

Contents

Preface	XXIII
List of Abbreviations	XXV
List of Contributors	XXXI

Part One Theory 1

1	Splicing in the RNA World	3
	<i>Emanuele Buratti, Maurizio Romano, and Francisco E. Baralle</i>	
1.1	Introduction: The Fascination of Alternative Pre-mRNA Splicing	3
1.2	RNA Can Adopt a Flexible Conformation	4
1.3	Enzymatic RNAs and the RNA World	5
1.4	Common Classes of Eukaryotic RNA	6
1.5	Alternative Pre-mRNA Splicing as a Central Element of Gene Expression	8
1.6	Increasing Numbers of Human Diseases are Associated with “Wrong” Splice Site Selection	9
	References	11
2	RNPs, Small RNAs, and miRNAs	13
	<i>Michaela Beitzinger and Gunter Meister</i>	
2.1	Introduction	13
2.2	Ribonuclease P (RNase P)	14
2.3	Small Nucleolar RNAs (snoRNAs)	15
2.4	Small Regulatory RNAs	16
2.4.1	Short Interfering RNAs (siRNAs)	16
2.4.2	MicroRNAs (miRNAs)	17
2.4.3	Piwi-Interacting RNAs (piRNAs)	17
2.5	7SL RNA	17
2.6	7SK RNA	18
2.7	U-Rich Small Nuclear RNAs (U snRNAs)	18
	References	18
3	RNA Elements Involved in Splicing	21
	<i>William F. Mueller and Klemens J. Hertel</i>	
3.1	Introduction	21
3.2	Splice Site Sequence	22
3.3	Intron/Exon Architecture	23
3.4	Splicing Regulatory Elements (SREs)	24
3.5	RNA Secondary Structure	26
3.6	Coupling between Transcription and RNA Processing	27
3.7	Combinatorial Effects of Splicing Elements	28
	References	29

4	A Structural Biology Perspective of Proteins Involved in Splicing Regulation	33
	<i>Antoine Cléry and Frédéric H.-T. Allain</i>	
4.1	Introduction	33
4.2	The RRM: A Versatile Scaffold for Interacting with Multiple RNA Sequences and also Proteins	35
4.2.1	RRM–RNA Interaction and Splicing Regulation	35
4.2.1.1	RNA Binding by Splicing Factors Containing a Single RRM	36
4.2.1.2	RNA Binding by Splicing Factors Containing Multiple RRMs	37
4.2.2	RRM–RRM and RRM–Protein Interactions in Splicing Regulation	41
4.2.2.1	RRM–Protein Interactions Without RNA Binding	41
4.2.2.2	RRM–Protein Interactions Allowing RNA Binding	41
4.2.2.3	Impact of RRM–RRM Interactions on Splicing Mechanism	42
4.3	The Zinc Finger Domain	42
4.4	The KH Domain	44
4.5	Conclusions and Perspectives	45
	References	46
5	The Spliceosome in Constitutive Splicing	49
	<i>Patrizia Fabrizio and Reinhard Lührmann</i>	
5.1	Introduction	49
5.2	The Mechanism of Splicing	49
5.3	The Stepwise Assembly Pathway of the Spliceosome	51
5.4	Dynamics of the Spliceosomal RNA–RNA Rearrangements	53
5.5	Splice-Site Recognition and Pairing Involves the Coordinated Action of RNA and Proteins	55
5.6	Driving Forces and Molecular Switches Required During the Spliceosome’s Activation and Catalysis	55
5.7	A Conformational Two-State Model for the Spliceosome’s Catalytic Center	56
5.8	Compositional Dynamics and Complexity of the Spliceosome	57
5.9	Reconstitution of Both Steps of <i>S. cerevisiae</i> Splicing with Purified Spliceosomal Components	61
5.10	Evolutionarily Conserved Blueprint for Yeast and Human Spliceosomes	61
5.11	Concluding Remarks	63
	References	63
6	The Use of <i>Saccharomyces cerevisiae</i> to Study the Mechanism of pre-mRNA Splicing	65
	<i>Brian C. Rymond</i>	
6.1	Introduction	65
6.2	The Basics of Splicing	65
6.3	Yeast Intron–Exon Organization	66
6.4	The Yeast Spliceosome	67
6.5	Defining the Constellation of Yeast Splicing Factors: Primary Screens and Genomic Inspection	68
6.6	Reporter Genes as Readouts of Splicing Efficiency	69
6.7	Genetic Interaction: Dosage Suppression or Antagonism	70
6.8	Extragenic Suppressors	70
6.9	Synthetic Lethality	71
6.10	Systematic Approaches to Define the Interactome	72
	References	74
7	Challenges in Plant Alternative Splicing	79
	<i>Andrea Barta, Yamile Marquez, and John W.S. Brown</i>	
7.1	Introduction	79

7.2	Plant Introns	80
7.3	The Plant Spliceosome	81
7.4	Plant Spliceosomal Proteins	86
7.5	Alternative Splicing in Plants	88
	References	90
8	Alternative Splice Site Selection	93
	<i>Miguel B. Coelho and Christopher W.J. Smith</i>	
8.1	Introduction	93
8.2	The Players: Splicing Regulators	95
8.3	The Stage: The Splicing Complex Assembly and Exon Definition	96
8.4	Switching Splicing Patterns	99
8.5	Src N1 Exon: A Model of Combinatorial Splicing Regulation	101
8.6	The Global View: Towards a Splicing Code	103
	References	104
9	Integration of Splicing with Nuclear and Cellular Events	109
	<i>Aparna K. Sapra and Karla Neugebauer</i>	
9.1	Introduction	109
9.2	Overview	109
9.3	Nuclear Structure and Distribution of Splicing Factors	110
9.3.1	Cajal Bodies (CBs)	111
9.3.2	Splicing Factor Compartments (SFCs)/Speckles	111
9.3.3	Paraspeckles	111
9.4	Integration of Splicing with Nuclear and Cellular Processes	111
9.4.1	Splicing and Transcription	112
9.4.2	Splicing and mRNA Capping	112
9.4.3	Splicing and 3' End Processing	113
9.4.4	Splicing and Export	113
9.4.5	Splicing and Translation	114
9.4.6	Splicing and Nonsense-Mediated Decay (NMD)	114
9.4.7	Splicing and Chromatin Structure	115
	References	115
10	Splicing and Disease	119
	<i>Emanuele Buratti and Francisco E. Baralle</i>	
10.1	Introduction	119
10.2	Splicing and Disease	120
10.3	Therapeutic Approaches	121
10.4	The Generation of Aberrant Transcripts	122
10.5	Exon Skipping	122
10.6	Cryptic Splice Site Activation	122
10.7	Intron Retention	123
10.8	Pseudoexon Inclusion	124
10.9	Unexpected Splicing Outcomes Following the Disruption of Classical Splicing Sequences	124
10.10	Conclusions	125
	References	125
11	From Bedside to Bench: How to Analyze a Splicing Mutation	129
	<i>Marco Baralle and Diana Baralle</i>	
11.1	Introduction	129
11.2	From Clinical Evaluation to Mutation Testing	130
11.3	An Example of an Uncertain Diagnosis	132
11.4	Mutation Testing Procedures	133
11.4.1	<i>In-Vitro</i> Splicing	134
11.4.2	Minigene Splicing	134

- 11.5 Concluding Remarks 136
References 137

Part Two Basic Methods 139

- 12 Analysis of Common Splicing Problems 141**
Stefan Stamm
- 12.1 Introduction 141
12.2 Is a Mutation Causing a Change in AS? 142
12.3 How is a Splicing Event Regulated, and How Can it be Influenced? 143
12.4 Is There a Difference in Alternative pre-mRNA Processing Between Two Cell Populations? 144
References 145
- 13 Ultracentrifugation in the Analysis and Purification of Spliceosomes Assembled *In Vitro* 147**
Klaus Hartmuth, Maria A. van Santen, and Reinhard Lührmann
- 13.1 Theoretical Background 147
13.2 Protocol 148
13.2.1 Preparation of the Gradient 148
13.2.1.1 Manual Gradient Formation 148
13.2.1.2 Automatic Gradient Formation with the Gradient Master 149
13.2.2 Preparing the Run 150
13.2.2.1 Loading the Sample 150
13.2.2.2 Sedimentation Markers 150
13.2.3 The Ultracentrifuge Run 150
13.2.4 Harvesting the Gradient 150
13.3 Example Experiment 151
13.3.1 Purification of the Spliceosomal B Complex 151
13.3.1.1 Preliminaries 151
13.3.1.2 Preparation of the Spliceosomal B Complex 151
13.4 Troubleshooting 153
References 153
- 14 Chemical Synthesis of RNA 155**
Claudia Höbartner
- 14.1 Theoretical Background 155
14.1.1 RNA Solid-Phase Synthesis 155
14.1.2 RNA Modifications 158
14.1.2.1 RNA Modification During Solid-Phase Synthesis 158
14.1.2.2 Post-Synthetic RNA Modification 158
14.1.3 Combined Chemical and Enzymatic Strategies 158
14.2 Representative Protocols 160
Protocol 1: Incorporation of Modified Phosphoramidites During Solid-Phase Synthesis 160
Protocol 2: Coupling of Biophysical Probes to Aliphatic Amino Groups on RNA 160
Protocol 3: Enzymatic Ligation of RNA fragments using T4 RNA or T4 DNA Ligase 160
14.3 Troubleshooting 161
References 161
- 15 RNA Interference (siRNA, shRNA) 165**
Daphne S. Cabianca and Davide Gabellini
- 15.1 Theoretical Background 165
15.1.1 RNAi 165
15.1.2 siRNAs and shRNAs 165

- 15.1.3 Lentiviral-Mediated RNAi 167
- 15.2 Protocol 167
- 15.2.1 Map of pLKO.1 Puro 167
- 15.2.2 Oligonucleotide Design 167
 - 15.2.2.1 Determining the Optimal 21-mer Targets in the Gene 167
 - 15.2.2.2 Ordering Oligos Compatible with pLKO.1 168
- 15.2.3 Generating the pLKO.1 Puro with a shRNA Construct 169
 - 15.2.3.1 Annealing of the Oligonucleotides 169
 - 15.2.3.2 Preparation of pLKO.1 TRC for Cloning 169
 - 15.2.3.3 Ligating and Transforming into Bacteria 169
 - 15.2.3.4 Screening for Inserts 169
- 15.2.4 Production of Lentiviral Particles 169
- 15.2.5 Lentiviral Infection 170
- 15.3 Example Experiment 171
- 15.4 Troubleshooting 172
- References 172

- 16 Expression and Purification of Splicing Proteins 175**
James Stévenin and Cyril F. Bourgeois
- 16.1 Theoretical Background 175
- 16.2 Protocol 1: The Preparation of Total HeLa SR Proteins 175
 - 16.2.1 Example Experiment 176
 - 16.2.2 Troubleshooting and Important Points 177
- 16.3 Protocol 2: The Purification of Individual SR Proteins 177
 - 16.3.1 Expression of SR Proteins in *Escherichia coli* and Purification 177
 - 16.3.2 Preparation of SR Proteins Using a Baculovirus System 178
 - 16.3.3 Example Experiment 178
 - 16.3.4 Troubleshooting and Important Points 178
 - 16.3.5 Production and Purification of Individual SR Proteins in Mammalian Cells 179
- References 179

- 17 Detection of RNA–Protein Complexes by Electrophoretic Mobility Shift Assay 183**
Manli Shen and Michael G. Fried
- 17.1 Theoretical Background 183
 - 17.1.1 Choice of RNA Substrate 184
 - 17.1.2 Detection and Quantitation of Binding 184
 - 17.1.3 Fluorescence 185
 - 17.1.4 Chromogenic and Chemiluminescent Detection Methods 186
 - 17.1.5 Stability of RNA–Protein Complexes During Electrophoresis 186
 - 17.1.6 Competing Nucleic Acids and Polyanions 187
 - 17.1.7 Binding Stoichiometry 187
 - 17.1.8 Measurement of Binding Activity 187
 - 17.1.9 Measurement of Dissociation Constants 188
 - 17.1.10 Binding Competition 190
- 17.2 Protocol 190
 - 17.2.1 Equipment 191
 - 17.2.2 Reagents 191
 - 17.2.3 Gel Preparation 191
 - 17.2.4 Pre-Electrophoresis 192
 - 17.2.5 Sample Preparation 192
 - 17.2.6 Electrophoresis and Imaging 193
- 17.3 Example Experiment 194
- 17.4 Troubleshooting 195
- References 196

18	Functional Analysis of Large Exonic Sequences Through Iterative <i>In Vivo</i> Selection 201 <i>Ravindra N. Singh and Natalia N. Singh</i>
18.1	Theoretical Background 201
18.1.1	Spinal Muscular Atrophy 202
18.2	Protocol 202
18.2.1	Minigene, Cell Culture, Transfection, and <i>In Vivo</i> Splicing Assay 202
18.2.2	Generation of a Partially Random Exon 204
18.2.3	<i>In vivo</i> Selection 204
18.2.4	Analysis of Sequences 204
18.3	Example Experiment 205
18.3.1	Generating the Initial Pool of Splicing Cassettes 206
18.3.2	<i>In Vivo</i> Selection Procedure 207
18.4	Troubleshooting 209 References 209
19	Identification of Splicing <i>cis</i>-Elements Through an Ultra-Refined Antisense Microwalk 211 <i>Natalia N. Singh, Joonbae Seo, and Ravindra N. Singh</i>
19.1	Theoretical Background 211
19.2	Protocol 212
19.2.1	Oligonucleotide Design and Synthesis 212
19.2.2	Cell Culture, Transfection, and <i>In Vivo</i> Splicing Analysis 212
19.2.3	Validation of Specificity 212
19.3	Example Experiment 214
19.4	Troubleshooting 216 References 217
20	Genomic SELEX to Identify RNA Targets of Plant RNA-Binding Proteins 219 <i>Olga Bannikova, Maria Kalyna, and Andrea Barta</i>
20.1	Introduction 219
20.2	Protocols 221
20.3	Example Experiment 225
20.4	Troubleshooting 225 References 226

Part Three Detection of Splicing Events 227

21	Quantification of Alternative Splice Variants 229 <i>Miriam Llorian and Christopher W.J. Smith</i>
21.1	Theoretical Background 229
21.1.1	PCR for Splice Variant Quantification 229
21.1.2	Conventional RT-PCR 230
21.1.3	Real-Time PCR: Chemistry and Quantification Methods 230
21.1.4	Primer Design 232
21.2	Protocol 233
21.2.1	RNA Extraction 233
21.2.2	DNase Treatment 233
21.2.3	Reverse Transcriptase Reaction 233
21.2.4	Radiolabeled PCR 233
21.2.5	qPCR Reactions 234
21.2.6	Data Analysis 234
21.3	Example Experiment: Microarray Validation of PTB-Regulated Events 234

- 21.3.1 Primer Design 235
- 21.4 Troubleshooting 235
- References 236

- 22 High-Throughput Analysis of Alternative Splicing by RT-PCR 239**
Roscoe Klinck, Benoit Chabot, and Sherif Abou Elela
- 22.1 Theoretical Background 239
- 22.1.1 Endpoint PCR for the Detection of Alternative Splicing Events 239
- 22.1.2 Computational Identification of Alternative Splicing Events 241
- 22.1.3 Primer Design 241
- 22.1.4 Capillary Electrophoresis 242
- 22.1.5 Data Analysis 242
- 22.1.6 Validation of Microarray and RNASeq Data 242
- 22.1.7 Tissue-Specific Annotation from Sequence Databases 243
- 22.2 Protocol 243
- 22.2.1 Primer Design 243
- 22.2.2 RNA Preparation 243
- 22.2.3 RT and QC of cDNA 244
- 22.2.4 PCR Reactions and Amplicon Detection 244
- 22.3 Example Experiment 245
- 22.4 Troubleshooting 245
- References 246

- 23 Monitoring Changes in Plant Alternative Splicing Events 249**
Craig G. Simpson, Naeem Hasan Syed, Sujatha Manthri, John D. Fuller, Monika Maronova, Branislav Kusenda, Maria Kalyna, Andrea Barta, and John W.S. Brown
- 23.1 Theoretical Background 249
- 23.1.1 Alternative Splicing in Plants 249
- 23.1.2 Splicing Analysis 250
- 23.1.3 Establishing the RT-PCR Conditions 250
- 23.1.4 Characterizing Novel Alternatively Spliced Products 250
- 23.1.5 Identifying AS Events in Genes of Interest 251
- 23.2 Protocols 251
- 23.3 Example Experiments 256
- 23.4 Troubleshooting 257
- References 258

- 24 Array Analysis 261**
Pierre de la Grange
- 24.1 Theoretical Background 261
- 24.1.1 Microarrays: General Principles 261
- 24.1.2 Probe Design of Splicing Microarrays: Interest and Limitation 262
- 24.1.3 Available Splicing Microarrays 262
- 24.1.4 The Different Steps of the Microarray Data Treatment 263
- 24.2 Protocol 263
- 24.2.1 Normalization 264
- 24.2.2 Background Subtraction 264
- 24.2.3 Statistical Analysis 264
- 24.2.4 Visualization of Data 265
- 24.2.5 Functional Analysis of Results 265
- 24.3 Example Experiment 265
- 24.4 Troubleshooting 266
- References 267

25	The CLIP Method to Study Protein–RNA Interactions in Intact Cells and Tissues 269
	<i>James Tollervey and Jernej Ule</i>
25.1	Theoretical Background 269
25.2	Protocols 270
25.2.1	Material and Reagents 270
	References 277
26	RNA–Protein Crosslinking and Immunoprecipitation (CLIP) in <i>Schizosaccharomyces pombe</i> 281
	<i>Branislav Kusenda and Andrea Barta</i>
26.1	Introduction 281
26.2	Protocol 282
26.2.1	Materials 282
26.2.2	Growth Conditions and Cell Harvest 283
26.2.3	Crosslinking 283
26.2.4	Sonication of the Cells 284
26.2.5	Immunoprecipitation 284
26.2.6	DNase Treatment 284
26.2.7	RNase Treatment 284
26.2.8	Dephosphorylation 284
26.2.9	L3 Linker Ligation 284
26.2.10	Phosphorylation of the 5' End of RNA 285
26.2.11	L5 Linker Ligation 285
26.2.12	Elution of the Protein–RNA Complex 285
26.2.13	cDNA Synthesis 285
26.2.14	PCR Amplification 285
26.2.15	Size Selection of DNA Fragments 286
26.2.16	Cloning Step: Generation of Overhangs 286
26.2.17	TOPO TA Cloning Reaction 286
26.2.18	Transformation of <i>Escherichia coli</i> 286
26.3	Example Experiment 286
26.4	Troubleshooting 287
	References 288
27	Identification of Proteins Bound to RNA 291
	<i>Emanuele Buratti</i>
27.1	Theoretical Background 291
27.2	Protocol 292
27.2.1	RNA Templates 292
27.2.2	Loading the Beads with RNA 293
27.2.3	Incubation with Protein Mix (Buffer A) 294
27.2.4	Incubation with Protein Mix (Buffer B) 294
27.3	Example Experiment 295
27.4	Troubleshooting 296
	References 296
28	Single-Cell Detection of Splicing Events with Fluorescent Splicing Reporters 299
	<i>Hidehito Kuroyanagi, Akihito Takeuchi, Takayuki Nojima, and Masatoshi Hagiwara</i>
28.1	Theoretical Background 299
28.1.1	Visualization of Alternative Splicing Patterns with Multiple Fluorescent Proteins 299
28.1.2	Designing Fluorescent Reporter Minigenes to Monitor Splicing Patterns 300
28.1.3	Constructing Fluorescent Reporter Minigenes 302

- 28.1.3.1 MultiSite Gateway System 302
- 28.1.3.2 Other Aspects for Consideration in Minigene Construction 303
- 28.2 Protocols 303
- 28.2.1 Constructing Genomic DNA Fragment Cassettes in “Entry” Vectors 303
- 28.2.1.1 Primer Design 304
- 28.2.1.2 Performing PCR 304
 - Protocol 1: Two-Step PCR Amplification of *attB*-DNA Fragments 304
 - ‘BP’ Recombination Reaction and the Selection of “Entry” Clones 305
 - Protocol 2: BP Clonase II Reaction and Selection of Appropriate “Entry” Clones 305
 - Modification of “Entry” Clones (Optional) 305
 - ‘LR’ Recombination Reaction and Selection of “Expression” Clones 305
 - Protocol 3: LR Clonase II Plus Reaction and Selection of Appropriate “Expression” Clones 306
 - Transfection of Cultured Cells and Generation of Transgenic Animals 306
 - Checking the Splicing Pattern of the Minigene-Derived mRNAs 306
 - Protocol 4: RT-PCR Analysis of Minigene-Derived mRNAs 306
- 28.3 Example Experiments 307
- 28.4 Troubleshooting 308
- References 308

Part Four Analysis of Splicing *In Vitro* 311

- 29 The Preparation of HeLa Cell Nuclear Extracts 313**
Klaus Hartmuth, Maria A. van Santen, Tanja Rösler, Berthold Kastner, and Reinhard Lührmann
- 29.1 Theoretical Background 313
- 29.2 Protocols 314
- 29.3 Example Experiment 318
- 29.4 Troubleshooting 318
- References 318

- 30 *In Vitro* Splicing Assays 321**
Akila Mayeda and Adrian R. Krainer
- 30.1 Theoretical Background 321
- 30.2 Protocols 321
- 30.2.1 Preparation of Pre-mRNA Substrates 321
- 30.2.1.1 Minigene Construction in a Vector for *In Vitro* Transcription 321
- 30.2.1.2 ³²P-Labeled Pre-mRNA Preparation by *In Vitro* Transcription 322
- 30.2.2 *In Vitro* Splicing of Pre-mRNAs 323
- 30.2.2.1 Preparation of Reagents 323
- 30.2.2.2 Splicing Reactions 324
- 30.2.3 Analysis of Splicing Products 325
- 30.2.3.1 Preparation of Reagents 325
- 30.2.3.2 Denaturing PAGE and Autoradiography 326
- 30.3 Example Experiment 327
- 30.4 Troubleshooting 327
- References 328

- 31 Assembly and Isolation of Spliceosomal Complexes *In Vitro* 331**
Klaus Hartmuth, Maria A. van Santen, Peter Odenwälder, and Reinhard Lührmann
- 31.1 Theoretical Background 331
- 31.2 Protocols 332
- 31.3 Example Experiment 338

- 31.4 Troubleshooting 339
- References 340

- 32 Analysis of Site-Specific RNA–Protein Interactions 343**
Nathalie Marmier-Gourrier, Audrey Vautrin, Christiane Branlant, and Isabelle Behm-Ansmant
- 32.1 Theoretical Background 343
- 32.2 Protocols 345
- 32.2.1 UV Crosslinking and Immunoselection 345
- 32.2.1.1 Chemicals and Enzymes 345
- 32.2.1.2 Buffers 345
- 32.2.1.3 UV Crosslinking 345
- 32.2.2 RNA Secondary Structure and Footprint Experiments 347
- 32.2.2.1 Probes Used and Properties 347
- 32.2.2.2 Chemicals and Enzymes 348
- 32.2.2.3 Buffers and Reagents 348
- 32.2.2.4 RNA Synthesis 349
- 32.2.2.5 RNA Transcript Renaturation 349
- 32.2.2.6 EMSA 349
- 32.2.3 Supershift 352
- 32.2.3.1 Chemicals and Enzymes 352
- 32.2.3.2 Buffers 352
- 32.2.3.3 Supershift Experiments 352
- 32.3 Example Experiments 353
- 32.4 Troubleshooting 355
- References 355

- 33 Immunoprecipitation and Pull-Down of Nuclear Proteins 359**
Natalya Benderska, Chiranthani Sumanasekera, and Stefan Stamm
- 33.1 Theoretical Background 359
- 33.1.1 Immunoprecipitation 359
- 33.1.2 Pull-Down of Proteins 360
- 33.2 Protocols 360
- 33.3 Example Experiments 362
- 33.4 Troubleshooting 363
- References 364

- 34 Analysis of Protein (-RNA) Complexes by (Quantitative) Mass Spectrometric Analysis 367**
Carla Schmidt and Henning Urlaub
- 34.1 Theoretical Background 367
- 34.1.1 Mass Spectrometry-Based Identification of Proteins (Proteomics) 367
- 34.1.2 Sample Preparation and Separation 368
- 34.1.2.1 Additional Separation of Generated Peptides 368
- 34.1.2.2 Separation of Proteins 368
- 34.1.3 Liquid Chromatography-Coupled Mass Spectrometry (LC-MS) 369
- 34.1.4 Quantification by Mass Spectrometry 370
- 34.1.4.1 Introduction 370
- 34.1.4.2 Relative Quantification 370
- 34.1.4.3 Absolute Quantification 371
- 34.1.5 Detection of Post-Translational Modifications by Mass Spectrometry 372
- 34.1.5.1 Introduction 372
- 34.1.5.2 Phosphorylation 372
- 34.1.6 Detection of Protein–RNA Interactions by Mass Spectrometry 373
- 34.2 Protocols 373
- 34.3 Example Experiment 377
- 34.4 Troubleshooting 377
- References 378

Part Five Analysis of Splicing *In Vivo* 381

- 35 Fast Cloning of Splicing Reporter Minigenes 383**
Zhaiyi Zhang, Amit Khanna, and Stefan Stamm
- 35.1 Theoretical Background 383
 - 35.1.1 Detection of Alternative Splicing Using Minigenes 383
 - 35.1.2 Site-Specific Recombination 384
 - 35.1.2.1 DNA Recombination Sites 384
 - 35.1.2.2 Recombination Proteins 385
 - 35.1.3 pSpliceExpress 385
 - 35.2 Protocol 386
 - 35.2.1 Ordering a BAC Clone 386
 - 35.2.2 PCR Primer Design 386
 - 35.2.2.1 Forward Primer 386
 - 35.2.2.2 Reverse Primer 386
 - 35.2.2.3 Nested Primers 386
 - 35.2.3 Performing the PCR 387
 - 35.2.4 Recombination Reaction 387
 - 35.2.5 Transformation of Cells 388
 - 35.2.6 Identification of Positive Clones 388
 - 35.2.7 Transfection of the Minigene 388
 - 35.2.7.1 Transfection of HEK293 Cells with Reporter Gene Constructs 389
 - 35.3 Example Experiment 389
 - 35.4 Troubleshooting 390
 - References 391
- 36 *In Vivo* Analysis of Splicing Assays 393**
Isabel C. López-Mejía and Jamal Tazi
- 36.1 Theoretical Background 393
 - 36.1.1 Studying an Alternative Splicing Event 393
 - 36.1.2 Transfection of Adherent Cell Lines 394
 - 36.2 Protocol 395
 - 36.2.1 Reagents and Solutions 395
 - 36.2.2 Materials and Equipment 395
 - 36.3 Example Experiment 398
 - 36.4 Troubleshooting 398
 - References 399
- 37 Coupled Promoter Splicing Systems 401**
Manuel J. Muñoz, Manuel de la Mata, and Alberto R. Kornblihtt
- 37.1 Theoretical Background 401
 - 37.2 Protocol 402
 - 37.2.1 Choosing the Promoter of the Minigene: Constitutive Versus Inducible Promoters 402
 - 37.2.2 The Role of Pol II on Alternative Splicing 403
 - 37.2.3 Transfection of the Alternative Splicing Reporter Minigene 404
 - 37.2.4 RNA Extraction and RT-PCR 405
 - 37.3 Example Experiment 406
 - 37.3.1 Effect of a Slow Pol II Mutant (hC4) in EDI Alternative Splicing 406
 - 37.4 Troubleshooting 407
 - References 407
- 38 Stable Cell Lines with Splicing Reporters 409**
Christian Kroun Damgaard, Søren Lykke-Andersen, and Jørgen Kjems
- 38.1 Theoretical Background 409
 - 38.1.1 Plasmid-Based Site-Specific Integration of Splicing Reporters 410

- 38.1.2 The Flp Recombinase 410
- 38.1.3 Induction of Gene Expression 410
- 38.2 Protocol 411
- 38.2.1 Cloning 411
- 38.2.2 Transfection of Recipient Cell Line (HEK293 Flp-In T-REx) 411
- 38.3 Example Experiment 412
- 38.3.1 Splicing the Phenotype of Two HIV-1 Minigenes 412
- 38.4 Troubleshooting 413
- References 414

- 39 Splicing Factor ChIP and ChRIP: Detection of Splicing and Splicing Factors at Genes by Chromatin Immunoprecipitation 417**
Aparna K. Sapra, Fernando Carrillo Oesterreich, Marta Pabis, Imke Listerman, Nicole Bardehle, and Karla M. Neugebauer
- 39.1 Theoretical Background 417
- 39.1.1 Cotranscriptional Splicing 417
- 39.1.2 Chromatin Immunoprecipitation 418
- 39.1.3 Application of ChIP to Splicing Studies 419
- 39.1.4 Quantitation of the Immunoprecipitated Nucleic Acids 419
- 39.2 Protocol 419
- 39.2.1 Splicing Factor ChIP in *S. cerevisiae* 419
- 39.2.1.1 Buffers and Chemicals Used for ChIP 421
- 39.2.2 Splicing Factor ChIP in Mammalian Cells 421
- 39.2.2.1 Buffers and Chemicals Used for ChIP 423
- 39.2.3 ChRIP for Analysis of Cotranscriptional RNA Processing 423
- 39.2.3.1 Additional Buffers and Chemicals Used for ChRIP 424
- 39.2.4 Data Analysis Using qPCR 424
- 39.3 Example Experiment 424
- 39.4 Troubleshooting 426
- References 427

- 40 Yeast Genetics to Investigate the Function of Core Pre-mRNA Splicing Factors 429**
Raymond T. O'Keefe and Jean D. Beggs
- 40.1 Theoretical Background 429
- 40.1.1 The Use of Yeast *Saccharomyces cerevisiae* as a Model System for Studying Spliceosomal Factors 429
- 40.1.2 Yeast Genetics 429
- 40.1.3 Synthetic Genetic Interactions 430
- 40.2 Protocol 431
- 40.2.1 Plasmid Shuffle for Functional Analysis and Production of Conditional Mutations in Essential Yeast Genes 431
- 40.2.1.1 Construction of Complementing *URA3* Plasmid and a Plasmid for Mutagenesis 431
- 40.2.1.2 Transformation of Diploid Knockout Strain 432
- 40.2.1.3 Yeast Sporulation and Tetrad Dissection 432
- 40.2.1.4 Functional Analysis of Essential Yeast Genes by Plasmid Shuffle 433
- 40.2.2 Genetic Interaction Analysis 433
- 40.2.2.1 Construction of Double-Gene *URA3* Plasmid 433
- 40.2.2.2 Construction of Double-Knockout Strains 433
- 40.2.2.3 Genetic Screens 434
- 40.3 Example Experiment 434
- 40.4 Troubleshooting 435
- References 435

- 41 Analysis of HIV-1 RNA Splicing 439**
Simon Duffy and Alan Cochrane
- 41.1 Theoretical Background 439
 - 41.1.1 Virus RNA Splicing 439
 - 41.1.2 HIV-1 RNA Splicing 440
 - 41.1.3 Quantitative Reverse Transcription PCR (qRT-PCR) 440
 - 41.2 Protocols 441
 - 41.2.1 qRT-PCR Primer Design 441
 - 41.2.1.1 Primers for Analysis of HIV-1 Transcripts 441
 - 41.2.2 Transfection of Virus/Provirus Vector DNA into Target Cells 441
 - 41.2.3 Secreted Alkaline Phosphatase (SEAP) Assay 442
 - 41.2.4 RNA Isolation and cDNA Synthesis 442
 - 41.2.5 qRT-PCR Reaction Conditions and Analysis 443
 - 41.2.6 Analysis of Splice Site Utilization 444
 - 41.3 Example Experiment 446
 - 41.4 Troubleshooting 447
 - References 448
- 42 In Vivo Analysis of Plant Intron Splicing 451**
Craig G. Simpson, Michele Liney, Diane Davidson, Dominika Lewandowska, Maria Kalyna, Sean Chapman, Andrea Barta, and John W.S. Brown
- 42.1 Theoretical Background 451
 - 42.1.1 Plant Splicing Analysis *In Vivo* 451
 - 42.1.2 Splicing of Plant and Animal Introns in Reciprocal Systems 451
 - 42.1.3 Plant Splicing Reporter Constructs 452
 - 42.1.4 Expression of trans-Acting Factors 453
 - 42.2 Protocols 453
 - 42.2.1 Transfection of Plasmid DNA into Plant Protoplasts 453
 - 42.2.2 Analyzing the Results of *In vivo* Splicing Analysis 459
 - 42.2.2.1 RNA Extraction 459
 - 42.2.2.2 RT-PCR Analysis 459
 - 42.2.2.3 Western Analysis 460
 - 42.2.2.4 Visualizing GFP Expression 460
 - 42.3 Example Experiment 461
 - 42.4 Troubleshooting 461
 - References 462
- 43 Modification State-Specific Antibodies 465**
Jordan B. Fishman, Olga Kelemen, and Eric A. Berg
- 43.1 Theoretical Background 465
 - 43.2 Protocol 466
 - 43.2.1 Selection of the Target Site and Peptide Immunogen Design 466
 - 43.2.2 Peptide Synthesis 467
 - 43.2.3 Peptide Immunogen–Carrier Protein Conjugation 469
 - 43.2.4 Immunization, Antibody Purification, and Immunodepletion 469
 - 43.2.5 The Human SMAD2 Protein C-Terminal Domain 470
 - 43.3 Example Experiment 471
 - 43.4 Troubleshooting 472
 - References 473
- 44 Analysis of Alternative Splicing in *Drosophila* Genetic Mosaics 475**
Shihuang Su, Diana O’Day, Shanzhi Wang, and William Mattox
- 44.1 Theoretical Background 475
 - 44.1.1 Reporter Genes for Splicing in Living Organisms 475
 - 44.1.2 The Introduction of Splicing Reporters into the *Drosophila* Genome 475
 - 44.1.3 The Application of Genetic Mosaics to the Analysis of Alternative Pre-mRNA Splicing 476

- 44.2 Protocol 476
- 44.2.1 Transgenes Used in Flip-Out Studies 476
- 44.2.2 Generation of Mosaic Flies 478
- 44.2.3 Immunostaining of *Drosophila* Tissues 478
- 44.3 Example Experiment 479
- 44.4 Troubleshooting 479
- References 479

Part Six Manipulation of Splicing Events 481

- 45 Antisense Derivatives of U7 Small Nuclear RNA as Modulators of Pre-mRNA Splicing 483**
Kathrin Meyer and Daniel Schümperli
- 45.1 Theoretical Background 483
- 45.1.1 What Makes U7 snRNA a Suitable *In Vivo* Splicing Modulation Tool? 483
- 45.1.2 Strategic Considerations 485
- 45.1.3 Gene Transfer and Regulated Expression 487
- 45.2 Protocols 488
- 45.3 Example Experiment 492
- 45.3.1 Mutagenic PCR (Protocol 1) with Four Different Primers 492
- 45.4 Troubleshooting 492
- References 493

- 46 Screening for Alternative Splicing Modulators 497**
Peter Stoilov
- 46.1 Theoretical Background 497
- 46.1.1 Overview of *In Vivo* Splicing Reporters 497
- 46.1.2 Two-Color Fluorescent Reporters for Splicing and Translation 498
- 46.1.3 Data Acquisition and Processing 499
- 46.1.4 Hit Validation 500
- 46.2 Protocols 500
- 46.2.1 Equipment and Materials 501
- 46.2.1.1 Equipment 501
- 46.2.1.2 Materials 501
- 46.2.2 Compound Plate Layout 501
- 46.2.3 Cell Plating and Compound Addition 502
- 46.2.4 Imaging 502
- 46.2.5 Image Processing and Data Analysis 502
- 46.2.6 RT-PCR Validation 503
- 46.2.6.1 RNA Preparation 503
- 46.2.6.2 First-Strand cDNA Synthesis 503
- 46.2.6.3 PCR Amplification 504
- 46.2.6.4 Capillary Electrophoresis 504
- 46.3 Example Experiment 504
- 46.4 Troubleshooting 505
- References 507

- 47 Use of Oligonucleotides to Change Splicing 511**
Annemieke Aartsma-Rus and Judith C.T. van Deutekom
- 47.1 Theoretical Background 511
- 47.1.1 Antisense-Mediated Modulation of Splicing 511
- 47.1.2 AON Design 513
- 47.1.3 AON Chemistry 514
- 47.1.4 Transfection in Cultured Cells 514
- 47.1.5 RNA Analysis 515
- 47.2 Protocol 515
- 47.2.1 AONs 515

- 47.2.2 Transfection 516
- 47.2.3 RNA Isolation 517
- 47.2.4 RT-PCR Analysis 517
- 47.2.5 Determining Exon Skipping Efficiency 518
- 47.3 Example Experiment 518
- 47.4 Troubleshooting 518
- References 519

- 48 Changing Signals to the Spliceosome 523**
Denise R. Cooper and Niketa A. Patel
- 48.1 Theoretical Background 523
- 48.1.1 Identification of Extracellular Stimulated Signaling Pathways Altering Spliceosomal Proteins 523
- 48.1.2 Identification of *cis*-Elements and *trans*-Factors Targeted by Kinase Pathways 525
- 48.1.3 To Establish Whether a Kinase Regulating an Alternative Exon is Activated by a Hormone 525
- 48.1.4 Use of siRNA to Establish the Role of a Kinase and Spliceosomal Protein in a Splicing Event 526
- 48.1.5 Use of 2'-O-Methoxyethyl (MOE) Antisense Oligonucleotides to Identify Relevant *cis*-Elements in the Sequence 526
- 48.1.6 Identifying the Minimum Boundaries of the Genomic Sequence Regulated by Stimuli for Cloning into a Heterologous Minigene 528
- 48.1.7 Physiological Readout of Splice Variant Function 528
- 48.1.8 Prediction of Splicing Relevance Using Knockout Tissue or Tissue from Mutant or Disease States 528
- 48.2 Protocols 528
- 48.2.1 Cell Culture 528
- 48.2.2 Transfections 529
- 48.2.3 Western Blot Analysis 529
- 48.2.4 Immunoprecipitation 530
- 48.2.4.1 Buffer solutions 530
- 48.2.5 Minigenes 530
- 48.2.6 RT-PCR Analysis 531
- 48.2.7 Inhibitors 532
- 48.3 Example Experiment 532
- 48.4 Troubleshooting 533
- References 533

Part Seven Bioinformatic Analysis of Splicing 535

- 49 Overview of Splicing Relevant Databases 537**
Pierre de la Grange
- 49.1 Theoretical Background 537
- 49.1.1 Alternative Splicing Databases: Interest 537
- 49.1.2 Alternative Splicing Databases: Common Strategy 537
- 49.1.3 Description of Alternative Splicing Databases 538
- 49.1.4 The UCSC Genome Browser 538
- 49.2 Protocol 538
- 49.3 Example Experiment 541
- 49.4 Troubleshooting 541
- References 542

- 50 Analysis of RNA Transcripts by High-Throughput RNA Sequencing 545**
Paolo Ribeca, Vincent Lacroix, Michael Sammeth, and Roderic Guigó
- 50.1 Theoretical Background 545
- 50.1.1 Reads, Mapping, and Mappability 545

50.1.2	Quantification of AS Events and Read Deconvolution	546
50.2	Protocol	548
50.2.1	Experimental Protocol	548
50.2.2	Pre-Processing of the Reads	548
50.2.3	Mapping of the Reads	550
50.2.3.1	Reference Indexing	550
50.2.4	Quantification of Abundances	551
50.3	Example Experiment	552
50.4	Troubleshooting	553
	References	554
51	Identification of Splicing Factor Target Genes by High-Throughput Sequencing	557
	<i>Chaolin Zhang and Michael Q. Zhang</i>	
51.1	Theoretical Background	557
51.1.1	Transcriptome Analysis by mRNA-Seq	557
51.1.2	Available NGS Platforms	558
51.1.3	Short Reads Mapping	558
51.1.4	Single-End versus Paired-End mRNA-Seq	560
51.2	Protocol	560
51.2.1	mRNA-Seq Experiment Design	560
51.2.2	Sequence Alignment	561
51.2.3	Read Counting and Statistical Analysis	561
51.2.4	Visualization of Data	562
51.3	Example Experiment	562
51.4	Troubleshooting	563
	References	563
52	Bioinformatic Analysis of Splicing Events	567
	<i>Zhaiyi Zhang and Stefan Stamm</i>	
52.1	Theoretical Background	567
52.1.1	General Databases	567
52.1.2	Splice Site Analysis	568
52.1.3	Exonic and Intronic Splicing Elements	568
52.1.4	Secondary Structure	570
52.2	Protocol	570
52.2.1	Detecting a New Sequence Using BLAT	570
52.2.2	Determine Regulatory Elements in RNA Sequence	570
52.3	Example Experiment	571
52.4	Troubleshooting	572
	References	573
53	Analysis of Pre-mRNA Secondary Structures and Alternative Splicing	575
	<i>Michael Hiller</i>	
53.1	Theoretical Background	575
53.1.1	Alternative Splicing and Secondary Structures	575
53.1.2	Computational Prediction of Secondary Structures	576
53.2	Protocol	576
53.2.1	Input Sequences	576
53.2.2	Predicting Single-Stranded Regions	577
53.2.2.1	Computing Base-Pair Probability Plots	577
53.2.2.2	Computing PU Values	577
53.2.3	Predicting if a Mutation Leads to Structural Changes	578
53.2.3.1	Comparing Base-Pair Probability Plots	578
53.2.3.2	Computing PU Values	579

53.2.4	Finding Alternative Splice Events that Overlap Evolutionarily Conserved Secondary Structures	579
53.3	Example Experiment	579
53.4	Troubleshooting	580
	References	580
54	Structure Prediction for Alternatively Spliced Proteins	583
	<i>Lukasz Kozlowski, Jerzy Orłowski, and Janusz M. Bujnicki</i>	
54.1	Theoretical Background	583
54.2	Protocol	585
54.2.1	Primary Structure Analysis	585
54.2.2	Predicting Disordered Regions	585
54.2.3	Predicting Transmembrane Helices, Coiled-Coils, and Repeats	586
54.2.4	Protein Fold Recognition	586
54.2.5	Target–Template Alignment	586
54.2.6	Template-Based Modeling	587
54.2.7	Model Quality Assessment	587
54.2.8	Is the Same Possible for RNA 3-D Structure Prediction?	588
54.3	Example Experiment	588
54.4	Troubleshooting	590
	References	590
55	Comparative Genomics Methods for the Prediction of Small RNA-Binding Sites	593
	<i>Rym Kachouri-Lafond and Mihaela Zavolan</i>	
55.1	Theoretical Background	593
55.1.1	snoRNAs	594
55.1.2	miRNAs	595
55.2	Protocol	596
55.3	Example Experiment	598
55.4	Troubleshooting	599
	References	600
	Appendices	603
	Appendix A1: Yeast Nomenclature Systematic	
	Open Reading Frame (ORF) Designations	605
	<i>Min Chen and Brian Rymond</i>	
A1.1	Protein-Coding Genes	605
A1.2	Recombinant Derivatives	606
A1.3	Proteins	606
A1.4	Noncoding Genes, Genes Not Encoded by Nuclear Chromosomal DNA, and other Chromosomal Features	606
A1.5	Yeast Strains	607
	References	607
	Appendix A2: Glossary	609
Index		615

