Analysis of Small Endogenous RNAs

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ABSTRACT

Eukaryotic cells express small noncoding RNAs to silence target genes or regulate chromatin domains. MicroRNAs (miRNA) are endogenous small RNAs that are thought to each regulate multiple mRNA targets. To gain a deeper understanding of processes regulated by small noncoding RNAs, techniques are required to identify and detect them. This unit describes standard laboratory methods for and comments about high-throughput technologies for the identification and detection of small RNAs. This unit also outlines a cell-based reporter gene assay for assessing the regulatory potential of a microRNA on a candidate mRNA target. *Curr. Protoc. Mol. Biol.* 81:26.7.1-26.7.15. © 2008 by John Wiley & Sons, Inc.

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INTRODUCTION

Since the discovery that exogenous double-stranded RNAs in eukaryotes are converted into ~20- to 24-nucleotide small RNAs (Hamilton and Baulcombe, 1999; Zamore et al., 2000), there has been an intense pursuit to uncover new endogenous small RNAs in organisms. Although the first known endogenous small RNAs in animals, *lin-4* and *let-7*, were identified by forward genetics (Lee et al., 1993; Reinhart et al., 2000), cloning of small RNA molecules (*UNIT 26.4*) has greatly accelerated the discovery and characterization of other endogenous small RNAs from animals, plants, fungi, and protists. Currently known classes of small endogenous RNAs are listed in Table 26.7.1.

Although the different classes of small endogenous RNAs exhibit distinct functional roles, they share the unifying theme of participating in the gene silencing phenomenon known as RNA interference (RNAi). RNAi is an ancient gene regulatory mechanism; core proteins that mediate the production and function of the small RNAs are conserved from single-cell eukaryotes like fission yeast and algae to complex organisms like plants and animals (reviewed in Zamore and Haley, 2005). The general role of small RNAs is to guide and localize effector proteins to specific RNA or DNA targets via base-pairing between the small RNA and the nucleic acid target. A stable interaction between the small RNA/effector protein and the nucleic acid target can result in a variety of outcomes, depending on the context of the target and the small RNA, as illustrated in Figure 26.7.1.

Studies of small RNAs in the RNAi pathway generally begin with the identification and detection of the small RNAs. This unit introduces a technique for identifying populations of small endogenous RNAs in a non-sequence-specific manner (see Basic Protocol) and allowing for separation of small RNAs from other noncoding RNAs like tRNAs and rRNAs. Detection of an identified small RNA is often accomplished by northern blot, and this unit provides an updated method that increases the sensitivity of detection for less abundant small RNAs by chemically cross-linking the RNA to the membrane (see Support Protocol 1). Finally, this unit describes a general technique for assaying the regulatory potential between a candidate mRNA target and a microRNA (miRNA; see Support Protocol 2).

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Table 26.7.1 Classes of Endogenous Small RNAs

Class	Full name	Length (nt)	Detected in	Precursors	Roles
miRNA	MicroRNA	~19–23	Plants and animals	Transcript containing a fold-back structure resembling a hairpin	Often directs target mRNA degradation (plants); imperfect base-pairing with 3′-UTR of mRNAs, causing translation repression and sometimes RNA instability (most animal miRNAs)
siRNA	Small interfering RNA	~20–24	Eukaryotes	Long dsRNA formed by transcription from convergent promoters from two distinct transcripts with perfect complementarity, or conversion of ssRNA to dsRNA by an RNA-dependent RNA polymerase (RdRP)	Directs specific cleavage of target protein-coding RNA at site perfectly complementary to the small RNA, often leading to mRNA instability and degradation; can theoretically also target precursor ssRNA before conversion into dsRNA
tasiRNA	*Trans*-acting siRNA	~24	Plants	Noncoding transcript with two surrounding miRNA binding sites, which triggers an RdRP to convert transcript into dsRNA	Directs in *trans* the degradation of other mRNAs with complementarity to tasiRNA, rather than precursor ssRNA transcript
natsiRNA	Natural antisense transcripts siRNA	~21–24	Plants	Coding transcripts from convergent promoters overlapping to form a region of dsRNA	Allows regulation of other genes when expression is induced by stress conditions
tncRNA	Tiny noncoding RNA	~20–21	Nematodes	May have double-stranded element, because biogenesis requires Dicer and rde-4, a dsRNA binding protein	No known function

continued

Table 26.7.1 Classes of Endogenous Small RNAs, *continued*

Class	Full name	Length (nt)	Detected in	Precursors	Roles
21U RNA	21U RNA	21	Nematodes	Unknown, although an upstream motif present for each and every 21U RNA suggests automonous expression	No known function
scnRNA	Scan RNA	~28	Ciliate protists	dsRNA from convergent promoters or conversion of repetitive transcripts into dsRNA by RdRP; biogenesis Dicer-dependent	Signals formation of heterochromatin at repetitive loci within a new replicated macronucleus genome; heterochromatic loci are then physically eliminated from the cell
h-rasiRNA	Heterochromatic-repeat-associated siRNA	~20–24	Fungi, plants, and animals	dsRNA from repetitive loci made via convergent promoters or conversion of repetitive transcripts into dsRNA by RdRP	Signals formation of heterochromatin at repetitive loci, via histone modifications (yeast) or DNA modifications (plants); animal rasiRNAs roles intersect with piRNAs
piRNAs	Piwi-interacting RNA	~24–31	Animals	Long ssRNA transcripts yielding clusters of mammalian single-copy piRNA and other animal piRNAs; repeat-associated piRNAs may arise from a putative double-stranded precursor	Not fully clear, but may serve a genome surveillance role in animal germ cells, perhaps to suppress transposon mobilization

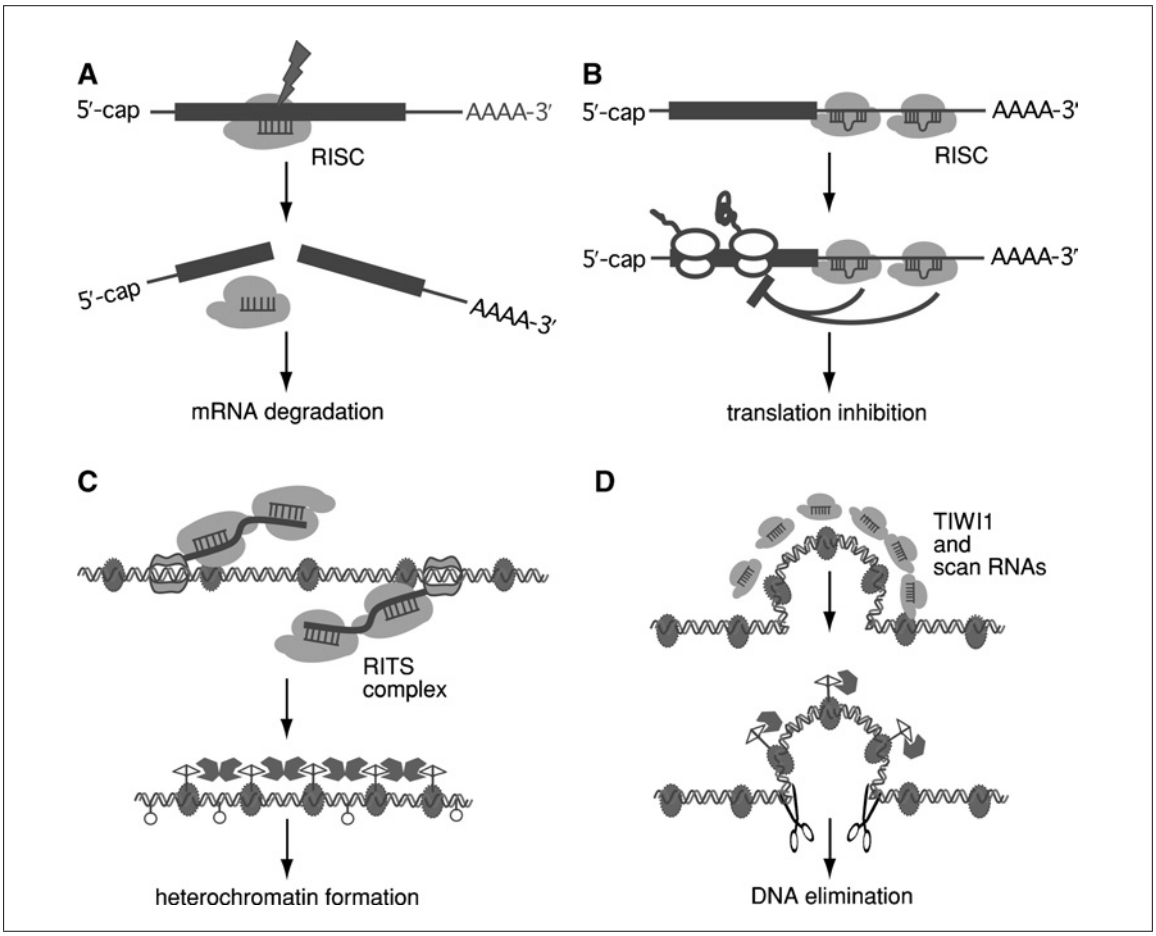

Figure 26.7.1 Gene silencing mechanisms of small RNAs. Panels (**A**) and (**B**) show post-transcriptional gene silencing of target mRNAs recognized by the small RNA within the RNA-induced silencing complex (RISC) of plants and animals. Perfect base-pairing between the small RNA and target results in mRNA degradation (A), while imperfect base-pairing seen in microRNA targets often results in translation inhibition (B). (**C**) In fungi and plants, the RNA-induced transcriptional silencing (RITS) complex can direct heterochromatin formation. (**D**) Ciliate protists utilize scan RNAs and the TIWI1 protein to delete entire regions of DNA.

BASIC PROTOCOL

SIMPLE CHROMATOGRAPHY AND DETECTION OF SMALL-RNA RIBONUCLEOPROTEINS

This protocol allows for the separation of ribonucleoproteins containing small RNAs from other long and noncomplexed RNAs based on the tighter binding of the latter species to the positively charged Q ion-exchange column. Bound proteins shield the charge of the small RNAs, which either flow through the column or elute at lower salt concentrations than long and noncomplexed RNAs. Following chromatography, the small RNA species are separated from protein and radioactively end-labeled for detection by gel electrophoresis and autoradiography. This method improves the construction of small-RNA libraries (*UNIT 26.4*) because degradation products of abundant tRNAs and rRNAs are greatly reduced in the small-RNA sample.

Materials

Cells of interest, cultured or from tissue
0.1 M and 1 M potassium acetate in base buffer (see recipe)
25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol (*UNIT 2.1A*)
Chloroform
10 mg/ml glycogen from blue mussel
3 M sodium chloride
Absolute and 70% (v/v) ethanol

Yeast poly-A polymerase and 5× poly-A polymerase buffer (USB)
3′-Deoxyadenosine 5′-[α-^{32}P]-triphosphate (cordycepin 5′-[α-^{32}P]-triphosphate, 5000 Ci/mmol, 185 TBq/mmol; Perkin Elmer)
Formamide loading dye (*UNIT 4.9*)

5-ml Hitrap Q column (GE Healthcare)
FPLC instrument with fraction collector
85°C heating block or water bath
Phosphorimager scanner or photographic film

Additional reagents and equipment for extracting soluble proteins from cells (*UNIT 12.1*), dialyzing extracts (*APPENDIX 3C*), extracting and precipitating nucleic acids (*UNIT 2.1A*), and performing denaturing polyacrylamide gel electrophoresis with RNA (*UNIT 4.9*)

Prepare RNA sample

1. Create soluble protein extracts from cultured cells or cells homogenized from tissues, e.g., following protocols in *UNIT 12.1*.
2. Dialyze the extract (*APPENDIX 3C*) against 0.1 M potassium acetate in base buffer.

   Extracts must be dialyzed to 100 mM salt for noncomplexed RNAs and other ribonucleoproteins to bind efficiently to the column.

3. Equilibrate a 5-ml Hitrap Q column with 3 column volumes of 0.1 M potassium acetate in base buffer.
4. Add 1 to 5 mg total protein from cell extract to the column, and wash with 2 column volumes of 0.1 M potassium acetate in base buffer. Collect 1-ml fractions of the flowthrough.
5. Elute ribonucleoproteins with a linear gradient of 0.1 to 1 M potassium acetate in base buffer in 4 column volumes, and collect 1-ml fractions.

   The elution gradient can be varied between 2 and 5 column volumes, while fraction volumes can be varied between 0.5 and 2 ml.

6. Deproteinize 500 µl of flowthrough and eluate fractions (half of each 1-ml fraction) in 1.7-ml microcentrifuge tubes by extracting with 500 µl of 25:24:1 phenol/chloroform/isoamyl alcohol (*UNIT 2.1A*).
7. Re-extract the aqueous (upper) phase containing nucleic acids with chloroform to remove trace phenol.
8. Precipitate the nucleic acid in the 500-µl fractions by adding 1 µl of 10 mg/ml glycogen, 50 µl of 3 M sodium chloride, and 2 volumes (1 ml) of absolute ethanol. Mix and allow the tubes to incubate ~1 hr at –20°C.
9. Microcentrifuge the RNA 20 min at maximum speed, 4°C, and wash the pellet with 100 µl of 70% ethanol. Centrifuge and resuspend the RNA pellet in 5 µl water.

Detect small RNAs

10. Perform 3′ end-labeling of the RNA in each fraction by adding 0.5 U of yeast poly-A polymerase, 0.5 µl of cordycepin 5′-[α-^{32}P]-triphosphate, and 2 µl of 5× poly-A polymerase buffer in a total volume of 10 µl. Incubate 1 hr at 37°C.

    By using yeast poly-A polymerase and cordycepin, only a single radioactive molecule is added to the 3′ end of RNA. A cheaper but less sensitive alternative for the radiolabeling reaction can be performed with T4 RNA ligase and radiolabeled pCp (see UNIT 3.15).

11. Stop the reaction by adding 10 µl formamide loading dye and heat the samples in a heating block 5 min at 85°C.

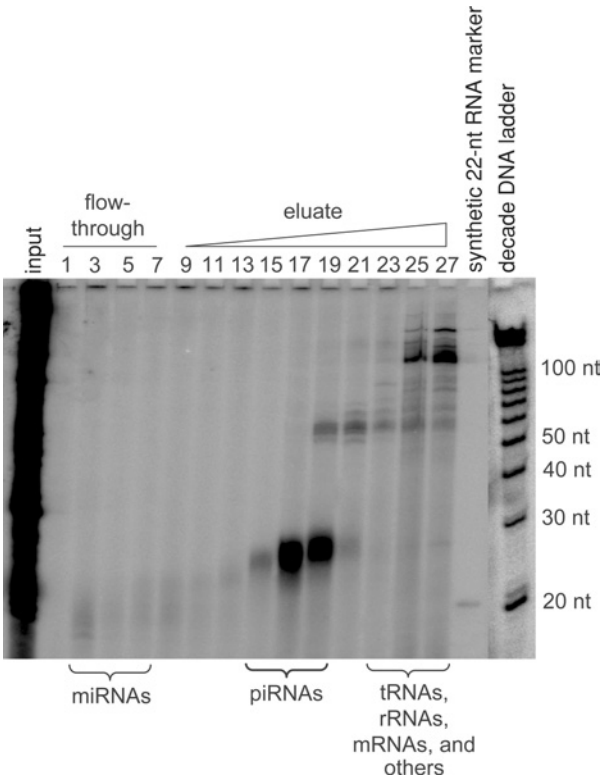

Figure 26.7.2 Fractionation of a rat testis RNA extract on a Hitrap Q column (Basic Protocol) and detection of RNAs by radiolabeling with cordycepin 5′-[α-^{32}P]-triphosphate.

12. Resolve the RNAs on an 8 M urea/15% polyacrylamide gel (*UNIT 4.9*).

13. Expose the gel to a phosphorimaging plate or film.

A typical result is illustrated in Figure 26.7.2.

SUPPORT PROTOCOL 1

INCREASING SMALL-RNA NORTHERN BLOT SENSITIVITY BY CHEMICAL CROSS-LINKING

Northern blots tailored for small-RNA detection often use charged membranes and cross-linking by UV light to immobilize the RNA on the membrane, and these blots are sufficient for detection of individual abundant small RNAs. However, this procedure can damage the RNA or affect the accessibility of probe hybridization, such that rare small RNAs are unable to bind sufficient amounts of probe for detection. This protocol, originally developed by Andrew Hamilton's laboratory (Pall et al., 2007), circumvents the problem of UV damage by utilizing a cross-linking chemical that attaches the 5′-terminal phosphates of small RNAs to the neutral nylon membrane and leaves other nucleotides intact.

Materials

Small-RNA samples (e.g., see Basic Protocol)
12.5 M 1-methylimidazole
RNase-free water
1 M HCl
1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)

Neutral nylon membrane (e.g., Hybond NX, GE Healthcare)
3MM blotting paper, slightly larger than membrane (20 × 16–cm maximum)
Glass plate

Plastic wrap (e.g., Saran Wrap)
50°C to 60°C incubator

Additional reagents and equipment for performing denaturing polyacrylamide gel electrophoresis and northern blots (*UNIT 4.9*)

1. Carry out denaturing polyacrylamide gel electrophoresis of small-RNA samples as detailed in *UNIT 4.9*, Basic Protocol 2.
2. Transfer the RNA from the gel to the membrane as in *UNIT 4.9*, Basic Protocol 2, but use a neutral nylon membrane such as Hybond NX.

   It is critical to use a neutral nylon membrane, because charged membranes cross-link poorly with EDC.
3. Dilute 245 μl of 12.5 M stock 1-methylimidazole into 24 ml RNAse-free water. Adjust the pH to 8.0 with 1 M HCl (~300 μl).
4. Dissolve 0.753 g EDC into this solution
5. Place a 3MM blotting paper sheet slightly larger than the nylon membrane onto a clean glass plate. Soak the paper with the EDC solution.
6. Disassemble the transfer sandwich, noting which side of the membrane is contacting the gel (the side to which RNA is transferred).
7. Place the wet membrane, RNA side up, onto the EDC-saturated blotting paper on the glass plate. Wrap the glass plate assemblage in plastic wrap.
8. Incubate 2 hr at 50°C to 60°C.
9. Remove the blot and wash with RNAse-free water.
10. Carry out probe hybridization, washes, and detection as in *UNIT 4.9*, Basic Protocol 2.

ASSAYING REGULATORY POTENTIAL OF PREDICTED miRNA TARGET ELEMENTS

SUPPORT PROTOCOL 2

MicroRNAs are hypothesized to each regulate a significant number of genes post-transcriptionally (Farh et al., 2005; Lim et al., 2005). Empirical and evolutionary data suggest that miRNAs recognize mRNA targets through critical "seed" interactions, which are base-pairing interactions at nucleotides 2 to 7 in the 5′ portion of the miRNA (Lewis et al., 2003; Lim et al., 2005). Incomplete base pairing in the 3′ portion of the miRNA with the target is less critical for regulation of protein expression, but can impact whether message degradation occurs (see Fig. 26.7.1). In animals, miRNA-binding sites of target mRNAs tend to reside in the 3′-untranslated region (UTR) and can be present in single or multiple copies (Grimson et al., 2007). Typically, multiple miRNA-binding sites exhibit greater gene silencing effects than single sites.

Sequence gazing and more sophisticated bioinformatics tools have yielded many candidate mRNAs as targets of miRNA regulation (Wightman et al., 1993; Lewis et al., 2003). Although genetic and in vivo analyses are ultimately required to fully validate the candidacy of the target messages, a simple in vitro cell-based assay detailed here can assess the regulatory potential of a 3′-UTR that may be targeted by a miRNA. By using PCR and site-directed mutagenesis, wild-type (wt) and mutant fragments of a 3′-UTR can be amplified and cloned behind a reporter gene such as luciferase (see Fig. 26.7.3). One can assess the degree of gene silencing conferred by the miRNA on the 3′-UTR by transfecting into cultured mammalian cells the reporter gene with the 3′-UTR element, a reference control reporter gene, and a short RNA duplex that mimics an endogenous duplex of a miRNA and its passenger strand (referred to as the miRNA∗ sequence). In

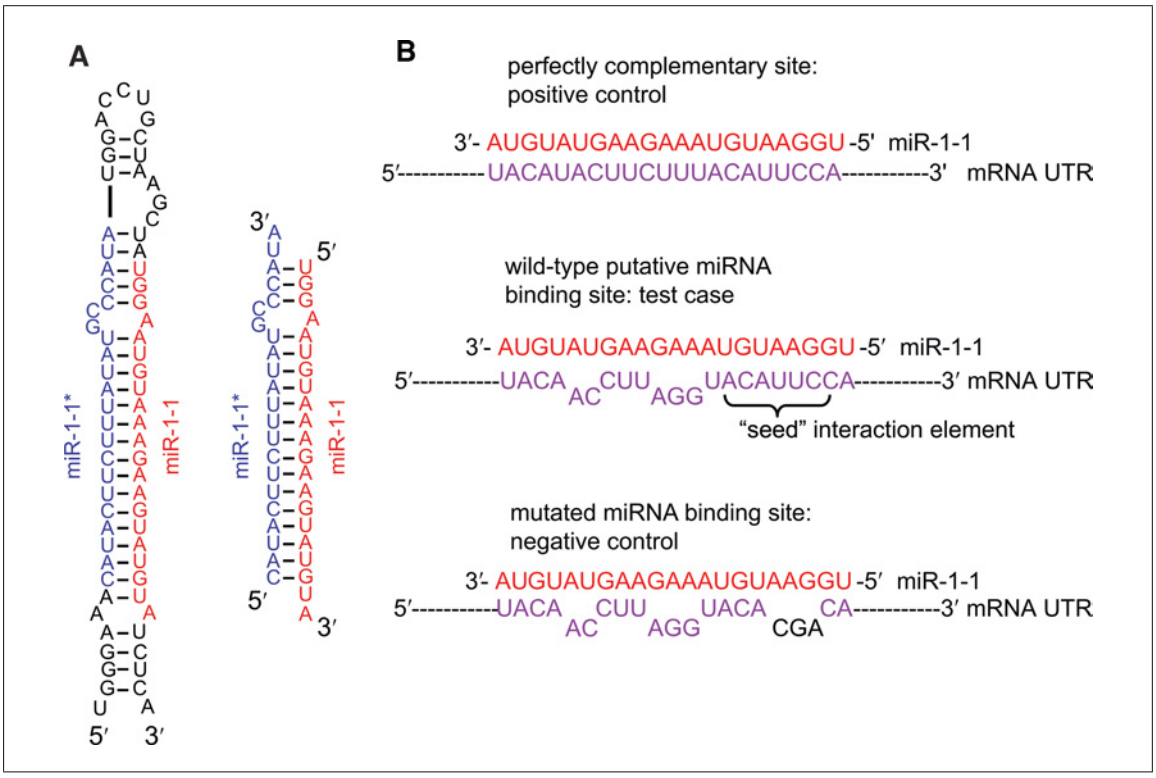

Figure 26.7.3 In vitro assay for miRNA regulatory potential. (**A**) An siRNA duplex that mimics the endogenous miRNA:miRNA* duplex of human miRNA 1–1. (**B**) Examples of perfectly complementary, wild-type, and mutant sites in UTR elements that can be fused behind a reporter gene. For the color version of this figure go to *http://www.currentprotocols.com*.

the assay described here, two different luciferase genes (*Renilla* and firefly) are used as reporters, and a commercial dual-luciferase detection kit is used to assay expression. Data analysis and comparison are achieved by normalizing reporter gene levels of wt and mutant UTR constructs (*Renilla*) to the reference control reporter gene (firefly). In cases where the miRNA being studied is endogenously expressed by the cell line, the synthetic miRNA duplex can be omitted from the experiment.

The transfection method in this assay is very similar to the one described in *UNIT 26.2*, especially the protocol for cotransfection of siRNAs and reporter plasmids. A more general description of transfection using cationic lipids can be found in *UNIT 9.4*.

Materials

Synthetic miRNA duplexes: experimental and noncognate control (completely different sequence)
Genomic DNA or cDNA containing wildtype (wt) and mutant UTR elements (see *UNIT 8.5*)
TK– or SV40–*Renilla* luciferase reporter gene plasmid (Promega)
Mammalian cells (e.g., HeLa cells)
Lipofectamine 2000 (Invitrogen)
SV40–firefly luciferase control plasmid (Promega)
pUC19 (carrier DNA plasmid)
PBS (*APPENDIX 2*)
Commercial cell lysis buffer (e.g., Promega)
Dual-luciferase luminescence detection kit (Promega)

24-well culture plate
Luminometer

Additional reagents and equipment for PCR (*UNIT 15.1*), subcloning (*UNIT 3.16*), sequencing (Chapter 7), purifying by anion-exchange chromatography (*UNIT 2.1B*), growing mammalian cells in culture (*APPENDIX 3F*), and cotransfecting siRNA and plasmids (*UNIT 26.2*)

Design and synthesize siRNA duplexes

1. Design and synthesize an siRNA duplex mimicking a miRNA:miRNA∗ duplex. Include a noncognate small-RNA duplex as a control.

Design and amplify UTR elements

2. Design PCR primers for amplifying the UTR element containing the putative miRNA-binding sites.

3. Use PCR (*UNIT 15.1*) to amplify the wt and mutant UTR elements from genomic DNA (assuming that the fragment does not contain splice junctions) or a cDNA clone.

   To mutate UTR elements at miRNA-binding sites, it is best to clone the wt element and then perform PCR-mediated site mutagenesis (UNIT 8.5).

4. Subclone wt and mutant amplicons into the UTR region of a TK– or SV40–*Renilla* luciferase reporter gene plasmid (*UNIT 3.16*).

5. Sequence constructs to confirm the identity of the amplicons (Chapter 7).

6. Purify transfection-grade plasmids on a maxi- or midi-prep scale by anion-exchange chromatography (*UNIT 2.1B*).

Transfect mammalian cells

7. Seed mammalian cells (e.g., HeLa cells) into a 24-well plate and allow the cells to grow overnight to 50% confluency by the next day.

   siRNAs transfect more effectively when the cells are 50% confluent at transfection.

8. For each set of transfection experiments (cognate miRNA and wt UTR, cognate miRNA and mutant UTR, and noncognate small RNA and wt UTR), set up a transfection mix suitable for transfecting at least three wells of cells. Following the instructions for the transfection reagent Lipofectamine 2000, prepare the transfection mix in a volume of 3 ml medium containing the following amounts:

   75 pmol miRNA duplex (25 nM final concentration per well)
   75 ng SV40–firefly luciferase reporter plasmid (transfection control; 25 ng/ml final concentration per well)
   3 mg pUC19 plasmid (carrier; 1 mg/ml final concentration per well)
   150 ng TK–*Renilla* luciferase (50 ng/ml final concentration per well) or 30 ng SV40–*Renilla* luciferase (10 ng/ml final concentration per well) reporter plasmid with UTR element.

   Different promoter strengths and the sensitivity of the luminometer determine the amount of Renilla and firefly luciferase constructs to be transfected.

   Carrier plasmid DNA is required to reduce the amount of reporter plasmid being transfected while maintaining the optimal amount of nucleic acid for efficient liposome complex formation.

9. Aspirate off the medium from the cells and add 1 ml transfection mix to each of the three wells of cells being tested.

   To generate enough measurements to carry out meaningful statistical analyses of gene repression levels, triplicate measurements should be taken (i.e., three wells from a shared transfection mix), and the entire experiment should be repeated independently at least three or four times.

10. Incubate the cells 24 to 48 hr in a standard tissue culture incubator (37°C, 5% CO_2).

11. Wash the cells once with 1 ml of 1× PBS and lyse cells using a commercial lysis buffer according to manufacturer's instructions.

12. Follow the instructions for a dual-luciferase luminescence assay kit, with detection on a luminometer.

Analyze results

13. Normalize all *Renilla* luciferase activity (wt and mutant) to firefly luciferase activity in the same well and calculate the geometric mean of the replicates.

14. Further normalize reporter gene readouts to the noncognate control transfection.

15. Compare wt and mutant values and express as an *x*-fold repression.

    A candidate site displaying miRNA regulation should display decreased reporter expression with the wt UTR, and restoration of reporter expression in the mutant UTR. See Critical Parameters and Troubleshooting for further discussion of data analysis.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

Base buffer

10 mM HEPES, pH 7.9
10% glycerol
0.1 mM EDTA
1.5 mM magnesium acetate
Store indefinitely at 4°C
Just before use add:
0.2 mM phenylmethylsulfonyl fluoride (PMSF)
1.0 mM dithiotreitol (DTT)
1× protease inhibitor cocktail (Roche)

Use nuclease-free water to make this solution.

COMMENTARY

Background Information

Eukaryotic genomes can encode a rich number of small endogenous RNAs. The nematode *Caenorhabditis elegans* contains at least 112 miRNAs and numerous other endogenous small RNAs, while mammals express at least 400 miRNAs and possibly over 100,000 different Piwi-interacting RNAs (piRNA; Griffiths-Jones, 2006; Girard et al., 2006; Lau et al., 2006; Aravin et al., 2007; Pang et al., 2007). Small-RNA expression profiles vary between different cell types and disease states, so methodologies to evaluate small-RNA expression in a high-throughput manner are becoming essential to understanding biological processes dependent on small-RNA function (Lu et al., 2005b). Although not detailed as a protocol, this unit will comment below on particular commercial technologies that have significantly advanced the analytical capabilities of small-RNA research.

Currently, there are three main commercial platforms for high-throughput small-RNA analysis: massively parallel sequencing (MPS), multiplexed bead-based flow cytometry, and microarrays tailored for small-RNA detection. A sample comparison of these technologies and links to the vendors are listed in Table 26.7.2. The MPS technology requires creation of a cDNA library of small RNAs, which is described in *UNIT 26.4*. Vendors have conversion protocols for adapting small-RNA cDNA libraries to be compatible with the sequencing technology. Since so many reads are obtained, the relative frequency of reads can reflect molecular abundance. Sequencing technology offers exquisite sensitivity and the

additional benefit of discovery of new small-RNA species (Lu et al., 2005a; Girard et al., 2006; Lau et al., 2006; Ruby et al., 2006; Aravin et al., 2007). However, the high up-front costs and relatively extensive turnaround times can limit experimental replicates.

Multiplex bead-based flow cytometry and microarrays can detect a defined set of known small-RNA sequences in a rapid manner (Goff et al., 2005; Lu et al., 2005b; Castoldi et al., 2006; Wang et al., 2007). Probes with antisense sequence to small RNAs are immobilized on a bead or a glass slide array, while the small RNAs can be directly labeled with fluorescent moieties for detection. Many bead sets or microarrays can be purchased, and special labeling kits are designed specifically for small RNAs, which lack the sequence length needed for conventional kits for mRNA microarrays. Profiles of different miRNA expression patterns show promise as a phenotypic signature for classifying different tumorigenic states of biopsies (Lu et al., 2005b). Some custom printing services provide microarrays that contain a large number of probes tiled across genomic regions. These arrays allow the interrogation of small RNAs that arise from clusters, such as piRNAs and rasiRNAs (Vagin et al., 2006).

The Basic Protocol for simple chromatographic enrichment of small-RNA ribonucleoproteins can improve the construction of small-RNA cloning libraries for MPS, and may reduce nonspecific hybridization of degradation products on microarray or bead-based detection platforms. With the discovery of new small-RNA species that are low in abundance, the alternative cross-linking procedure for northern blots can aid in validation of the expression of the small RNAs. Newly discovered miRNAs and predicted mRNA targets can be tested for possible regulation via a cell-based reporter assay.

Critical Parameters and Troubleshooting

For the procedure to enrich for and detect small RNAs in extracts, one should start with a whole-cell extract that has a protein concentration of ~1 to 10 mg/ml. Extracts must be dialyzed to 100 mM salt for noncomplexed RNAs and ribonucleoproteins to bind efficiently to the column. The salt anion must be compatible with ethanol precipitation of nucleic acids (i.e., chloride or acetate), so sulfate and phosphate salts must be thoroughly dialyzed away before ethanol precipitation steps. Attention must also be paid to the binding capacity of the column. If this is exceeded, excess degradation RNA products may appear in the flowthrough. A post-run 3 M sodium chloride wash that clears large nucleic acids is necessary for regenerating the ion-exchange column.

If RNAs are phenol-extracted from fractions prior to being radioactively end-labeled enzymatically, one should back-extract the fractions with chloroform prior to ethanol precipitation, so that residual phenol will not affect the labeling reaction. When labeling multiple fractions, one can use a master mix containing radiolabel and enzyme; this mix should be kept on ice during the setup of reactions. Residual phenol does not affect detection of RNAs on a gel stained with SYBR Gold.

The success of the chemical cross-linking procedure for northern blots of small RNAs depends upon utilizing a neutral nylon membrane, which contains the necessary free amine groups for linking to the 5′-phosphate of the small RNA. Some endogenous small RNAs may lack a 5′-phosphate, in which case treatment of RNA samples with kinase and ATP (*UNIT 3.10*) will make these RNAs suitable for cross-linking. Care should be taken not to wash the neutral membranes after transfer and before cross-linking because this may cause RNAs to diffuse. The membrane should be cross-linked immediately after transfer of the RNAs. See *UNIT 4.9* for additional considerations for northern blotting.

The relative stoichiometry of a miRNA binding to target mRNA sites may influence gene silencing effects, and the reporter gene constructs must be effectively titrated to expression levels that more appropriately reflect the endogenous expression level of a putative mRNA. To achieve this titration in transfection experiments, carrier plasmid DNA is included in the transfection mix so that reporter gene plasmids can be diluted. Because transfection experiments can exhibit experimental variability, technical and experimental replicates are necessary for solid statistical analysis of the gene silencing levels. As outlined in Support Protocol 2, this is achieved using using three wells for each transfection and repeating the entire experiment three times. Mutated reporter elements and a noncognate small RNA are necessary controls for comparison of the gene-silencing levels that may be imparted by the putative miRNA-target combination. The technical considerations for the cell-based assay for examining regulatory

Table 26.7.2 Commercial Platforms for High-Throughput Detection of Small RNAs

Platform	Company	System and throughput	Technology	Web site and reference
Massively parallel sequencing (MPS)	454 Life Sciences (Roche Diagnostics)	Genome Sequencer 20 System: ~200,000 reads per run, ~100 bp long	Individual molecules of a small-RNA cDNA library coupled to and amplified on beads and loaded onto a picoliter plate; microfluidic pyrosequencing and detection	*http://www.454.com*; Margulies et al. (2005)
Massively parallel sequencing (MPS)	Illumina (formerly Solexa)	1 G Genome Analyzer: ~3,000,000 reads per run, ~30 bp long	cDNA molecules amplified into clusters on a high-density primer lawn; reversible dye-terminator sequencing chemistry and laser detection on the cDNA clusters	*http://www.illumina.com*; Lu et al. (2005a)
Bead-based flow cytometry	Luminex	FlexmiR Kit and Luminex Analyzer: 100 multiplex samples per run, >1000 runs per day	Beads in 100 different fluorescent colors coupled to specific locked nucleic acid (LNA) probes; beads pair with a 3'-fluorophore-labeled miRNA; detected by flow cytometry	*http://www.luminexcorp.com/microrna*; Lu et al. (2005b)

continued

Table 26.7.2 Commercial Platforms for High-Throughput Detection of Small RNAs, *continued*

Platform	Company	System and throughput	Technology	Web site and reference
Microarray	Agilent	Custom array design or preprinted human miRNA arrays; each array can accommodate up to 244,000 probes	60-nt DNA probes directly synthesized onto array slide with ink-jet fluidics; T4 RNA ligase in special reaction buffer directly couples fluorophore to small RNA	*http://www.chem.agilent.com*; Wang et al. (2007)
Microarray	Exiqon	miRCURY LNA Arrays: ~1500 probes for current set of all known miRNAs for all organisms	T_m balanced LNA probes are spotted onto microarrays; high binding affinity of LNA bases allows for greater specificity	*http://www.exiqon.com*; Castoldi et al. (2006)
Microarray	LC Sciences	Custom array design of small oligo probes directly synthesized onto array chip	Hybridization in a μParaFlo microfluidics chip with probes of balanced T_m (due to base modifications)	*http://www.lcsciences.com*; Vagin et al. (2006)
Microarray	Invitrogen	NCode miRNA Analysis Array: ~500 probes for human, mouse, rat, *Drosophila*, *C. elegans*, and zebrafish miRNAs	T_m balanced dimer probes spotted onto epoxide glass slides; miRNAs labeled via polyA tailing and binding of fluorescent detection oligos	*http://www.invitrogen.com*; Goff et al. (2005)

potential of a miRNA upon a UTR element are similar to the parameters discussed for gene silencing techniques for siRNAs in mammalian cells (*UNIT 26.2*). Other considerations that accompany cell transfection experiments apply (e.g., see *UNIT 9.4*).

Proper analysis of UTR-reporter constructs will require spreadsheet calculations (e.g., using Microsoft Excel) and can be performed according to the methodology described in Farh et al. (2005) and Grimson et al. (2007). In order to control for transfection efficiency, the ratio of *Renilla* luciferase activity to firefly luciferase activity should be calculated for each well. To combine replicate values, the geometric mean of replicate values should be calculated. Repression for the target site of interest (expressed as *x*-fold repression) can then be calculated by comparing the geometric mean of normalized *Renilla* luciferase values between the wt and mutant UTRs, each cotransfected with miRNA cognate to the wt.

As an important control, calculate the same ratio for wt and mutant UTRs cotransfected with noncognate miRNA (this should be ~1). Combine values from at least three or four independent experiments. Statistical significance is assessed with nonparametric (Wilcoxon rank-sum; see *APPENDIX 3I*) tests, comparing normalized values for wt UTR with cognate siRNA to values for mutant UTR with cognate siRNA; a significant difference indicates significant repression for the wt sites.

Anticipated Results

Positive ion-exchange chromatography of small-RNA ribonucleoproteins generally resolves miRNAs into the flowthrough fractions. In germ cell tissues, such as testis or certain animal ovaries, piRNAs bind to a Q column, but elute under mild salt conditions (~300 mM potassium acetate; Lau et al., 2006).

The chemical cross-linking procedure for small-RNA northern blots can increase sensitivity with end-labeled oligonucleotide probes 5- to 100-fold (Pall et al., 2007).

Although some gene targets regulated by miRNAs can be silenced more than 10-fold (Wightman et al., 1993; Reinhart et al., 2000; Lewis et al., 2003), the majority of UTR target elements in the cell-based reporter assay respond to miRNA silencing by reduction levels of 2- to 5-fold (Farh et al., 2005; Lim et al., 2005; Grimson et al., 2007). Lack of gene silencing in an experiment may indicate that a predicted target does not pass muster as an actual regulatory target of a miRNA.

Time Considerations

Creating an extract from cells or tissues will take 1 to 2 days, including dialysis. Fractionation of the cell extract and extraction of RNAs from the fractions take another day. RNA labeling and detection can be completed in <1 day.

The chemical cross-linking procedure for small-RNA northern blots adds 3 hr to the standard northern analysis procedure.

The cell-based reporter assay for miRNA regulation begins with several days to a few weeks of molecular cloning techniques to establish the reporter gene elements. The actual transfection experiment and the readout of reporter gene levels take 3 to 4 days to complete.

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