Cat. # M212

For Research Use

TaKaRa

Anti-Human Ago2, Monoclonal (Clone 1B1) for Immunoprecipitation

Product Manual

v1106

Table of Contents

I. Description

Eukaryotes have sequence-specific gene expression control mechanisms called RNA interference (RNAi) or RNA silencing that rely on single-stranded RNA molecules (microRNAs, abbreviated as miRNAs) ranging in length from 21-24 nt. In RNAi, Argonaute proteins, which are major components of RNA-Induced Silencing Complexes (RISC), form complexes with miRNAs to facilitate sequence-dependent inhibition of gene expression in which miRNA serve as guide molecules[1)].

The human Argonaute protein family consists of 8 members classified into 2 subfamilies, the Ago subfamily (Ago1-4) and the piwi subfamily (PIWIL1-4)[2)]. Members of the Ago subfamily (Ago1-4) contribute to inhibition of mRNA translation[3)]. In particular, Argonaute 2 (Ago2) exhibits endonuclease activity and plays an important role in RNAi by facilitating the degradation of specific mRNA transcripts by RISCs[4)].

This product includes a biotinylated mouse monoclonal antibody that binds to the N terminal region (amino acids 1-148) of human Argonaute 2 (Ago2) (NP_036286.2) with high specificity. Avidin resin can be used to capture the antibody-Ago2 complex for immunoprecipitation experiments.

Additionally, RNA extraction may be performed on the antigen-antibody complexes to analyze miRNAs present in the immunoprecipitate.

II. Kit Components

(1) Biotinylated Human Ago2 Antibody (Clone 1B1) (Lyophilized)	0.1 mg
(2) Lysis and IP Buffer (Cell lysis and wash buffer for IP)	60 ml x 2

III. Materials Required but not Provided

1. Reagents

- Avidin-conjugated magnetic beads or avidin-conjugated resin
  e.g., Magnosphere MS300/Streptavidin (Cat. #5325)
- miRNA purification reagent
  e.g., RNAiso Plus (Cat. #9108, not available in the U.S. or Europe), NucleoSpin miRNA (Clontech Cat. #740971.10)
- solution for preparation of antibody dilutions
  e.g., 1% BSA/PBS, Blocker Casein in PBS (Thermo Fisher Cat. #37528), Block Ace (AbD Serotec)
- PBS (sterilized)
- Antibody for Western blot analysis (as needed)
  Anti-Human Ago2, Monoclonal (Unlabeled) (Cat. #M211)
- Recombinant RNase Inhibitor (Cat. #2313A) (as needed)

2. Materials

- Magnetic stand (For immobilization of Magnosphere MS300/Streptavidin complexes)
  Magnetic Stand (6 tubes) (Clontech Cat. #631964)
- Refrigerated microfuge

IV. Storage

- Biotinylated Human Ago2 Antibody : 4℃
  This product does not contain preservatives. Once reconstituted, stock solution can be dispensed into aliquots and stored at -20℃ for up to 1 year. Alternately, a preservative (such as 0.1% sodium azide) can be added and stock solution can be stored at 4℃ for up to 3 months. Avoid repeated freezing and thawing.
- Lysis and IP Buffer : 4℃

V. Protocol

V-1. Overview of miRNA Analysis by Ago2 Immunoprecipitation

Step 1 Prepare cell extracts using the buffer provided for cell solubilization (Lysis and IP Buffer＊).

Step 2 Bind the biotin-labeled antibody (Biotinylated Human Ago2 Antibody＊) to the avidin magnetic beads (or resin) and immobilize the antibody-bead complex.

Step 3 Mix the cell extracts with the biotinylated antibody-bead complex to form the immunoprecipitate.

Step 4 Purify miRNA from the immunoprecipitate.

Step 5 Analyze the miRNA.

＊: Included in this product

Caution: The Biotinylated Human Ago2 Antibody cannot be used as the primary antibody for Western blot analysis.
Please use Anti-Human Ago2, Monoclonal (Cat. #M211) (Unlabeled) for Western blotting.

V-2. Reagent Preparation

1. Biotinylated Human Ago2 Antibody (See Section V-4 below, "Immunoprecipitation")

   Reconstitute the Biotinylated Human Ago2 Antibody (lyophilized) in 100 µl of distilled water to prepare the 1.0 mg/ml stock solution＊. (Solution composition: 10 mM PBS containing 1% BSA, pH7.4)

   At the time of use, dilute to a concentration of 5 µg/ml using a protein-containing buffer solution (such as PBS with 1% BSA, Blocker™ Casein in PBS, or Block Ace™). Use 1 ml of diluted antibody solution (at a concentration of 5 µg/ml) per reaction.

   ＊: Once reconstituted, the stock solution can be dispensed into aliquots and stored at -20℃ for up to 1 year. Alternatively, a preservative (such as 0.1% sodium azide) can be added and the stock solution can be stored at 4℃ for up to 3 months. Use diluted antibody solutions immediately.

2. Lysis and IP Buffer (See Section V-3, "Preparation of Cell Extract," and Section V-4, "Immunoprecipitation")

   This buffer is used for cell solubilization and wash steps during immunoprecipitation. To avoid contamination, dispense aliquots of buffer into a separate tubes prior to use. When conducting miRNA analysis, add RNase inhibitor as necessary. When doing so, dispense only the buffer volume that will be used immediately and add the RNase inhibitor just before use (See Section V-3, "Preparation of Cell Extract").

3. Avidin-conjugated beads: Magnosphere MS300/Streptavidin (Cat. #5325) (Used in Section V-4 below, "Immunoprecipitation").

   For each reaction, transfer 50 µl of Magnosphere MS300/Streptavidin (referred to as "beads" below) to a microtube.
   Using the Magnetic Stand to immobilize beads, wash beads with 1 ml of PBS. Remove PBS after washing.
   50 µl of Magnosphere MS300/Streptavidin is equivalent to 0.5 mg of beads. The binding capacity is 400 - 600 pmol biotin/mg of beads.

V-3. Preparation of Cell Extract

1. Prepare the cells.
   - Cells such as HeLa S3 (Human cervical cancer), HepG2 (Human liver cancer) or MCF7 (Human breast cancer) can be used as Ago2 protein-positive cells.

     Note: If other cells are used, confirm that they are Ago2-positive before use by Western blot analysis with Anti-Human Ago2, Monoclonal (Unlabeled). (See VII, Appendix, "Western Blot Analysis of Ago2 Protein-Positive Cells.")
   - 5×10^6 - 1×10^7 cells are necessary for each immunoprecipitation.＊1

     ＊1 : Approximate cell numbers when each indicated cell type is grown to 95% confluency in a 10 cm plate.

HeLa S3	2.4×10^7 cells	for 5 reactions
HepG2	1.1×10^7 cells	for 2 reactions
MCF7	5.6×10^6 cells	for 1 reaction

   - In addition to the plate used for immunoprecipitation, prepare a duplicate plate cultured under the same conditions for quantifying cell count.
2. Remove the cells using a cell scraper＊2. Adjust the volume of culture supernatant as needed to aid collection.

   ＊2 : Do not perform trypsin treatment when collecting cells. Doing so may affect immunoprecipitation results.
3. Transfer cell suspension to a 50 ml centrifuge tube. Centrifuge at room temperature for 5 minutes at 1500 rpm (440 X g).
4. Discard the supernatant. Add 10 ml of sterilized PBS to the cell pellet. Centrifuge at room temperature for 5 minutes at 1500 rpm (440 X g).
5. Discard the supernatant. Add 10 ml of sterilized PBS, then resuspend the cell pellet. Aliquot the resuspended cell pellet into separate tubes for each reaction (5×10^6 - 1×10^7 cells per reaction). Centrifuge at room temperature for 5 minutes at 1500 rpm (440 X g)＊3.

   ＊3 : • After removing the supernatent (prior to solubilizing), pelleted cells may be stored at -80℃ for up to 1 month.

   • When processing multiple reactions at the same time, it is also possible to not aliquot the cells before solubilization, but instead to divide samples after solubilization by adding the Lysis and IP Buffer (See Step 6 below).
6. Discard the supernatant. Add 1 ml of Lysis and IP Buffer＊4 to each tube and incubate on ice for 10 minutes. Solubilize the cells by pipetting.

   ＊4 : Add RNase Inhibitor to the Lysis and IP Buffer as needed. Use approximately 400 units of Recombinant RNase Inhibitor (Cat. #2313A) per 1 ml of Lysis and IP Buffer.
7. Transfer 1 ml of the solubilized cell extract to a 1.5 ml microtube. Centrifuge for 20 minutes at 4℃ at 14,500 rpm (20,000 X g) using a microcentrifuge. Use supernatent for immunoprecipitation as described below＊5.

   ＊5 : Cell extracts that have been solubilized using Lysis and IP Buffer should be used for immunoprecipitation on the same day.

   For protein quantification by BCA Protein Assay of cell extracts prepared with Lysis and IP Buffer, dilute assay samples more than 5-fold to minimize buffer interference with the BCA assay.

V-4. Immunoprecipitation

1. Mix 1 ml of diluted (5 µg/ml) Biotinylated Human Ago2 Antibody and 50 µl of washed Magnosphere MS300/Streptavidin (beads). Conduct the biotin-avidin binding reaction by subjecting tubes to inversion mixing for 1 hour at 4℃.
2. Remove supernatant after immobilizing the beads using the magnetic stand. Add 1 ml of Lysis and IP Buffer and vortex briefly. Remove the buffer and recover beads using the magnetic stand.
3. Add 1 ml of the cell extract prepared in Section VI-3 "Preparation of Cell Extract," to the beads. Conduct the antigen-antibody binding reaction by subjecting tubes to inversion mixing for 1 hour at 4℃. The use of a rotary shaker is recommended.
4. Remove the supernatant after immobilizing the beads using the magnetic stand. Wash beads by adding 1 ml of Lysis and IP Buffer and vortex briefly. Remove the buffer and recover beads using the magnetic stand.

   Perform this wash step 3 times. After washing, remove all traces of buffer.

   Depending on downstream analysis to be performed on the immunoprecipitate (e.g., RNA preparation or Western blot analysis), it may be desirable to resuspend the beads in 50 µl of Lysis and IP Buffer and aliquot as appropriate.

   Note: The choice of procedure for the next step (Step 5 below) differs depending on whether RNA preparation or Western blot analysis is desired.

For RNA Preparation

5. Add RNAiso Plus (Cat. #9108 - not available in the U.S. or Europe) or Lysis Buffer ML from NucleoSpin® miRNA (Clontech Cat. #740971.10) to the immunoprecipitate (beads). Prepare the RNA (miRNA) according to the appropriate product protocol. When preparing with RNAiso Plus, use Dr. GenTLE® Precipitation Carrier (Cat. #9094) at the isopropanol precipitation step. Final volume of the purified RNA solution will be approximately 50 µl per sample.

For Western Blot

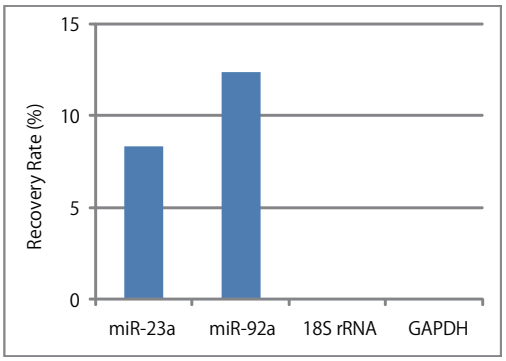
5. Add 50 µl of sample buffer for SDS-PAGE (with reducing agent) to the immunoprecipitate (beads) to extract proteins. Perform Western blot analysis using approximately 10 - 20 µl of the extract per lane.

   Anti-Human Ago2, Monoclonal (Cat. #M211) (Unlabeled) can be used as primary antibody. Use at a concentration of 5 µg antibody/ml incubation solution for 1 hour at room temperature.
6. Use anti-mouse IgG peroxidase-labelled secondary antibody and an appropriate luminescent or colorimetric substrate.

VI. Experimental Example : Preparation of miRNA from Ago2 Immunoprecipitate and Analysis by Real Time PCR

<Method>

miRNA was purified using 1 ml of RNAiso Plus on immunoprecipitate obtained from HeLa S3 cells (5 x 10^6 cells) with this product. The miRNAs miR-23a and miR-92a in the miRNA-enriched fraction and in the purified total RNA fraction obtained from cell extract were detected using the Mir-X miRNA qRT-PCR SYBR® Kit (Clontech Cat. #638314). Recovery rates of each miRNA in the miRNA-enriched fraction are shown below. Additionally, the purification efficiency for the miRNA fraction was assessed by detecting the 18S rRNA and GAPDH mRNA contained in each RNA sample using the PrimeScript RT Reagent Kit (Perfect Real Time) (Cat. #RR037A) and SYBR® *Premix Ex Taq* (Perfect Real Time) (Cat. #RR041A).



Analysis of miRNA in immunoprecipitate by real time PCR.

<Results>

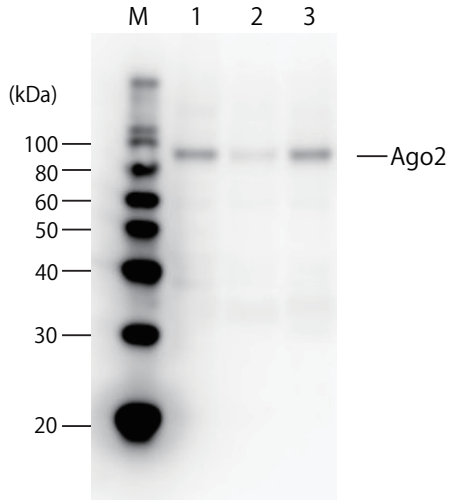
The microRNAs miR-23a and miR-92a were recovered from the Ago2 immunoprecipitate fraction of HeLa S3 cells with high efficiency. Furthermore, 18S rRNA and GAPDH mRNA were not detected, confirming the purity of the miRNA fraction.

VII. Appendix

1. Western Blot Analysis of Ago2 Protein-Positive Cells

<Method>

Cell extracts were prepared from 5 x 10^6 cells of three types of Ago2 protein-positive cells (HeLa S3, HepG2 and MCF7). Western blot detection of the Ago2 protein was carried out using Anti-Human Ago2, Monoclonal (Unlabeled) primary antibody at a concentration of 5 µg/ml, anti-mouse IgG peroxidase-labelled secondary antibody, and a luminescent substrate.



M: Protein marker
1: HeLa S3
2: HepG2
3: MCF7

<Results>

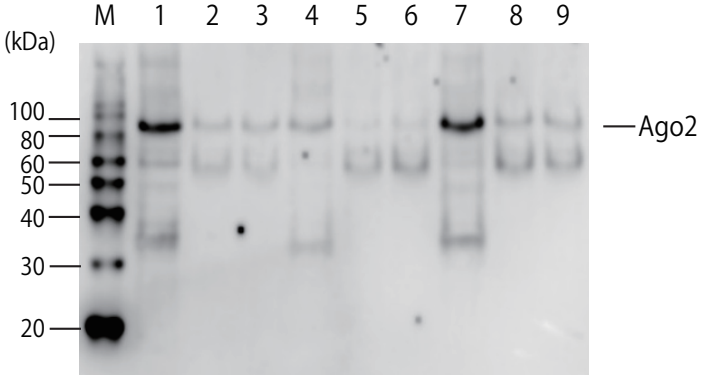
Western blot analysis confirmed differential levels of Ago2 protein detected in each cell type.

2. High-Throughput Immunoprecipitation on an Avidin-Coated 96-Well Plate

An Avidin-Coated 96-well plate was used to carry out immunoprecipitation on multiple samples using Biotinylated Ago2 antibody.

<Method>

1. Biotinylated Human Ago2 Antibody (1 mg/ml) was diluted to 10 µg/ml with Blocker Casein in PBS (Thermo Fisher). 50 µl of the diluted antibody were added to each well of an avidin plate (BioBind streptavidin-coated strip plate, Thermo Scientific Inc., Code 95029 263) and then the Ago2 antibody was immobilized. Each well was washed three times with sterilized PBS.
2. 5 x 10^6 cells per cell type (HeLa S3, HepG2, or MCF7) were solubilized using 1 ml of Lysis and IP Buffer and then centrifuged. 50 µl of the supernatant were dispensed into each of 4 wells and the plate was incubated in darkness for 1 hour at 4℃. Wells were then washed with sterilized PBS 4 times and the plate was inverted on a paper towel to completely remove the PBS.
3. To recover protein from each of the 4 identical wells per cell type, 50 µl of sample buffer for SDS-PAGE (with reducing agent) was added to the first of the 4 wells, then transferred to each of the remaining 3 wells in turn.
4. Duplicates of immunoprecipitate samples (20 µl per lane) were analyzed by Western blot using Anti-Human Ago2, Monoclonal (Unlabeled).



M:	Protein marker	
1:	HeLa S3 cell extract	5 X 10^6 cells/ml, 10 µl
2:	HeLa S3 immunoprecipitate	96 well plate, 2 well
3:	HeLa S3 immunoprecipitate	96 well plate, 2 well
4:	HepG2 cell extract	5 X 10^6 cells/ml, 10 µl
5:	HepG2 immunoprecipitate	96 well plate, 2 well
6:	HepG2 immunoprecipitate	96 well plate, 2 well
7:	MCF7 cell extract	5 X 10^6 cells/ml, 10 µl
8:	MCF7 immunoprecipitate	96 well plate, 2 well
9:	MCF7 immunoprecipitate	96 well plate, 2 well

<Results>

Western blot analysis was performed using the immunoprecipitate from 2 wells of a 96-well plate. Plate-based immunoprecipitation can be used for high-throughput screening of Ago2 protein-containing complexes from multiple samples.

VIII. References

1) Gregory RI, Chendrimada TP, Cooch N, Shiekhattar R: Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. *Cell* 2005, **123**:631-640.
2) Sasaki T, Shiohama A, Minoshima S, Shimizu N: Identification of eight members of the Argonaute family in the human genome. *Genomics* 2003, **82**:323-330.
3) Pillai RS, Artus CG, Filipowicz W: Tethering of human Ago proteins to mRNA mimics the miRNA-mediated repression of protein synthesis. *RNA* 2004, **10**:1518-1525.
4) Meister G, Landthaler M, Patkaniowska A, Dorsett Y, Teng G, Tuschl T: Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. *Mol Cell* 2004, **15**:185-197.

IX. Related Products

Anti-Human Ago2, Monoclonal (Cat. #M211)
Magnosphere MS300/Streptavidin (Cat. #5325)
Magnetic Stand (6 tubes) (Clontech Cat. #631964)
Dr. GenTLE® Precipitation Carrier (Cat. #9094)
NucleoSpin® miRNA (Clontech Cat. #740971.10)
Mir-X™ miRNA qRT-PCR SYBR® Kit (Clontech Cat. #638314)