

CONTENTS

<i>Contributors</i>	xv
<i>Preface</i>	xxi
<i>Volumes in Series</i>	xxiii

Section I. Decapping Analyses 1

1. Analysis of mRNA Decapping 3

Shin-Wu Liu, Xinfu Jiao, Sarah Welch, and Megerditch Kiledjian

1. Introduction	4
2. Measuring Decapping Activities of Recombinant and Endogenous Dcp2	6
3. Measuring DcpS Activity	15
4. Migration of Cap Analogs with Various Thin-layer Chromatography Running Buffers	17
Acknowledgment	19
References	19

2. A Kinetic Assay to Monitor RNA Decapping Under Single-Turnover Conditions 23

Brittnee N. Jones, Duc-Uy Quang-Dang, Yuko Oku, and John D. Gross

1. Introduction	24
2. Kinetic Equations	25
3. Materials	28
4. Kinetic Assay	30
5. Summary	36
Acknowledgments	37
References	38

3. Purification and Analysis of the Decapping Activator Lsm1p-7p-Pat1p Complex from Yeast 41

Sundaresan Tharun

1. Introduction	42
2. Purification of the Lsm1p-7p-Pat1p Complex	43

3. Analysis of RNA Binding by the Lsm1p-7p-Pat1p Complex	50
4. Concluding Remarks	53
Acknowledgments	53
References	53
4. Reconstitution of Recombinant Human LSM Complexes for Biochemical, Biophysical, and Cell Biological Studies	57
Bozidarka L. Zaric and Christian Kambach	
1. Introduction	58
2. Cloning	61
3. Protein Expression and Purification	65
4. LSM Complex Reconstitution	68
5. LSM Complex Functional Assays	70
6. Outlook	72
References	72
Section II. Polyadenylation/Deadenylation Analyses	75
5. Regulated Deadenylation <i>In Vitro</i>	77
Aaron C. Goldstrohm, Brad A. Hook, and Marvin Wickens	
1. Introduction	78
2. <i>In Vitro</i> Deadenylation Systems	80
3. Troubleshooting	100
References	101
6. Cell-Free Deadenylation Assays with <i>Drosophila</i> Embryo Extracts	107
Mandy Jeske and Elmar Wahle	
1. Introduction	107
2. Preparation of <i>Drosophila</i> Embryo Extracts	110
3. Preparation of Substrate RNA	112
4. Deadenylation Assay with <i>Drosophila</i> Embryo Extracts	113
5. Characterization of Sequence-Dependent Deadenylation in <i>Drosophila</i> Embryo Extracts	115
Acknowledgments	116
References	116
7. Measuring CPEB-Mediated Cytoplasmic Polyadenylation-Deadenylation in <i>Xenopus laevis</i> Oocytes and Egg Extracts	119
Jong Heon Kim and Joel D. Richter	
1. Introduction	120
2. Monitoring Polyadenylation of Cyclin B1 RNA in <i>Xenopus laevis</i> Oocytes	121

3. Detection of Nuclear and Cytoplasmic Polyadenylation of <i>X. laevis</i> Cyclin B1 mRNA	126
4. Monitoring Deadenylation and Re-adenylation of <i>X. laevis</i> Cyclin B1 RNA	128
5. Detection of Endogenous Cytoplasmic Poly(A) Polymerase Activity	130
6. Overexpression of Exogenous mRNAs and Measuring Polyadenylation-Deadenylation	132
7. Preparation of <i>X. laevis</i> Egg Extracts and Measuring Polyadenylation-Deadenylation with Cyclin B1 RNA	133
Acknowledgments	137
References	137

8. The Preparation and Applications of Cytoplasmic Extracts from Mammalian Cells for Studying Aspects of mRNA Decay **139**

Kevin J. Sokoloski, Jeffrey Wilusz, and Carol J. Wilusz

1. Introduction	140
2. Preparation of HeLa-Cell Cytoplasmic Extracts	142
3. Preparation of RNA Substrates	148
4. Evaluating mRNA Decay with Cytoplasmic Extracts	152
5. Concluding Remarks	161
Acknowledgments	162
References	162

Section III. Nucleases in mRNA Decay **165**

9. *In Vitro* Assays of 5' to 3' Exoribonuclease Activity **167**

Olivier Pellegrini, Nathalie Mathy, Ciarán Condon, and Lionel Bénard

1. Introduction	168
2. Purification of <i>Xrn1</i>	170
3. <i>In Vitro</i> RNA Substrate Synthesis	171
4. Degradation of RNA by <i>XRN1</i> Depends on the Nature of the 5'-End	173
5. Determining the Directionality of Decay	176
6. Conclusions and Prospects	180
References	181

10. Reconstitution of RNA Exosomes from Human and *Saccharomyces cerevisiae*: Cloning, Expression, Purification, and Activity Assays **185**

Jaclyn C. Greimann and Christopher D. Lima

1. Introduction	186
2. Cloning Strategies for Recombinant Protein Expression	190
3. PCR and Subcloning Protocols	191
4. PCR and Subcloning Protocols for Yeast cDNA	191
5. PCR and Subcloning Protocols for Human cDNA	194

6. Expression and Purification of Yeast Exosome Proteins	195
7. Expression and Purification of Human Exosome Proteins	198
8. Reconstitution and Purification of Human and Yeast Exosomes	201
9. Exoribonuclease Assays	206
10. Comparative Exoribonuclease Assays with Different RNA Substrates	207
11. Conclusions	208
Acknowledgments	208
References	209
11. Biochemical Studies of the Mammalian Exosome with Intact Cells	211
Geurt Schilders and Ger J. M. Pruijn	
1. Introduction	212
2. Identifying Protein-Protein Interactions by the Mammalian Two-Hybrid System	213
3. Characterization of Different Exosome Subsets by Glycerol Sedimentation	218
4. Studying Exosome Function with RNAi	222
References	224
12. Determining <i>In Vivo</i> Activity of the Yeast Cytoplasmic Exosome	227
Daneen Schaeffer, Stacie Meaux, Amanda Clark, and Ambro van Hoof	
1. Introduction	228
2. Is My Favorite RNA Degraded and/or Processed by the Exosome?	229
3. Is the Cytoplasmic Exosome Active in my Mutant or Under my Conditions?	232
References	238
13. Approaches for Studying PMR1 Endonuclease-Mediated mRNA Decay	241
Yuichi Otsuka and Daniel R. Schoenberg	
1. Introduction	242
2. Identification of Endonuclease Cleavage Sites within mRNA	244
3. Analysis of PMR1-Containing Complexes	251
4. Affinity Recovery of PMR1-Containing Complexes	257
5. Analysis of PMR1 Activity <i>In Vivo</i> and <i>In Vitro</i>	260
6. Summary	262
Acknowledgments	262
References	262

Section IV. Measuring mRNA Half-life *In Vivo* 265**14. Methods to Determine mRNA Half-Life in *Saccharomyces cerevisiae*** 267

Jeff Collier

1. Introduction	268
2. The Use of Inducible Promoters	269
3. Measuring mRNA Decay by Use of Thermally Labile Alleles of RNA Polymerase II	276
4. Measuring mRNA Decay with Thiolutin	277
5. RNA Extractions	277
6. Northern Blot Analysis	280
7. Loading Controls	280
8. Determination of mRNA Half-Lives	280
9. Concluding Remarks	282
Acknowledgments	282
References	282

15. mRNA Decay Analysis in *Drosophila melanogaster*: Drug-Induced Changes in Glutathione S-Transferase D21 mRNA Stability 285

Bünyamin Akgül and Chen-Pei D. Tu

1. Introduction	286
2. Materials and Methods	287
3. Concluding Remarks	295
Acknowledgment	296
References	296

16. Measuring mRNA Stability During Early *Drosophila* Embryogenesis 299

Jennifer L. Semotok, J. Timothy Westwood, Aaron L. Goldman, Ramona L. Cooperstock, and Howard D. Lipshitz

1. Maternal mRNAs and Early <i>Drosophila</i> Development	300
2. Gene-by-Gene Analysis of mRNA Decay	308
3. Genome-Wide Analysis of mRNA Decay	318
4. Concluding Remarks	331
Acknowledgments	331
References	332

17. Messenger RNA Half-Life Measurements in Mammalian Cells	335
Chyi-Ying A. Chen, Nader Ezzeddine, and Ann-Bin Shyu	
1. Introduction	336
2. General Considerations of mRNA Half-Life Measurements	337
3. Determining mRNA Decay Constant	338
4. Methods for Measuring mRNA Half-Life	339
5. Concluding Remarks	354
Acknowledgments	355
References	355
18. Trypanosomes as a Model to Investigate mRNA Decay Pathways	359
Stuart Archer, Rafael Queiroz, Mhairi Stewart, and Christine Clayton	
1. Introduction	359
2. Genetic Manipulation in Trypanosomes: Down-Regulating Expression of Proteins Involved in mRNA Decay	361
Acknowledgments	375
References	375
19. Cell Type-Specific Analysis of mRNA Synthesis and Decay <i>In Vivo</i> with Uracil Phosphoribosyltransferase and 4-thiouracil	379
Michael D. Cleary	
1. Introduction	380
2. Experimental Design Considerations	382
3. Materials	386
4. Methods	388
Acknowledgments	405
References	405
Section V. Defining Degradative Activities	407
20. Analysis of Cytoplasmic mRNA Decay in <i>Saccharomyces cerevisiae</i>	409
Dario O. Passos and Roy Parker	
1. Introduction	410
2. Measuring mRNA Half-Life	410
3. Determination of mRNA Decay Pathways	415
4. Combination of the Preceding Approaches	425
References	425

21. Transcriptome Targets of the Exosome Complex in Plants	429
Dmitry Belostotsky	
1. Exosome: At the Nexus of the Cellular RNA Transactions	429
2. Unique Features of the Plant Exosome	432
3. Resources for the Mutational Analyses of the Plant Exosome	434
4. Transcriptome-wide Mapping of Targets of the Plant Exosome Complex	436
Acknowledgments	440
References	440
22. Sensitive Detection of mRNA Decay Products by Use of Reverse-Ligation-Mediated PCR (RL-PCR)	445
Thierry Grange	
1. Introduction	446
2. Footprinting of RNA-Protein Interaction	450
3. RL-PCR with Ligation of an RNA Linker	452
4. Circularization RL-PCR to Analyze mRNA Decay Involving Modification of the 5' and 3'-Ends	458
5. Concluding Remarks	465
Acknowledgments	465
References	465
23. Tethering Assays to Investigate Nonsense-Mediated mRNA Decay Activating Proteins	467
Niels H. Gehring, Matthias W. Hentze, and Andreas E. Kulozik	
1. Introduction	468
2. Plasmid Cloning	470
References	480
24. Assays for Determining Poly(A) Tail Length and the Polarity of mRNA Decay in Mammalian Cells	483
Elizabeth L. Murray and Daniel R. Schoenberg	
1. Introduction: Poly(A) Tail Length Assays	484
2. Introduction: Invader RNA Assay	492
Acknowledgments	504
References	504

Section VI. Cell Biology of RNA Decay (i.e., Translational Repression/P Bodies)	505
25. Analyzing P-bodies in <i>Saccharomyces cerevisiae</i>	507
Tracy Nissan and Roy Parker	
1. Introduction	508
2. Determining Whether a Specific Protein Can Accumulate in P-Bodies	508
3. Monitoring Messenger RNA in P-Bodies	514
4. Determining Whether a Mutation or Perturbation Affects P-Body Size and Number	515
5. Quantification of P-Body Size and Number	518
Acknowledgments	519
References	519
26. Real-Time and Quantitative Imaging of Mammalian Stress Granules and Processing Bodies	521
Nancy Kedersha, Sarah Tisdale, Tyler Hickman, and Paul Anderson	
1. Introduction	522
2. Experimental Rationale	524
3. Experimental Considerations	524
4. Selection Criteria	527
5. Transfection	528
6. Properties of Representative Stable Lines	533
7. Environmental Control	541
8. Microscope Hardware: Widefield vs Confocal	543
9. Useful Microscopy Internet Resources	547
10. Sample Protocols	547
11. Conclusions	551
References	551
27. Cell Biology of mRNA Decay	553
David Grünwald, Robert H. Singer, and Kevin Czaplinski	
1. Introduction	554
2. FISH Probe Design	556
3. Hybridization	557
4. Image Acquisition	557
5. FISH Protocol	558
6. Colabeling Protein with IF and RNA with FISH	560
7. IF-FISH Protocol	561
8. Following mRNA in Living Cells	562
9. Live Single-Molecule Detection	563

10. Single mRNA Data Analysis; What You Can Observe	564
11. How Do You Know That You See Single Molecules?	566
12. The Secret to Getting Good Data: More Photons, Less Noise	568
13. Setting Up a Microscope for Single Molecule Detection	569
14. Conclusion	571
Acknowledgments	571
References	575

<i>Author Index</i>	579
<i>Subject Index</i>	597