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Detection and Verification of Mammalian Mirtrons by Northern Blotting

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Abstract

microRNAs (miRNAs) have vital roles in regulating gene expression–contributing to major diseases like cancer and heart disease. Over the last decade thousands of miRNAs have been discovered through high throughput sequencing-based annotation. Different classes have been described, as well as a great dynamic range of expression levels. While sequencing approaches provide insight into biogenesis and allow confident identification, there is a need for additional methods for validation and characterization. Northern blotting was one of the first techniques used for studying miRNAs, and remains one of the most valuable as it avoids enzymatic manipulation of miRNA transcripts. Blotting can also provide insight into biogenesis by revealing RNA processing intermediates. Compared to sequencing, however, northern blotting is a relatively insensitive technology. This creates a challenge for detecting low expressed miRNAs, particularly those produced by inefficient, non-canonical pathways. In this chapter, we describe a strategy to study such miRNAs by northern blotting that involves ectopic expression of both miRNAs and miRNA-binding Argonaute (Ago) proteins. Through use of epitope tags, this strategy also provides a convenient method for verification of small RNA competency to be loaded into regulatory complexes.

Keywords

miRNA; mirtron; tailed-mirtron; low-abundance detection; RNA-immunoprecipitation; northern blot

1. Introduction

Small non-coding RNAs are common features of eukaryotic transcriptomes that require specialized detection methods due to their small size. Moreover many species are present in low abundance, which further complicates detection [1]. microRNAs are an ~23 nucleotide (nt) class of small RNAs that have vital roles in gene regulation and their dysregulation is associated with numerous diseases [2,3]. Animal microRNAs are a highly diverse class of genes. Some miRNAs are ancient, predating the birth of metazoans, while other are newly

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evolved and species specific [4]. Accordingly, microRNAs are a highly-diversified class of transcript [5]. High throughput sequencing has been instrumental in small RNA discovery [6]. Indeed, thousands of miRNAs have been discovered and deposited into databases [7]. Yet, compared to the number of miRNAs annotated, there have been very few functional studies, suggesting there is a need for additional strategies for detection.

miRNA biogenesis typically involves cleavage from hairpin RNAs precursors (premiRNAs). For most miRNAs the Drosha/DGCR8-containing microprocessor complex excises pre-miRNA from primary transcripts [8]. Final processing is then carried-out by Dicer, yielding miRNA/miRNA* duplexes [9]. One strand then is loaded into Argonuate (Ago) proteins where it participates in targeting and repression of transcripts [10]. Beyond this canonical pathway, there are many atypical pathways. Multiple varieties of miRNAs have been found to bypass Drosha/Dgcr8 processing like mirtrons, shRNAs, snoRNA-derived miRNAs, tRNA-derived miRNAs, and rRNA-derived miRNAs (figure 1)[11–15]. In this article, we will focus on the detection of mirtrons. They are derived from small introns that after splicing and debranching can fold into small hairpins [2,16,17].

Hundreds of mirtrons have been discovered in the human genome through inspecting hairpins coincident with splice sites from which small RNA sequencing reads are derived [18]. Mirtrons are classified in three categories: conventional, 3’-tailed, and 5’-tailed. In conventional, both hairpin ends correspond to splice sites and after splicing can become dicer substrates. In “tailed” varieties only one side of the hairpin is at a splice site, while the other side has a stretch of nt’s, or tail, between the hairpin base and splice site [19,20]. Studies in *Drosophila* have also found that mirtrons are commonly modified by Tailor, a TUTase (terminal Uridyltransferase), which uridylates hairpins to inhibit their biogenesis by abrogating Dicer processing [21,22]. Thus, mirtrons more so than other types of miRNAs are less robustly expressed than canonical miRNAs.

Comparing expression of human canonical miRNAs (deep conserved or newly evolved) to mirtrons inferred from small RNA sequencing data meta-analysis reveals that mirtrons have on average several fold lower abundance (Figure 2) [23]. Furthermore, the distribution of mirtrons is bimodal, showing that many species accumulate at the lowest levels of expression observed for miRNAs (Figure 2). The type of mirtron in the lowest abundance population are tailed varieties, consistent with the requirement for additional processing during tail removal. Therefore, detection strategies capable of detecting tailed-mirtrons via northern blotting will be applicable for studying all types of low abundance miRNAs.

Northern blotting was the first technique used to detect a miRNA transcript [24]. Many techniques have been developed in the interim which frequently rely on reverse transcription and PCR, which can introduce bias, artifacts, and mostly can’t distinguish between biogenesis intermediates [25,26]. The challenge in using northern blotting is relatively low sensitivity, making detection of tailed-mirtrons problematic. Despite this difficulty, it is possible to detect tailed-mirtrons using ectopic expression of the miRNA and Ago proteins [18]. In this chapter, we describe a protocol for applying this technique to study mammalian tailed-mirtrons. This process involves transfection of two constructs, one to express the mirtron and the other to express a myc-tagged human Ago2 (Figure 3a). In this system, the

mirtron is cloned into the open-reading frame of GFP, producing a fluorescent reporter for verification that splicing has occurred efficiently. This strategy also takes advantage of a cell culture phenomena where increased rates of miRNA biogenesis are observed in cells grown to high confluency [27]. Thus, it is critical to use a cell line that does not exhibit contact-inhibition. The protocol described here uses Hela cells. Following transfection RNAs can either be bulk isolated by a routine RNA purification method like TriZol extraction or through immunoprecipitation targeting the myc epitope fused to Ago2. Northern blotting, as previously described, involving urea-PAGE separation and transfer to nylon membranes can then be used to visualize RNAs in conjunction with oligonucleotide probes (Figure 3b) [28].

2. Materials

2.1) Plasmid Constructs

Mirtrons are amplified and inserted into expression vectors within opening reading frame of GFP [29]. The protocol also utilizes a myc-tagged hAgo2 (Addgene: Plasmid #19872). Plasmid DNAs are isolated via commonly-used commercial purification kits like the Qiagen®Endo-free Maxi Prep Kit.

2.2) Transfection Reagents

2.2.1) Lipofectamine 3000 ® Invitrogen reagent. Keep at 4°C.

2.2.2) Opti-Mem 1X reduced serum medium (Thermo Ficsher Scientific)

2.3) RNA Immunoprecipitation

2.3.1) Phospate buffered Saline, lysis buffer (5% Glycerol, 150mM sodium chloride, 2mM Magnesium Acetate, 20mM, Tris 7.5 pH, 0.5% NP-40, 2mM DTT, 40 units/mL RNase Out ® Invitrogen, and Complete mini Protease Inhibitor Cocktail ® Roche). Lysis buffer should be used within several days of mixing and kept at 4°C.

2.3.2) Protein G Dynabeads ® Invitrogen, magnetic stand, and tween-20.

2.3.3) Anti-myc (clone 9E10, Thermofisher) and a control IgG (Jackson Immunoresearch) antibodies

2.3.4) Bead preparation and RNA isolation: TRIzol ® Invitrogen, or 400 mM sodium chloride, acid phenol chloroform (Ambion), glycogen co-precipitant, isopropanol, 70% ethanol, and sodium acetate pH 5.3.

2.4) Northern Blotting

2.4.1) RNA loading buffer II (ambion), Tris-Boric Acid EDTA (TBE) running buffer

2.4.2) 12% urea PAGE gel mixed from the UreaGel system (National Diagnostics). Ammonium persulfate (10 % solution in water) (APS), and N,N,N,N’ Tetramethylethylenediamine (TEMED)

2.4.3) Positively Charged Nylon membrane (Hybond-N, GE-lifesciences), Filter paper, Semi-dry electroblotting apparatus.

2.4.4) 5X SSC, 1mM EDTA, 2x Denhardt's, 1% SDS, 2% Dextran Sulfate, 30ug/ml. Combine all components and boil until completely dissolved.

2.4.5) Probes: T4 polynucleotide kinase (PNK) (NEB), DNA oligonucleotides complementary to the miRNA of interest, gamma-P^{32}ATP 6000uCi/mL (Perkin Elmer), and Sephadex G-25 columns (GE-Life Sciences).

2.4.6) Decade Marker (Ambion).

2.4.7) Wash Buffers: High stringency (2X SSC, 0.1% SDS) and low stringency (0.2 X SSC, 0.1% SDS).

2.4.8) X-ray film or Phosphor-imaging system.

Methods

3.1) Hela cells are maintained in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% FBS and 1% penicillin/Streptomycin using standard cell culture methodology. Hela cells are efficiently transfected with Lipofectamine 3000 (Invitrogen) to deliver mirtron and myc-Ago2 encoding plasmids, as described above. Transfection and splicing efficiency is determined by visualizing GFP fluorescence.

3.1.1) Grow cells to 100% confluency, and change media 3 hours before transfection with antibiotics free media.

3.1.2) Warm OptiMEM to room temperature. Make a mixture of OptiMEM, Plasmids, and Lipofectamine 3000 based on the scale of transfection in accordance with manufacturer recommendations. For this protocol six well plates are typically used.

3.1.3) Incubate the mixture at room temperature for 20–25 minutes.

3.1.4) Add the transfection mixture to the cells dropwise, without touching the plate wall.

3.1.5) Swirl the dish gently and incubate for 2 days (37°C, 5% CO_2).

3.1.6) Check transfection efficiency two days after transfection.

3.2) RNA isolation

Following the two-day incubation RNA is harvested. This could be done using the TRIzol method, following manufacturer protocols, or immunoprecipitation to isolate Ago-miRNA complexes.

3.2.1) TRIzol extraction (optional)

3.2.1.1) Add TRIzol directly to tissue culture plates. Cells should immediately become opaque, and easily dislodged by micropipetting. Remove cells and place in microcentrifuge tube.

3.2.1.2) Incubate TRIzol and cells for 10 mins at room temperature.

3.2.1.3) Add 200 μl of chloroform to every 1 mL of TRIzol and incubate at room temperature for 5 mins.

3.2.1.4) Separate phases at 4°C for 15 mins by spinning at 13,000 rpm.

3.2.1.5) Transfer the clear aqueous phase to an equal volume of isopropanol and incubate overnight at −20°C.

3.2.1.6) Spin precipitations at 4°C for 30 mins, resuspend pellets in RNase free water, and measure concentration via spectrophotometry.

3.2.2) Immunoprecipitation of miRNA-Ago Complexes (optional).

3.2.2.1) Wash transfected cells with ice-cold PBS twice while in culture dish. Add lysis buffer to cells and incubate 20 mins on ice. Cell layer should begin to detach from the bottom of the dish with gentle micropipetting. Transfer to microcentrifuge tube, mix well with pipette to eliminate clumps, and spin down at 13000 rpm for 20 minutes at 4°C to clarify lysate. Transfer supernatant to new tube.

3.2.2.2) Prepare beads for incubation with lysate. Add protein G magnetic beads to tubes (two tubes for each sample). Place the tubes on a magnetic stand and remove the supernatant. Wash tubes twice with PBS containing 0.02% tween-20. In PBS-tween add Anti-myc or control IgG antibodies.

3.2.2.3) Remove PBS tween from beads and add cell lysate from step 3.2.1.1. Reserve 10% of the volume to be used as input control. Place on rocker for 2 h, 4°C

3.2.2.4) Wash beads with lysis buffer five times using magnetic stand to remove beads from solution.

3.2.2.5) Add equal volumes of 400mM NaCl and acid phenol chloroform to beads. Aggressively vortex. This procedure should also be performed on the reserved 10% volume of lysate set aside for input control.

3.2.2.6) Separate phases at 4°C for 15 mins by spinning at 13,000 rpm

3.2.2.7) Add clear aqueous phase to an equal volume of isopropanol and incubate overnight at −20°C.

3.2.2.8) The following day spin precipitations at 4°C for 30 mins, resuspend pellets in RNA II loading buffer.

3.3) Northern Blotting

3.3.1) Sample Preparation. If immunoprecipitation was performed samples are ready to load on the gel. If TRIzol extraction was used, 20μg of RNA should be loaded in each lane.

3.3.1.1) Reprecipitate 20μg of RNAs by adding the appropriate volume of RNA to 100% ethanol containing 300 mM sodium acetate.

3.3.1.2) Incubate the RNA at −20°C for at least one hour.

3.3.1.3) Spin precipitations at 4°C for 30 mins, resuspend pellets in RNA II loading buffer.

3.3.1.4) RNAs dissolved in RNA II loading buffer are denatured by incubating at 94°C for 3 minutes and then immediately placed on ice.

3.3.2) Prepare and Run Gel using a vertical gel rig and the UreaGel system.

3.3.2.1) Mix UreaGel diluent, buffer, and concentrate, then add APS and TEMED.

3.3.2.2) Quickly, using a large volume pipette pour gel mixture between glass plates, insert comb, and lay horizontally until gel is set.

3.3.2.3) Place gel on apparatus and fill buffer tanks with TBE. Pre-run the gel at 250 V for 30 minutes.

3.3.2.4) Using long gel loading tips transfer RNA dissolved in RNA II loading buffer to the wells of the gel. Prior to loading samples, wells should be washed out to remove urea that has diffused into the space. Radio-labeled decade marker should also be loaded on the gel.

3.3.2.5) Run the gel at 250–350 V until the fastest moving, bromophenol blue dye band has migrated approximately eight inches.

3.3.3) Blotting of RNAs onto nylon membranes

3.3.3.1) Soak filter paper in 0.5X TBE buffer and place onto wet-dry transfer rig.

3.3.3.2) Crack the gel using a separator wedge, and cut the region of the gel to be blotted. The lower dye band corresponds to ~10 nt RNAs. The gel should be cut approximately one inch below the lower band in order to capture all the RNA molecules.

3.3.3.3) Place the gel onto the 0.5X TBE soaked filter paper.

3.3.3.4) Prepare the membrane by quickly rinsing in water, followed by briefly soaking in 0.5 X TBE. Once the membrane sinks in the buffer it is ready to be placed on top of the gel.

3.3.3.5) Place another presoaked piece of filter paper on top of the membrane.

3.3.3.6) Place the lid on the rig. The filter paper/gel/membrane should be sandwiched tightly between the electrodes.

3.3.3.7) Transfer the RNAs to the membrane for 30 mins at 10V at 4°C.

3.3.3.8) Remove membrane from transfer apparatus, and air dry briefly on a paper towel.
3.3.3.9) Crosslink the RNAs to the membrane with UV exposure.

3.3.3.8) Bake the blot at 80°C for 30 mins

3.3.4) Probing and visualizing membranes.

3.3.4.1) Blots are place into cylindrical hybridization tubes along with enough hybridization buffer to keep the blot wet.

3.3.4.2) Prehybridize blots for >1 hour at 40–45°C, in a rotating hybridization oven.

3.3.4.3) Prepare radio-labeled probes by mixing water, complementary DNA oligo, 10X PNK buffer, PNK, and gamma-P^{32}ATP 6000uCi/mL.

3.3.4.4) Incubate the labeling reaction at 37°C for > 1hour.

3.3.4.5) Stop the reaction by adding EDTA.

3.3.4.6) Remove unincorporated radionucleotides using G-25 sephadex columns. Remove buffer from column by spinning at 2000rpm for 4 min. Transfer the probe-labeling reaction to the column and spin again for 2000rpm, 4 min. The probe is in the column flowthrough.

3.3.4.7) Add the radiolabeled probe to the hybridization solution. Avoid touching the membrane with concentrated probe.

3.3.4.8) Incubate the membrane overnight at 40–45°C in a rotating hybridization oven.

3.3.5) Wash and expose the blot.

3.3.5.1) Wash the membrane twice with high stringency wash buffer at the hybridization temperature for one hour.

3.3.5.2) Wash the membrane twice with low stringency wash buffer at the hybridization temperature for one hour.

3.3.5.3) Remove membranes from tubes, and dab on filter paper. Do not over-dry the membrane. The objective is to remove excess liquid.

3.3.5.4) Wrap the membrane with cling wrap and expose to X-ray film or to phosphorimager screens.

3.3.5.5) Develop films or scan screens after one day.

3.4) Reprobing membranes

3.4.1) Signal can be stripped from blots by boiling membranes in 0.1% SDS. Blots should be placed in boiling solution and left to cool for three hours. This should be repeated once.

3.4.2) Following the stripping procedure, blots are placed into hybridization buffer and the procedure repeated starting at step 3.3.4.2.

3.4.3) Blots can be subject to multiple reprobings before the integrity of the immobilized RNA is compromised.

4.) Notes

4.1) The protocol described here relies on a radiolabel to visualize the interaction of probes with RNAs immobilized on blots. There are alternatives to this type of detection, which are untried with mirtrons [30]. These procedures involve an epitope labeled probe such as biotin followed by antibody detection leading to visualization through chemiluminescence. For labs that want to avoid isotope this could be a viable alternative.

4.2) This protocol calls for DNA oligonucleotide probes, but is also appropriate for use with LNA-based probes. These molecules offer greater specificity compare to DNA probes. To use this alternative chemistry the hybridization buffer should be substituted with a formulation containing formamide.

4.2.1) LNA hybridization buffer: 50% Formamide, 5X SSPE, 5x Denhardt's, 0.5% SDS, 20ug/ml ssDNA.

4.3) Avoid touch nylon membranes with gloves, and then only on the edges. This will avoid the appearance of blotting artifacts that might obscure signal.

4.4) Care should be taken to load the optimal amount of RNA ladder. The Decade Marker protocol calls for a single μl of gamma-P^{32}ATP 6000uCi/ml, but this could lead to innapropriatly intense bands that obscure signal if not properly diluted. When fresh ATP is used, the marker should be serially diluted in RNA II loading buffer. 1 μl of reaction should be diluted in 30 μl followed by a second dilution in of 1 μl in 30 μl. One μl of the final dilution should be loaded on the gel.

4.4) Following the final wash step it is typical to observe very little signal emanating from the blot via Geiger counter. However, when signals are very weak longer exposure times may be required. Experimenters could extend exposure times to a week for the lowest signals.

4.5) When removing combs from UreaGels the wells should be washed aggressively with water to prevent residual acrylamide from polymerizing and causing unevenness in the bottom of the well which will result in RNA migration artifacts.

4.6) Moderation of voltage used during PAGE separation of RNAs is recommended. This prevents bending of bands.

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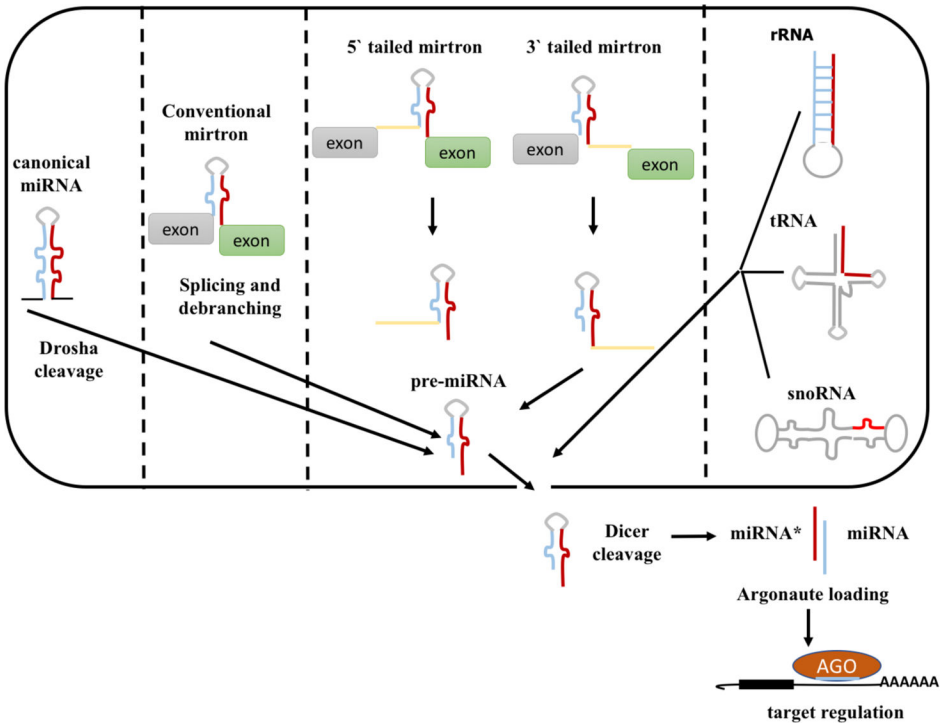

Figure 1:
Classes of miRNAs defined by biogenesis pathways

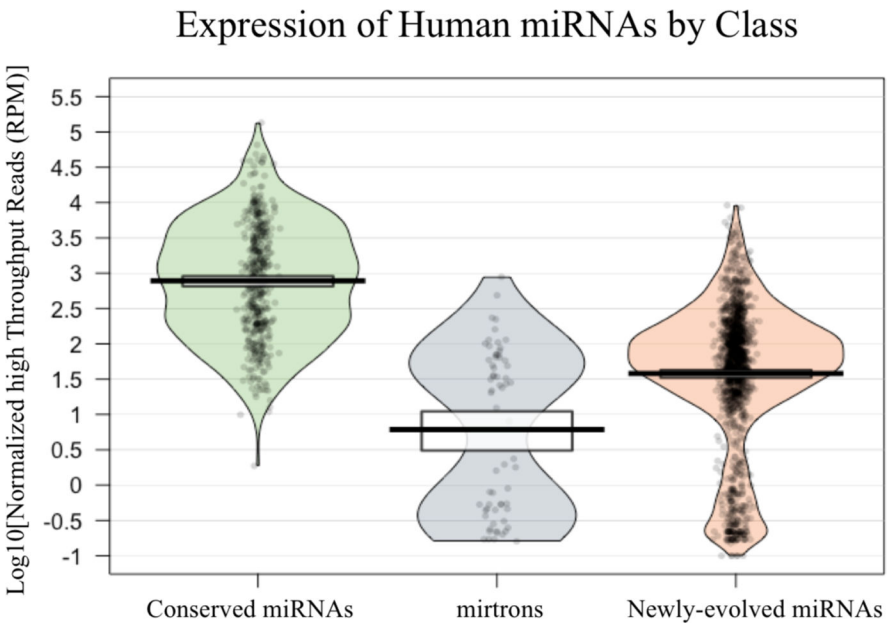

Figure 2:
RDI plot showing the distribution of normalized (Reads per million) miRNA expression separated by class. Values were taken were taken from miRbase.

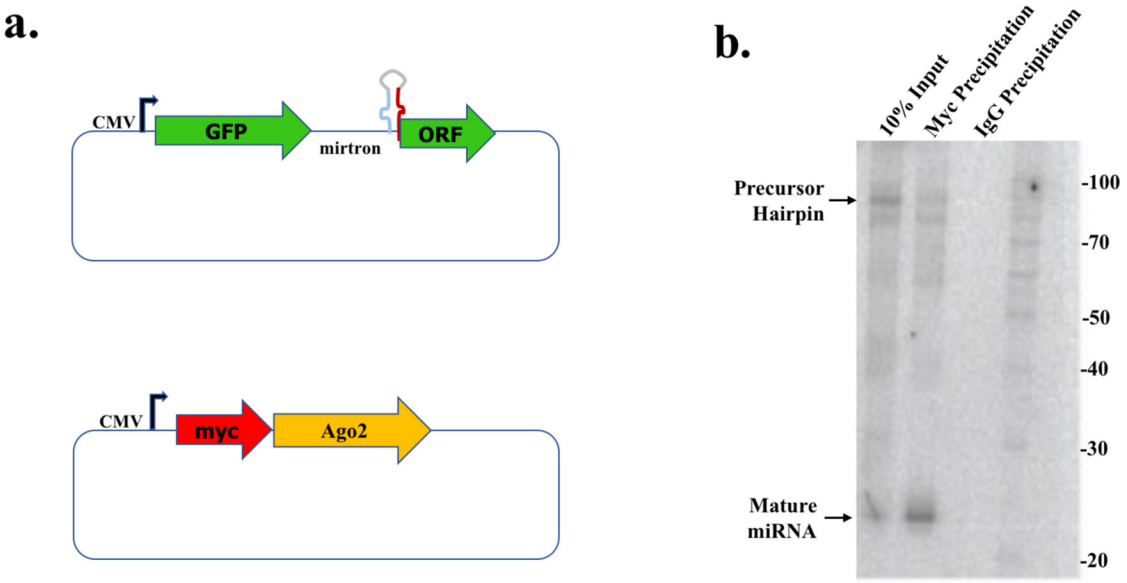

Figure 3:

System for detecting and verifying mammalian mirtrons a) Constructs for cotransfection. Top vector configured to couple mirtron splicing to GFP expression, by interrupting the GFP ORF with the mirtron-contianing intron. Bottom vector for ectopic expression of Ago2-myc b) Northern Blotting reveals expression of miR-5010, a 5'-tailed mirtron, using the vectors in (a) in conjunction with an immunoprecipitation procedure.