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 *Saccharomyces cerevisiae* 37
Zachary Britton, Carissa Young, Özge Can, Patrick McNeely, Andrea Naranjo, and Anne Skaja Robinson

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 Modifi cations 43
2.3.1.1 Glycosylation 43
2.3.1.2 Disulfi de 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: *Pichia pastoris* 75
Fatima Alkhalfoui, Christel Logez, Olivier Bornert, and Renaud Wagner

3.1 Introduction 75
3.2 A (Brief) Summary on the (Long) History of *P. pastoris* 75
3.3 Introducing *P. pastoris* as a Biotechnological Tool: Its (Extended) Strengths and (Limited) Weaknesses 76
3.4 Basics of the *P. pastoris* 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
  P. pastoris 81
  3.5.1 P. pastoris for Membrane Protein Expression 81
  3.5.2 Common Trends for an Efficient Expression of Membrane Proteins in
  P. pastoris 92
3.6 Guidelines for Optimizing Membrane Protein Expression in
  P. pastoris 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 H3 Receptor 118
    4.2.3.1 Solubilization of the Histamine H3 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
## Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.2.2 Gene Delivery and Expression in Mammalian Systems</td>
<td>143</td>
</tr>
<tr>
<td>5.2.2.1 High Transfection Efficiency in Adherent Cell Cultures with Cationic Liposome</td>
<td>143</td>
</tr>
<tr>
<td>5.2.3 Post-Translational Modifications in Mammalian Systems</td>
<td>147</td>
</tr>
<tr>
<td>5.2.3.1 Glycosylation</td>
<td>148</td>
</tr>
<tr>
<td>5.2.3.2 Protein Lipidation</td>
<td>149</td>
</tr>
<tr>
<td>5.3 Case Studies</td>
<td>150</td>
</tr>
<tr>
<td>5.3.1 Increasing Membrane Protein Expression by Virus Vectors</td>
<td>150</td>
</tr>
<tr>
<td>5.3.2 Anti-apoptosis Engineering for Increasing Membrane Protein</td>
<td>152</td>
</tr>
<tr>
<td>5.3.3 Increasing Membrane Protein Expression by Chaperones</td>
<td>156</td>
</tr>
<tr>
<td>5.3.4 Membrane Protein Expression in Cancer Cell Lines</td>
<td>157</td>
</tr>
<tr>
<td>5.3.5 Membrane Proteins as Biotherapeutics</td>
<td>158</td>
</tr>
<tr>
<td>5.4 Conclusions</td>
<td>159</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>160</td>
</tr>
<tr>
<td>References</td>
<td>161</td>
</tr>
</tbody>
</table>

### 6 Membrane Protein Production Using Photosynthetic Bacteria: A Practical Guide 167

*Philip D. Laible, Donna L. Mielke, and Deborah K. Hanson*

#### 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 E. coli 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 *Rhodobacter* via Conjugal Mating 180
6.3.1 Transformation of *E. coli* S17-1 180
6.3.2 Conjugation of *E. coli* with *R. sphaeroides* 180
6.4 Small-Scale Screening for Expression and Localization of Target Protein in *Rhodobacter* 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 Electroblotting 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 *Rhodobacter* 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* 205
7.2.1.2 Case 2: Group 1B Secreted Phospholipase A2 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  
*Alexei Yeliseev and Krishna Vukoti*  
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  
*David Salom and Krzysztof Palczewski*  
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 E. coli 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
Igor Dodevski and Andreas Plückthun
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
Fabio Casagrande, Klaus Maier, Hans Kiefer, Stanley J. Opella,
and Sang Ho Park
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
12  **Solubilization, Purification, and Characterization of Integral Membrane Proteins**  317

_Víctor Lórenz-Fonfría, Alex Perálvarez-Marín, Esteve Padrós, and Tzvetana Lazarova_

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
## Contents

### 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

Mark Lorch and Rebecca Batchelor

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

Susan Schlegel, Mirjam Klepsch, Dimitra Gialama, David Wickström, David Drew, and Jan-Willem de Gier

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-His₈ 403
14.5 Conclusions 404
Acknowledgments 405
Abbreviations 405
References 405

Index 407