

Contents

About the Authors	x	3. Completion of Vector Preparation and Polymerase Chain Reaction Amplification of <i>egfp</i>	23
Preface	xiii	I. Introduction	23
Acknowledgements	xv	II. Laboratory Exercises	26
Note to Instructors	xvii	A. Clean-Up of Digested pET-41a Vector	26
Nomenclature	xix	B. Agarose Gel Electrophoresis	27
Introduction	xxi	C. Polymerase Chain Reaction Amplification of <i>egfp</i>	29
		Discussion Questions	30
Part I		4. Preparation of Insert DNA (<i>egfp</i>) PCR Product	31
Manipulation of DNA		I. Introduction	31
Advanced Alternatives Within Part I	3	II. Laboratory Exercises	31
1. Getting Oriented; Practicing With Micropipettes	7	A. Spin Column Clean-up of PCR Product	31
I. Equipment Checklist	7	B. Quantification of Purified <i>egfp</i> PCR Product	31
II. Micropipetting	8	C. Restriction Digestion of <i>egfp</i> PCR Product	31
III. Laboratory Exercises	10	D. Removing Enzymes and Cleaning Digested DNA Using a Spin Column	32
A. Micropipetting Self-Test	10	E. Check PCR Reaction and Purification on an Agarose Gel	32
B. Preparing Bovine Serum Albumin Dilutions	11	F. Calculations for Next Week	33
C. Performing a Nitrocellulose Spot Test	11	Discussion Questions	33
Discussion Questions	12	5. DNA Ligation and Transformation of <i>Escherichia coli</i>	35
2. Purification and Digestion of Plasmid (Vector) DNA	13	I. Introduction	35
I. Introduction	13	A. Ligation	35
A. Alkaline Lysis	13	B. Transformation	36
B. Silica Adsorption	13	II. Laboratory Exercises	37
C. DNA Quantification	14	A. Ligations and Ligation Controls	37
II. Introduction to Expression Vectors	14	B. Divalent Cation-Mediated Transformation	38
A. Principles of Gene Expression	14	C. Electrophoresis of Ligation Reactions	39
B. Expression Vectors	15	Discussion Questions	39
C. Orientation and Reading Frame	15		
D. Orientation	15		
E. Reading Frame	17		
III. Laboratory Exercises	17		
A. Alkaline Lysis and Silica Adsorption Protocol	17		
B. DNA Quantification	19		
C. Restriction Digestion of Expression Vector DNA pET-41a, a GST Fusion Protein Vector	20		
Discussion Questions	22		

Part II Screening Transformants Advanced Alternatives Within Part II

6. Screening of Transformants, Part I	45
6a. Interim Laboratory Session	46
COUNTING TRANSFORMANTS, REPLICA PLATING, AND INOCULATING OVERNIGHT CULTURES	46
I. Introduction	46
II. Laboratory Exercises	46
A. Counting Transformants and Transformation Efficiency	46
B. Replica Plating and Inoculation of Overnight Cultures	47
6b. Isolation of Miniprep DNA From Potential Transformants	48
I. Introduction	48
II. Laboratory Exercise	48
6c. PCR Screening	50
I. Introduction	50
II. Laboratory Exercise	50
6d. Visualization of Green Fluorescent Protein, Part I	52
I. Introduction	52
II. Laboratory Exercise	52
Discussion Questions	52
7. Screening of Transformants, Part II	53
7a. Restriction Digestion of DNA	54
I. Introduction	54
II. Laboratory Exercise	54
7b. Analysis of PCR Screen and Miniprep Digestions	56
I. Introduction	56
II. Laboratory Exercise	56
7c. Visualization of Enhanced Green Fluorescent Protein, Part II	57
I. Introduction	57
II. Laboratory Exercise	57
7d. Analysis of DNA Sequence from a Positive Clone, Part I	58
I. Introduction	58
II. Laboratory Exercise	59
III. Compiled Screening Data	61
Discussion Questions	62

8. Analysis of DNA Sequence From a Positive Clone, Part II	63
I. Introduction	63
II. Laboratory Exercise	67
Discussion Questions	69

Part III Expression, Detection, and Purification of Recombinant Proteins from Bacteria Advanced Alternatives Within Part III

9. Expression of Fusion Protein from Positive Clones, SDS-PAGE and Western Blot: Part I	75
9a. Interim Laboratory Session	76
INOCULATE CULTURE FOR SDS-PAGE	76
I. Laboratory Exercise	76
9b. Expression of Fusion Protein from Positive Clones, SDS-PAGE and Western Blot, Part I	77
I. Introduction	77
II. Laboratory Exercises	78
A. Sample Preparation	78
B. Loading Samples on the Gel	79
C. Electrophoresis	80
D. Stopping Electrophoresis	80
E. Transferring to Nitrocellulose	80
F. Disassemble and Store	82
Discussion Questions	82
10. Expression of Fusion Protein from Positive Clones, SDS-PAGE, and Western Blot: Part II	83
I. Introduction	83
II. Laboratory Exercises	84
A. SDS-PAGE and Western Blot, Part II	84
B. Replica Plate Positive Clone	85
Discussion Questions	86
11. Extraction of Recombinant Protein From <i>Escherichia coli</i> Using a Glutathione Affinity Column	87
11a. Interim Laboratory Session	88
INOCULATE CULTURES FOR PROTEIN PURIFICATION	88
I. Laboratory Exercise	88

11b. Extraction of Recombinant Protein from <i>Escherichia coli</i> and Purification Using a Glutathione Affinity Column	89	II. Laboratory Exercises	115
I. Introduction	89	A. Reverse Transcription	115
II. Laboratory Exercises	91	B. Quantitative PCR	116
A. Growing Bacterial Suspension Cultures for Fusion Protein Purification	91	Discussion Questions	119
B. Harvesting IPTG-Induced Cultures	91	15. Analysis of <i>gst::egfp</i> mRNA Levels by RT-qPCR: Part II	121
C. Breaking Open Bacterial Cells	92	I. Introduction	121
D. Removing Insoluble Debris From the Crude Homogenate	92	II. Laboratory Exercise	123
E. Purifying Protein by Affinity Chromatography	93	Discussion Questions	125
Discussion Questions	94	16. Analysis of <i>gst::egfp</i> mRNA Levels by Semiquantitative RT-PCR: Part I	127
12. Analysis of Purification Fractions	95	I. Introduction	127
12a. Analysis of Purification Fractions	96	II. Laboratory Exercises	128
I. Introduction	96	A. Reverse Transcription	128
A. Protein Quantitation by Fluorescence	96	B. Semiquantitative Polymerase Chain Reaction	128
B. Protein Quantification by Bradford Assay	97	Discussion Questions	130
II. Laboratory Exercises	97	17. Analysis of <i>gst::egfp</i> mRNA Levels by Semiquantitative RT-PCR: Part II	131
A. Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Purified Fusion Protein	97	I. Introduction	131
B. Protein Quantification Assays	98	II. Laboratory Exercises	131
12b. Replica Plating (Only if Performing Lab Session 13)	102	A. Agarose Gel Electrophoresis	131
I. Laboratory Exercise	102	B. Quantification	131
Discussion Questions	102	Discussion Questions	133
Part IV		Part V	
Analysis of mRNA Levels		Modulation of Gene Expression	
13. Total RNA Purification	105	18. Culturing Mammalian Cells	137
13a. Interim Laboratory Session	106	I. Introduction to Sterile Technique	137
I. Laboratory Exercise	106	II. Laboratory Exercises	139
13b. Total RNA Purification	107	A. Operating and Using a Laminar Flow Hood	139
I. Introduction	107	B. Other Considerations: Using Incubators and Microscopes Within a Cell Culture Facility	140
II. Laboratory Exercises	109	C. Counting and Plating Mammalian Cells	140
A. Purification of Total RNA	109	Discussion Questions	142
B. Sample Preparation	110	19. Transient Transfection of Mammalian Cells	143
C. DNase Digestion	110	19a. Interim Laboratory Session	144
D. Quantification of RNA	111	TRANSIENT TRANSFECTION OF PEGFP-N1 IN HEK293 CELLS USING FUGENE HD	144
Discussion Questions	111	I. Introduction	144
14. Analysis of <i>gst::egfp</i> mRNA Levels by RT-qPCR: Part I	113	II. Laboratory Exercise	145
I. Introduction	113		
A. Reverse Transcription	113		
B. Quantitative PCR	114		

19b. Analysis of EGFP Expression Using Fluorescence Microscopy	148	23c. Interim Laboratory Session	177
I. Introduction	148	EXPANSION OF TRANSFECTED CELLS	177
II. Laboratory Exercise	149	I. Introduction	177
19c. Creating Stable EGFP Expressing HEK293 Cells	152	II. Laboratory Exercise	177
I. Introduction	152	24. CRISPR-Mediated Knockout of EGFP: Part II	179
II. Laboratory Exercises	152	I. Introduction	179
A. Performing a Kill Curve	152	II. Laboratory Exercise	179
B. Selecting for Stables	152	Discussion Questions	180
Discussion Questions	153	25. Advanced CRISPR: Part I	181
20. RNAi-Mediated Knockdown of EGFP: Part I	155	I. Introduction	181
I. Introduction	155	A. sgRNA Design	181
II. Laboratory Exercise	157	B. Annealed Oligo Cloning	182
Discussion Questions	160	C. PCR Primer Design Basics	183
21. RNAi-Mediated Knockdown of EGFP: Part II	161	II. Laboratory Exercises	185
I. Introduction	161	A. sgRNA Design and Analysis	185
21a. Counting and Plating EGFP Expressing Mammalian Cells	162	B. Primer Design	186
I. Laboratory Exercise	162	C. Restriction Enzyme Digestion and Dephosphorylation of CRISPR Vector	188
21b. Interim Laboratory Session	163	D. Agarose Gel Electrophoresis	188
TRANSIENT TRANSFECTION OF SIRNAS	163	E. Gel Extraction of Digested CRISPR Vector Backbone	189
I. Laboratory Exercise	163	F. DNA Concentration Determination	190
22. RNAi-Mediated Knockdown of EGFP: Part III	165	Discussion Questions	190
I. Introduction	165	26. Advanced CRISPR: Part II	191
II. Laboratory Exercise	165	I. Introduction	191
Discussion Questions	168	II. Laboratory Exercises	191
23. CRISPR-Mediated Knockout of EGFP: Part I	169	A. sgRNA Oligo Annealing and Phosphorylation	191
23a. CRISPR-Mediated Knockout of <i>egfp</i>, Part I	170	B. Ligation of sgRNAs to Digested CRISPR Vector	192
I. Introduction	170	C. Transformation Into <i>E.coli</i>	193
II. Laboratory Exercise	172	Discussion Questions	194
23b. Interim Laboratory Session	174	27. Interim Laboratory Session	195
TRANSFECTION OF CRISPR VECTORS	174	27a. Interim Laboratory Session	196
I. Introduction	174	COUNTING TRANSFORMANTS AND INOCULATING CULTURES FOR MINIPREP DNA	196
II. Laboratory Exercise	175	I. Introduction	196
Discussion Questions	176	II. Laboratory Exercise	196

27b. Advanced CRISPR: Part III	197	II. Laboratory Exercises	205
I. Introduction	197	A. Harvesting Cells	206
II. Laboratory Exercises	197	B. Lysis and Extraction	207
A. Isolation of Miniprep DNA	197	C. Pouring Agarose Gels	207
B. Preparation for Sequencing	198	D. PCR	207
Discussion Questions	198	Discussion Questions	208
28. Advanced CRISPR: Part IV	199	30. Advanced CRISPR: Part VI	209
28a. Advanced CRISPR: Part IV	200	I. Introduction	209
I. Introduction	200	II. Laboratory Exercises	211
II. Laboratory Exercises	200	A. Verifying the PCR Product	211
A. Computational Analysis of DNA Sequences	200	B. Denaturing and Reannealing Reactions	211
B. Counting and Plating EGFP Expressing Mammalian Cells	201	C. Enzyme Digestion	212
Discussion Questions	202	D. Gel Analysis	212
28b. Interim Laboratory Session	203	Discussion Questions	213
TRANSFECTION OF CRISPR VECTORS		Appendix A: Equipment	215
I. Introduction	203	Appendix B: Prep List	217
II. Laboratory Exercise	203	Appendix C: Preparation of Competent <i>Escherichia coli</i> Cells	247
29. Advanced CRISPR: Part V	205	Appendix D: Pre-Lab Questions	249
I. Introduction	205	Index	265