GENE CLONING AND DNA ANALYSIS
## Contents in Brief

### Part I The Basic Principles of Gene Cloning and DNA Analysis

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Why Gene Cloning and DNA Analysis are Important</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>Vectors for Gene Cloning: Plasmids and Bacteriophages</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>Purification of DNA from Living Cells</td>
<td>25</td>
</tr>
<tr>
<td>4</td>
<td>Manipulation of Purified DNA</td>
<td>47</td>
</tr>
<tr>
<td>5</td>
<td>Introduction of DNA into Living Cells</td>
<td>75</td>
</tr>
<tr>
<td>6</td>
<td>Cloning Vectors for <em>Escherichia coli</em></td>
<td>93</td>
</tr>
<tr>
<td>7</td>
<td>Cloning Vectors for Eukaryotes</td>
<td>111</td>
</tr>
<tr>
<td>8</td>
<td>How to Obtain a Clone of a Specific Gene</td>
<td>135</td>
</tr>
<tr>
<td>9</td>
<td>The Polymerase Chain Reaction</td>
<td>157</td>
</tr>
</tbody>
</table>

### Part II The Applications of Gene Cloning and DNA Analysis in Research

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Sequencing Genes and Genomes</td>
<td>175</td>
</tr>
<tr>
<td>11</td>
<td>Studying Gene Expression and Function</td>
<td>201</td>
</tr>
<tr>
<td>12</td>
<td>Studying Genomes</td>
<td>225</td>
</tr>
</tbody>
</table>

### Part III The Applications of Gene Cloning and DNA Analysis in Biotechnology

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>Production of Protein from Cloned Genes</td>
<td>247</td>
</tr>
<tr>
<td>14</td>
<td>Gene Cloning and DNA Analysis in Medicine</td>
<td>269</td>
</tr>
<tr>
<td>15</td>
<td>Gene Cloning and DNA Analysis in Agriculture</td>
<td>291</td>
</tr>
<tr>
<td>16</td>
<td>Gene Cloning and DNA Analysis in Forensic Science and Archaeology</td>
<td>311</td>
</tr>
</tbody>
</table>

Glossary 329

Index 345
Preface to the Seventh Edition  xvii
About the Companion Website  xix

Part I The Basic Principles of Gene Cloning and DNA Analysis  1

1 Why Gene Cloning and DNA Analysis are Important  3
  1.1 The early development of genetics  3
  1.2 The advent of gene cloning and the polymerase chain reaction  4
  1.3 What is gene cloning?  5
  1.4 What is PCR?  6
  1.5 Why gene cloning and PCR are so important  7
     1.5.1 Obtaining a pure sample of a gene by cloning  7
     1.5.2 PCR can also be used to purify a gene  8
  1.6 How to find your way through this book  11
  Further reading  12

2 Vectors for Gene Cloning: Plasmids and Bacteriophages  13
  2.1 Plasmids  13
     2.1.1 Size and copy number  14
     2.1.2 Conjugation and compatibility  16
     2.1.3 Plasmid classification  16
     2.1.4 Plasmids in organisms other than bacteria  17
  2.2 Bacteriophages  17
     2.2.1 The phage infection cycle  18
     2.2.2 Lysogenic phages  19
        Gene organization in the λ DNA molecule  19
        The linear and circular forms of λ DNA  19
        M13 – a filamentous phage  22
     2.2.3 Viruses as cloning vectors for other organisms  24
  Further reading  24
3 Purification of DNA from Living Cells 25

3.1 Preparation of total cell DNA 25
- 3.1.1 Growing and harvesting a bacterial culture 26
- 3.1.2 Preparation of a cell extract 28
- 3.1.3 Purification of DNA from a cell extract 29
  - Removing contaminants by organic extraction and enzyme digestion 29
  - Using ion-exchange chromatography to purify DNA from a cell extract 30
  - Using silica to purify DNA from a cell extract 30
- 3.1.4 Concentration of DNA samples 32
- 3.1.5 Measurement of DNA concentration 33
- 3.1.6 Other methods for the preparation of total cell DNA 34

3.2 Preparation of plasmid DNA 35
- 3.2.1 Separation on the basis of size 35
- 3.2.2 Separation on the basis of conformation 37
  - Alkaline denaturation 37
  - Ethidium bromide–cesium chloride density gradient centrifugation 38
- 3.2.3 Plasmid amplification 39

3.3 Preparation of bacteriophage DNA 40
- 3.3.1 Growth of cultures to obtain a high λ titre 41
- 3.3.2 Preparation of non-lysogenic λ phages 41
- 3.3.3 Collection of phages from an infected culture 43
- 3.3.4 Purification of DNA from λ phage particles 43
- 3.3.5 Purification of M13 DNA causes few problems 43

Further reading 45

4 Manipulation of Purified DNA 47

4.1 The range of DNA manipulative enzymes 48
- 4.1.1 Nucleases 48
- 4.1.2 Ligases 50
- 4.1.3 Polymerases 51
- 4.1.4 DNA-modifying enzymes 52

4.2 Enzymes for cutting DNA: Restriction endonucleases 53
- 4.2.1 The discovery and function of restriction endonucleases 54
- 4.2.2 Type II restriction endonucleases cut DNA at specific nucleotide sequences 55
- 4.2.3 Blunt ends and sticky ends 55
- 4.2.4 The frequency of recognition sequences in a DNA molecule 57
- 4.2.5 Performing a restriction digest in the laboratory 58
- 4.2.6 Analysing the result of restriction endonuclease cleavage 59
  - Separation of molecules by gel electrophoresis 59
  - Visualizing DNA molecules in an agarose gel 60
- 4.2.7 Estimation of the sizes of DNA molecules 61
- 4.2.8 Mapping the positions of different restriction sites in a DNA molecule 62
6.2 Cloning vectors based on \(\lambda\) bacteriophage 99

6.2.1 Segments of the \(\lambda\) genome can be deleted without impairing viability 99

6.2.2 Natural selection was used to isolate modified \(\lambda\) that lack certain restriction sites 100

6.2.3 Insertion and replacement vectors 102

6.2.4 Cloning experiments with \(\lambda\) insertion or replacement vectors 103

6.2.5 Long DNA fragments can be cloned using a cosmid 103

6.2.6 \(\lambda\) and other high-capacity vectors enable genomic libraries to be constructed 104

6.3 Cloning vectors for the synthesis of single-stranded DNA 106

6.3.1 Vectors based on M13 bacteriophage 107

6.3.2 Hybrid plasmid–M13 vectors 108

6.4 Vectors for other bacteria 109

Further reading 110

7 Cloning Vectors for Eukaryotes 111

7.1 Vectors for yeast and other fungi 111

7.1.1 Selectable markers for the 2 \(\mu\)m plasmid 112

7.1.2 Vectors based on the 2 \(\mu\)m plasmid: Yeast episomal plasmids 112

7.1.3 A YEp may insert into yeast chromosomal DNA 113

7.1.4 Other types of yeast cloning vector 115

7.1.5 Artificial chromosomes can be used to clone long pieces of DNA in yeast 116

7.1.6 Vectors for other yeasts and fungi 118

7.2 Cloning vectors for higher plants 119

7.2.1 \textit{Agrobacterium tumefaciens}: nature’s smallest genetic engineer 119

Using the Ti plasmid to introduce new genes into a plant cell 120

Production of transformed plants with the Ti plasmid 122

The Ri plasmid 123

Limitations of cloning with \textit{Agrobacterium} plasmids 123

7.2.2 Cloning genes in plants by direct gene transfer 124

Direct gene transfer into the nucleus 125

Transfer of genes into the chloroplast genome 125

7.2.3 Attempts to use plant viruses as cloning vectors 126

Caulimovirus vectors 127

Geminivirus vectors 127

7.3 Cloning vectors for animals 127

7.3.1 Cloning vectors for insects 128

P elements as cloning vectors for \textit{Drosophila} 128

Cloning vectors based on insect viruses 129
7.3.2 Cloning in mammals 130
   Viruses as cloning vectors for mammals 130
   Gene cloning without a vector 131

Further reading 132

8 How to Obtain a Clone of a Specific Gene 135
8.1 The problem of selection 135
   8.1.1 There are two basic strategies for obtaining the clone you want 136
8.2 Direct selection 137
   8.2.1 Marker rescue extends the scope of direct selection 138
   8.2.2 The scope and limitations of marker rescue 139
8.3 Identification of a clone from a gene library 140
   8.3.1 Gene libraries 140
      Not all genes are expressed at the same time 140
      mRNA can be cloned as complementary DNA 142
8.4 Methods for clone identification 143
   8.4.1 Complementary nucleic acid strands hybridize to each other 143
   8.4.2 Colony and plaque hybridization probing 144
      Labelling with a radioactive marker 145
      Non-radioactive labelling 146
   8.4.3 Examples of the practical use of hybridization probing 146
      Abundancy probing to analyse a cDNA library 147
      Oligonucleotide probes for genes whose translation products have been characterized 148
      Heterologous probing allows related genes to be identified 150
      Southern hybridization enables a specific restriction fragment containing a gene to be identified 151
   8.4.4 Identification methods based on detection of the translation product of the cloned gene 153
      Antibodies are required for immunological detection methods 153
      Using a purified antibody to detect protein in recombinant colonies 154
      The problem of gene expression 155

Further reading 155

9 The Polymerase Chain Reaction 157
9.1 PCR in outline 157
9.2 PCR in more detail 159
   9.2.1 Designing the oligonucleotide primers for a PCR 159
   9.2.2 Working out the correct temperatures to use 162
9.3 After the PCR: Studying PCR products 164
   9.3.1 Gel electrophoresis of PCR products 164
   9.3.2 Cloning PCR products 166
   9.3.3 Problems with the error rate of Taq polymerase 167
9.4 Real-time PCR enables the amount of starting material to be quantified 169
  9.4.1 Carrying out a quantitative PCR experiment 169
  9.4.2 Real-time PCR can also quantify RNA 171

Further reading 171

Part II The Applications of Gene Cloning and DNA Analysis in Research 173

10 Sequencing Genes and Genomes 175
  10.1 Chain-termination DNA sequencing 176
    10.1.1 Chain-termination sequencing in outline 176
    10.1.2 Not all DNA polymerases can be used for sequencing 178
    10.1.3 Chain-termination sequencing with Taq polymerase 179
    10.1.4 Limitations of chain-termination sequencing 180
  10.2 Next-generation sequencing 182
    10.2.1 Preparation of a next-generation sequencing library 182
      DNA fragmentation 183
      Immobilization of the library 184
      Amplification of the library 184
    10.2.2 Next-generation sequencing methods 185
      Reversible terminator sequencing 186
      Pyrosequencing 187
    10.2.3 Third-generation sequencing 188
    10.2.4 Directing next-generation sequencing at specific sets of genes 188
  10.3 How to sequence a genome 189
    10.3.1 Shotgun sequencing of prokaryotic genomes 190
      Shotgun sequencing of the Haemophilus influenzae genome 190
      Shotgun sequencing of other prokaryotic genomes 193
    10.3.2 Sequencing of eukaryotic genomes 194
      The hierarchical shotgun approach 194
      Shotgun sequencing of eukaryotic genomes 196
      What do we mean by ‘genome sequence’? 198

Further reading 198

11 Studying Gene Expression and Function 201
  11.1 Studying the RNA transcript of a gene 202
    11.1.1 Detecting the presence of a transcript and determining its nucleotide sequence 203
    11.1.2 Transcript mapping by hybridization between gene and RNA 204
    11.1.3 Transcript analysis by primer extension 205
    11.1.4 Transcript analysis by PCR 206
11.2 Studying the regulation of gene expression 207
11.2.1 Identifying protein binding sites on a DNA molecule 209
Gel retardation of DNA–protein complexes 209
Footprinting with DNase I 210
Modification interference assays 212
11.2.2 Identifying control sequences by deletion analysis 212
Reporter genes 213
Carrying out a deletion analysis 215

11.3 Identifying and studying the translation product of a cloned gene 216
11.3.1 HRT and HART can identify the translation product of a cloned gene 216
11.3.2 Analysis of proteins by in vitro mutagenesis 216
Different types of in vitro mutagenesis techniques 218
Using an oligonucleotide to create a point mutation in a cloned gene 220
Other methods of creating a point mutation in a cloned gene 220
The potential of in vitro mutagenesis 223

Further reading 223

12 Studying Genomes 225
12.1 Genome annotation 225
12.1.1 Identifying the genes in a genome sequence 226
Searching for open reading frames 226
Simple ORF scans are less effective at locating genes in eukaryotic genomes 227
Gene location is aided by homology searching 228
Comparing the sequences of related genomes 229
Identifying the binding sites for regulatory proteins in a genome sequence 230
12.1.2 Determining the function of an unknown gene 231
Assigning gene function by experimental analysis requires a reverse approach to genetics 231
Specific genes can be inactivated by homologous recombination 232

12.2 Studies of the transcriptome and proteome 233
12.2.1 Studying the transcriptome 234
Studying transcriptomes by microarray or chip analysis 234
Studying a transcriptome by SAGE 235
Sequencing a transcriptome by RNA-seq 236
Advantages of the different methods for transcriptome analysis 237
12.2.2 Studying the proteome 237
Separating the proteins in a proteome 238
Identifying the individual proteins after separation 239
12.2.3 Studying protein–protein interactions 240
Phage display 241
The yeast two-hybrid system 242

Further reading 243
Part III  The Applications of Gene Cloning and DNA Analysis in Biotechnology 245

13  Production of Protein from Cloned Genes 247

13.1 Special vectors for the expression of foreign genes in E. coli 249
  13.1.1 The promoter is the critical component of an expression vector 251
  The promoter must be chosen with care 251
  Examples of promoters used in expression vectors 253
  13.1.2 Cassettes and gene fusions 254

13.2 General problems with the production of recombinant protein in E. coli 257
  13.2.1 Problems resulting from the sequence of the foreign gene 257
  13.2.2 Problems caused by E. coli 258

13.3 Production of recombinant protein by eukaryotic cells 259
  13.3.1 Recombinant protein from yeasts and filamentous fungi 260
    Saccharomyces cerevisiae as the host for recombinant protein synthesis 260
    Other yeasts and fungi 261
  13.3.2 Using animal cells for recombinant protein production 262
    Protein production in mammalian cells 262
    Protein production in insect cells 262
  13.3.3 Pharming: Recombinant protein from live animals and plants 263
    Pharming in animals 263
    Recombinant proteins from plants 265
    Ethical concerns raised by pharming 265

Further reading 266

14  Gene Cloning and DNA Analysis in Medicine 269

14.1 Production of recombinant pharmaceuticals 269
  14.1.1 Recombinant insulin 270
    Synthesis and expression of artificial insulin genes 270
  14.1.2 Synthesis of human growth hormones in E. coli 271
  14.1.3 Recombinant factor VIII 274
  14.1.4 Synthesis of other recombinant human proteins 275
  14.1.5 Recombinant vaccines 275
    Producing vaccines as recombinant proteins 276
    Recombinant vaccines in transgenic plants 277
    Live recombinant virus vaccines 279

14.2 Identification of genes responsible for human diseases 280
  14.2.1 How to identify a gene for a genetic disease 282
    Locating the approximate position of the gene in the human genome 282
    Linkage analysis of the human BRCA1 gene 283
    Identification of candidates for the disease gene 284
14.3 Gene therapy 286
  14.3.1 Gene therapy for inherited diseases 286
  14.3.2 Gene therapy and cancer 288
  14.3.3 The ethical issues raised by gene therapy 288
Further reading 290

15 Gene Cloning and DNA Analysis in Agriculture 291
15.1 The gene addition approach to plant genetic engineering 292
  15.1.1 Plants that make their own insecticides 292
    The δ-endotoxins of Bacillus thuringiensis 292
    Cloning a δ-endotoxin gene in maize 293
    Cloning δ-endotoxin genes in chloroplasts 295
    Countering insect resistance to δ-endotoxin crops 296
  15.1.2 Herbicide-resistant crops 298
    ‘Roundup Ready’ crops 298
    A new generation of glyphosate-resistant crops 299
  15.1.3 Other gene addition projects 300
15.2 Gene subtraction 302
  15.2.1 Antisense RNA and the engineering of fruit ripening in tomato 302
    Using antisense RNA to inactivate the polygalacturonase gene 302
    Using antisense RNA to inactivate ethylene synthesis 304
  15.2.2 Other examples of the use of antisense RNA in plant genetic engineering 304
15.3 Problems with genetically modified plants 305
  15.3.1 Safety concerns with selectable markers 305
  15.3.2 The terminator technology 306
  15.3.3 The possibility of harmful effects on the environment 307
Further reading 308

16 Gene Cloning and DNA Analysis in Forensic Science and Archaeology 311
16.1 DNA analysis in the identification of crime suspects 312
  16.1.1 Genetic fingerprinting by hybridization probing 312
  16.1.2 DNA profiling by PCR of short tandem repeats 312
16.2 Studying kinship by DNA profiling 315
  16.2.1 Related individuals have similar DNA profiles 315
  16.2.2 DNA profiling and the remains of the Romanovs 315
    STR analysis of the Romanov bones 315
    Mitochondrial DNA was used to link the Romanov skeletons with living relatives 317
    The missing children 318
16.3 Sex identification by DNA analysis 318
  16.3.1 PCRs directed at Y chromosome-specific sequences 318
  16.3.2 PCR of the amelogenin gene 319
16.4 Archaeogenetics: Using DNA to study human prehistory 320
  16.4.1 The origins of modern humans 320
    DNA analysis has challenged the multiregional hypothesis 321
    DNA analysis shows that Neanderthals are not the direct ancestors of modern Europeans 322
    The Neanderthal genome sequence suggests there was interbreeding with *H. sapiens* 323
  16.4.2 DNA can also be used to study prehistoric human migrations 324
    Modern humans may have migrated from Ethiopia to Arabia 324
    Colonization of the New World 325

**Further reading** 328

Glossary 329
Index 345
A nyone who works with DNA is well aware of the dramatic changes that have taken place during the past few years in DNA sequencing methodology. To reflect these advances, in this new edition of *Gene Cloning and DNA Analysis: An Introduction* I have completely remodelled the chapter on DNA sequencing to give the new ‘next-generation’ methods equal prominence alongside the traditional approaches to DNA sequencing, and also to modernize the description of the ways in which genome sequences are generated. Elsewhere, I have stressed the importance of RNA-seq as a means of studying transcriptomes, and ChIP-seq for locating protein-binding sites. These changes correct the major weakness of the Sixth Edition, which was written just before these methods came into mainstream use.

Elsewhere, I have made the usual updates, especially in Part III where I have tried to keep pace with the increasingly rapid developments in the applications of gene cloning and DNA analysis in industry, medicine and agriculture. I have also rewritten the last part of the final chapter, on archaeogenetics, in order to present some of the new information on the human past that has been revealed by the Neanderthal and Denisovan genome sequences. As always, my primary aim is to ensure that *Gene Cloning* remains an introductory text that begins at the beginning and does not assume that the reader has any prior knowledge of the techniques used to study genes and genomes.

For the n-th time I must thank my wife Keri for the unending support that she has given to me in my decision to use up evenings and weekends writing this and other books.

T.A. Brown
University of Manchester
This book is accompanied by a companion website:

www.wiley.com/go/brown/cloning

The website includes:

- Powerpoints of all figures from the book for downloading
- PDFs of tables from the book
PART I

The Basic Principles of Gene Cloning and DNA Analysis

1 | Why Gene Cloning and DNA Analysis are Important  3
2 | Vectors for Gene Cloning: Plasmids and Bacteriophages 13
3 | Purification of DNA from Living Cells 25
4 | Manipulation of Purified DNA 47
5 | Introduction of DNA into Living Cells 75
6 | Cloning Vectors for Escherichia coli 93
7 | Cloning Vectors for Eukaryotes 111
8 | How to Obtain a Clone of a Specific Gene 135
9 | The Polymerase Chain Reaction 157
Chapter 1

Why Gene Cloning and DNA Analysis are Important

In the middle of the 19th century, Gregor Mendel formulated a set of rules to explain the inheritance of biological characteristics. The basic assumption of these rules is that each heritable property of an organism is controlled by a factor, called a gene, that is a physical particle present somewhere in the cell. The rediscovery of Mendel’s laws in 1900 marks the birth of genetics, the science aimed at understanding what these genes are and exactly how they work.

1.1 The early development of genetics

For the first 30 years of its life this new science grew at an astonishing rate. The idea that genes reside on chromosomes was proposed by W. Sutton in 1903, and received experimental backing from T.H. Morgan in 1910. Morgan and his colleagues then developed the techniques for gene mapping, and by 1922 had produced a comprehensive analysis of the relative positions of over 2000 genes on the four chromosomes of the fruit fly, Drosophila melanogaster.

Despite the brilliance of these classical genetic studies, there was no real understanding of the molecular nature of the gene until the 1940s. Indeed, it was not until the
experiments of Avery, MacLeod, and McCarty in 1944, and of Hershey and Chase in 1952, that anyone believed that deoxyribonucleic acid (DNA) is the genetic material. Up until then it was widely thought that genes were made of protein. The discovery of the role of DNA was a tremendous stimulus to genetic research, and many famous biologists (Delbrück, Chargaff, Crick, and Monod were among the most influential) contributed to the second great age of genetics. During the 14 years between 1952 and 1966, the structure of DNA was elucidated, the genetic code cracked, and the processes of transcription and translation described.

1.2 The advent of gene cloning and the polymerase chain reaction

These years of activity and discovery were followed by a lull, a period of anticlimax when it seemed to some molecular biologists (as the new generation of geneticists styled themselves) that there was little of fundamental importance that was not understood. In truth, there was a frustration that the experimental techniques of the late 1960s were not sophisticated enough to allow the gene to be studied in any greater detail.

Then, in the years 1971–1973 genetic research was thrown back into gear by what at the time was described as a revolution in experimental biology. A whole new methodology was developed, enabling previously impossible experiments to be planned and carried out, if not with ease, then at least with success. These methods, referred to as recombinant DNA technology or genetic engineering, and having at their core the process of gene cloning, sparked another great age of genetics. They led to rapid and efficient DNA sequencing techniques that enabled the structures of individual genes to be determined, reaching a culmination at the turn of the century with the massive genome sequencing projects, including the human project which was completed in 2000. They led to procedures for studying the regulation of individual genes, which have allowed molecular biologists to understand how aberrations in gene activity can result in human diseases such as cancer. The techniques spawned modern biotechnology, which puts genes to work in production of proteins and other compounds needed in medicine and industrial processes.

During the 1980s, when the excitement engendered by the gene cloning revolution was at its height, it hardly seemed possible that another, equally novel and equally revolutionary process was just around the corner. According to DNA folklore, Kary Mullis invented the polymerase chain reaction (PCR) during a drive along the coast of California one evening in 1985. His brainwave was an exquisitely simple technique that acts as a perfect complement to gene cloning. PCR has made easier many of the techniques that were possible but difficult to carry out when gene cloning was used on its own. It has extended the range of DNA analysis and enabled molecular biology to find new applications in areas of endeavour outside of its traditional range of medicine, agriculture, and biotechnology. Archaeogenetics, molecular ecology, and DNA forensics are just three of the new disciplines that have become possible as a direct consequence of the invention of PCR, enabling molecular biologists to ask questions about human evolution and the impact of environmental change on the biosphere, and to bring their powerful tools to bear in the fight against crime. Forty years have passed since the dawning of the age of gene cloning, but we are still riding the rollercoaster and there is no end to the excitement in sight.
1.3 What is gene cloning?

What exactly is gene cloning? The easiest way to answer this question is to follow through the steps in a gene cloning experiment (Figure 1.1):

1. A fragment of DNA, containing the gene to be cloned, is inserted into a circular DNA molecule called a **vector**, to produce a **recombinant DNA molecule**.
2. The vector transports the gene into a host cell, which is usually a bacterium, although other types of living cell can be used.
3. Within the host cell the vector multiplies, producing numerous identical copies, not only of itself but also of the gene that it carries.
4. When the host cell divides, copies of the recombinant DNA molecule are passed to the progeny and further vector replication takes place.
5. After a large number of cell divisions, a colony, or **clone**, of identical host cells is produced. Each cell in the clone contains one or more copies of the recombinant DNA molecule. The gene carried by the recombinant molecule is now said to be cloned.

**Figure 1.1**
The basic steps in gene cloning.
1.4 What is PCR?

The polymerase chain reaction is very different from gene cloning. Rather than a series of manipulations involving living cells, PCR is carried out in a single test tube simply by mixing DNA with a set of reagents and placing the tube in a thermal cycler, a piece of equipment that enables the mixture to be incubated at a series of temperatures that are varied in a preprogrammed manner. The basic steps in a PCR experiment are as follows (Figure 1.2):

1. The mixture is heated to 94 °C, at which temperature the hydrogen bonds that hold together the two strands of the double-stranded DNA molecule are broken, causing the molecule to denature.

**Figure 1.2**
The basic steps in the polymerase chain reaction.

1. Denaturation of the template DNA at 94°C
2. Annealing of the oligonucleotide primers at 50–60°C
3. Synthesis of new DNA at 74°C
4. Repeat the cycle 25–30 times
2 The mixture is cooled down to 50–60 °C. The two strands of each molecule could join back together at this temperature, but most do not because the mixture contains a large excess of short DNA molecules, called oligonucleotides or primers, which anneal to the DNA molecules at specific positions.

3 The temperature is raised to 74 °C. This is a good working temperature for the Taq DNA polymerase that is present in the mixture. We will learn more about DNA polymerases on p. 51. All we need to understand at this stage is that the Taq DNA polymerase attaches to one end of each primer and synthesizes new strands of DNA, complementary to the template DNA molecules, during this step of the PCR. Now we have four stands of DNA instead of the two that there were to start with.

4 The temperature is increased back to 94 °C. The double-stranded DNA molecules, each of which consists of one strand of the original molecule and one new strand of DNA, denature into single strands. This begins a second cycle of denaturation–annealing–synthesis, at the end of which there are eight DNA strands. By repeating the cycle 30 times the double-stranded molecule that we began with is converted into over 130 million new double-stranded molecules, each one a copy of the region of the starting molecule delineated by the annealing sites of the two primers.

1.5 Why gene cloning and PCR are so important

As can be seen from Figures 1.1 and 1.2, gene cloning and PCR are relatively straightforward procedures. Why, then, have they assumed such importance in biology? The answer is largely because both techniques can provide a pure sample of an individual gene, separated from all the other genes in the cell.

1.5.1 Obtaining a pure sample of a gene by cloning

To understand exactly how cloning can provide a pure sample of a gene, consider the basic experiment from Figure 1.1, but drawn in a slightly different way (Figure 1.3). In this example, the DNA fragment to be cloned is one member of a mixture of many different fragments, each carrying a different gene or part of a gene. This mixture could indeed be the entire genetic complement of an organism – a human, for instance. Each of these fragments becomes inserted into a different vector molecule to produce a family of recombinant DNA molecules, one of which carries the gene of interest. Usually, only one recombinant DNA molecule is transported into any single host cell, so that although the final set of clones may contain many different recombinant DNA molecules, each individual clone contains multiple copies of just one molecule. The gene is now separated away from all the other genes in the original mixture, and its specific features can be studied in detail.

In practice, the key to the success or failure of a gene cloning experiment is the ability to identify the particular clone of interest from the many different ones that are obtained. If we consider the genome of the bacterium Escherichia coli, which contains just over 4000 different genes, we might at first despair of being able to find just one gene among all the possible clones (Figure 1.4). The problem becomes even more overwhelming when we remember that bacteria are relatively simple organisms and that the human genome contains about five times as many genes. As explained in Chapter 8, a variety
of different strategies can be used to ensure that the correct gene can be obtained at the end of the cloning experiment. Some of these strategies involve modifications to the basic cloning procedure, so that only cells containing the desired recombinant DNA molecule can divide and the clone of interest is automatically selected. Other methods involve techniques that enable the desired clone to be identified from a mixture of lots of different clones.

Once a gene has been cloned there is almost no limit to the information that can be obtained about its structure and expression. The availability of cloned material has stimulated the development of many different analytical methods for studying genes, with new techniques being introduced all the time. Methods for studying the structure and expression of a cloned gene are described in Chapters 10 and 11, respectively.

1.5.2 PCR can also be used to purify a gene

The polymerase chain reaction can also be used to obtain a pure sample of a gene. This is because the region of the starting DNA molecule that is copied during PCR