Ribonomics

MBL

Printed April 14, 2012
Version 2.1

For Research Use Only. Not for use in diagnostic procedures.

RiboCluster Profiler™

RIP-Assay Kit *for microRNA*

10 assays

CODE No. RN1005

MBL MEDICAL & BIOLOGICAL LABORATORIES CO., LTD.
TEL: (052) 238-1904, E-mail: support@mbl.co.jp
URL: https://ruo.mbl.co.jp/je/rip-assay/

Contents

I. Introduction

Please read these instructions carefully before beginning the assay.

It is very important to isolate "high-quality RNA" from various materials to validate experiments such as reverse transcription polymerase chain reaction (RT-PCR) and gene expression analysis based on microarray technology (Chip analysis) because experimental results may be sensitive to RNA quality. In order to obtain "high-quality RNA", and reduce the chance of RNase contamination, gloves should be worn when proceeding RIP-Assay, and RNase-free microcentrifuge tubes and pipette tips should be used for the assay.

1. Background and Introduction

Discovery of RNA interference (RNAi) has given a great boost to research on functional RNA and post-transcriptional regulation. RNAi, which plays a central role in sequence-specific gene silencing in eukaryotic cells, depends on the functions of RNA-induced silencing complex (RISC) composed of small non-coding RNAs (ncRNAs) and proteins. The main classes of small ncRNA are short interfering RNAs (siRNAs), microRNAs (miRNAs) and PIWI-interacting RNAs (piRNAs). siRNAs are generated by cleavage of exogenous long double-stranded RNA precursors in response to viral infection or artificial introduction. In contrast, miRNAs are generated from endogenous transcripts containing stem-loop structures. The siRNAs and miRNAs processed by Dicer, which functions as a ribonuclease III enzyme, are incorporated into the RISC in order to silence the specific mRNAs based on partial sequence complementarity between the small RNAs and the 3' untranslated regions (UTRs) of the mRNAs. Argonaute family proteins are a core component of RISC and divided into the AGO and PIWI subfamilies. siRNAs and miRNAs are loaded onto AGO proteins whereas piRNAs are loaded onto PIWI proteins. Each member of the family proteins functions as a silencer to inactivate their target mRNAs.

Hundreds of miRNA species have been discovered in animals and plants, many of which exhibit temporally and spatially controlled expression. One approach to investigate the biological functions of miRNAs has been to identify their targets. The miRNA target predictions are based on computational analyses of complementary sequence elements that are refined by considering evolutionary homologies across multiple species. While a variety of computational approaches and algorithms have been used to make such predictions, it is not certain that each miRNA gains functional access to these targeted mRNAs in the cell under a given set of conditions.

RIP-Chip (ribonucleoprotein immunoprecipitation-microarray profiling) is a biochemical approach to identify the composition and organization of endogenous mRNAs, miRNAs and RNA binding proteins (RBPs) within messenger ribonucleoprotein (mRNP) complexes. RIP-Chip has been successfully employed to isolate AGO-containing RNPs by immunopurification with anti-AGO antibodies. When the co-isolated miRNA and mRNA subpopulations are analyzed using the computational predictions of conserved seed sequences, this approach provides a powerful tool to identify functional miRNA targets based on their physical interaction *in vivo*. RBPs have been reported to bind to mRNAs that encode

functionally related proteins, and coordinately regulate these mRNAs during cellular processes. The RIP-Chip approach can isolate functionally related mRNAs. Since miRNAs can be co-immunoprecipitated with those mRNAs, the RIP-Chip approach can also isolate miRNAs that regulate specific group of mRNAs that are functionally related.

2. Product Description

RIP-Assay Kit for microRNA is optimized to immunochemically isolate endogenous miRNAs, mRNAs and RBPs within mRNP complexes. The kit is designed to isolate cellular miRNAs that being incorporated into the RISC and/or to isolate unique group of miRNAs that bind to specific group of mRNAs encoding functionally related proteins.

Approach 1: RISC immunoprecipitation to isolate miRNAs and their target mRNAs in a global manner.

Mammalian miRNAs associate with members of the Argonaute (AGO) family proteins, the core of the RISC, and bind to partially complementary sequences in the 3' UTR of specific target mRNAs. Therefore, using an antibody against one of the RISC components, such as *RIP-Certified Anti-EIF2C2/AGO2 Antibody* provided from MBL, researchers can isolate miRNAs that are incorporated into the RISC. This approach gives a global view of which miRNAs are fully processed and incorporated into the RISC for binding to their specific target mRNAs.

Approach 2: RBP immunoprecipitation to isolate a unique group of miRNAs that bind to a specific group of mRNAs encoding functionally related proteins.

mRNAs encoding functionally related proteins are coordinately regulated during cellular processes such as proliferation, differentiation or drug treatment. RBPs and miRNAs constitute the primary regulators of eukaryotic post-transcriptional mRNA expression. When using *RIP-Certified RBP Antibodies* provided from MBL, researchers can isolate mRNAs that encode functionally related RNAs and proteins. Using the MBL specific RBP Immunoprecipitation approach, researchers can isolate functionally related miRNAs as well as their target mRNAs that encode functionally related proteins.

In the RIP-Assay protocol, mRNP complexes are isolated from cell extracts by immunoprecipitation with *RIP-Certified RBP Antibodies* provided from MBL. mRNAs and miRNAs are isolated from mRNPs using the proprietary buffers provided in this *RIP-Assay Kit for microRNA*. MBL provides customers with three different protocols to isolate mRNAs and miRNAs depending on the customer's needs. One protocol can provide the best recovery of both mRNAs and miRNAs, and the other protocol is simpler and quicker but provides a more moderate recovery of mRNAs and miRNAs.

Once purified, the mRNAs and miRNAs present in the complexes are analyzed to identify the target mRNAs of miRNAs using various molecular biology tools such as RT-PCR, gene expression analysis based on a suitable microarray platform (Chip analysis), or direct sequencing.

3. Licensing Opportunity

The RIP-Assay uses patented technology (US patent No. 6,635,422, US patent No. 7,504,210, Canadian patent No. 2,396,058 and world-wide patents pending) of Ribonomics, Inc. MBL manufactures

and distributes this product under license from Ribonomics, Inc. Researchers may use this product for their own research. Researchers are not allowed to use this product or RIP-Assay technology for commercial purpose without a license. For commercial use, please contact us for licensing opportunities at RIP@mbl.co.jp

4. Kit Components 10 assays

1.	**mi-Lysis Buffer**	26 mL × 1 bottle
2.	**mi-Wash Buffer**	35 mL × 2 bottles
3.	**Normal Rabbit IgG**	0.33 mL × 1 vial: Negative control: 330 μg of normal rabbit IgG in 330 μL of phosphate buffered saline (PBS) containing 0.09 % sodium azide as a preservative.
4.	**High-Salt Solution**	6 mL × 1 vial: In some cases, addition of this solution to both mi-Lysis Buffer and mi-Wash Buffer is required. Please refer to the datasheet of *RIP-Certified Antibody* (See **Related Products**).
5.	**mi-Solution I**	0.26 mL × 1 vial: enzyme solution
6.	**mi-Solution II†**	6 mL × 1 vial: diluent for Solution I
7.	**mi-Solution III‡**	4 mL × 1 vial: protein dissolvent Solution III can dissolve proteins and dissociate immunocomplex.
8.	**mi-Solution IV**	200 μL × 1 vial: co-precipitator Solution IV can increase RNA precipitation efficiently.
9.	**Gel Extraction Buffer**	25 mL × 1 vial: This solution is suitable for recovering the small RNA fraction from a slice of a denaturing (7 M urea) polyacrylamide gel.
10.	**3 M NaOAc**	1 mL × 2 vials: 3 M sodium acetate This solution contributes to effective alcohol precipitation.
11.	**miSPIKE™ ***	100 pmoles × 1 vial: size control for small RNA (lyophilized product) Centrifuge the vial prior to opening. Reconstitute in 12 μL of nuclease-free water. In order to keep the quality, reconstituted miSPIKE™ should be stored at -20ºC or below just before use. This reagent is manufactured by Integrated DNA Technologies, Inc. (IDT, Inc.)

Note: † Solution II may become turbid when stored for long-term at 2–8ºC. Turbidity does not affect performance. If Solution II is turbid, equilibrate to room temperature (15–25ºC) and mix well before use.

‡ Precipitates may appear when Solution III is stored for long-term at 2–8ºC. If Solution III contains precipitates, dissolve them by equilibrating the solution to room temperature (15–25ºC) and mix well before use.

‡ This reagent contains guanidine hydrochloride; this is a potentially hazardous substance and should be used with appropriate caution.

***** miSPIKE™ is a 21-mer RNA designed specifically to assist in small RNA cloning. miSPIKE™ functions as a size control for small RNA or indicator for successful ligation of small RNA with 3' cloning linker. This RNA oligonucleotide lacks a 5' phosphate so it cannot be 5' linkered. The miSPIKE™ sequence does not have any significant homology to any currently known small RNA as determined via BLAST against RNAdb, GenBank and miRBase. miSPIKE™ sequence is described below.
Sequence: 5'- rCrUrCrArGrGrArTrGrGrCrGrGrArGrCrGrGrUrCrU - 3'

5. Storage and Stability

RIP-Assay Kit for microRNA is stable for two years from the date of manufacture when stored at 4ºC. Do not freeze.

6. Materials Required but Not Provided

Reagents

1. RIP-Certified Antibody (See **Related Products**)
2. Protease inhibitor (molecular biology grade)*
   Commercial reagent
   Aprotinin
   Leupeptin
   Phenylmethylsulfonyl fluoride (PMSF)
3. RNase inhibitor*
4. Dithiothreitol (DTT)*
5. Protein A or Protein G agarose beads**
6. 100% Ethanol (molecular biology grade)
7. 100% 2-Propanol (molecular biology grade)
8. Nuclease-free PBS
9. Nuclease-free water
10. Isotype control IgG (if necessary)***
11. Urea (molecular biology grade)****
12. 40 (w/v) %-Acrylamide/Bis Mixed Solution (19:1)****
13. N,N,N',N'-Tetramethylethylenediamine****
14. Ammonium peroxodisulfate (APS)****
15. Tris-Borate-EDTA (TBE) buffer (Nuclease-free)****
16. GelStar® Nucleic Acid Stain (Takara Bio, Inc.)
17. MOPS Buffer (pH 7.0)
18. RNA ladder for small RNA (if necessary)
19. Xylene cyanol FF (molecular biology grade) (if necessary)
20. Sucrose (molecular biology grade) (if necessary)

21. Bromophenol blue (molecular biology grade) (if necessary)

Equipment

22. Microcentrifuge capable of 15,000 × *g*
23. Microcentrifuge tubes (1.5 mL or 2 mL) (Nuclease-free)
    (Recommendation; use low-adhesion tube for RIP-Assay)
24. Centrifuge capable of 2,000 × *g*
25. Centrifuge tubes (15 mL or 50 mL)
26. Pipettes (5 mL, 10 mL, 25 mL) (Nuclease-free)
27. Pipette tips (10 μL, 20–100 μL, 200 μL, and 1,000 μL) (Nuclease-free)
    (Recommendation; use low-adhesion pipette tip for RIP-Assay)
28. Disposable pestle (Nuclease-free)
29. Ultra low temperature freezer (-80ºC)
30. Freezer (below -20ºC)
31. End-over-end rotator
32. Vortex mixer
33. Gloves
34. Slab gel electrophoresis device
35. UV transilluminator
36. Power supply

Note: * Recommended concentration of each reagent is shown in **Appendix**.

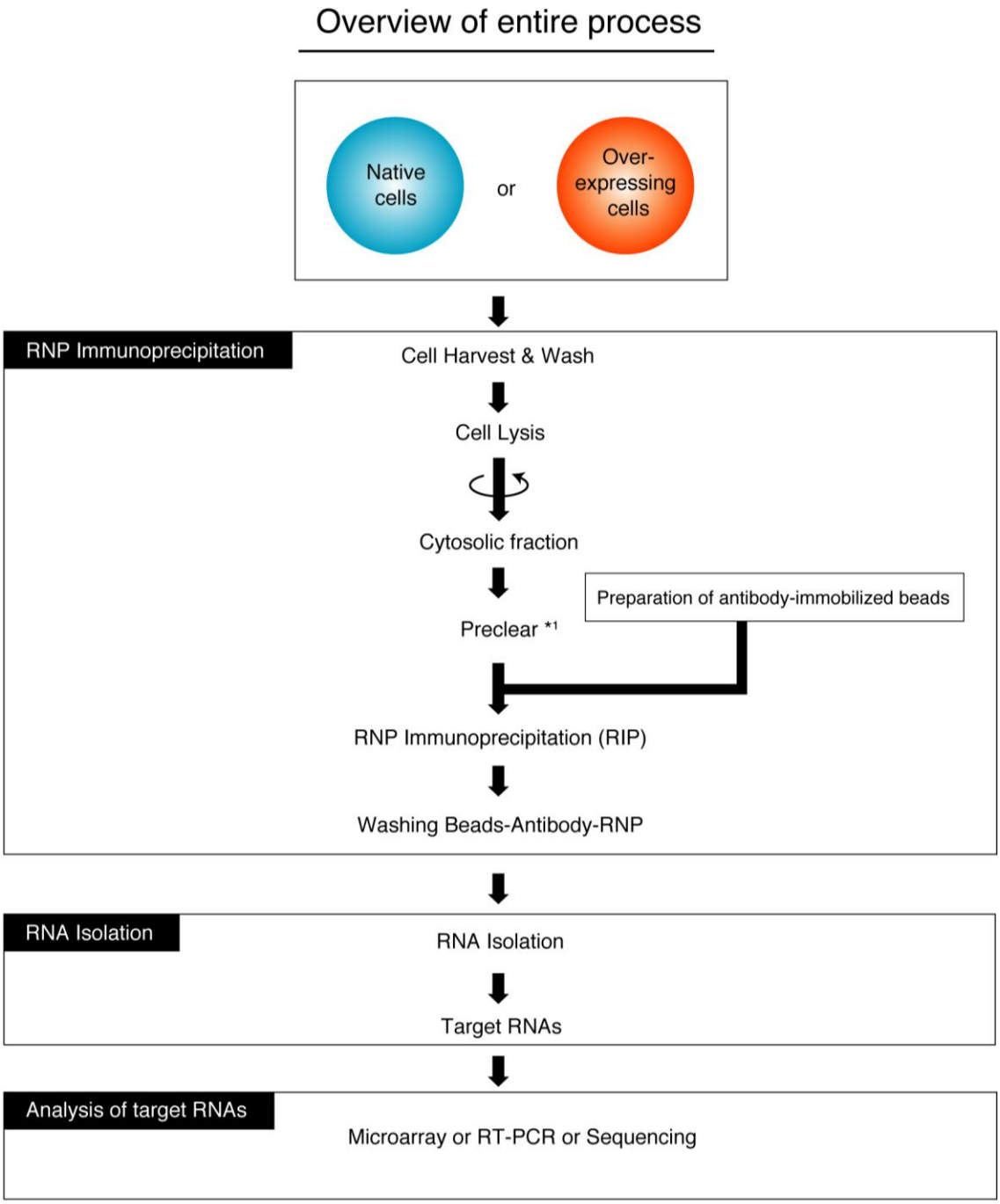
** Commercially available reagents confirmed to work with *RIP-Assay Kit for microRNA* are shown in **Appendix**.

*** In the case of using monoclonal antibodies to RNP immunoprecipitation, the isotype control IgG should be prepared as negative controls. Please refer to the **Related Products**.

**** Preparation of denaturing (7 M urea) polyacrylamide gel is shown in **Appendix**.

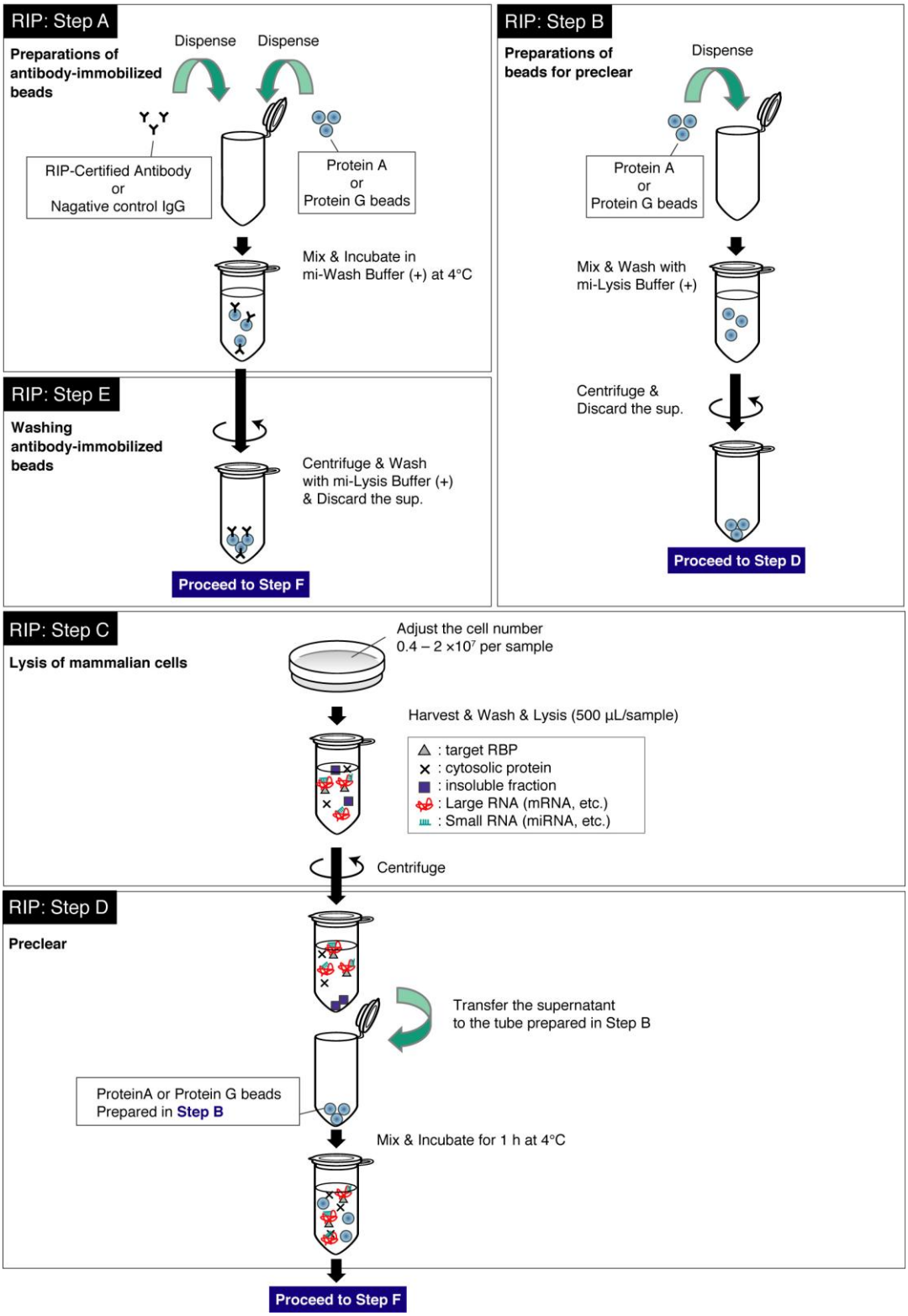
II. RIP-Assay Kit *for microRNA* Procedure

1. Procedure Summary

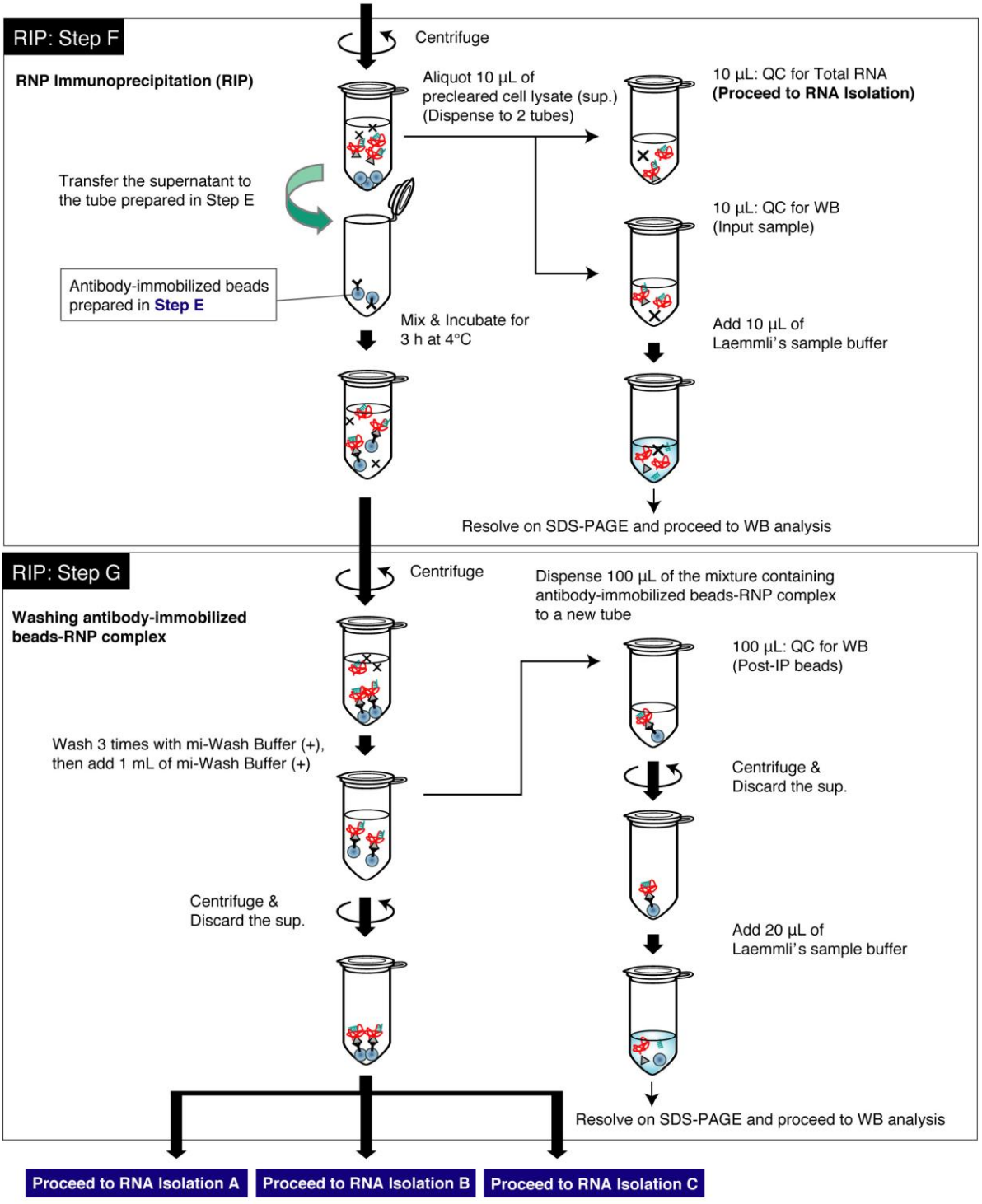


*1; Preclear the cell lysate by pre-incubating the prepared lysate with the beads

RIP-Assay process by step



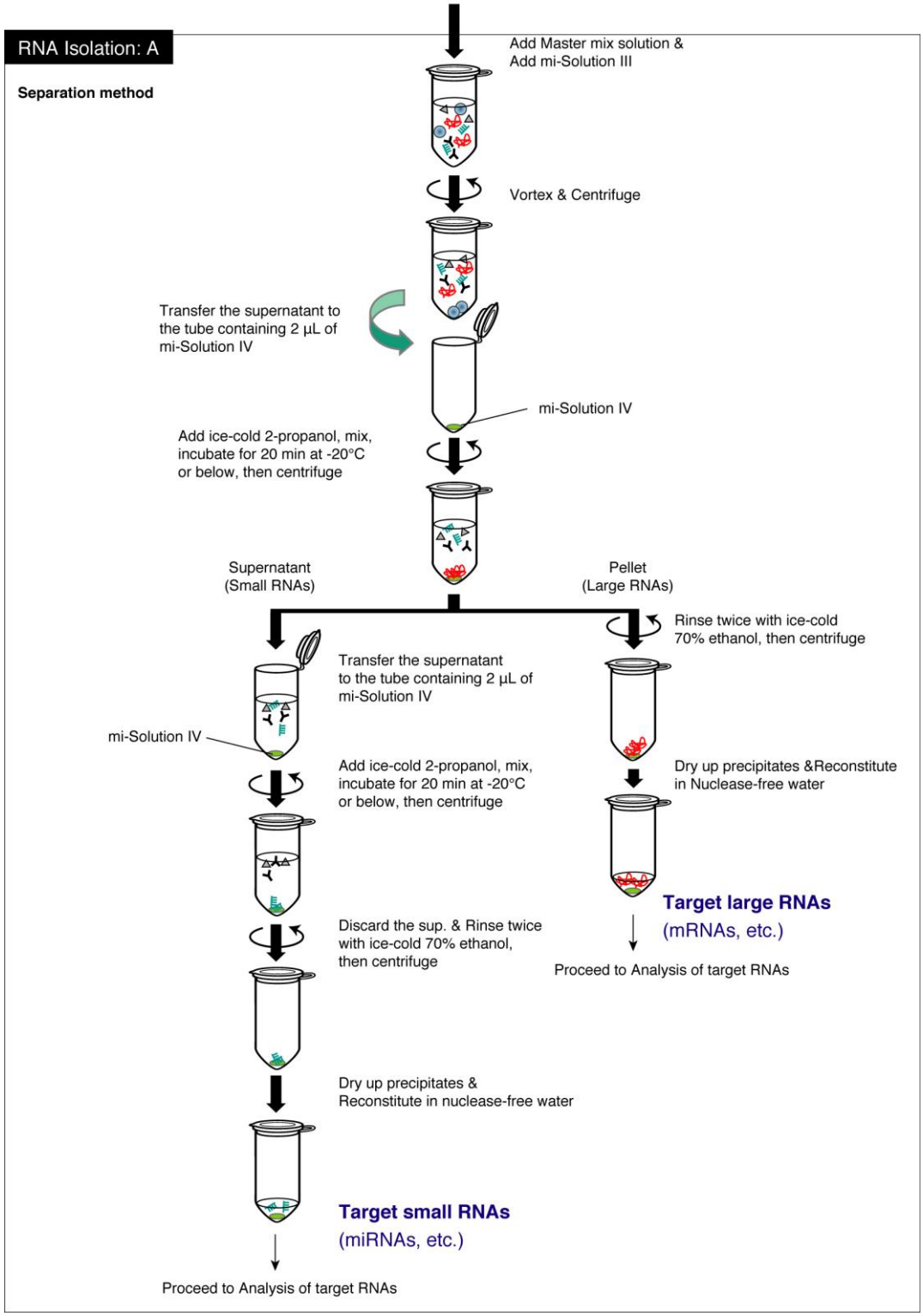
RIP-Assay process by step-cont.



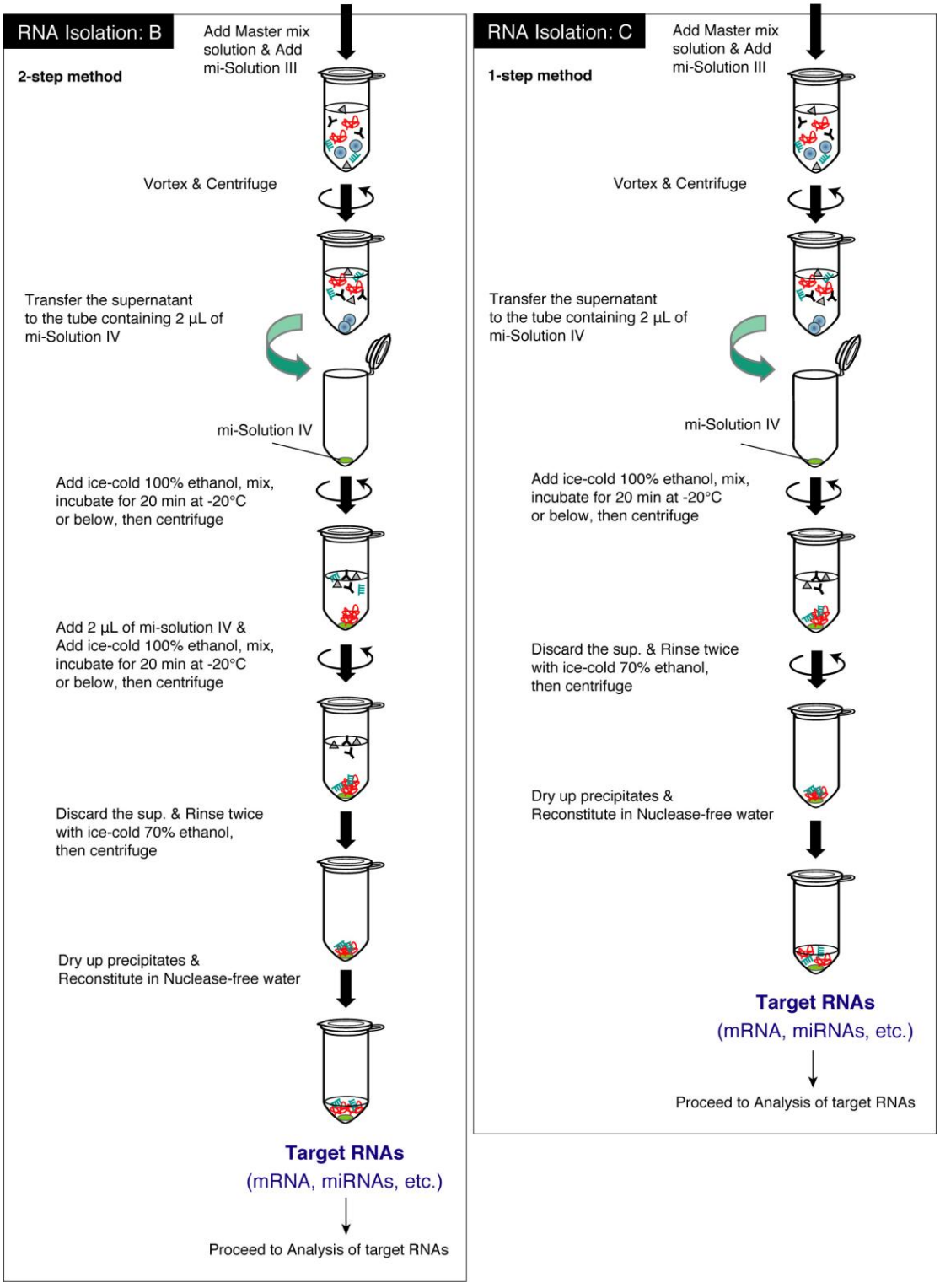
MBL provides three different methods to isolate mRNAs and miRNAs depending on the customer's needs.

Please see "Comparative table of 3 RNA isolation methods" on page 16.

RIP-Assay process by step-cont.



RIP-Assay process by step-cont.



Gel Extraction process

Gel Extraction

Prepare samples for denaturing urea PAGE using the loading buffer

Resolve small RNA on a 10% denaturing Polyacrylamide Gel

50
40
30
20

miSPIKE™

Excise the small RNA fragment from the gel and Place it in the tube

Crush the gel slice into small pieces with a disposable pestle

Add the Gel Extraction Buffer, break up the gel slice with the pestle, and vortex, incubate with rotation, then centrifuge

Transfer the supernatant to the tube containing 2 µL of Solution IV and 1/10 volume of 3 M NaOAc

mi-Solution IV

Add ice-cold 100% ethanol, mix, incubate, then centrifuge

Discard the sup & Rinse twice with ice-cold 70% ethanol, then centrifuge

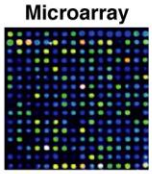
Dry up precipitates & Reconstitute in Nuclease-free water

Target small RNAs
(miRNAs, etc.)

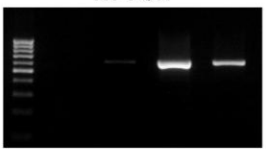
Proceed to Analysis of target RNAs

Analysis of target RNAs

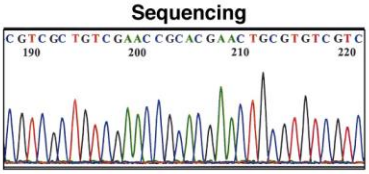
Microarray



RT-PCR



Sequencing



2. Buffer Preparation

1. mi-Lysis Buffer

   Add appropriate concentrations of protease inhibitors, RNase inhibitor, and dithiothreitol (DTT) to mi-Lysis Buffer just before use. mi-Lysis Buffer containing these reagents is described as mi-Lysis Buffer (+) in the following protocols. The final and optimal concentration of each reagent for RIP-Assay is shown in **Appendix**.

2. mi-Wash Buffer

   Add appropriate concentration of dithiothreitol (DTT) to mi-Wash Buffer just before use. mi-Wash Buffer containing DTT is described as mi-Wash Buffer (+) in the following protocols. The final and optimal concentration of the reagent for RIP-Assay is shown in **Appendix**.

(Precaution: Additional Buffer Preparation)

In some cases, both mi-Lysis Buffer (+) and mi-Wash Buffer (+) require the addition of appropriate volumes of High-Salt Solution (in these cases, add 30 µL of High-Salt Solution to each mL of mi-Lysis Buffer and mi-Wash Buffer). Please refer to the datasheet of *RIP-Certified Antibody* (See **Related Products**).

3. Protocols For RNP Immunoprecipitation Assay (RIP-Assay)

The following protocol is for the isolation of RNA from RNP complex expressed in various cells. Expression level of the target RBP may vary. Accordingly, adjust the number of cells used for this assay between 4 million to 20 million per sample.

◆ RNP Immunoprecipitation (RIP)

(A. Pre-step: Preparation of Antibody-immobilized Protein A or Protein G agarose beads)

1. Wash the Protein A or Protein G agarose beads 3 times with equal amount of nuclease-free PBS (centrifuge; 2,000 × *g* for 1 minute at 4ºC).
2. Aliquot 30 µL of the 50% beads slurry to each new microcentrifuge tube.
3. Add 1 mL of mi-Wash Buffer (+) to each tube.
4. Add 15–25 µg of Antibody (Normal Rabbit IgG as a negative control or *RIP-Certified Antibody* for target RBP, respectively) to each tube.
5. Incubate the tube with rotation for at least 30 minutes at 4ºC. If necessary, this incubation can be extended to overnight.

(B. Pre-step: Preparation of Protein A or Protein G agarose beads for preclear)

6. Wash the Protein A or Protein G agarose beads 3 times with equal amount of nuclease-free PBS (centrifuge; 2,000 × *g* for 1 minute at 4ºC).
7. Aliquot 30 µL of the 50% beads slurry to each new microcentrifuge tube.
8. Add 500 µL of mi-Wash Buffer (+) to each tube, and mix briefly.
9. Centrifuge the tube at 2,000 × *g* for 1 minute at 4ºC.
10. Discard the supernatant carefully.
11. Leave the beads at 4ºC or on ice until starting **Preclear step**.
12. Just before **Preclear step**, wash the beads once with 500 µL of mi-Lysis Buffer (+).

13. Centrifuge the tube at 2,000 × *g* for 1 minute at 4ºC.
14. Discard the supernatant carefully. Use these Protein A or Protein G agarose beads washed once with mi-Lysis Buffer (+) for **preclear step** (step 28).

(C. Lysis of Mammalian Cells)

Note: In order to obtain "high-quality RNA", freshly cultured cells should be used in RIP-Assay.

15. Detach the cells from the culture dish by pipetting or using a cell scraper, if necessary. Collect the cell suspension into centrifuge tube.
16. Centrifuge the cell suspension at 300 × *g* for 5 minutes at 4ºC to pellet the cells. Carefully remove and discard the supernatant.
17. Wash the cells by resuspending the cell pellet with ice-cold PBS.
18. Centrifuge the cell suspension at 300 × *g* for 5 minutes at 4ºC to pellet the cells. Carefully remove and discard the supernatant.
19. Wash the cells once again using steps 17–18.
20. Wash the cells by resuspending the cell pellet with ice-cold nuclease-free PBS.
21. Centrifuge the cell suspension at 300 × *g* for 5 minutes at 4ºC to pellet the cells. Carefully remove and discard the supernatant.
22. Wash the cells by resuspending the cell pellet with ice-cold nuclease-free PBS.
23. Aliquot the cell suspension to each new microcentrifuge tube.
24. Centrifuge the cell suspension at 300 × *g* for 5 minutes at 4ºC to pellet the cells. Carefully remove and discard the supernatant.
25. Add 500 µL of mi-Lysis Buffer (+) to each tube containing the cell pellet, and vortex thoroughly.
26. Incubate the tube for 10 minutes at 4ºC or on ice.
27. Centrifuge the cell suspension at 12,000 × *g* for 5 minutes at 4ºC.

(D. Preclear step)

28. Transfer the supernatant (cell lysate) to the tube (prepared in step 14) containing Protein A or Protein G agarose beads washed once with mi-Lysis Buffer (+); that were prepared in steps 6–14.
29. Incubate the tube with rotation for 1 hour at 4ºC.

(E. Washing the Antibody-immobilized Protein A or Protein G agarose beads)

During **Preclear step**, wash once the Antibody-immobilized Protein A or Protein G agarose beads with 1 mL of mi-Lysis Buffer (+).

30. Centrifuge the tube (prepared in step 5) containing Antibody-immobilized Protein A or Protein G agarose beads at 2,000 × *g* for 1 minute at 4ºC.
31. Discard the supernatant carefully.
32. Add 1 mL of mi-Lysis Buffer (+), and mix briefly, then centrifuge the tube at 2,000 × *g* for 1 minute at 4ºC.
33. Discard the supernatant carefully.

(F. Preparation of Antibody-immobilized Protein A or Protein G agarose beads-RNP complex)

34. Centrifuge the tube (prepared in step 29) containing cell lysate and Protein A or Protein G agarose beads at 2,000 × *g* for 1 minute at 4ºC.

Note*: Preparation of Quality Check (QC) sample

In order to confirm whether RIP-Assay is running properly, we recommend to perform a quality check. Collect QC samples and check the protein and RNA expression level at two points: precleared cell lysate (RIP-step F) and post-IP beads (RIP-step G). Use one of the aliquots of precleared cell lysate (Input sample) and Post-IP beads for analysis of RBP expression level by Western blotting. Use the other aliquots of precleared cell lysate for analysis of Total RNA (See **Example of RIP-Assay Results**).

- **Preparation of Input sample (for Western blotting)**
  - i) Add 10 µL of Laemmli's sample buffer to 10 µL of precleared cell lysate, boil for 3–5 minutes, mix well, and centrifuge.
  - ii) Resolve 20 µL of the prepared sample on SDS-PAGE, and proceed to Western blotting analysis.
- **Preparation of Total RNA (for quality check of Total RNA)**
  - i) Place 10 µL of precleared cell lysate at -80ºC until starting RNA isolation.
  - ii) After RNP immunoprecipitation, use the lysate to prepare Total RNA sample according to **RNA Isolation protocol** (See below).

35. Transfer 500 µL of the precleared cell lysate to the tube (prepared in step 33) containing Antibody-immobilized Protein A or Protein G agarose beads washed once with mi-Lysis Buffer (+); that were prepared in steps 30–33.
36. Incubate the tube with rotation for 3 hours at 4ºC.

(G. Wash of Antibody-immobilized Protein A or Protein G agarose beads-RNP complex)

37. Centrifuge the tube (prepared in step 36) containing Antibody-immobilized Protein A or Protein G agarose beads-RNP complex at 2,000 × *g* for 1 minute at 4ºC.
38. Discard the supernatant carefully.
39. Add 1 mL of mi-Wash Buffer (+), mix briefly, and centrifuge the tube at 2,000 × *g* for 1 minute at 4ºC.
40. Discard the supernatant carefully.
41. Wash the Antibody-immobilized beads-RNP complex twice using steps 39–40.
42. For fourth wash, add 1 mL of mi-Wash Buffer (+), then mix well and dispense 100 µL of the mixture to new microcentrifuge tube for QC sample (post-IP beads). Use those aliquots for quality check by Western blotting (See **Example of RIP-Assay Results**).

Note*: Preparation of QC sample (for post-IP beads)

- **Preparation of post-IP beads sample (for Western blotting)**
  - i) Centrifuge the tube containing 100 µL of the mixture at 2,000 × *g* for 1 minute at 4ºC.
  - ii) Discard the supernatant carefully.
  - iii) Resuspend the precipitated beads in 20 µL of Laemmli's sample buffer, boil for 3–5 minutes, mix well and centrifuge the tube at 2,000 × *g* for 1 minute.

iv) Resolve 20 µL of the prepared sample on SDS-PAGE, and proceed to Western blotting analysis.

43. Centrifuge the tube containing Antibody-immobilized Protein A or Protein G agarose beads-RNP complex at 2,000 × *g* for 1 minute at 4ºC.
44. Discard the supernatant carefully.
45. Proceed to **RNA Isolation** (See below).

◆ RNA Isolation

(from Antibody-immobilized Protein A or Protein G agarose beads-RNP complex)

Solution II and Solution III should be equilibrated to room temperature before use.

Reagents should be briefly but thoroughly mixed before use.

§ **Please use one of the three methods described bellow.**

A. Separation method:

Large RNAs and small RNAs are divided into individual microcentrifuge tubes. By this method, RNAs are split into large and small RNAs based on their length. This method is recommended for predicting analysis of interaction sites between miRNA and target mRNA.

B. 2-step method:

Both large RNAs and small RNAs are simultaneously isolated into one microcentrifuge tube. The advantage of this method is that the recovery rates for both RNAs are higher than the other 2 methods. Please note that the RNAs isolated by this method are not suitable for visualization by silver staining following denaturing urea PAGE because of high background.

C. 1-step method:

This is a simplified method for isolating small RNAs, but not suitable for isolating large RNAs because the recovery for large RNAs is inefficient. The advantage of this method is that the time required for RNA isolation is short compared with the other 2 methods. Please note that RNAs isolated by this method are mainly small RNAs, while co-purification of large RNAs is observed (~40% of large RNAs).

§ **Comparative table of 3 RNA isolation methods**

	Separation method	**2-step method**	**1-step method**
Collectable RNA species	large RNA small RNA (in individual tubes)	large RNA small RNA (in one tube)	small RNA (a small amount of large RNA)
Recovery rate for large RNA	>90%	>90%	<40%
Recovery rate for small RNA	>80%	>90%	>90%
Classification by nucleotide length	Yes (large RNA: >60-80 nt) (small RNA: <60-80 nt)	No	No
Assay time	75 min.	75 min.	45 min.
Background (silver staining)	Low	High	Moderate
Advantage	Available for multiple applications	High-recovery rate for large/small RNA	Short assay time
Disadvantage	A little loss in recovery of small RNA compared to the other 2 methods	Not suitable for visualization by silver staining following denaturing PAGE	Low-recovery rate for large RNA (~40% of large RNAs)

The data described above represents a typical result obtained from the following three protocols when using a mixture of four synthetic miRNAs or total RNA sample containing mainly large RNAs. Result may vary depending on the samples and experimental conditions.

A. Separation method

1. Prepare Master mix solution by diluting 10 μL of mi-Solution I with 240 μL of mi-Solution II per sample.
2. Dispense 2 μL of mi-Solution IV to each new microcentrifuge tube for step 5.
3. Add 250 μL of Master mix solution to each tube (prepared in RIP-step 44) containing Antibody-immobilized Protein A or Protein G agarose beads-RNP complex (obtained in previous **RNP Immunoprecipitation**), vortex thoroughly, then spin-down.
4. Add 150 μL of mi-Solution III to each tube, vortex thoroughly, then centrifuge the tube at 2,000 × *g* for 2 minutes at room temperature.
5. Carefully transfer the supernatant to the tube containing 2 μL of mi-Solution IV prepared in step 2. (Avoid to remove the Protein A or Protein G agarose beads from the pellet. Contamination of the beads may affect following steps.)
6. Add 300 μL of ice-cold 2-propanol to each tube, vortex briefly but thoroughly, then spin-down.
7. Incubate the tube at -20ºC or below for 20 minutes (or for overnight, if necessary). During incubation, dispense 2 μL of mi-Solution IV to each new microcentrifuge tube for step 9.
8. Centrifuge the tube at 12,000 × *g* for 10 minutes at 4ºC. At this point, the pellet is mainly composed of large RNAs, while small RNAs remain in the supernatant.
9. Transfer the supernatant, which contains small RNAs, to the tube containing 2 μL of mi-Solution IV prepared in step 7. Isolation method for small RNAs from the supernatant is described in the following steps 10–18.
   In case of purification of large RNAs in the pellet, skip to step 13.

   ✧ **Additional protocol: isolation for small RNAs**

   10. Add 500 μL of ice-cold 2-propanol to the supernatant containing small RNAs prepared in step 9, vortex briefly but thoroughly, then spin-down.
   11. Incubate the tube at -20ºC or below for 20 minutes (or for overnight, if necessary).
   12. Centrifuge the tube at 12,000 × *g* for 10 minutes at 4ºC, then aspirate the supernatant carefully.
13. Rinse the pellet with 500 μL of ice-cold 70% ethanol, and mix briefly.
14. Centrifuge the tube at 12,000 × *g* for 3 minutes at 4ºC, then aspirate the supernatant carefully.
15. Rinse the pellet once again using steps 13–14.
16. Dry up the pellet by aspirating excess ethanol followed by evaporation for 5–15 minutes at room temperature. Avoid RNase contamination. (Evaporation in clean bench is recommended.)
17. Reconstitute the pellet containing large RNAs in 20 μL of nuclease-free water and the pellet containing small RNAs in 10 μL of nuclease-free water.
18. Store at -80ºC until starting following analysis.

In order to obtain QC sample, isolate Total RNA by using 10 μL of precleared cell lysate (prepared in RIP-step 34) and isolate the RNA following steps 1–18 above.

B. 2-step method

1. Prepare Master mix solution by diluting 10 µL of mi-Solution I with 240 µL of mi-Solution II per sample.
2. Dispense 2 µL of mi-Solution IV to each new microcentrifuge tube for step 5.
3. Add 250 µL of Master mix solution to each tube (prepared in RIP-step 44) containing Antibody-immobilized Protein A or Protein G agarose beads-RNP complex (obtained in previous **RNP Immunoprecipitation**), vortex thoroughly, then spin-down.
4. Add 150 µL of mi-Solution III to each tube, vortex thoroughly, then centrifuge the tube at 2,000 × *g* for 2 minutes at room temperature.
5. Carefully transfer the supernatant to the tube containing 2 µL of mi-Solution IV prepared in step 2. (Avoid to remove the Protein A or Protein G agarose beads from the pellet. Contamination of the beads may affect following steps.)
6. Add 400 µL of ice-cold 100% ethanol to each tube, vortex briefly but thoroughly, then spin-down.
7. Incubate the tube at -20ºC or below for 20 minutes (or for overnight, if necessary).
8. Centrifuge the tube at 12,000 × *g* for 10 minutes at 4ºC, then add 2 µL of mi-Solution IV to the supernatant in the same tube.
9. Add 400 µL of 100% ethanol to each tube, vortex briefly but thoroughly, then spin-down.
10. Incubate the tube at -20ºC or below for 20 minutes (or for overnight, if necessary).
11. Centrifuge the tube at 12,000 × *g* for 10 minutes at 4ºC, then aspirate the supernatant carefully.
12. Rinse the pellet with 500 µL of ice-cold 70% ethanol, and mix briefly.
13. Centrifuge the tube at 12,000 × *g* for 3 minutes at 4ºC, then aspirate the supernatant carefully.
14. Rinse the pellet once again using steps 12–13.
15. Dry up the pellet by aspirating excess ethanol followed by evaporation for 5–15 minutes at room temperature. Avoid RNase contamination. (Evaporation in clean bench is recommended.)
16. Reconstitute the pellet in 10 µL of nuclease-free water.
17. Store at -80ºC until starting following analysis.

In order to obtain QC sample, isolate Total RNA by using 10 µL of precleared cell lysate (prepared in RIP-step 34) and isolate the RNA following steps 1–17 above.

C. 1-step method

1. Prepare Master mix solution by diluting 10 µL of mi-Solution I with 240 µL of mi-Solution II per sample.
2. Dispense 2 µL of mi-Solution IV to each new microcentrifuge tube for step 5.
3. Add 250 µL of Master mix solution to each tube (prepared in RIP-step 44) containing Antibody-immobilized Protein A or Protein G agarose beads-RNP complex (obtained in previous **RNP Immunoprecipitation**), vortex thoroughly, then spin-down.
4. Add 150 µL of mi-Solution III to each tube, vortex thoroughly, then centrifuge the tube at 2,000 × *g* for 2 minutes at room temperature.
5. Carefully transfer the supernatant to the tube containing 2 µL of mi-Solution IV prepared in step 2. (Avoid to remove the Protein A or Protein G agarose beads from the pellet. Contamination of the beads may affect following steps.)
6. Add 800 µL of ice-cold 100% ethanol to each tube, vortex briefly but thoroughly, then spin-down.
7. Incubate the tube at -20ºC or below for 20 minutes (or for overnight, if necessary).
8. Centrifuge the tube at 12,000 × *g* for 10 minutes at 4ºC, then aspirate the supernatant carefully.
9. Rinse the pellet with 500 µL of ice-cold 70% ethanol, and mix briefly.
10. Centrifuge the tube at 12,000 × *g* for 3 minutes at 4ºC, then aspirate the supernatant carefully.
11. Rinse the pellet once again using steps 9–10.
12. Dry up the pellet by aspirating excess ethanol followed by evaporation for 5–15 minutes at room temperature. Avoid RNase contamination. (Evaporation in clean bench is recommended.)
13. Reconstitute the pellet in 10 µL of nuclease-free water.
14. Store at -80ºC until starting following analysis.

In order to obtain QC sample, isolate Total RNA by using 10 µL of precleared cell lysate (prepared in RIP-step 34) and isolate the RNA following steps 1–14 above.

Additional Procedure: Analysis of isolated RNA

We recommend qualitative and quantitative analysis of isolated RNAs prior to downstream analysis such as RT-PCR, microarray and sequencing. These technologies may be useful for profiling RNAs in the target mRNP complex.

- **Quality control for large RNAs**

  Quantify the isolated large RNAs with NanoDrop (Thermo Fisher Scientific Inc.), and characterize the RNAs with Bioanalyzer (Agilent Technologies, Inc.). It is very important for comprehensive analysis such as microarray to retain high-quality RNA because experimental results may be sensitive to RNA quality.

- **Quality control for small RNAs**

  Quantification of isolated small RNAs with a spectrophotometer is not recommended because absorbance at 260 nm is not measurable in most cases. We recommend visualization of small RNAs by staining method following denaturing urea polyacrylamide gel electrophoresis (PAGE). After visualization, to obtain the enriched small RNA fraction for following analysis, cut out the polyacrylamide gel corresponding to small RNA fragment and recover the RNA from the gel slice. RNA recovery method is shown in **Gel Extraction** (See below).

4. Gel Extraction

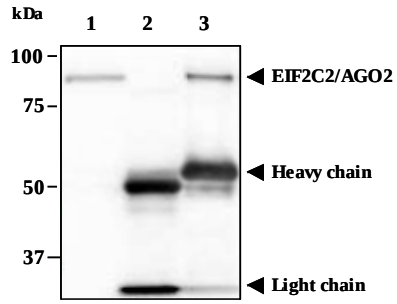
This protocol is designed based on miRCat™ (IDT, Inc.) with some modifications. Please refer to the datasheet of miRCat™ if necessary.

1. Prepare the loading sample by mixing 8 μL of RNA solution with 12 μL of loading buffer for denaturing PAGE. In addition, prepare the size control by diluting 1 μL of reconstituted miSPIKE™ solution with 10 μL of the loading buffer.
2. Incubate the sample at 65ºC for 10 minutes, then quench the sample at 4ºC or on ice for 5 minutes.
3. Resolve both the prepared RNA sample and diluted miSPIKE™ on a 10% polyacrilamide gel with 7 M urea.
4. Stain the gel with GelStar® Nucleic Acid Stain (Takara Bio, Inc.) according to manufacturer's instructions and visualize the RNA fragment under medium wavelength of UV illumination (around 310 nm).
   Please note that small RNA fragment may be invisible when RNA quantity is too low.
5. Select small RNA fragment by utilizing miSPIKE™ as an indicator, and then excise the fragment from the gel.
6. Place the gel slice in a new microcentrifuge tube and crush it with a disposable pestle.
7. Add 500 μL of Gel Extraction Buffer and break up the gel slice with a disposable pestle as small as possible, then vortex thoroughly, incubate at 4ºC for 10 minutes with rotating. During incubation, dispense both 2 μL of mi-Solution IV and 40 μL of 3 M NaOAc to a new microcentrifuge tubes for step 9.
8. Vortex thoroughly, then centrifuge the tube at 2,000 × *g* for 5 minutes.
9. Carefully transfer the supernatant (about 400 μL) to the tube containing 2 μL of mi-Solution IV and 40 μL of 3 M NaOAc prepared in step 7.
10. Add 800 μL of ice-cold 100% ethanol to each tube, vortex briefly but thoroughly, then spin-down.
11. Incubate the tube at -20ºC or below for 20 minutes (or for overnight, if necessary).
12. Centrifuge the tube at 12,000 × *g* for 10 minutes at 4ºC, then aspirate the supernatant carefully.
13. Rinse the pellet with 500 μL of ice-cold 70% ethanol, and mix briefly.
14. Centrifuge the tube at 12,000 × *g* for 3 minutes at 4ºC, then aspirate the supernatant carefully.
15. Rinse the pellet once again using steps 13–14.
16. Dry up the pellet by aspirating excess ethanol followed by evaporation for 5–15 minutes at room temperature. Avoid RNase contamination. (Evaporation in clean bench is recommended.)
17. Reconstitute the pellet in 10 μL of nuclease-free water.
18. Store at -80ºC until starting following analysis.

III. Example of RIP-Assay Results

1. RIP-Assay Results for EIF2C2/AGO2

A. Quality check: Analysis of RBP expression level by Western blotting.

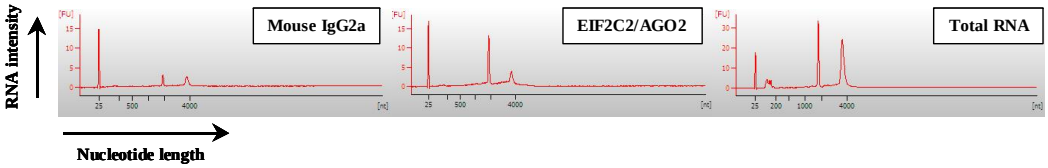


Lane 1: Input sample
Lane 2: post-IP beads of Mouse IgG2a Isotype control
Lane 3: post-IP beads of Anti-EIF2C2/AGO2 monoclonal antibody (Code No. RN003M)

Western blotting (IB: *Anti-EIF2C2/AGO2 monoclonal antibody*, Code No. RN003M)

Quality check of immunoprecipitated endogenous EIF2C2/AGO2 expressed in Jurkat cells. 10 µL of precleared cell lysate (Input sample, Protocol step 34) contained detectable level of target RBP (EIF2C2/AGO2) (Lane 1). The RNP complex was successfully concentrated by RIP-Assay because no EIF2C2/AGO2 was detected in the post-IP beads coated with Mouse IgG2a Isotype control (Code No. M076-3), but EIF2C2/AGO2 was detected in the post-IP beads coated with anti-EIF2C2/AGO2 monoclonal antibody (lanes 2 and 3, respectively).

B. Quality check: Characterization of isolated RNA with Bioanalyzer.



Characterization of isolated RNA with Bioanalyzer

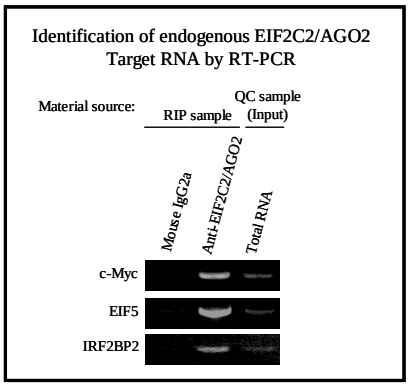
Cellular EIF2C2/AGO2-associated RNA in Jurkat cells was isolated by *RIP-Assay Kit for microRNA* and *RIP-Certified Anti-EIF2C2/AGO2 monoclonal antibody* (Code No. RN003M). Endogenous EIF2C2/AGO2-associated RNA was analyzed on a Bioanalyzer RNA pico chip (Agilent Technologies, Inc.) according to manufacturer's instructions. RNA isolated from the EIF2C2/AGO2 complex containing mRNP showed a different migration profile compared with that isolated from the Mouse IgG2a Isotype control complex (negative control). Total RNA was also isolated from Jurkat cells. The migration profile of the Total RNA sample showed 2 main peaks at around 2,000 and 4,000 nucleotides corresponding to 18S and 28S ribosomal RNA, respectively.

Note:

If an RNA component of the target mRNP is known, it is recommended to analyze the RNA by RT-PCR to determine whether the target mRNAs have been immunoprecipitated into the post-RIP samples. Migration pattern of the isolated large RNAs on a Bioanalyzer may look similar between the post-RIP sample isolated by anti-EIF2C2/AGO2 monoclonal antibody and the control sample (isolated by Mouse IgG2a Isotype control), since the concentration of isolated mRNAs in post-RIP sample may be

low.

C. Identification of target RNAs isolated from cellular RNP complex by RT-PCR.



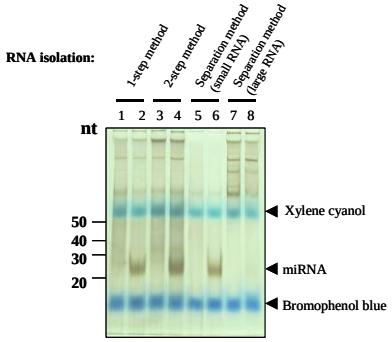
Identification of endogenous EIF2C2/AGO2 target RNA by RT-PCR

The association of endogenous EIF2C2/AGO2 with endogenous target mRNAs (in this case, c-Myc, EIF5, IRF2BP2) in Jurkat cells was tested by RIP-Assay, followed by detection of the target transcripts of interest by RT-PCR of RIP materials. PCR products were visualized by electrophoresis in ethidium bromide-stained 2% agarose gels to ensure correct size. RT-PCR was performed using the large RNAs isolated by **Separation method**.

Identification of target RNA isolated from cellular EIF2C2/AGO2 containing mRNPs by RT-PCR
Cellular EIF2C2/AGO2-associated RNA in Jurkat cells was isolated with *RIP-Assay Kit for microRNA* and *RIP-Certified Anti-EIF2C2/AGO2 monoclonal antibody* (Code No. RN003M). An equal amount of Mouse IgG2a Isotype control (Code No. M076-3) was used as a negative control. RNA in the RIP products was analyzed for the presence of specific target mRNA by RT-PCR using gene-specific primer pairs. Compared with Mouse IgG2a Isotype control, the expression levels of the EIF2C2/AGO2-target mRNAs: c-Myc, EIF5, and IRF2BP2 in the anti-EIF2C2/AGO2 monoclonal antibody-immunoprecipitates were enriched.

D. Analysis of isolated small RNA by silver staining and sequencing.

a). Visualization of isolated small RNA by silver staining following denaturing PAGE.

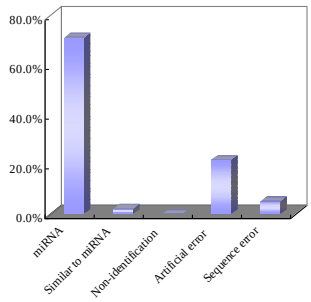


Lane 1, 3, 5, 7 : post-RIP sample of Mouse IgG2a Isotype control
Lane 2, 4, 6, 8 : post-RIP sample Anti-EIF2C2/AGO2 monoclonal antibody (Code No. RN003M)

Visualization of isolated RNA by silver staining
Cellular EIF2C2/AGO2-associated RNA in Jurkat cells was isolated with *RIP-Assay Kit for microRNA* and *RIP-Certified Anti-EIF2C2/AGO2 monoclonal antibody* (Code No. RN003M). Endogenous EIF2C2/AGO2-associated RNA was visualized by silver staining following 10% denaturing PAGE. The band at 20-30 nt corresponding to functional small non-coding RNA such as miRNA was detected in the post-RIP beads coated with anti-EIF2C2/AGO2 monoclonal antibody (lanes 2, 4, 6, and 8), whereas no band was detected in the post-RIP beads coated with Mouse IgG2a Isotype control (Code No. M076-3) (lanes 1, 3, 5, and 7).

Recommendation*: RNA isolation according to **Separation method** is suitable for visualization by silver staining because of low-background (lane 6 compared with lanes 2 and 4).
The following data were obtained by **Separation method.**

b). Identification of target miRNA isolated from cellular RNP complex by sequencing.



Analysis of isolated small RNA by sequencing.

Cellular EIF2C2/AGO2-associated RNA in Jurkat cells was isolated with *RIP-Assay Kit for microRNA* and *RIP-Certified Anti-EIF2C2/AGO2 monoclonal antibody* (Code No. RN003M). Cloning of small RNAs were carried out by the miRCat™ (IDT, Inc.) according to manufacturer's instructions with some modifications.

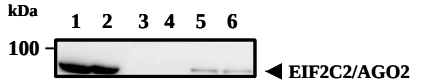
A total of 96 clones were sequenced with an ABI 3130/Genetic Analyzer (Life Technologies Inc.).

Analysis of cloned small RNA by sequencing

68 potential miRNA were identified by BLAST search against miRBase (coverage >90%, identity >90%). 2 clones were similar to known miRNAs (coverage >80%, identity >80%). Non-identification was not observed in this experiment, and artificial errors such as PCR error were evident in 21 clones. Sequencing errors were evident in 5 clones (coverage <50%, identity <50%). Non-identification may include *de novo* sequence information because the nature of RNA is not completely elucidated.

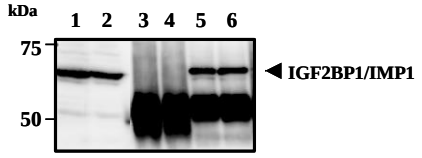
2. RIP-Assay Results for IGF2BP1/IMP1

A. Quality check: Analysis of RBP expression level by Western blotting.



IB: anti-EIF2C2/AGO2 monoclonal antibody (Code No. RN003M)

Lane 1, 2: Input sample
Lane 3, 4: post-IP beads of Normal Rabbit IgG
Lane 5, 6: post-IP beads of Anti-IGF2BP1/IMP1 polyclonal antibody (Code No. RN007P)



IB: anti-IGF2BP1/IMP1 polyclonal antibody (Code No. RN007P)

Lane 1, 2: Input sample
Lane 3, 4: post-IP beads of Normal Rabbit IgG
Lane 5, 6: post-IP beads of Anti-IGF2BP1/IMP1 polyclonal antibody (Code No. RN007P)

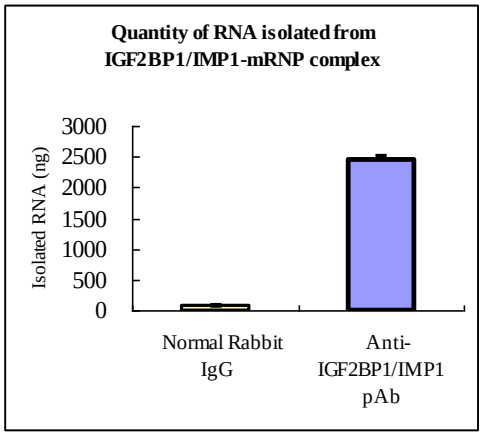
Western blotting

(IB: anti-IGF2BP1/IMP1 pAb (lower panel) or anti-EIF2C2/AGO2 mAb (upper panel))

Quality check of immunoprecipitated endogenous IGF2BP1/IMP1 expressed in K562 cells. 10 µL of precleared cell lysate (Input sample, Protocol step 34) contained detectable level of target RBP (IGF2BP1/IMP1) (lanes 1 and 2). The RNP complex was successfully enriched by RIP-Assay because IGF2BP1/IMP1 was detected in the post-IP beads coated with anti-IGF2BP1/IMP1 polyclonal antibody (lanes 5 and 6) while no IGF2BP1/IMP1 was detected in the post-IP beads coated with Normal Rabbit IgG (lanes 3 and 4).

In addition, EIF2C2/AGO2 was found to co-immunoprecipitate with IGF2BP1/IMP1, suggesting that IGF2BP1/IMP1 may interact with EIF2C2/AGO2 through mRNA.

B. Quality check: Quantification of isolated RNA with NanoDrop.



Average quantity of isolated RNA (n=2)

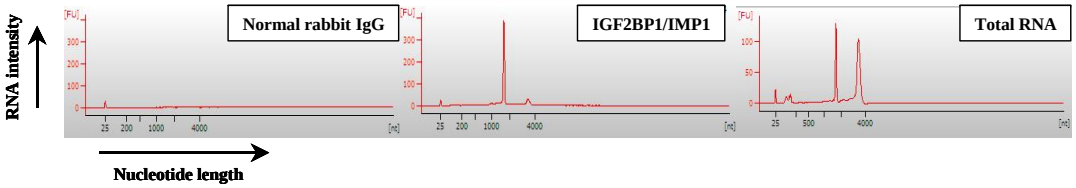
Antibody	RNA (ng)
Normal Rabbit IgG	71.4
Anti-IGF2BP1/IMP1 pAb	2453.8
Total RNA	262520

Note: Quantity of RNA was calculated based on volume ratio used for RNA isolation. Quantity of Total RNA represents whole amount of RNA in precleared cell lysate.

Quantification of isolated RNA with NanoDrop

The RNA isolated from the endogenous IGF2BP1/IMP1-mRNA complex expressed in K562 cells was quantified with a spectrophotometer (NanoDrop) according to manufacturer's instructions (Thermo Fisher Scientific Inc.). In comparison with the quantity of RNA isolated from the Normal Rabbit IgG complexes (negative control), the RNA obtained from the anti-IGF2BP1/IMP1 polyclonal antibody-immunoprecipitates was significantly enriched.

C. Quality check: Characterization of isolated RNA with Bioanalyzer.

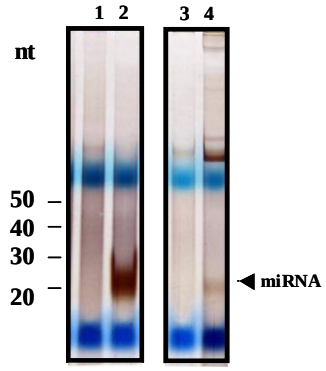


Characterization of isolated RNA with Bioanalyzer

Cellular IGF2BP1/IMP1-associated RNA in K562 cells was isolated by *RIP-Assay Kit for microRNA* and *RIP-Certified Anti-IGF2BP1/IMP1 polyclonal antibody* (Code No. RN007P). Endogenous IGF2BP1/IMP1-associated RNA was analyzed on a Bioanalyzer RNA pico chip (Agilent Technologies, Inc.) according to manufacturer's instructions. RNA isolated from the IGF2BP1/IMP1 complex containing mRNP showed a different migration profile compared with that isolated from the Normal Rabbit IgG complex (negative control). Total RNA was also isolated from K562 cells. The migration profile of the Total RNA sample showed 2 main peaks at around 2,000 and 4,000 nucleotides corresponding to 18S and 28S ribosomal RNA, respectively.

D. Analysis of isolated small RNA by silver staining and sequencing.

a) Visualization of isolated small RNA by silver staining following denaturing PAGE.



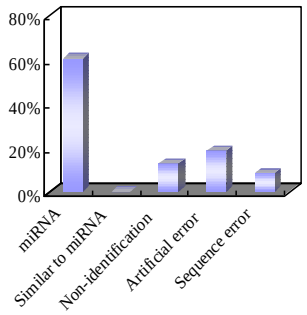
Lane 1: post-RIP sample of Mouse IgG2a Isotype control
Lane 2: post-RIP sample of Anti-EIF2C2/AGO2 monoclonal antibody (Code No. RN003M)
Lane 3: post-RIP sample of Normal Rabbit IgG
Lane 4: post-RIP sample of Anti-IGF2BP1/IMP1 polyclonal antibody (Code No. RN007P)

RNA isolation for small RNA: Separation method

Visualization of isolated RNA by silver staining

Cellular IGF2BP1/IMP1-associated RNA in K562 cells was isolated by *RIP-Assay Kit for microRNA* and *RIP-Certified Anti-IGF2BP1/IMP1 polyclonal antibody* (Code No. RN007P). Endogenous IGF2BP1/IMP1-associated RNA was visualized by silver staining following 10% denaturing PAGE. The band at around 20-25 nt corresponding to functional small non-coding RNA such as miRNA was detected in the post-RIP beads coated with anti-IGF2BP1/IMP1 polyclonal antibody, whereas no band was detected in the post-RIP beads coated with Normal Rabbit IgG (lanes 3 and 4).

b). Identification of target miRNA isolated from cellular RNP complex by sequencing.



Analysis of isolated small RNA by sequencing.

Cellular IGF2BP1-associated RNA in K562 cells was isolated by *RIP-Assay Kit for microRNA* and *RIP-Certified Anti-IGF2BP1 polyclonal antibody* (Code No. RN007P). Cloning of small RNAs were carried out by the miRCat™ (IDT, Inc.) according to manufacturer's instructions with some modifications.

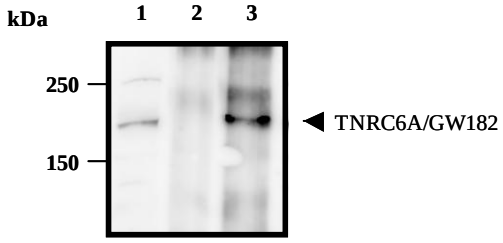
A total of 48 clones were sequenced with an ABI 3130/Genetic Analyzer (Life Technologies Inc.).

Analysis of cloned small RNA by sequencing

As a result of sequencing analysis, 29 potential miRNA were identified by BLAST search against miRBase (coverage >90%, identity >90%). 6 clones were not identified, and artificial errors such as PCR error were evident in 9 clones. Sequencing errors were evident in 4 clones (coverage <50%, identity <50%). Non-identification may include *de novo* sequence information because the nature of RNA is not completely elucidated.

3. RIP-Assay Results for TNRC6A/GW182

A. Quality check: Analysis of RBP expression level by Western blotting.

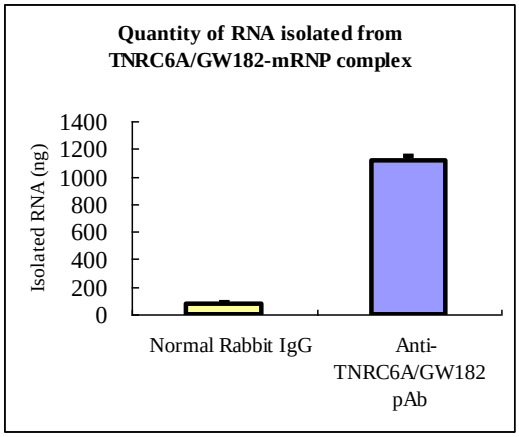


Lane 1: Input sample
Lane 2: post-IP beads of Normal Rabbit IgG
Lane 3: post-IP beads of Anti-TNRC6A/GW182 (Code No. RN033P)

Western blotting (IB: anti-TNRC6A/GW182 polyclonal antibody, Code No. RN033P)

Quality check of immunoprecipitated endogenous TNRC6A/GW182 expressed in K562 cells. 10 μL of precleared cell lysate (Input sample, Protocol step 34) contained detectable level of target RBP (TNRC6A/GW182) (lane 1). The RNP complex was successfully concentrated by RIP-Assay because no TNRC6A/GW182 was detected in the post-IP beads coated with Normal Rabbit IgG, but TNRC6A/GW182 was detected in post-IP beads coated with anti-TNRC6A/GW182 polyclonal antibody (lanes 2 and 3, respectively).

B. Quality check: Quantification of isolated RNA with NanoDrop.



Average quantity of isolated RNA (n=2)

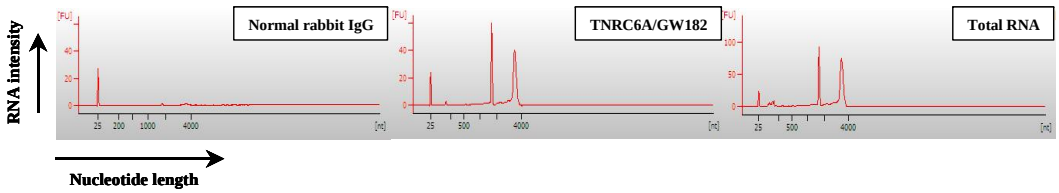
Antibody	RNA (ng)
Normal Rabbit IgG	71.4
Anti-TNRC6A/GW182 pAb	1144.5
Total RNA	262520

Note: Quantity of RNA was calculated based on volume ratio used for RNA isolation. Quantity of Total RNA represents whole amount of RNA in precleared cell lysate.

Quantification of isolated RNA with NanoDrop

RNA isolated from endogenous TNRC6A/GW182-mRNA complex expressed in Jurkat cells was quantified with spectrophotometer (NanoDrop) according to manufacture's instructions (Thermo Fisher Scientific Inc.). Compared with Normal Rabbit IgG (negative control), isolated RNA from anti-TNRC6A/GW182 polyclonal antibody-immunoprecipitates was significantly enriched.

C. Quality check: Characterization of isolated RNA with Bioanalyzer.

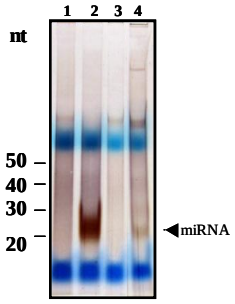


Characterization of isolated RNA with Bioanalyzer

Cellular TNRC6A/GW182-associated RNA in K562 cells was isolated by *RIP-Assay Kit for microRNA* and *RIP-Certified Anti-TNRC6A/GW182 polyclonal antibody* (Code No. RN033P). Endogenous TNRC6A/GW182-associated RNA was analyzed on a Bioanalyzer RNA pico chip (Agilent Technologies, Inc.) according to manufacturer's instructions. RNA isolated from the TNRC6A/GW182 complex containing mRNP showed a different migration profile compared with that isolated from the Normal Rabbit IgG complex (negative control). Total RNA was also isolated from K562 cells. The migration profile of the Total RNA sample showed 2 main peaks at around 2,000 and 4,000 nucleotides corresponding to 18S and 28S ribosomal RNA, respectively.

D. Analysis of isolated small RNA by silver staining and sequencing.

a) Visualization of isolated small RNA by silver staining following denaturing PAGE.



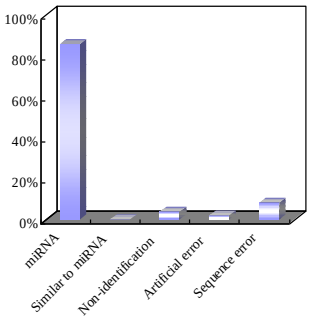
Lane1: post-RIP sample of Mouse IgG2a Isotype control
Lane2: post-RIP sample of Anti-EIF2C2/AGO2 monoclonal antibody (Code No. RN003M)
Lane3: post-RIP sample of Normal Rabbit IgG
Lane4: post-RIP sample of Anti-TNRC6A/GW182 polyclonal antibody (Code No. RN033P)

RNA isolation for small RNA: Separation method

Visualization of isolated RNA by silver staining

Cellular TNRC6A/GW182-associated RNA in K562 cells was isolated by *RIP-Assay Kit for microRNA* and *RIP-Certified Anti-TNRC6A/GW182 polyclonal antibody* (Code No. RN033P). Endogenous TNRC6A/GW182-associated RNA was visualized by silver staining following 10% denaturing PAGE. The band at around 20-25 nt corresponding to functional small non-coding RNA such as miRNA was detected in the post-RIP beads coated with anti-TNRC6A/GW182 polyclonal antibody, whereas no band was detected in the post-RIP beads coated with Normal Rabbit IgG (lanes 3 and 4).

b) Identification of target miRNA isolated from cellular RNP complex by sequencing.



Analysis of isolated small RNA by sequencing.

Cellular TNRC6A/GW182-associated RNA in K562 cells was isolated by *RIP-Assay Kit for microRNA* and *RIP-Certified Anti-TNRC6A/GW182 polyclonal antibody* (Code No. RN033P). Cloning of small RNAs were carried out by the miRCat™ (IDT, Inc.) according to manufacturer's instructions with some modifications.

A total of 48 clones were sequenced with an ABI 3130/Genetic Analyzer (Life Technologies Inc.).

Analysis of cloned small RNA by sequencing

As a result of sequencing analysis, 41 potential miRNA were identified by BLAST search against miRBase (coverage >90%, identity >90%). 2 clones were not identified, and artificial errors such as PCR error were evident in 1 clone. Sequencing errors were evident in 4 clones (coverage <50%, identity <50%). Non-identification may include *de novo* sequence information because the nature of RNA is not completely elucidated.

IV. Related Products

RIP-Certified Antibody

RN001P	Anti-EIF4E (polyclonal)
RN002P	Anti-EIF4G1 (polyclonal)
RN003M	Anti-EIF2C2/AGO2 (monoclonal)
RN004P	Anti-ELAVL1/HuR (polyclonal)
RN007P	Anti-IGF2BP1/IMP1 (polyclonal)
RN015P	Anti-YBX1 (polyclonal)
RN033P	Anti-TNRC6A/GW182 (polyclonal)

Other RIP-Certified Antibodies are also available.
Please visit our website at https://ruo.mbl.co.jp/je/rip-assay/

Isotype Control Antibody

Various isotype control antibodies for mouse and rat are available.
Please visit our website at https://ruo.mbl.co.jp/je/rip-assay/

RIP-Assay Starter Kit

RIP-Assay Starter Kit contains 40 μg of RIP-Certified Antibody and RIP-Assay Kit.

RN001PK	RIP-Assay Starter Kit EIF4E (polyclonal)
RN002PK	RIP-Assay Starter Kit EIF4G1 (polyclonal)
RN003PK	RIP-Assay Starter Kit EIF4G2 (polyclonal)
RN004PK	RIP-Assay Starter Kit ELAVL1/HuR (polyclonal)
RN005PK	RIP-Assay Starter Kit ELAVL2/HuB (polyclonal)
RN006PK	RIP-Assay Starter Kit ELAVL3/HuC (polyclonal)
RN007PK	RIP-Assay Starter Kit IGF2BP1/IMP1 (polyclonal)
RN008PK	RIP-Assay Starter Kit IGF2BP2/IMP2 (polyclonal)
RN009PK	RIP-Assay Starter Kit IGF2BP3/IMP3 (polyclonal)
RN010PK	RIP-Assay Starter Kit MSI1/Musashi1 (polyclonal)

Other RIP-Assay Starter Kits are also available.
Please visit our website at https://ruo.mbl.co.jp/je/rip-assay/

RBP Antibody

RBP Antibody works on WB and/or IP, but not certified for working on RIP-Assay.

RN023PW	Anti-PABPN1 (polyclonal)
RN028PW	Anti-EIF2C1/AGO1 (polyclonal)
RN029PW	Anti-EIF2C2/AGO2 (polyclonal)
RN030PW	Anti-DICER1 (polyclonal)
RN031PW	Anti-ZFP36 (polyclonal)
RN034PW	Anti-CUGBP1 (polyclonal)
RN035PW	Anti-CUGBP2 (polyclonal)

Other RBP Antibodies are also available.
Please visit our website at https://ruo.mbl.co.jp/je/rip-assay/

V. Appendix

1. The following commercially available reagents have been confirmed to work with *RIP-Assay Kit for microRNA* at indicated final concentration.

Protease inhibitor		Final concentration
	Aprotinin	10 μg/mL
	Leupeptin	5 μg/mL
	PMSF	0.5 mM

Reducing agent		Final concentration
	DTT	1.5 mM

RNase inhibitor		Distribution source	Code No.	Final concentration
	RNase OUT	Invitrogen	10777-019	50−200 U/mL

Protein A beads		Distribution source	Code No.
	Protein A Sepharose CL-4B	GE Healthcare	17-0780-01

Protein G beads		Distribution source	Code No.
	Immobilized Protein G Plus	Pierce	22852

2. Preparation of denaturing (7 M urea) polyacrylamide gel

10% denaturing Polyacrylamide Gel (7 M Urea)		Amount
	Urea	4.2 g
	40 (w/v) %-Acrylamide/Bis Mixed Solution (19:1)	2.5 mL
	10×TBE Buffer	1 mL
	N,N,N',N'-Tetramethylethylenediamine	10 μL
	10% APS	100 μL
	Nuclease-free water	~10 mL*

*Fill nuclease-free water to a volume of 10 mL.
10 mL is enough for preparation of one mini-gel (size: 85mm (W) × 70mm (H)× 1mm (T)).

3. Preparation of loading buffer for denaturing PAGE

Loading buffer		Amount
	10 × TBE Buffer	1.5 mL
	Urea	6 g
	Sucrose	1 g
	5% w/v Xylene Cyanol	100 μL
	5% w/v Bromophenol Blue	100 μL
	Nuclease-free water	~10 mL*

*Fill nuclease-free water to a volume of 10 mL.