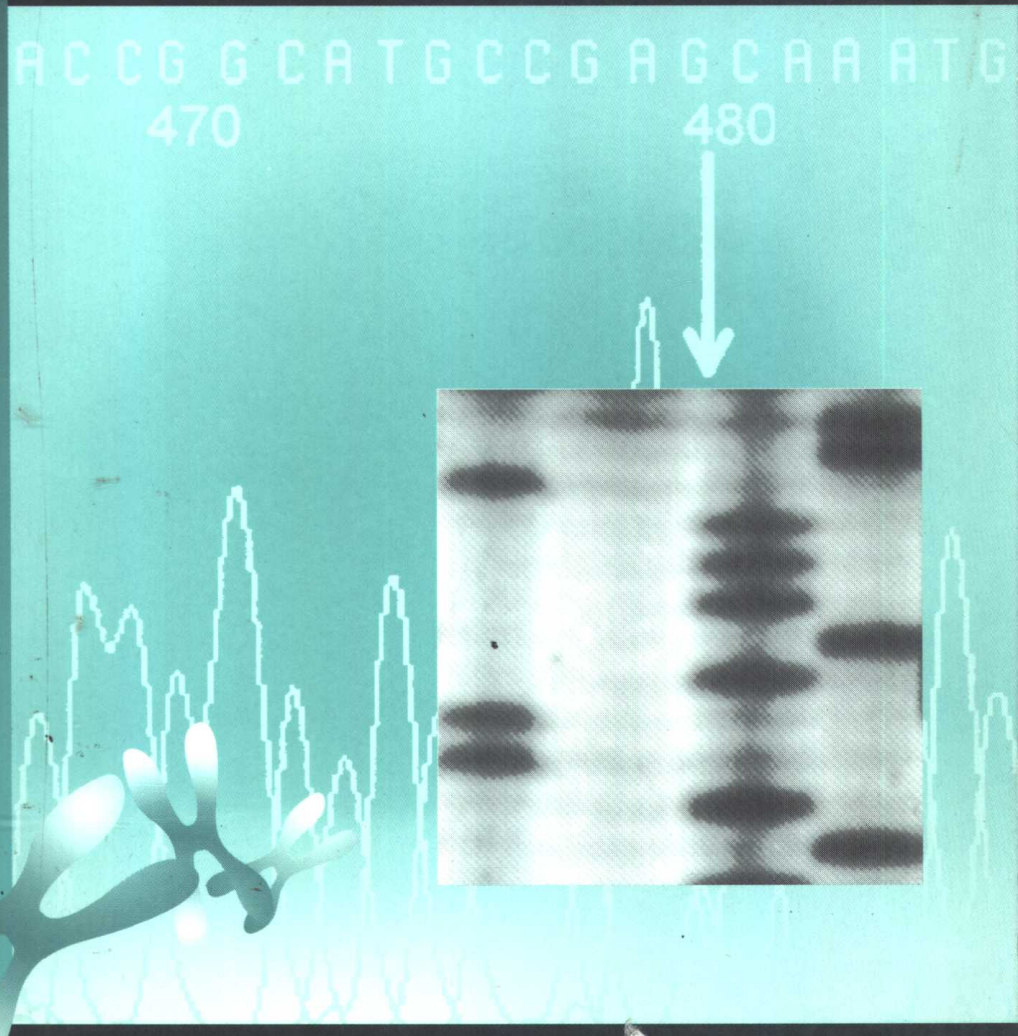
INTRODUCTION TO BIOTECHNIQUES

LUKE ALPHEY

DNA Sequencing

FROM EXPERIMENTAL METHODS TO BIOINFORMATICS



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Abbreviations

BLAST	Basic Local Alignment Search Tool
BSA	bovine serum albumin
CASP	critical assessment of structure prediction
CCD	charge-coupled device
cDNA	complementary DNA
DDBJ	DNA Databank of Japan
DDGE	double-strand denaturing gel electrophoresis
ddNTP	2′, 3′-dideoxynucleotide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EBI	European Bioinformatics Institute
EDTA	ethylenediamine tetraacetic acid
EMBL	European Molecular Biology Laboratory
EPD	Eukaryotic Promoter Database
EST	expressed sequence tag
ExoIII	exonuclease III
ftp	file transfer protocol
GCG	Genetics Computing Group
HPLC	high-performance liquid chromatography
HSSP	homology-derived structures of proteins
IUB	International Union of Biochemistry
IUPAC	International Union of Pure and Applied Chemistry
MCS	multiple cloning site
5-MeC	5-methylcytosine
mRNA	messenger RNA
NCBI	National Center for Biotechnology Information
NMR	nuclear magnetic resonance
NP-40	Nonidet P-40
OMIM	Online Mendelian Inheritance in Man
ORF	open reading frame
PC	personal computer
PCR	polymerase chain reaction
PDB	protein databank
PEG	polyethyleneglycol
PNK	poynucleotide kinase
REBASE	Restriction Enzyme Database
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RT–PCR	reverse transcriptase–polymerase chain reaction
SCOP	structural classification of proteins
SRS	Sequence Retrieval System

SSCP	single-strand conformation polymorphism
STS	sequence tagged site
TBE	Tris–borate–EDTA
TE	Tris-EDTA
TEMED	N,N,N′,N′-tetramethylethylenediamine
TES	2-[Tris(hydroxymethyl)methylamino]-1-ethanesulfonic acid
TREMBL	Translated EMBL
TTE	Tris–taurine–EDTA

Preface

In the 20 years since the current methods were first introduced, DNA sequencing has been at the heart of modern molecular biology. The sequence databases have been growing at an exponential rate, and even that rate of increase is improving, with doubling time down from about 22 months to 9 months. Whole new areas of research have been opened up by this technology, from molecular genetics to molecular taxonomy. With the advent of whole genome sequencing, exciting new vistas are emerging.

This book is intended as a practical guide, particularly at the strategic level. It aims to explain the options available and their relative merits, to allow the reader to decide which is most suitable for their application. The book covers the whole process of DNA sequencing, from planning the approach, through data acquisition, to extracting useful biological information from the data.

The book is aimed primarily at those new to DNA sequencing, but I hope that it will also prove a useful text for more experienced sequencers and that the information provided will be useful as a source of further information on familiar techniques and as a reference for less common ones. Part 1 describes the basic methods in detail, including manual and automated sequencing and the various pitfalls that may be encountered on the way. The equipment required is discussed, together with the advantages and disadvantages of each option.

Part 2 details the major applications of DNA sequencing: confirmatory sequencing to check a particular construct or mutant; sequencing PCR products; and strategies for sequencing large fragments of uncharacterized DNA. Part 3 covers Bioinformatics – the analysis of the sequence data to extract useful information. This section was contributed by Dr Andy Brass, Senior Lecturer in Bioinformatics at the University of Manchester, UK. It covers sequence analysis from checking and compiling the raw data through to homology searches and structural predictions.

Luke Alphey

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Safety

Certain reagents indicated for use in this book are chemically hazardous or radioactive. The researcher is cautioned to exercise care with these reagents and with the equipment (e.g. electrophoresis equipment) used in these procedures, strictly following the manufacturer's safety recommendations. Disposal of waste (including waste chemicals and radioactive materials), must comply with all local, national and other applicable regulations. These procedures may also be governed by other relevant regulations, for example those covering the containment and use of genetically modified micro-organisms. While every care has been taken to ensure that the experimental details discussed in this book are accurate and safe, the author accepts no liability for any loss or injury howsoever caused.

Many of the procedures discussed in this book are protected by patents or other legal protection. The reader is hereby notified that the purchase of this book does not convey any license or authorization to practise any of these procedures.

Contents

1 What is DNA Sequencing?

1.1 An introduction

DNA sequencing is the determination of all or part of the nucleotide sequence of a specific deoxyribonucleic acid (DNA) molecule. The ability to sequence DNA lies at the heart of the molecular biology revolution. Techniques to sequence DNA were developed only quite recently; the original papers describing the modern methods were published in 1977 [1, 2]. The rate at which new sequence information is determined has increased rapidly over the last 20 years. It is still accelerating, to the extent that the entire human genome sequence of approximately 3×10^9 base pairs will be determined within the next few years, as will the genome sequences of a considerable number of other organisms of medical, agricultural or scientific importance.

The fundamental reasons for wishing to know the sequence of a DNA molecule are:

- to make predictions about its function;
- to facilitate manipulation of the molecule.

The aim of this book is to show how DNA sequence information is obtained and analyzed, and some of the major reasons for doing so. Chapters 2–8 describe the sequencing methods in common usage, with particular emphasis on the relative merits and pitfalls of each approach. Chapters 9–11 describe the major applications of these methods. Subsequent chapters cover the computer-based analysis of sequence data.

Before discussing the principles behind DNA sequencing, we must first consider the structure of a DNA molecule.

1.2 Nucleic acid structure

The normal conformation of DNA is as a double helix (see *Figure 1.1*). This helix comprises two DNA strands running antiparallel to each other, each strand being a chain of bases, each base covalently linked to the next. The bases are each attached to deoxyribose, a sugar molecule, and each sugar molecule is linked to the adjacent sugar molecule via a phosphate group. The basic repeat unit of DNA therefore comprises a base, a sugar and a phosphate group, and is known as a nucleotide (see *Figures 1.2–1.5*).

The structure of a four-nucleotide segment of DNA is shown in *Figure 1.6*. Note that only one strand is shown. Note also the numbering of the carbon atoms in the deoxyribose (sugar) part of the molecule. These each have a 'prime', for example 5′ and 3′, to distinguish them from the atoms of the bases. It is the 5′ and 3′ carbons of adjacent sugars that are linked via the phosphate groups, so each covalently linked DNA strand will have a 5′ end and a 3′ end, as shown in

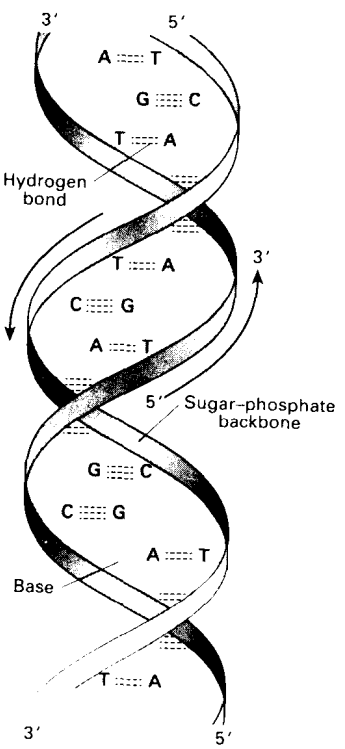

***FIGURE 1.1**: The DNA double helix. Reproduced from Williams* et al. *(1993)* Genetic Engineering, *BIOS Scientific Publishers Ltd.*

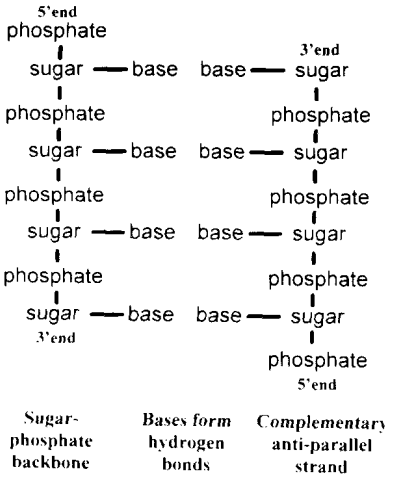

FIGURE 1.2: *Components of a DNA helix. A single strand of nucleic acid has a sugar–phosphate backbone to which the bases are attached. These linkages are all covalent. The other strand runs antiparallel. The two strands are held together by hydrogen bonds formed between complementary bases.*

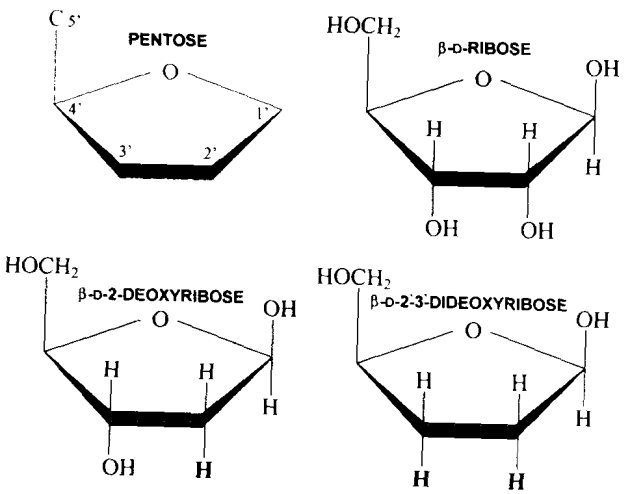

FIGURE 1.3: *Sugar structures of rNTPs and ddNTPs. The sugar–phosphate backbone of RNA contains the 5-carbon sugar ribose, whereas that of DNA contains 2' -deoxyribose. ddNTPs which are used in DNA sequencing by the chain termination method (Chapter 3) contain the synthetic analog 2', 3'-dideoxyribose. The standard numbering system for the carbon atoms is shown for a generic 5-carbon sugar (pentose). Carbon atoms in the sugar part of nucleotides are designated 1', 2', etc. to distinguish them from the atoms in the base.*

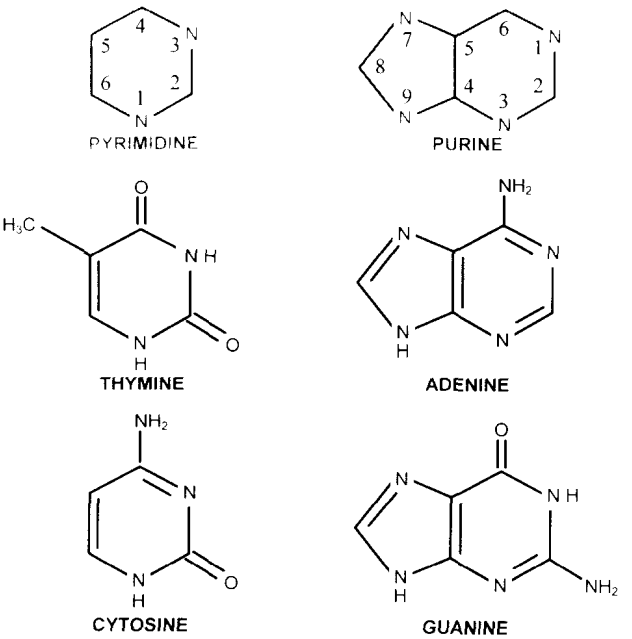

FIGURE 1.4: *The structure of the bases found in DNA. Thymine and cytosine are pyrimidines, adenine and guanine are purines. The numbering system for the ring atoms is shown. Pyrimidines are linked to the sugar at N_1, purines at N_9.*

Figure 1.6. In a linear double-stranded molecule, the 5′ end of one strand is complementary to the 3′ end of the other strand.

The two strands of the double helix are held together noncovalently by hydrogen bonds. The hydrogen bonds form between the complementary bases: adenine (A) pairs with thymine (T) and guanine (G) with cytosine (C) (see *Figure 1.7*).

Nucleoside = base + sugar

	base	*nucleoside*
A	adenine	adenosine
C	cytosine	cytidine
G	guanine	guanosine
T	thymine	thymidine
U	uracil	uridine

Nucleotide = base + sugar + phosphate

ATP	adenosine triphosphate
dATP	deoxyadenosine triphosphate
ddATP	dideoxyadenosine triphosphate
dGTP	deoxyguanosine triphosphate
dNTP	deoxynucleoside triphosphate
ddNTP	dideoxynucleoside triphosphate

FIGURE 1.5: *Nomenclature of nucleic acid precursors. The abbreviations A, C, G, T, etc. are usually clearer than the full names. dNTP and ddNTP can contain any base and are often used to refer to an equimolar mix of all four (di)deoxynucleoside triphosphates.*

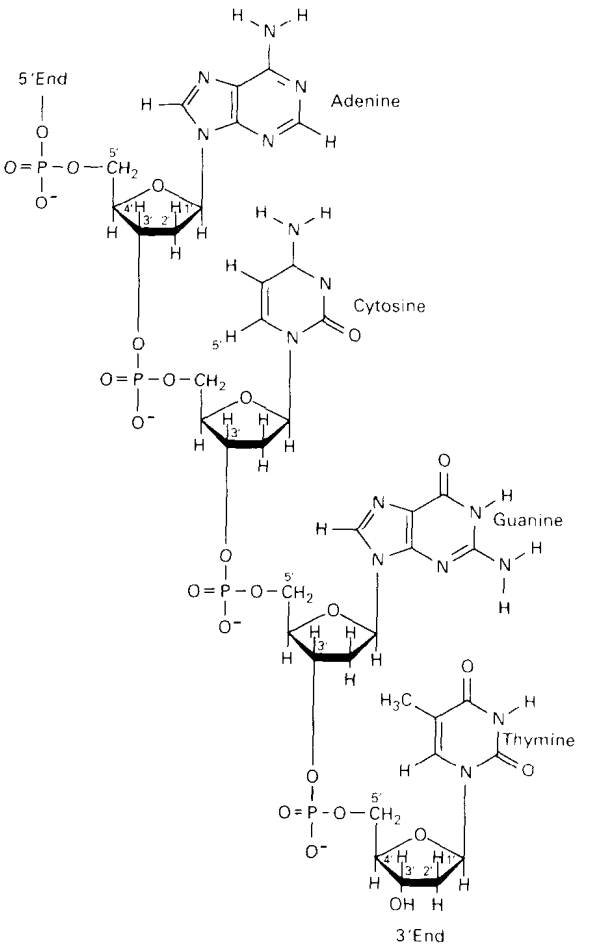

FIGURE 1.6: *A single-stranded DNA molecule four nucleotides in length. Reproduced from Newton and Graham (1994)* PCR, *BIOS Scientific Publishers Ltd.*

The related nucleic acid RNA (ribonucleic acid) differs from DNA in that the sugar in the sugar–phosphate backbone is ribose, rather than deoxyribose (see *Figure 1.3*), and uracil (U) is used in place of T.

1.3 DNA sequencing

Methods for sequencing RNA were developed earlier than for DNA, but now RNA is rarely sequenced directly. Instead, a complementary

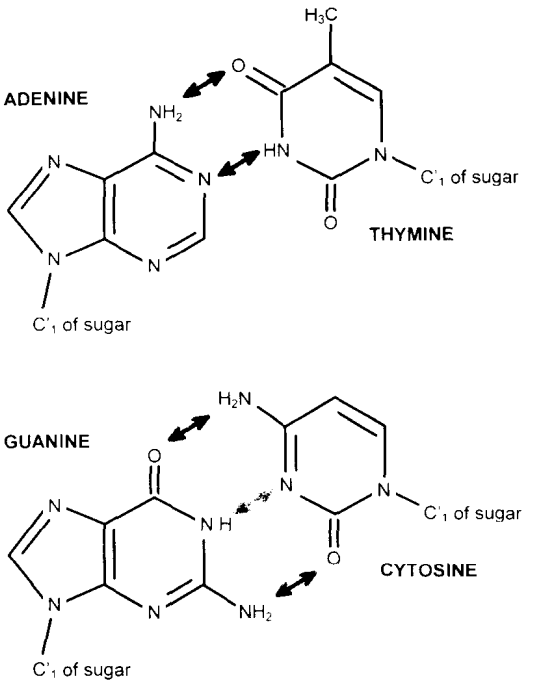

FIGURE 1.7: *Base pairing in DNA. Adenine (A) pairs with its complementary base thymine (T) and guanine (G) with cytosine (C). In RNA, uracil (U), replaces thymine. Note that the separation between the glycosidic bonds and the sugars are exactly the same (10.85 Å) for each base pair.*

DNA (cDNA) copy is synthesized. This cDNA is then sequenced, and the sequence of the original RNA deduced from this.

DNA sequencing is the determination of the base sequence of all or part of a DNA molecule. In the case of the molecule shown in *Figure 1.6*, DNA sequencing would advance our knowledge from 'a DNA fragment about four bases long' to 'a DNA fragment whose sequence is ACGT' (see *Figure 1.8*). Of course most, DNA molecules of biological interest are considerably longer than this!

The informational content of DNA is encoded in the order of the bases (A, C, G and T) in much the same way as binary information is stored in a computer as a string of 1s and 0s (*Figure 1.9*). The purpose of DNA sequencing is to determine the order (sequence) of these bases in a given DNA molecule. However, knowing the DNA sequence of a gene does not necessarily tell us what that gene does, any more than knowing the binary code of a computer program will necessarily tell