## Contents

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	Clo	ning	and	DNA	<b>Analysis</b>
	are li	mport	ant	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 71.5.1 Obtaining a pure sample of a gene by cloning 71.5.2 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  $\lambda$  DNA molecule 19
    The linear and circular forms of  $\lambda$  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-caesium 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  $\lambda$  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  $\lambda$  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

4.2.9	Special gel	electrophoresis	methods	for	separating	larger
	molecules	63				

#### 4.3 Ligation: Joining DNA molecules together 66

- 4.3.1 The mode of action of DNA ligase 66
- 4.3.2 Sticky ends increase the efficiency of ligation 67
- 4.3.3 Putting sticky ends on to a blunt-ended molecule 67
  Linkers 68
  Adaptors 68
  Homopolymer tailing 70
- 4.3.4 Blunt end ligation with a DNA topoisomerase 71 Further reading 74

#### 5 Introduction of DNA into Living Cells 75

- 5.1 Transformation: The uptake of DNA by bacterial cells 76
  - 5.1.1 Not all species of bacteria are equally efficient at DNA uptake 77
  - 5.1.2 Preparation of competent E. coli cells 78
  - 5.1.3 Selection for transformed cells 78
- 5.2 Identification of recombinants 79
  - 5.2.1 Recombinant selection with pBR322: Insertional inactivation of an antibiotic resistance gene 80
  - 5.2.2 Insertional inactivation does not always involve antibiotic resistance 81
- 5.3 Introduction of phage DNA into bacterial cells 83
  - 5.3.1 Transfection 83
  - 5.3.2 In vitro packaging of  $\lambda$  cloning vectors 83
  - 5.3.3 Phage infection is visualized as plaques on an agar medium 86
- 5.4 Identification of recombinant phages 86
  - 5.4.1 Insertional inactivation of a *lacZ'* gene carried by the phage vector 87
  - 5.4.2 Insertional inactivation of the λ cl gene 87
  - 5.4.3 Selection using the Spi phenotype 88
  - 5.4.4 Selection on the basis of  $\lambda$  genome size 88
- 5.5 Introduction of DNA into non-bacterial cells 88
  - 5.5.1 Transformation of individual cells 88
  - 5.5.2 Transformation of whole organisms 90 Further reading 90

#### 6 Cloning Vectors for Escherichia coli 93

- 6.1 Cloning vectors based on E. coli plasmids 94
  - 6.1.1 The nomenclature of plasmid cloning vectors 94
  - 6.1.2 The useful properties of pBR322 94
  - 6.1.3 The pedigree of pBR322 95
  - 6.1.4 More sophisticated *E. coli* plasmid cloning vectors 95 pUC8: A Lac selection plasmid 97 pGEM3Z: *In vitro* transcription of cloned DNA 98

6.2	Clonin	ng vectors based on λ 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

6.2.3 Insertion and replacement vectors 102
Insertion vectors 102
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 λ and other high-capacity vectors enable genomic libraries to be constructed 104
- 6.3 Cloning vectors for the synthesis of single-stranded DNA 1066.3.1 Vectors based on M13 bacteriophage 1076.3.2 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 µm plasmid: Yeast episomal plasmids
  - 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
     The structure and use of a YAC vector 116
     Applications for YAC vectors 118
  - 7.1.6 Vectors for other yeasts and fungi 118
- 7.2 Cloning vectors for higher plants 119
  - 7.2.1 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 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 *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	
Furthe	er reading 132	

#### 8 How to Obtain a Clone of a Specific Gene 135

- 8.1 The problem of selection 1358.1.1 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 1438.4.1 Complementary nucleic acid strands hybridize to each other143
  - 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
  - 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

15

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
Turther roughly and the same an
al I IDNIA I I I A I II
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
Further reading 308
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

16.3.1 PCRs directed at Y chromosome-specific sequences 318

16.3 Sex identification by DNA analysis 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