miRNA Tagging and Affinity-purification (miRAP)

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[Abstract] MicroRNAs(miRNAs) are a group of endogenously expressed 20~23 nt small noncoding RNAs, which can directly regulate mRNA stability or translation in a sequence specific manner by incomplete base pairing at the 3'UTR of target mRNA, or indirectly affect transcriptional network by regulating transcription factors. As key regulators of gene expression, miRNAs are involved in the control of diverse developmental and physiological processes, including embryogenesis, differentiation, developmental timing, organogenesis, growth control, and programmed cell death. Aberrant miRNA expression profiles have been observed in many pathological conditions, including cancers, psychiatric diseases, virus infection, etc. However, the underlying mechanisms have been difficult to study in part due to the cellular heterogeneity of complex tissue.

To systematically analyze miRNA expression in complex tissue, we present here a novel miRNA tagging and Affinity Purification method, miRAP, which can be applied to genetically defined cell types in any complex tissues in mice. This method is based on the fact that mature miRNAs are incorporated into RNA-induced silencing complex (RISC), in which the Argonaute protein AGO2 directly binds miRNAs and their mRNA targets. We demonstrate that epitope tagging of AGO2 protein allows direct purification of miRNAs from tissue homogenates using antibodies against the engineered molecular tag. We further established a Cre-loxP binary expression system to deliver epitope-tagged AGO2 (tAGO2) to genetically defined cell types.

Materials and Reagents

1. Mouse-anti-c-Myc (Santa Cruz, catalog number: sc-40)
2. Mouse-anti-Ago2 (Clone 2E12-1C9) (Abnova, catalog number: H00027161-M01)
3. Rabbit-anti-GFP (Rockland, catalog number: 600-401-215) or Chicken-anti-GFP (Rockland, catalog number: 600-901-215)
4. Mouse IgG1 negative control (clone Ci4) (EMD Millipore)
   Note: The above antibodies have been tested by the author and may be substituted with the antibodies desired by users.
5. Complete proteinase inhibitors (EDTA-free) (Roche Diagnostics)
6. Protein G Dynabeads (Life Technologies, Invitrogen™)

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7. RNasin (Life Technologies, Ambion®)
8. Proteinase K (Roche Diagnostics)
9. Acid phenochloroform (Life Technologies, Ambion®)
10. Chloroform (Life Technologies, Ambion®)
11. 3 M Sodium Acetate (pH 5.5) (Life Technologies, Ambion®)
12. Glycoblue (Life Technologies, Ambion®)
13. RNAzap (Life Technologies, Ambion®)
14. DEPC treated water or RNase free water (Life Technologies, Ambion®)
15. HEPES (pH 7.4)
16. KCl
17. $MaCl_2$
18. NP-40
19. DTT
20. EDTA
21. SDS
22. Lysis buffer (see Recipes)
23. Low salt NT2 buffer (see Recipes)
24. High salt NT2 buffer (see Recipes)
25. 0.5% NP-40 (see Recipes)
26. Proteinase K buffer (see Recipes)

Equipment

1. Glass douncer
2. Mortar and pestle
3. Ice bucket
4. Rotator
5. 4 °C centrifuge
6. Standard western blot set up

Procedure

A. Activate and validate tAGO2 expression in the cell of interest
   1. Set up appropriate Cre driver line breeding with LSL-tAgo2 reporter line (JAX stock number: 017626) to express tAgo2 in the cell of interest.

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2. Verify tAgo2 expression in the cell of interest by co-immunostaining of GFP tag within tAgo2 and markers identifying that cell type in tissue sections. Recommended dilution of GFP antibody is 1:800~1:1,000.
3. Euthanize the mouse and dissect out tissue of interest on ice as soon as possible.
4. Flash freeze tissue in liquid nitrogen (pause point: Tissue block can be stored in liquid nitrogen for at least half year).

B. miRAP sample preparation

Note: It is important to work in an RNase free environment from this part on. Gloves should be worn at all time. Bench top should be wiped with RNAzap. Glassware should be cleaned with RNAzap and rinsed with DEPC-treated water or RNase free water. RNase free pipette tips and tubes should be used when handling the samples. All reagents should be prepared in DEPC-treated water or RNase free water.

5. Cool down mortar and pestle in a liquid nitrogen containing ice bucket.
6. Pour appropriate amount of liquid nitrogen into the mortar (enough to immerse tissue block, but not too much that the liquid nitrogen will spill out), ground tissue into fine powder.
7. Transfer the tissue powder along with liquid nitrogen into a 50 ml falcon tube, loosely cap the tube, let it sit in room temperature for a few minutes until the liquid nitrogen completely evaporate.
8. Add 10 volume of lysis buffer, resuspend tissue powder quickly and transfer into pre-cooled glass douncer.
9. Homogenized tissue suspension using glass douncer by douncing 50-100 times. Certain tissue may take longer to lyse. Adjust the number of douncing according to your application.
10. Transfer tissue homogenates into 1.5 ml or 2.0 ml eppendorf tubes, centrifuge at 13,000 *x g* for 30 min, 4 °C to pellet cell debris and unsolubilized material.
11. Transfer supernatant to a new tube. This will be the sample to use for miRAP.

C. miRNA affinity purification

12. Prepare antibody conjugated protein G Dynabeads according to manufacturer's instruction. For cell type specific miRAP, use Myc antibody; for whole tissue control, use Ago2 antibody; for negative control, use mouse IgG1. The amount of beads to be used per sample and the antibody to beads ratio should be empirically determined. As a starting point, use 10 µg Myc antibody, 2.5 µg Ago2 antibody, and 10 µg IgG1 per 50 µl beads.
13. Add antibody conjugated beads to IP sample and incubate the mixture in 4 °C with end-over-end rotation for 4 h. The amount of beads to use per sample should be

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determined empirically. Western blot can be used to check the efficiency of IP. If there is still residue Ago2 or tAgo2 present in the supernatant after IP, use more beads.

14. Wash beads twice with low salt NT2 buffer and twice with high salt NT2 buffer, 15 min each, and treat the beads with 0.6 mg ml^{-1} proteinase K for 20 min at 55 °C.
15. Add equal volume of acid phenochloroform to the sample, mix well by vortexing, and spin at maximum speed in 4 °C for 15 min; transfer the upper phase to a new tube without disturbing the interphase. Repeat this extraction procedure twice with chloroform instead of phenochloroform to get rid of residue phenol. Add at least 3 volume of 100% ethanol, 1/10 volume of sodium acetate (pH 5.2), and 1 µl of glycoblue to the transferred upper phase, mix well, and precipitate RNA overnight at -80 °C.
16. Wash RNA pellet once with 75% ethanol and dissolve it in water for further application. (Pause point: RNA pellet can be stored in 75%-100% ethanol in -80 °C for years. RNA solution can be stored in -80 °C, but multiple freeze-thaw cycles should be avoided.)

D. Downstream applicationmiRNA purified from miRAP has high quality and can be directly used for deep sequencing, miRNA Taqman PCR, miRNA microarrary, Northern blot (which requires a high input and may not be possible for rare cell types or low expression miRNAs), *etc.*, following standard protocol.
Note: It is recommended to use Taqman PCR for a quick and sensitive examination of candidate miRNAs before you proceed to sequencing or microarray.

Recipes

1. Lysis buffer
   10 mM HEPES (pH 7.4)
   100 mM KCl
   5 mM $MaCl_2$
   0.5% NP-40
   1 mM DTT
   100 U/ml RNasin
   Roche Complete proteinase inhibitors EDTA-free (1 tab/10 ml).
2. Low salt NT2 buffer
   50 mM Tris-HCl (pH 7.5)
   150 mM NaCl
   1 mM $MgCl_2$
   0.5% NP-40

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1 mM DTT
100 U/ml RNasin

3. High salt NT2 buffer
   50 mM Tris-HCl (pH 7.5)
   600 mM NaCl
   1 mM $MgCl_2$
4. 0.5% NP-40
   1 mM DTT
   100 U/ml RNasin
5. Proteinase K buffer
   100 Mm Tris-HCl (pH 7.5)
   50 mM NaCl
   10 mM EDTA
   0.5% SDS
   10 mg/ml proteinase K

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