

Contents

Preface V

1	Introduction: RNA interference, the "Breakthrough of the Year 2002"	1
1.1	RNAi as a Tool for Functional Genomics	2
1.2	Mechanism of RNAi	3
1.3	Dicer – the Initiator to "Dice" the dsRNA?	3
1.4	miRNAs versus siRNAs: Two Classes of Small RNAs Using the RNAi Pathway?	7
1.5	RISC – the Effector to "Slice" the mRNA?	8
1.6	Are RNA-Dependent RNA Polymerases (RdRps) Responsible for the Catalytic Nature of RNAi?	12
1.7	Is RNAi Involved in the Regulation of Gene Expression?	13
1.8	RNAi in Mammals	14
1.9	Practical Approaches	17
1.10	References	17
2	RNAi in <i>Caenorhabditis elegans</i>	23
2.1	Introduction	23
2.2	Application of RNAi in <i>C. elegans</i>	26
2.3	Target Sequence Evaluation	27
2.4	dsRNA Synthesis	27
2.4.2	Generation of the DNA Template	28
2.4.2.1	Plasmid Templates	28
2.4.2.2	DNA Templates Derived by PCR/RT-PCR	30
2.4.2	In-vitro dsRNA Transcription	32
2.5	Delivery of dsRNA	36
2.5.1	General Information on the <i>C. elegans</i> Anatomy	36
2.5.2	<i>C. elegans</i> Strains for Silencing	38
2.5.3	Culturing the Worms	40
2.5.4	Microinjection Protocol	40
2.5.5	Soaking Protocol	41
2.5.6	RNAi Feeding Protocol	43
2.5.7	DNA Templates for dsRNA Expression in Feeding <i>E. coli</i>	44

2.5.8	DNA Templates for Hairpin RNA Expression	46
2.5.8.1	<i>C. elegans</i> Promoters	46
2.5.8.2	Inverted Repeat Constructs	47
2.6	Mounting Animals for Microscopy	52
2.7	Genome Wide Screens	55
2.7.1	<i>C. elegans</i> RNAi Library	56
2.8	Selected Literature on <i>C. elegans</i> Research	57
2.9	Useful <i>C. elegans</i> webpages	57
2.10	References	59
3	RNAi in <i>Drosophila</i>	61
3.1	Introduction	61
3.2	Application of RNAi in <i>Drosophila</i>	63
3.2.1	dsRNA from Linear DNA Templates	64
3.2.2	dsRNA from Inverted Repeat DNA	64
3.2.3	Inducible Expression in <i>Drosophila</i> Cell Lines	68
3.2.4	Limitations	70
3.3	dsRNA Synthesis	71
3.3.1	In-vitro dsRNA Transcription	72
3.3.2	Inverted Repeat DNA	76
3.4	Injections	82
3.4.1	Injection Services	82
3.4.2	Injection Method	82
3.4.3	DsRNA or Inverted Repeat DNA Preparation	83
3.4.4	Embryo Collection	83
3.5	Cell Lines	87
3.6	Protocols	89
3.6.1	Thawing and Maintenance of S2 Cells	89
3.6.2	Freezing Protocol	90
3.7	RNAi in S2 Cells	91
3.7.1	dsRNA Transfection Using the Calcium Phosphate Method	91
3.7.2	dsRNA Soaking of S2 Cells	92
3.8	High-Throughput Screens	94
3.8.1	<i>Drosophila</i> RNAi Library	95
3.9	Useful Webpages for <i>Drosophila</i> Research	96
3.10	Books and Literature on <i>Drosophila</i>	96
3.11	References	97
4	RNAi in Mammals	101
4.1	Introduction	101
4.2	Transient RNAi in Cell Culture	103
4.2.1	Chemical Synthesis and Modifications of siRNAs	103
4.2.1.1	Advantages	105
4.2.1.2	Limitations	107
4.2.2	Custom Synthesis of siRNA Oligos	107

4.2.3	siRNA Design Rules	108
4.2.3.1	siRNA Strand Bias and Off-Gene Targeting	108
4.2.3.2	Improvements in siRNA Stability	109
4.2.3.3	siRNA Design: Novel Modifications of the "Tuschl Rules"	111
4.2.3.4	Homology Search by BLAST, FASTA, or Smith-Waterman Algorithm	114
4.2.3.5	Troubleshooting	115
4.2.3.6	siRNA Design Programs and Algorithms	116
4.2.3.7	Preparation of siRNA Duplexes	117
4.2.4	Enzymatic Synthesis of siRNAs	118
4.2.4.1	Designing DNA Oligonucleotides	118
4.2.4.2	In-Vitro dsRNA Transcription	120
4.2.5	Processing of Long dsRNA	126
4.2.5.1	Expression of Dicer in Hi5 Insect Cells	127
4.2.5.2	DsRNA Digestion by Recombinant Dicer	131
4.2.5.3	Production of Recombinant RNaseIII from <i>E. coli</i>	132
4.3	Delivery of siRNAs into Cells	136
4.3.1	Transfection	136
4.3.2	Electroporation	138
4.3.3	Cell Penetration Peptides (CPPs) for siRNA Delivery	140
4.3.4	Cell-Penetrating Peptides (CPPs) or Protein Transduction Domains (PTDs)	141
4.3.4.1	Coupling of siRNA to CPPs	143
4.3.4.2	Chemical Synthesis of 5'-Thiol-Modified siRNAs	146
4.3.4.3	Enzymatic Synthesis of 5'-Thiol-Modified siRNAs	146
4.3.4.4	Coupling of Cys-modified CPPs to siRNAs	150
4.3.4.5	Treatment of Cells with pepsRNAs	152
4.4	Analysis of the siRNAs	154
4.4.1	PAGE of siRNAs	154
4.4.2	Determination of dsRNA and siRNAs by Agarose Gel Electrophoresis	158
4.4.2.1	Detection of dsRNA and siRNA Duplexes	159
4.4.2.2	Simultaneous Detection of siRNA and mRNA	159
4.5	RNAi with Short Hairpin RNAs (shRNA)	161
	Promoters	161
4.5.1	RNAi with shRNA-Expressing Vectors	162
4.5.1.1	Loop Structures	165
4.5.1.2	Interferon Response	165
4.5.1.3	Advantages	166
4.5.1.4	Limitations	166
4.5.1.5	Protocols	167
4.5.2	Lentiviral RNAi Approach	169
4.5.2.1	Delivery Issues	169
4.5.2.2	Limitations	172
4.5.2.3	Cloning of RNAi Cassette	173
4.5.2.4	Cloning Procedure	176
4.6	RNAi with Long Hairpin RNAs (lhrRNAs)	188

4.6.1	lhrnAs from Inverted Repeat DNA	190
4.6.1.1	Inverted Repeat DNA	191
4.6.2	Expression of lhrnA from Direct Repeat DNA	198
4.6.3	Inverted Repeat DNA Missing 5'-cap and Poly (A) Tail	201
4.6.4	RNAi versus dsRNA Deamination	205
4.7	Test for Interferon Response	206
4.7.1	Semi-Quantitative RT-PCR Analysis of 2'-5'-OAS Activation	208
4.7.2	DNA Fragmentation Analysis	209
4.7.3	PKR Activity and eIF2 α Phosphorylation	210
4.8	Inducible Persistent RNAi in Mammalian Cells	213
4.9	Application of RNAi in Mice	217
4.9.1	Hydrodynamic Tail Vein Injection	217
4.9.2	In-Vivo Electroporation	219
4.9.3	Infection of Adult Mice with Adenovirus	220
4.9.4	Generation of Transgenic Mice Using Lentiviral Infection	221
4.9.5	Generation of Transgenic Mice by Pronucleus Injection	221
4.10	Alternative Methods: Ribozyme Cleavage of dsRNA	224
4.11	High-Throughput Screens	226
4.11.1	Production of Bar Code-Supported shRNA Libraries	226
4.11.2	Enzymatically Retrieved Genome-Wide shRNA Libraries	228
4.11.3	siRNA Validation for Genome-Wide Screens	231
4.11.4	RNAi Microarrays	232
4.12	Useful Web Pages and Links	234
4.12.1	Academic Resources	234
4.12.2	Company Resources	234
4.12.3	Other Useful Links	235
4.13	References	235
	Appendix 1: Abbreviations	247
	Appendix 2: List of Protocols	255
	Appendix 3: Suppliers of RNAi-related Chemicals and Probes	257
	Appendix 4: Glossary of Terms	261
	Subject Index	301