

Contents

Preface XV

List of Contributors XVII

Introduction 1

Anne Skaja Robinson and Patrick J. Loll

Expression 2

Solubilization and Structural Methods 5

Abbreviations 7

References 7

Part One Expression Systems 11

1 Bacterial Systems 13

James Samuelson

1.1 Introduction 13

1.2 Understanding the Problem 14

1.3 Vector/Promoter Types 15

1.4 T7 Expression System 20

1.5 Tunable T7 Expression Systems 21

1.6 Other Useful Membrane Protein Expression Strains 22

1.7 Clone Stability 23

1.8 Media Types 24

1.9 Fusion Partners/Membrane Targeting Peptides 25

1.10 Chaperone Overexpression 26

1.11 Cautionary Notes Related to Chaperone Overexpression 27

1.12 Emerging Role of Quality Control Proteases 27

1.13 Tag Selection 28

1.14 Potential Expression Yield 29

1.15 Strategies to Overcome Protein Instability 30

Acknowledgments 31

Abbreviations 31

References 31

2	Membrane Protein Expression in <i>Saccharomyces cerevisiae</i>	37
	<i>Zachary Britton, Carissa Young, Özge Can, Patrick McNeely, Andrea Naranjo, and Anne Skaja Robinson</i>	
2.1	Introduction	37
2.2	Getting Started	38
2.2.1	Promoter Systems	38
2.2.1.1	Constitutive Promoters	38
2.2.1.2	Inducible Promoters	38
2.2.2	Host Strains, Selection Strategies, and Plasmids	39
2.2.2.1	Host Strains	39
2.2.2.2	Selection Strategies	40
2.2.2.3	Plasmids and Homologous Recombination	40
2.2.3	Expression Conditions	42
2.3	Special Considerations	43
2.3.1	Post-Translational Modifications	43
2.3.1.1	Glycosylation	43
2.3.1.2	Disulfide Bond Formation	44
2.3.2	Lipid Requirements	44
2.3.2.1	Glycerophospholipids	45
2.3.2.2	Sphingolipids	46
2.3.2.3	Sterols	46
2.3.3	Signal Sequences	47
2.3.4	Topology	47
2.3.5	Cellular Responses to Membrane Protein Expression	49
2.3.5.1	UPR	49
2.3.5.2	HSR	49
2.4	Case Studies	49
2.4.1	Ste2p	50
2.4.2	Pma1p	50
2.4.3	CFTR	60
2.5	Conclusions	61
	Abbreviations	62
	References	62
3	Expression Systems: <i>Pichia pastoris</i>	75
	<i>Fatima Alkhalifioui, Christel Logez, Olivier Bornert, and Renaud Wagner</i>	
3.1	Introduction	75
3.2	A (Brief) Summary on the (Long) History of <i>P. pastoris</i>	75
3.3	Introducing <i>P. pastoris</i> as a Biotechnological Tool: Its (Extended) Strengths and (Limited) Weaknesses	76
3.4	Basics of the <i>P. pastoris</i> Expression System	77
3.4.1	Methanol Utilization Pathway	77
3.4.2	Host Strains and Plasmids	78
3.4.3	Transformation and Clone Selection Strategies	80

3.4.4	Expression Conditions and Culturing Formats	80
3.5	Successful Large-Scale Expression of Membrane Proteins Using <i>P. pastoris</i>	81
3.5.1	<i>P. pastoris</i> for Membrane Protein Expression	81
3.5.2	Common Trends for an Efficient Expression of Membrane Proteins in <i>P. pastoris</i>	92
3.6	Guidelines for Optimizing Membrane Protein Expression in <i>P. pastoris</i> Using GPCRs as Models	94
3.6.1	Design and Selection of Enhanced Expression Clones	95
3.6.2	Optimization of the Expression Conditions	96
3.6.3	Yeast Cell Lysis	98
3.7	Conclusions and Future Directions	99
	Acknowledgments	99
	Abbreviations	99
	References	100
4	Heterologous Production of Active Mammalian G-Protein-Coupled Receptors Using Baculovirus-Infected Insect Cells	109
	Mark Chiu, Brian Estvander, Timothy Esbenshade, Steve Kakavas, Kathy Krueger, Marc Lake, and Ana Pereda-Lopez	
4.1	Introduction	109
4.2	Experimental	113
4.2.1	Generation of Recombinant Baculovirus	113
4.2.2	Baculovirus Infection of Insect Cells	115
4.2.3	Case Study: Histamine H ₃ Receptor	118
4.2.3.1	Solubilization of the Histamine H ₃ Receptor	125
4.2.3.2	Assay Validation	125
4.2.3.3	Competition Analysis of Solubilized versus Membrane-Bound Receptor	126
4.3	Conclusions and Future Perspectives	128
4.3.1	Executive Summary	130
4.3.2	Future Perspectives	130
	Abbreviation	131
	References	131
5	Membrane Protein Expression in Mammalian Cells	139
	Deniz B. Hizal, Erika Ohsfeldt, Sunny Mai, and Michael J. Betenbaugh	
5.1	Introduction	139
5.2	Mammalian Systems	140
5.2.1	Cell Culture Types and Media Optimization	140
5.2.1.1	Adherent Cell Culture	140
5.2.1.2	Suspension Cell Culture	141
5.2.1.3	Batch and Fed-Batch Culture	141
5.2.1.4	Perfusion Process	141
5.2.1.5	Media Optimization	141

5.2.2	Gene Delivery and Expression in Mammalian Systems	143
5.2.2.1	High Transfection Efficiency in Adherent Cell Cultures with Cationic Liposome	143
5.2.3	Post-Translational Modifications in Mammalian Systems	147
5.2.3.1	Glycosylation	148
5.2.3.2	Protein Lipidation	149
5.3	Case Studies	150
5.3.1	Increasing Membrane Protein Expression by Virus Vectors	150
5.3.2	Anti-apoptosis Engineering for Increasing Membrane Protein Expression	152
5.3.3	Increasing Membrane Protein Expression by Chaperones	156
5.3.4	Membrane Protein Expression in Cancer Cell Lines	157
5.3.5	Membrane Proteins as Biotherapeutics	158
5.4	Conclusions	159
	Abbreviations	160
	References	161
6	Membrane Protein Production Using Photosynthetic Bacteria: A Practical Guide	167
	<i>Philip D. Laible, Donna L. Mielke, and Deborah K. Hanson</i>	
6.1	Introduction	167
6.1.1	The Membrane Protein Problem	167
6.1.2	Exploiting the Physiology of Photosynthetic Bacteria	168
6.1.3	Expression Strategies	170
6.1.3.1	Design of the Expression Plasmids	170
6.1.3.2	Design of Expression Hosts	172
6.1.3.3	Autoinduction Conditions	173
6.1.4	Summary of Success Stories	174
6.2	Preparation of Expression Constructs	175
6.2.1	Platform Vector Preparation	175
6.2.1.1	Large-Scale Vector Preparation Protocol for Ligation-Dependent Cloning	175
6.2.1.2	Large-Scale Vector Preparation Protocol for LIC	176
6.2.2	Design of Oligonucleotide Primers for Gene Amplification and Cloning	177
6.2.2.1	Ligation-Dependent Cloning	177
6.2.2.2	LIC	177
6.2.3	Target Gene Preparation	178
6.2.3.1	PCR Amplification of Target Gene	178
6.2.3.2	Restriction Enzyme Digestion of PCR Amplicon	178
6.2.3.3	Digestion of PCR Amplicon to Generate LIC Overhangs	178
6.2.4	Cloning of Digested Amplicons	178
6.2.4.1	Generation of Recombinant Plasmids	178
6.2.4.2	Transformation of <i>E. coli</i> with Ligation or LIC Reactions	179
6.2.5	Screening for Successful Insertion of Target Gene into Platform Vector	179

6.3	Transfer of Plasmid DNA to <i>Rhodobacter</i> via Conjugal Mating	180
6.3.1	Transformation of <i>E. coli</i> S17-1	180
6.3.2	Conjugation of <i>E. coli</i> with <i>R. sphaeroides</i>	180
6.4	Small-Scale Screening for Expression and Localization of Target Protein in <i>Rhodobacter</i>	181
6.4.1	Small-Scale Growth and Preparation of Samples for SDS-PAGE	182
6.4.1.1	Growth and Harvest of Expression Strains	182
6.4.1.2	Preparing Whole-Cell Samples for SDS-PAGE	183
6.4.1.3	Preparing Membranes and the Soluble Fraction for SDS-PAGE	184
6.4.2	SDS-PAGE Followed by Electroblothing of Proteins to PVDF Membrane	185
6.4.3	Immunoblot Development	186
6.5	Large-Scale Culture	187
6.5.1	Growth and Harvest of Expression Culture	187
6.5.2	Cell Lysis	188
6.5.3	Membrane Isolation	188
6.6	Detergent Solubilization and Chromatographic Purification of Expressed Membrane Proteins	189
6.6.1	Solubilization of Membrane Proteins	190
6.6.2	Chromatography	190
6.6.2.1	Bench-Top Affinity Chromatography	190
6.6.2.2	Affinity Chromatography Using an ÄKTA-FPLC™	191
6.7	Protein Identification and Assessment of Purity	192
6.8	Preparations of Specialized <i>Rhodobacter</i> Membranes	192
	Appendix: Media and Buffer Formulations	194
	Abbreviations	196
	References	197

Part Two Protein-Specific Considerations 199

7	Peripheral Membrane Protein Production for Structural and Functional Studies	201
	Brian J. Bahnson	
7.1	Introduction	201
7.2	Case Studies of Peripheral Membrane Proteins	204
7.2.1	Electrostatic Interactions	204
7.2.1.1	Case 1: Cytochrome c_2	205
7.2.1.2	Case 2: Group IB Secreted Phospholipase A ₂	205
7.2.2	Hydrophobic Patch	207
7.2.2.1	Case 1: Plasma Platelet-Activating Factor Acetylhydrolase	208
7.2.2.2	Case 2: Human Serum Paraoxonase 1	210
7.2.3	Covalent Lipid Anchor	210
7.2.3.1	Case 1: Recoverin	211
7.2.3.2	Case 2: Intracellular Platelet-Activating Factor Acetylhydrolase Type II	212
7.2.4	Case 3: Palmitoylation of Human Proteins in Cell Culture	212

7.2.5	Lipid-Binding Domain	212
7.2.5.1	Case 1: Pleckstrin Homology Domain	213
7.2.5.2	Case 2: C2 Domain	213
7.3	Conclusions	214
	Acknowledgments	215
	Abbreviations	215
	References	215
8	Expression of G-Protein-Coupled Receptors	219
	<i>Alexei Yeliseev and Krishna Vukoti</i>	
8.1	Introduction	219
8.2	Bacterial Expression of GPCRs	220
8.3	Expression of GPCRs in Inclusion Bodies, and Refolding	228
8.4	Expression of GPCRs in Yeast	229
8.5	Expression of GPCRs in Insect Cells	231
8.6	Expression of GPCRs in Mammalian Cell Lines	234
8.7	Expression of GPCRs in Retina Rod Cells	234
8.8	Expression of GPCRs in a Cell-Free System	235
8.9	Stabilization of GPCRs during Solubilization and Purification	238
8.10	Conclusions	238
	Acknowledgments	239
	Abbreviations	239
	References	240
9	Structural Biology of Membrane Proteins	249
	<i>David Salom and Krzysztof Palczewski</i>	
9.1	Introduction	249
9.2	Folding and Structural Analysis of Membrane Proteins	249
9.2.1	Folding	249
9.2.2	Prediction Methods	251
9.2.3	Membrane Insertion	251
9.2.4	Estimating the Molecular Weight of Membrane Proteins	252
9.2.5	Amino Acid Composition	252
9.2.6	Transmembrane Helix Association Motifs and Membrane Protein Oligomerization	253
9.2.7	Post-Translational Modifications	254
9.2.7.1	Glycosylation	255
9.2.7.2	Palmitoylation	255
9.2.8	Sequence Modifications	256
9.2.9	Lipids and Water	258
9.2.10	Purity and Contaminants	260
9.2.11	Current Trends in the Crystallization of α -Helical Membrane Proteins	260
9.3	Test Cases	261
9.3.1	Rhodopsin	261

9.3.2	RPE65	264
9.3.2.1	Expression in <i>E. coli</i>	264
9.3.2.2	Expression in Sf9 Cells	264
9.3.2.3	Purification from Native Sources	264
9.3.3	Transmembrane Domain of M2 Protein from Influenza A Virus	265
	Acknowledgments	267
	Abbreviations	267
	References	267
	Part Three Emerging Methods and Approaches	275
10	Engineering Integral Membrane Proteins for Expression and Stability	277
	<i>Igor Dodevski and Andreas Plückthun</i>	
10.1	Introduction	277
10.2	Engineering Higher Expression	278
10.2.1	Directed Evolution of a GPCR for Higher Expression	280
10.2.2	Increasing Expression by Random Mutagenesis and Dot-Blot Based Screening	286
10.3	Engineering Higher Stability	288
10.3.1	Stabilizing a Prokaryotic IMP by Cysteine-Scanning, Random Mutagenesis, and Screening in a 96-Well Assay Format	289
10.3.2	Stabilizing GPCRs by Alanine-Scanning and Single-Clone Screening	290
10.3.3	Stabilizing GPCRs by Random Mutagenesis and Screening in a 96-Well Assay Format	291
10.4	Conclusions	294
	Abbreviations	295
	References	295
11	Expression and Purification of G-Protein-Coupled Receptors for Nuclear Magnetic Resonance Structural Studies	297
	<i>Fabio Casagrande, Klaus Maier, Hans Kiefer, Stanley J. Opella, and Sang Ho Park</i>	
11.1	Introduction: G-Protein-Coupled Receptor Superfamily	297
11.2	CXCR1	298
11.3	GPCR Structures	299
11.4	NMR Studies of GPCRs	300
11.5	Expression Systems	301
11.6	Cloning of CXCR1 into pGEX2a	303
11.7	Expression of CXCR1	304
11.8	Purification	305
11.9	Refolding and Reconstitution	306
11.10	Binding and Activity Measurements	307

11.10.1	NMR Samples	308
11.11	NMR Spectra	309
	Acknowledgments	310
	Abbreviations	311
	References	311
12	Solubilization, Purification, and Characterization of Integral Membrane Proteins	317
	<i>Victor L6renz-Fonfr6a, Alex Per6lvarez-Mar6n, Esteve Padr6s, and Tzvetana Lazarova</i>	
12.1	Introduction	317
12.2	Solubilization of IMPs	319
12.2.1	Physicochemical Characteristics of Detergents	319
12.2.2	Classification of Detergents	321
12.2.2.1	Nonionic Detergents	321
12.2.2.2	Ionic Detergents	321
12.2.2.3	Zwitterionic Detergents	322
12.2.2.4	Recently Developed Detergents	322
12.2.3	New Solubilizing Agents	322
12.2.4	Solubilization Process	332
12.2.5	The Means of a Successful Solubilization of IMPs	324
12.2.6	"All" or "Not All" Lipids and If "Purer Is Better"	325
12.2.7	Stability of the Protein-Detergent Solutions	325
12.3	IMP Purification	326
12.3.1	Strategy Definition	326
12.3.1.1	HTP Methods	327
12.3.2	Purification Process	328
12.3.2.1	Hydrophobicity	328
12.3.2.2	Charge	328
12.3.2.3	Size	329
12.3.2.4	Affinity	330
12.3.3	New Approaches and Advances in Purification	333
12.3.3.1	Magnetic Beads	333
12.3.3.2	Phase Separation Methods	334
12.4	Characterization of Solubilized IMPs	334
12.4.1	Sample Homogeneity and Protein Oligomeric State	334
12.4.1.1	SEC	334
12.4.1.2	Static Light Scattering (SLS)	335
12.4.1.3	Analytical Ultracentrifugation (AUC)	335
12.4.1.4	Blue-Native Electrophoresis (BN-PAGE)	336
12.4.2	Structural Characterization	336
12.4.2.1	Circular Dichroism (CD)	336
12.4.2.2	IR Spectroscopy	337
12.4.2.3	NMR Spectroscopy	337
12.4.3	Measurement and Characterization of Ligand Binding	339

12.4.3.1	Isothermal Titration Calorimetry (ITC)	339
12.4.3.2	Spectroscopic Methods	340
	Appendix	341
	Acknowledgments	348
	Abbreviations	348
	References	349
13	Stabilizing Membrane Proteins in Detergent and Lipid Systems	361
	<i>Mark Lorch and Rebecca Batchelor</i>	
13.1	Introduction	361
13.2	Choice of Detergent: Solubilization versus Stability	361
13.2.1	Detergents: General Characteristics	362
13.2.1.1	Ionic Detergents	363
13.2.1.2	Zwitterionic Detergents	363
13.2.1.3	Nonionic Detergents	364
13.2.1.4	Detergent-Like Phospholipids	364
13.2.2	Solubilization	365
13.3	Mitigating Protein Denaturation	366
13.3.1	Mixed Detergent Systems	366
13.3.1.1	Micelles	366
13.3.1.2	Bicelles	368
13.3.2	Detergent-Free Bilayer Systems	370
13.3.2.1	Lipid Nanodisk	370
13.3.2.2	Liposomes	372
13.3.3	Detergent-Mediated Reconstitution of Proteoliposomes	372
13.3.3.1	Dilution Method	373
13.3.3.2	Dialysis versus Hydrophobic Absorption	374
13.3.3.3	Detergent Saturation	375
13.3.4	Lipid Composition	375
13.3.4.1	Hydrophobic Mismatch	376
13.3.4.2	Curvature Elastic Stress	378
13.3.4.3	Specific Lipid Effects	380
13.4	Making or Selecting a Stable Protein	381
13.5	Conclusions	382
	Abbreviations	382
	References	383
14	Rapid Optimization of Membrane Protein Production Using Green Fluorescent Protein-Fusions and Lemo21(DE3)	391
	<i>Susan Schlegel, Mirjam Klepsch, Dimitra Gialama, David Wickström, David Drew, and Jan-Willem de Gier</i>	
14.1	Introduction	391
14.2	Main Protocol	392

14.2.1	Determination of Membrane Protein Topology and Selection of Expression Vector	392
14.2.2	Identification of the Optimal Expression Conditions in Lemo21(DE3) Using Whole-Cell and In-Gel Fluorescence	394
14.2.3	Scaling Up of Expression and Isolation of Membranes	396
14.2.4	Identification of a Suitable Detergent Using Fluorescence-Detection Size-Exclusion Chromatography	398
14.2.5	Purification of the Membrane Protein GFP-Fusion and Recovery of the Membrane Protein from the Fusion	399
14.3	Materials	402
14.3.1	Reagents	402
14.3.2	Equipment	403
14.4	Expression and Isolation of GFP-His ₈	403
14.5	Conclusions	404
	Acknowledgments	405
	Abbreviations	405
	References	405

Index	407
-------	-----