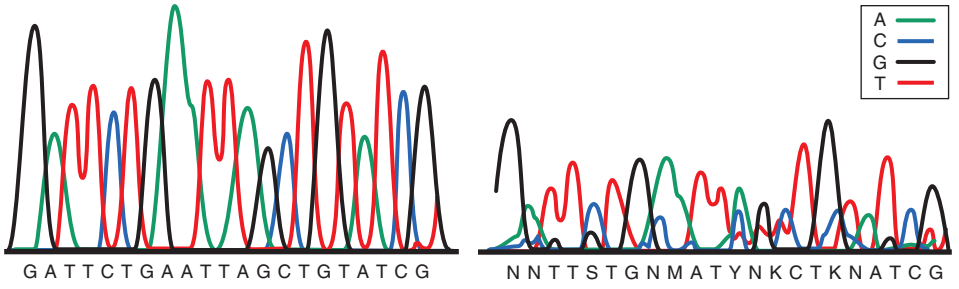
COLOR PLATE 3 Chromosome painting showing a derivative chromosome formed by the movement of a fragment of chromosome 12 (black) to an unidentified chromosome. See Figure 7.19 in the text.



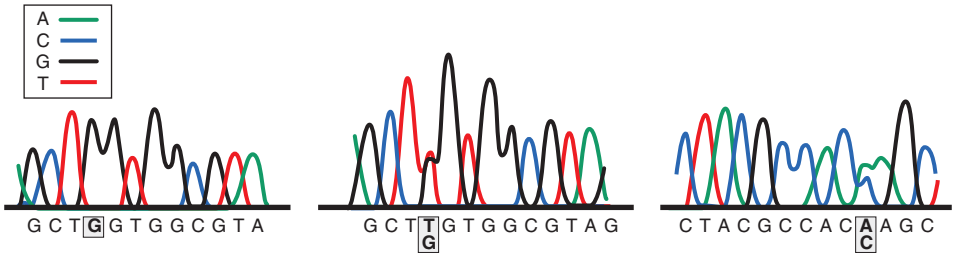
COLOR PLATE 4 Multicolor fluorescence in situ hybridization (FISH) analysis simultaneously reveals structural or numerical abnormalities in three loci. See Figure 7.21 in the text.



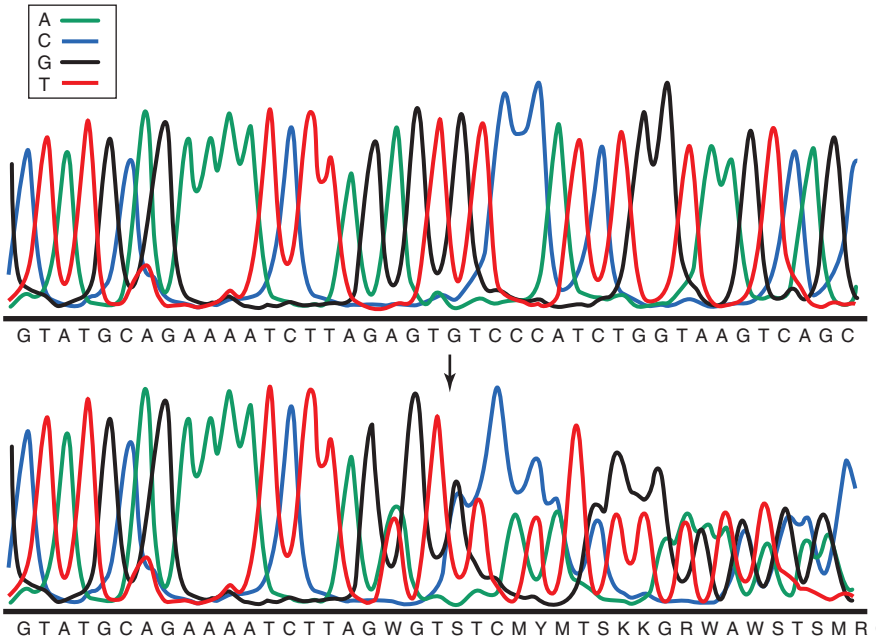
COLOR PLATE 5 Electropherogram showing a dye blob at the beginning of a sequence (nucleotide positions 9 to 15). The sequence read around this area is not accurate. See Figure 9.10 in the text.



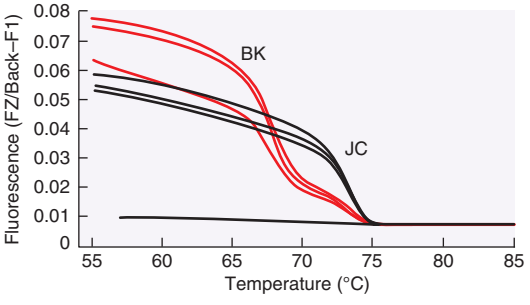
COLOR PLATE 6 Examples of good sequence quality *(left)* and poor sequence quality *(right)*. Note the clean baseline on the good sequence; that is, only one color peak is present at each nucleotide position. Automatic sequence-reading software will not accurately call a poor sequence. Compare the text sequences below the two scans. See Figure 9.11 in the text.

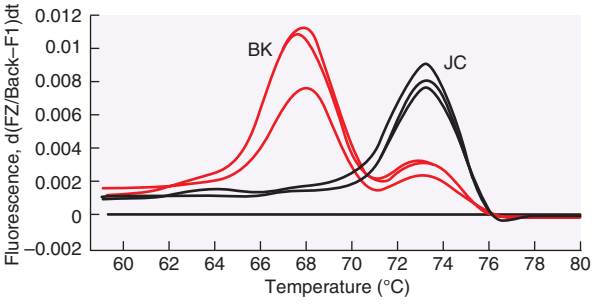


COLOR PLATE 7 Sequencing of a heterozygous G to T mutation in exon 12 of the *KRAS* gene. The normal codon sequence is GGT *(left)*. The heterozygous mutation (GT; *center*) is confirmed in the reverse sequence (CA; *right*). See Figure 9.12 in the text.



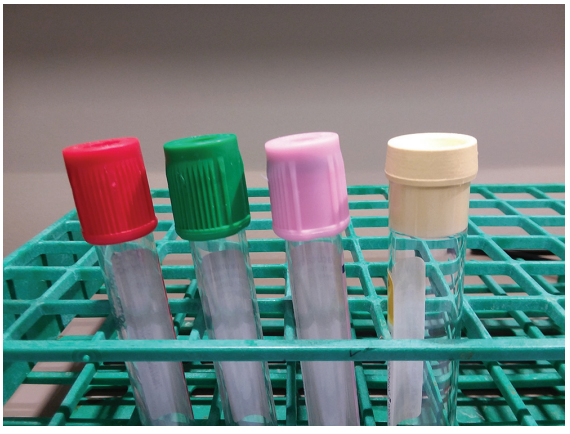
COLOR PLATE 8 The 187 delAG mutation in the *BRCA*1 gene detected by Sanger sequencing. This heterozygous dinucleotide deletion is evident in the lower panel where, at the site of the mutation, two sequences are overlaid: the normal sequence and the normal sequence minus two bases. See Figure 9.13 in the text.



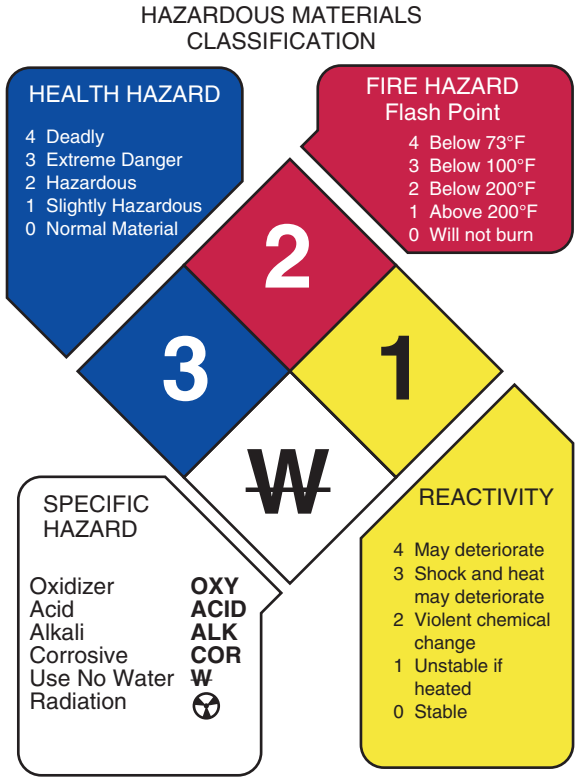


COLOR PLATE 9 Melt-curve analysis of BK and JC viruses. BK and JC are differentiated from one another by differences in the T_m* of the probe specific for each viral sequence. Fluorescence from double-stranded DNA decreases with increasing temperature and DNA denaturation to single strands *(top panel)*. Instrument software will present the derivative of the fluorescence *(bottom panel)* where the melting temperatures (T_m; 67°C to 68°C for BK and 73°C to 74°C for JC) are observed as peaks. See Figure 11.4 in the text.

COLOR PLATE 10 Biohazard stickers are required for cabinets, refrigerators, or freezers that contain potentially hazardous reagents or patient specimens. See Figure 15.1 in the text.



COLOR PLATE 11 For molecular analysis, blood or bone marrow specimens collected in ethylenediaminetetraacetic acid (EDTA; lavender-cap) or acid citrate dextrose (ACD; yellow-cap) tubes are preferred. Heparin (green cap) is used for cytogenetic tests. Immunoassays or mass-spectrometry methods may be performed on serum collected in tubes without coagulant (red-cap tubes). See Figure 15.3 in the text.

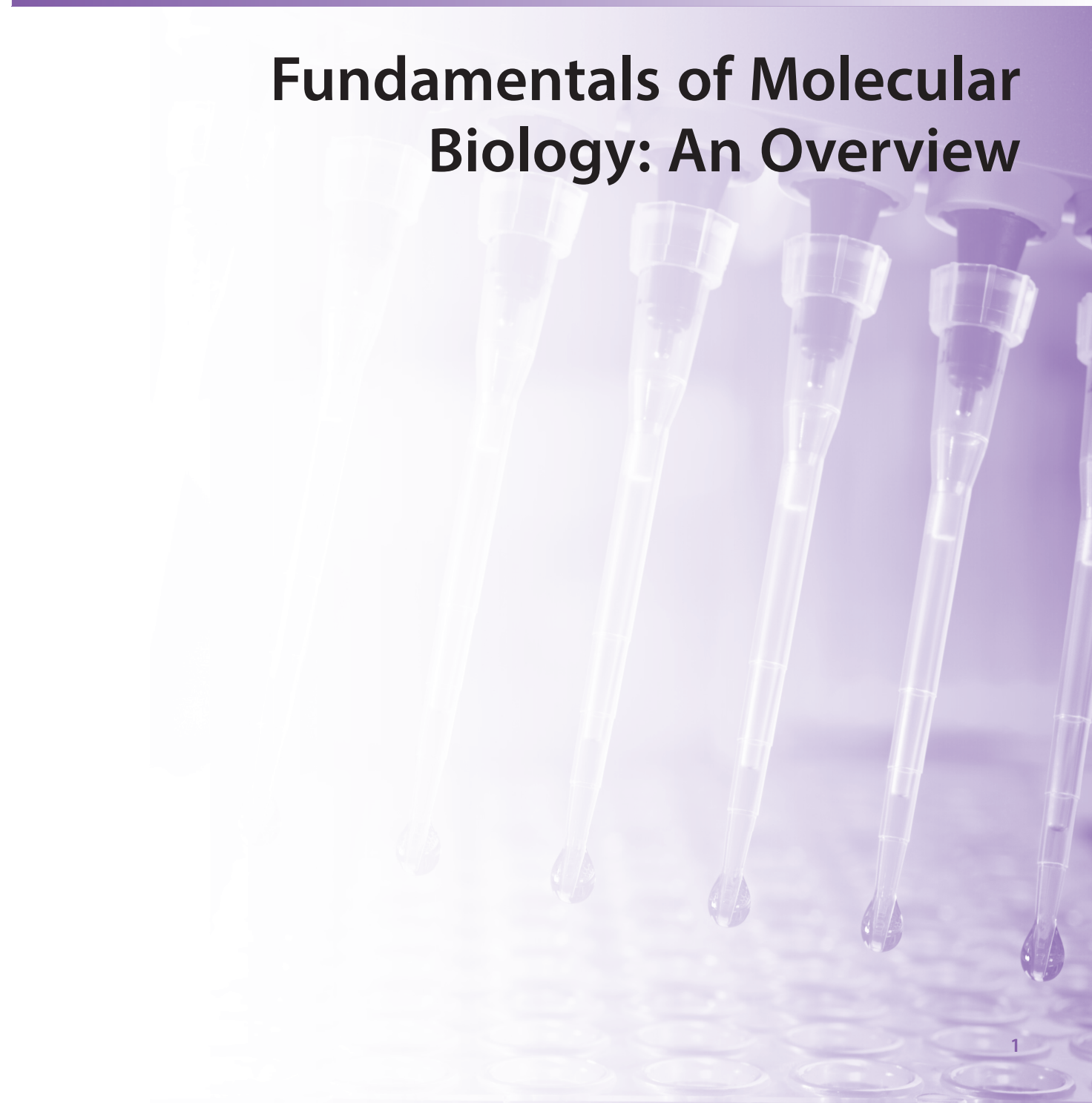


COLOR PLATE 12 National Fire Protection Association (NFPA) hazard labels have three parts, labeled with numbers 0 to 4, depending on the severity of the hazard, from none (0) to severe (4). The fourth section has two categories. OXY indicates a strong oxidizer, which greatly increases the rate of combustion. The W symbol indicates dangerous reactivity with water, which would prohibit the use of water to extinguish a fire in the presence of this chemical. See Figure 15.17 in the text.

COLOR PLATE 13 Rooms, cabinets, and equipment containing radioactive chemicals are identified with radiation safety labels. See Figure 15.18 in the text.

Section I

Fundamentals of Molecular Biology: An Overview



Chapter 1

Nucleic Acids and Proteins

Outline

DNA
DNA STRUCTURE
- *Nucleotides*
- *Nucleic Acid*

DNA REPLICATION
- *Polymerases*

ENZYMES THAT METABOLIZE DNA
- *Restriction Enzymes*
- *DNA Ligase*
- *Other DNA Metabolizing Enzymes*

RECOMBINATION IN SEXUALLY REPRODUCING ORGANISMS
RECOMBINATION IN ASEXUAL REPRODUCTION
- *Conjugation*
- *Transduction*
- *Transformation*

PLASMIDS
RNA
- *Transcription*
- *Transcription Initiation*
- *Transcription Elongation*
- *Transcription Termination*

TYPES/STRUCTURES OF RNA
- *Ribosomal RNA*
- *Messenger RNA*
  - Messenger RNA Processing
- *Small Nuclear RNA*
- *Transfer RNA*
- *Other RNAs*

RNA POLYMERASES
OTHER RNA-METABOLIZING ENZYMES
- *Ribonucleases*
- *RNA Helicases*

PROTEINS AND THE GENETIC CODE
- *Amino Acids*
- *Genes*
- *The Genetic Code*

TRANSLATION
- *Amino Acid Charging*
- *Protein Synthesis*

Objectives

1.1 Diagram the structure of nitrogen bases, nucleosides, and nucleotides.
1.2 Describe the nucleic acid structure as a polymer of nucleotides.
1.3 Demonstrate how deoxyribonucleic acid (DNA) is replicated such that the order or sequence of nucleotides is maintained (semiconservative replication).
1.4 Relate how ribonucleic acid (RNA) is synthesized (transcription) compared with DNA
1.5 List and describe types of RNA.

1.6 Explain the reaction catalyzed by polymerases that results in the phosphodiester backbone of the nucleic acid chains.
1.7 Note how the replicative process results in the antiparallel nature of complementary strands of DNA.
1.8 List the enzymes that modify DNA and RNA, and state their specific functions.
1.9 Explain mRNA processing, including capping, polyadenylation, and splicing.
1.10 Illustrate three ways in which DNA can be transferred between bacterial cells.
1.11 Define recombination, and sketch how new combinations of genes are generated in sexual and asexual reproduction.
1.12 Outline the structure and chemical nature of the 20 amino acids.
1.13 Show how the chemistry of the amino acids affects their chemical characteristics.
1.14 Give the definition of a gene.
1.15 Recount how the genetic code was solved.
1.16 Describe how amino acids are polymerized into proteins, using RNA as a guide (translation).
1.17 Relate protein function to the structural domains of the amino acid sequence.

When James Watson coined the term *molecular biology,*[1] he was referring to the biology of deoxyribonucleic acid (DNA). Of course, there are other molecules in nature. The term, however, is still used to describe the study of nucleic acids. In the medical laboratory, molecular techniques are designed for the handling and analysis of the nucleic acids, DNA and ribonucleic acid (RNA). Protein analysis and that of carbohydrates and other molecular species might also be categorized as "molecular" studies performed by flow cytometry, in situ histology, and tissue typing. The molecular biology laboratory, therefore, may be a separate entity or part of an existing clinical pathology unit. This chapter will address the nucleic acids.

Nucleic acids offer several characteristics that support their use for clinical purposes. Highly specific analyses can be carried out without the requirement for extensive physical or chemical selection of target molecules or organisms, allowing specific and rapid analysis from limited specimens. Furthermore, information carried in the order or sequence of the nucleotides that make up the nucleic acids is the basis for normal and pathological **traits** from microorganisms to humans and, as such, provides a powerful means of predictive analysis. Effective prevention and treatment of disease will result from the analysis of these molecules in the medical laboratory.

DNA

Deoxyribonucleic acid (DNA) is a macromolecule of carbon, nitrogen, oxygen, phosphorous, and hydrogen atoms. It is assembled in units of **nucleotides** that are composed of a phosphorylated ribose sugar and a **nitrogen base.** There are four nitrogen bases that make up the majority of DNA found in all organisms. These are **adenine, cytosine, guanine,** and **thymine.** Nitrogen bases are attached to a **deoxyribose** sugar, which forms a polymer with the deoxyribose sugars of other nucleotides through a **phosphodiester bond.** Linear assembly of the nucleotides makes up one strand of DNA. Two strands of DNA comprise the DNA double helix.

In 1871, Johann Friedrich Miescher published a paper on **nuclein,** the viscous substance extracted from cell nuclei. In his writings, he made no mention of the function of nuclein. Walther Flemming, a leading cell biologist, describing his work on the nucleus in 1882 admitted that the biological significance of the substance was unknown. We now know that the purpose of DNA, contained in the nucleus of the cell, is to store information. The information in the DNA storage system is based on the order or sequence of nucleotides in the nucleic acid polymer. Just as computer information storage is based on sequences of 0 and 1, biological information is based on sequences of A, C, G, and T. These four building blocks (with a few modifications) account for all of the biological diversity that makes up life on earth.

Historical Highlights

Johann Friedrich Miescher is credited with the discovery of DNA in 1869.[2] Miescher had isolated white blood cells out of seepage collected from discarded surgical bandages. He found that he could extract a viscous substance from the cells

in this material. Miescher also observed that most of the nonnuclear cell components could be lysed away with dilute hydrochloric acid, leaving the nuclei intact. Addition of extract of pig stomach (a source of pepsin to dissolve away contaminating proteins) resulted in a somewhat shrunken but clean preparation of nuclei. Extraction of these with alkali yielded the same substance isolated from the intact cells. It precipitated upon the addition of acid and redissolved in alkali. Chemical analysis of this substance demonstrated that it was 14% nitrogen and 2.5% phosphorus, different from any then-known group of biochemicals. He named the substance "nuclein." (Analytical data indicate that less than 30% of Miescher's first nuclein preparation was actually DNA.) He later isolated a similar viscous material from salmon sperm and noted: "If one wants to assume that a single substance . . . is the specific cause of fertilization, then one should undoubtedly first of all think of nuclein."

DNA STRUCTURE

The double helical structure of DNA (Fig. 1.1) was first described by James Watson and Francis Crick. Their molecular model was founded on previous observations of the chemical nature of DNA and physical evidence including diffraction analyses performed by Rosalind Franklin.[3] The helical structure of DNA results from the physicochemical demands of the linear array of nucleotides. Both the specific **sequence** (order) of nucleotides in the strand, as well as the surrounding chemical microenvironment, can affect the nature of the DNA helix.

Nucleotides

The four nucleotide building blocks of DNA are molecules of about 700 kd. Each nucleotide consists of a five-carbon sugar, the first carbon of which is covalently joined to a nitrogen base and the fifth carbon to a phosphate moiety (Fig. 1.2). A nitrogen base bound to an unphosphorylated sugar is a **nucleoside. Adenosine (A), guanosine (G), cytidine (C),** and **thymidine (T)** are nucleosides. If the ribose sugar is phosphorylated, the molecule is a nucleoside mono-, di-, or triphosphate or a nucleotide. For example, adenosine with one phosphate is adenosine monophosphate (AMP). Adenosine with three phosphates is adenosine triphosphate (ATP). Free nucleotides are deoxyribonucleoside triphosphates (e.g., dATP). They are routinely designated as A, C, G, and T in the DNA molecule. Nucleotides can be converted to nucleosides by hydrolysis.

The five-carbon sugar of DNA is deoxyribose, which is ribose with the number-two carbon of deoxyribose linked to a hydrogen atom rather than a hydroxyl group (see Fig. 1.2). The hydroxyl group on the third carbon is important for forming the phosphodiester bond that is the backbone of the DNA strand.

Nitrogen bases are planar carbon-nitrogen ring structures. The four common nitrogen bases in DNA are adenine, guanine, cytosine, and thymine. Amine and ketone substitutions, as well as the single or double bonds within the rings, distinguish the four bases that comprise the majority of DNA (Fig. 1.3). Nitrogen bases with a single-ring structure (thymine, cytosine) are **pyrimidines.** Bases with a double-ring structure (guanine, adenine) are **purines.**

The numbering of the positions in the nucleotide molecule starts with the ring positions of the nitrogen base, designated C or N 1, 2, 3, and so on. The carbons of the ribose sugar are numbered 1′ to 5′, distinguishing the positions of the sugar rings from those of the nitrogen base rings (Fig. 1.4).

Advanced Concepts

The double helix first described by Watson and Crick is DNA in its hydrated form (B-form) and is the standard form of DNA.[4] It has 10.5 steps or pairs of nucleotides (base pairs [bp]) per turn. Dehydrated DNA takes the A-form with about 11 bp per turn and the center of symmetry along the outside of the helix rather than down the middle as it is in the B-form. Both A- and B-form DNA are right-handed helices. Stress and torsion can throw the double helix into a Z-form. Z-DNA

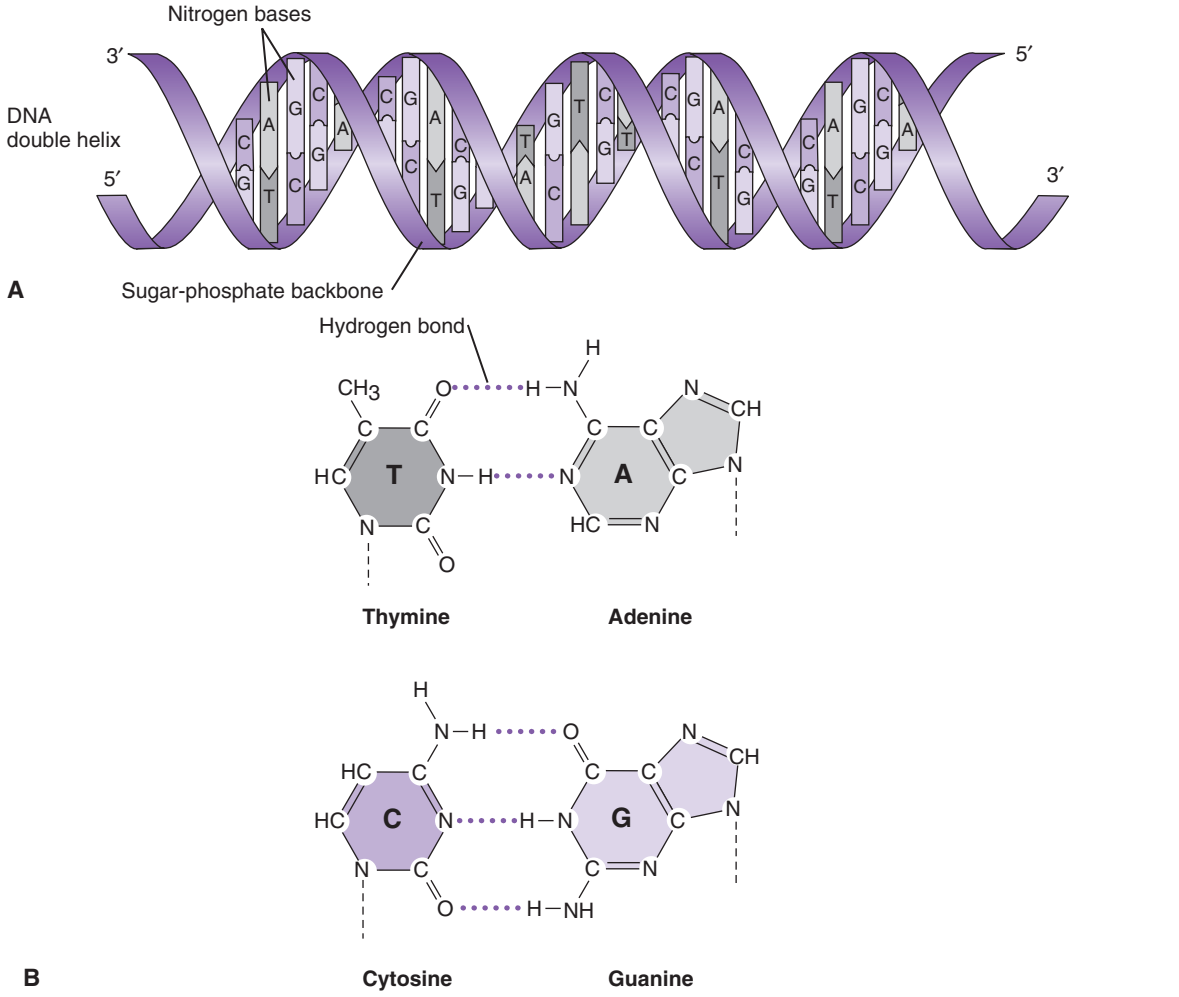


FIGURE 1.1 (A) The double helix. The phosphodiester backbones of the two nucleic acid chains form the helix. Nitrogen bases are oriented toward the center, where they hydrogen bond with homologous bases to stabilize the structure. (B) Two hydrogen bonds form between adenine and thymine. Three hydrogen bonds form between guanine and cytosine.

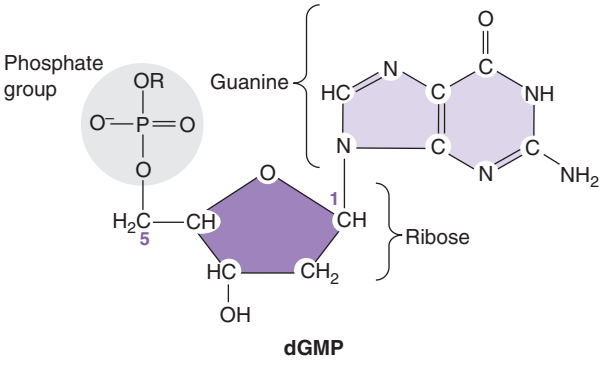


FIGURE 1.2 The nucleotide deoxyguanosine 5′ phosphate or guanosine monophosphate (dGMP). It is composed of deoxyribose covalently bound at its number 1 carbon to the nitrogen base, guanine, and at its number 5 carbon to a phosphate group. The molecule without the phosphate group is the nucleoside, deoxyguanosine.

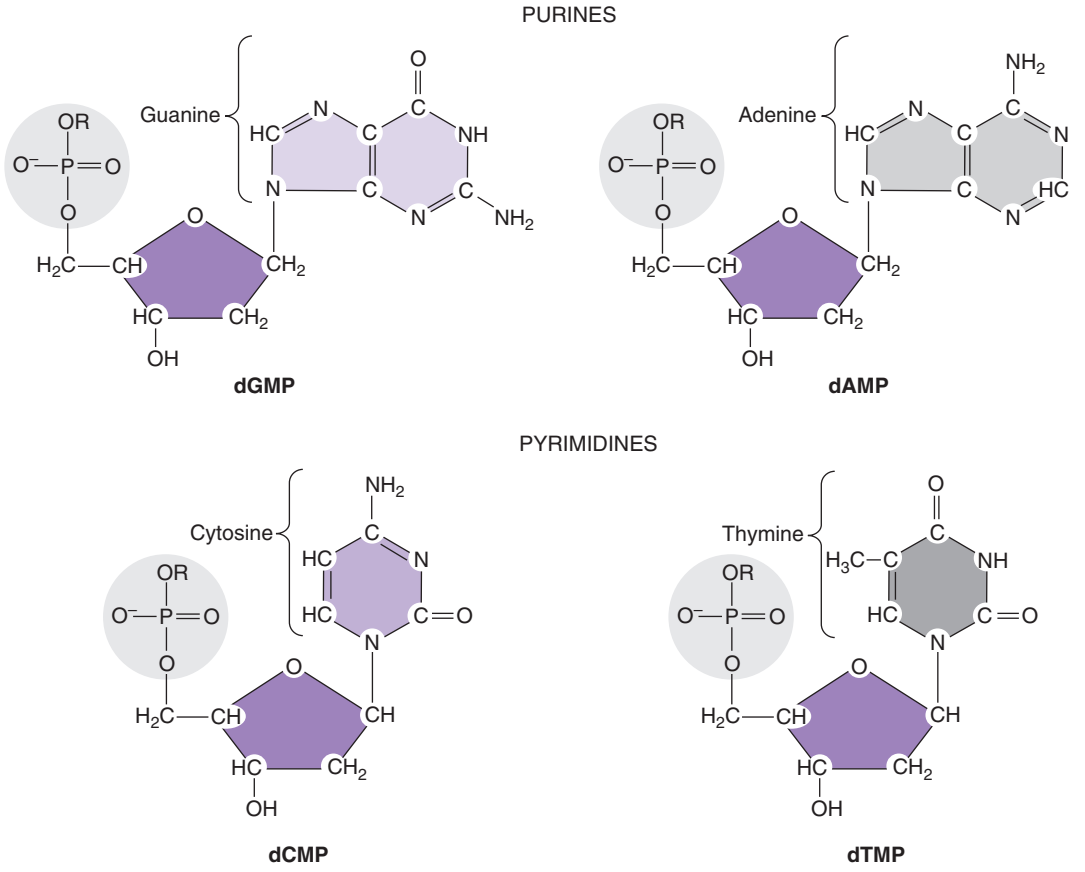


FIGURE 1.3 Nucleotides, deoxyguanosine monophosphate (dGMP), deoxyadenosine monophosphate (dAMP), deoxythymidine monophosphate (dTMP), and deoxycytidine monophosphate (dCMP), differ by the attached nitrogen bases. The nitrogen bases, guanine and adenine, have purine ring structures. Thymine and cytosine have pyrimidine ring structures. Uracil, the nucleotide base that replaces thymine in RNA, has the pyrimidine ring structure of thymine minus the methyl group and hydrogen bonds with adenine.

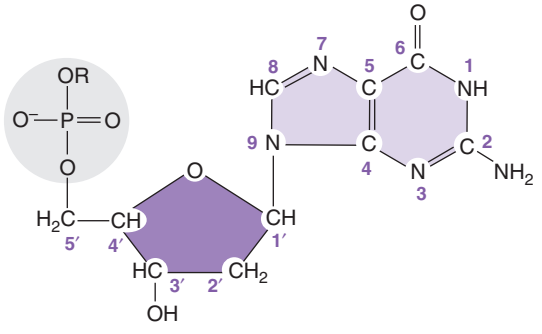


FIGURE 1.4 Carbon position numbering of a nucleotide monophosphate. The base carbons are numbered 1 through 9. The sugar carbons are numbered 1′ to 5′. The phosphate group on the 5′ carbon and the hydroxyl group on the 3′ carbon form phosphodiester bonds between bases. The 1′ carbon holds the nitrogen base.

is a left-handed helix with 12 bp per turn and altered geometry of the sugar-base bonds. Z-DNA has been observed in areas of chromosomes where the DNA is under torsional stress from unwinding for transcription or other metabolic functions.

Watson–Crick base pairing (purine:pyrimidine hydrogen bonding) is not limited to the ribofuranosyl nucleic acids, those found in our genetic system. Natural nucleic acid alternatives can also display the basic chemical properties of DNA (and RNA). Theoretical studies have addressed such chemical alternatives to DNA and RNA components. An example is the pentopyranosyl-(2′→4′) oligonucleotide system that exhibits stronger and more selective base pairing than DNA or RNA.[5] The study of nucleic acid alternatives has practical applications. For example, protein nucleic acids, which have a carbon-nitrogen peptide backbone replacing the sugar-phosphate backbone,[6,7] can be used in the laboratory as alternatives to DNA and RNA hybridization probes.[8,9] They have also been proposed as potential enzyme-resistant alternatives to RNA in antisense RNA therapies.[10]

Two DNA chains form **hydrogen bonds** with each other in a specific way. Guanines in one chain form three hydrogen bonds with cytosines in the opposite chain, and adenines form two hydrogen bonds with thymines (see Fig. 1.1B). In this way, single nucleic acid strands can bind or hybridize to single strands that have the corresponding bases. Hydrogen bonds between nucleotides are the key to the specificity of most nucleic acid–based tests used in the molecular laboratory. Specific hydrogen bond formation is also how the information held in the linear order of the nucleotides is maintained. As DNA is polymerized, each nucleotide to be added to the new DNA strand hydrogen bonds with the **complementary** nucleotide on the parental strand (A:T, G:C). In this way the parental DNA strand can be replicated without loss of the nucleotide order. Base pairs other than A:T and G:C or mismatches (e.g., A:C, G:T, A:A) can distort the DNA helix and disrupt the maintenance of sequence information.

Advanced Concepts

In addition to the four commonly occurring nucleotide bases, modified bases are also often found in nature. Base modifications have significant effects on phenotype. Some modified bases result from damage to DNA; others are naturally modified for specific functions or to affect gene expression, as will be discussed in later sections.

Modified nucleotides are used by bacteria and viruses as a primitive immune system that allows them to distinguish their own DNA from that of the host or invaders (restriction-modification [RM] system). Recognizing its own modifications, the host can target unmodified DNA for degradation.

Due to their effects on enzymes that metabolize DNA, modified nucleosides have been used effectively for clinical applications (Fig. 1.5). The anticancer drugs 5-bromouridine (5BrdU) and cytosine arabinoside (cytarabine, ara-C) are modified thymidine and cytosine

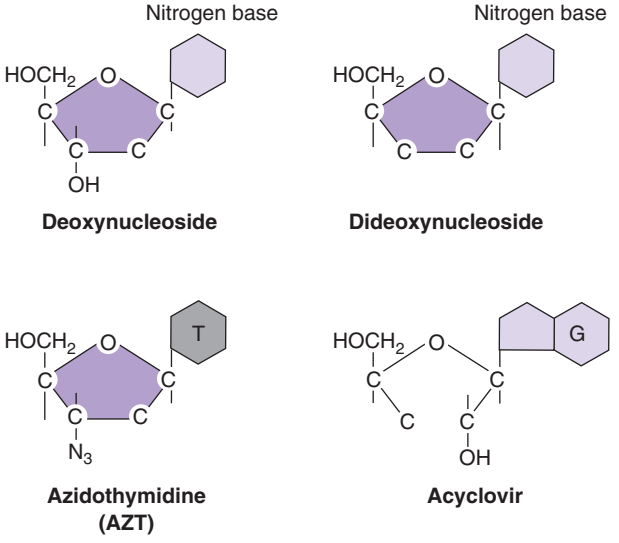


FIGURE 1.5 Substituted nucleosides used in the clinic and the laboratory. Dideoxynucleosides are used as laboratory reagents. Azidothymidine is an antiviral drug that inhibits the human immunodeficiency virus and is used to treat AIDS. Another antiviral, acyclovir, inhibits the growth of herpes viruses.

nucleosides, respectively. Azidothymidine (Retrovir, AZT), cytosine, 2′,3′-dideoxy-2′-fluoro (ddC), and 2′,3′-dideoxyinosine (Videx, ddI), drugs used to treat patients with human immunodeficiency virus (HIV) infections, are modifications of thymidine and cytosine and a precursor of adenine, respectively. An analog of guanosine, 2-amino-1,9-dihydro-9-[(2-hydroxyethoxy)methyl]-6*H*-purin-6-one (Acyclovir, Zovirax) is a drug used to combat herpes simplex virus and varicella-zoster virus.

In the laboratory, nucleosides can be modified for the purposes of labeling or detecting DNA molecules, sequencing, and other applications. The techniques used for these procedures will be discussed in later chapters.

Nucleic Acid

Nucleic acid is a macromolecule made of nucleotides bound together by the phosphate and hydroxyl groups on their sugars. A nucleic acid chain grows by the attachment of the 5′ phosphate group of an incoming nucleotide to the 3′ hydroxyl group of the last nucleotide on the growing chain (Fig. 1.6). Addition of nucleotides in this way gives the DNA chain a **polarity;** that is, it has a 5′ phosphate end and a 3′ hydroxyl end. We refer to DNA as oriented in a 5′ to 3′ direction, and the linear sequence of the nucleotides, by convention, is read in that order.

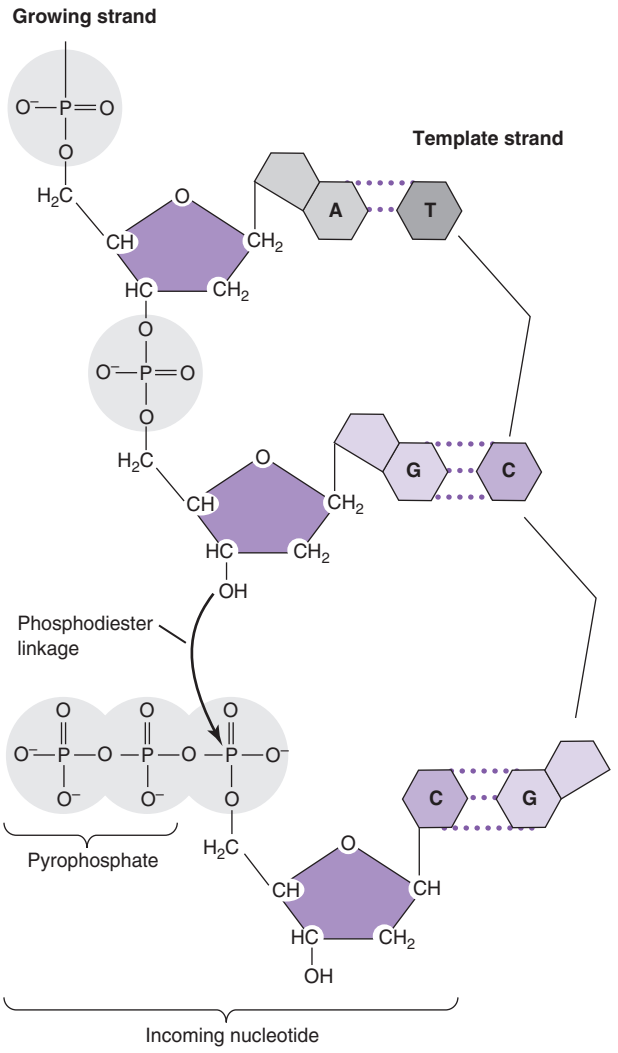


FIGURE 1.6 DNA replication is a template-guided polymerization catalyzed by DNA polymerase. The new strand is synthesized in the 5′ to 3′ direction, reading the template strand in the 3′ to 5′ direction.

Advanced Concepts

The sugar-phosphate backbones are arranged at specific distances from one another in the double helix (see Fig. 1.1). The two regions formed in the helix by the backbones are called the **major groove** and **minor groove.** The major and minor grooves are sites of interaction with the many proteins that bind to specific nucleotide sequences in DNA (binding or **recognition sites**). The double helix can also be penetrated by intercalating agents, molecules that slide transversely into the center of the helix. **Denaturing agents** such as formamide and urea displace the hydrogen bonds and separate the two strands of the helix.

DNA found in nature is mostly double stranded. Two strands exist in opposite 5′ to 3′/3′ to 5′ orientation, held together by the hydrogen bonds between their respective bases (A with T and G with C). The bases are positioned such that the sugar-phosphate chain that connects them **(sugar-phosphate backbone)** is oriented in a spiral or helix around the nitrogen bases (see Fig. 1.1).

The DNA double helix represents two versions of the information stored in the form of the order or sequence of the nucleotides on each chain. The sequences of the two strands that form the double helix are complementary, not identical (Fig. 1.7). They are in **antiparallel** orientation, with the 5′ end of one strand at the 3′ end of the other (Fig. 1.8). The formation of hydrogen bonds between two complementary strands of DNA is called **hybridization.** Single strands of DNA with identical sequences will not hybridize with each other. Later sections will describe the importance of this when designing assays.

PO 5′ **G T A G C T C G C T G A T** 3′ OH
HO 3′ **C A T C G A G C G A C T A** 5′ OP

FIGURE 1.7 Homologous sequences are not identical and are oriented in opposite directions.

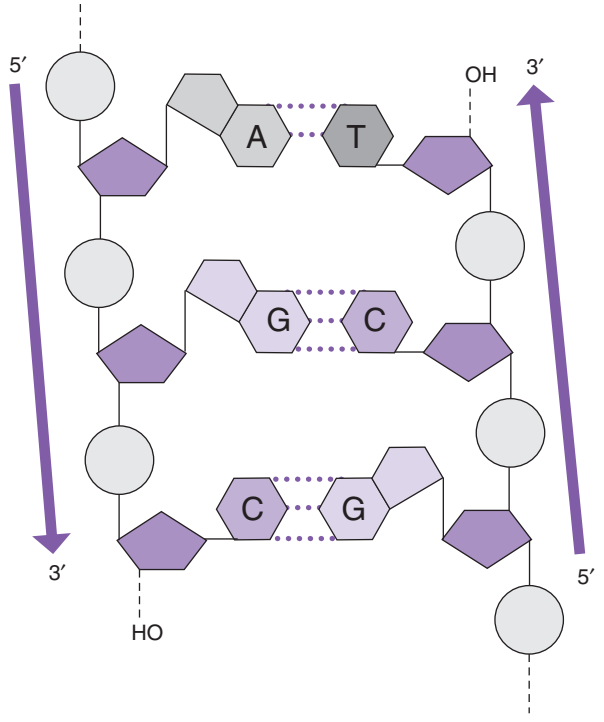


FIGURE 1.8 Because DNA synthesis proceeds from the 5′ phosphate group to the 3′ hydroxyl group and the template strand is copied in the opposite (3′ to 5′) direction, the new double helix consists of the template strand and the new daughter strand oriented in opposite directions from one another.

DNA REPLICATION

The two DNA strands of a double helix have an antiparallel orientation because of the way DNA is replicated. As DNA synthesis proceeds in the 5′ to 3′ direction, DNA polymerase, the enzyme responsible for polymerizing the nucleotide chains, uses a guide, or **template,** to determine which nucleotides to add to the chain. The enzyme reads the template in the 3′ to 5′ direction. The resulting double strand, then, will have a parent strand in one orientation and a newly synthesized strand arranged in the opposite orientation.

As Watson and Crick predicted, **semi-conservative** replication is the key to maintaining the sequence of the nucleotides in DNA through new generations. It is important that this information, in the form of the DNA sequence, be transferred faithfully at cell division. The replication apparatus is designed to copy the DNA strands in an orderly way with minimal errors before each cell division.

Historical Highlights

Before the double helix was determined, Erwin Chargaff[11] made the observation that the amount of adenine in DNA corresponded to the amount of thymine and the amount of cytosine to the amount of guanine. Upon the description of the double helix, Watson proposed that the steps in the ladder of the double helix were pairs of bases, thymine with adenine and guanine with cytosine. Watson and Crick, upon publication of their work, suggested that this arrangement was the basis for a copying mechanism. The complementary strands could separate and serve as guides or templates for producing complementary strands.

In the process of replication, DNA is first unwound from the helical duplex so that each single strand may serve as a template for the addition of nucleotides to the new strand (see Fig. 1.6). The new strand is elongated by hydrogen bonding of the complementary incoming nucleotide to the nitrogen base on the template strand and then a nucleophilic attack of the deoxyribose 3′ hydroxyl oxygen on a phosphorous atom of the phosphate group