Chapter 6

Loading of Argonaute Protein with Small Duplex RNA in Cellular Extracts

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Abstract

Argonaute (Ago) proteins are the minimum core proteins required for executing RNA interference (RNAi) mechanisms of gene regulation. For Ago proteins to regulate gene expression through RNAi they must be loaded, or "programmed," with a single strand of small RNA. Natural small RNAs are typically double-stranded duplexes that require additional factors for efficient and specific loading into Ago proteins. Here, a protocol is described for investigating RNAi programming through loading of human Ago2 using radio-labeled small interfering RNA (siRNA) and HeLa cell extracts. This protocol provides an Ago loading assay to study RNAi programming when starting with crude or partially purified cell extracts. The Ago loading assay should prove useful for studying other Ago proteins using a variety of mammalian cell extracts.

Key words RNA interference, RNAi programming, Argonaute loading, Small duplex RNA, Cell extract, In vitro, Ago2, Human, Mammalian

1 Introduction

RNA interference (RNAi) can be divided into at least two distinct phases, generically called "programming" and "execution" [1, 2]. First, an Argonaute (Ago) protein is loaded, or programmed, with small RNAs [2, 3]. Once loading has occurred, the ribonucleoprotein complex and associated proteins can then be guided to complementary target RNA through base-pairing with the small RNA that is bound to the Ago protein [4–6]. Interaction with the targeted RNA results in execution of RNAi by either target RNA cleavage and degradation or modulation of target RNA function, such as reduced translation of an mRNA [1, 2, 7–9]. Many Ago proteins have "slicer" activity, an RNase activity that breaks a specific phosphodiester bond of the targeted RNA [10–13]. In humans there are four Ago proteins, Ago1–4, but only the Ago2 protein is catalytic [14, 15].

The most common small RNAs in humans are microRNAs (miRNAs), which are imperfect duplexes that can vary in length from approximately 20–30 bases (Fig. 1a) [1, 16, 17]. RNAi not

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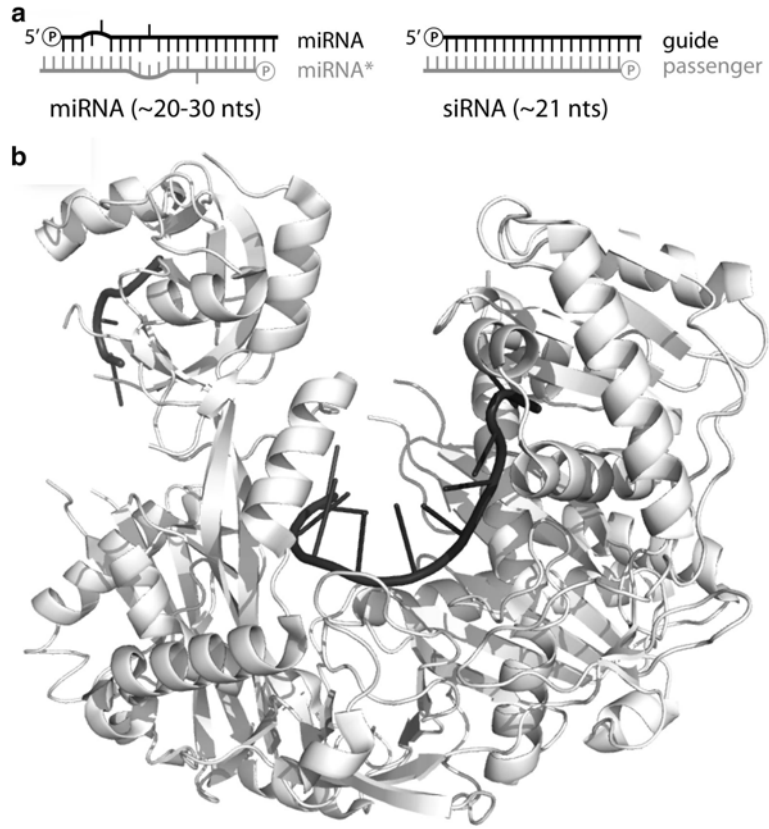

Fig. 1 Small RNAs and their loading into Argonaute protein. (a) Secondary structure of miRNAs and siRNAs. miRNAs are imperfect duplexes composed of a miRNA strand and a miRNA* strand, both of which can be loaded into Ago proteins and target different RNAs, although the miRNA* strand is of lower abundance [4]. siRNAs are perfect duplexes composed of guide and passenger strands, the latter of which is not usually loaded efficiently into the Ago protein. (b) Crystal structure of human Ago2 bound to miRNA miR20a (PDB ID 4F3T) [27]. Ago protein is shown in *light gray*. MiR20a was only partially resolved in this structure and is shown centered in *black*

only functions in natural regulation, but it can be redirected to regulate almost any cellular RNA by introducing small RNAs into cells [9, 18]. For biotechnology applications, small interfering RNAs (siRNAs) are commonly used, consisting of 19 nucleotide perfect duplexes with two nucleotide 3′ overhangs (Fig. 1a) [18–20]. RNAi has become a premier tool for genetic research and a promising future approach for therapeutic treatment of human disease [19, 21, 22]. RNAi continues to be intensely studied with the aim of improving biotechnology applications and understanding the cellular function and evolution of RNAi.

To initiate RNAi, Ago proteins are loaded with a single strand of short RNA [3, 12, 23]. Under in vitro conditions, purified Ago protein alone can usually bind and load small single-stranded nucleic acids of the appropriate size range [24, 25]. Once bound to Ago, the small RNA is displayed in a manner which presents its bases for specific recognition of complementary RNAs through Watson–Crick base-pairing (Fig. 1b) [26–28]. Naturally occurring small RNAs are typically double-stranded duplexes. Thus, loading is a regulated and multistep process whereby small RNA duplexes are unwound and one strand is selected for loading into the Ago protein [11, 29, 30]. The loaded strand is often referred to as the "guide" strand whereas the discarded strand is called the "passenger" strand [11].

Ago proteins alone do not appear to possess strong helicase activity for removing the passenger strand nor a clear mechanism for choosing the appropriate strand as the guide [11, 29, 31]. Therefore, these activities are primarily catalyzed and directed by other factors [30, 32]. One protein implicated in this process is heat shock protein 90 (Hsp90). Previous studies have shown that the ATP-dependent chaperoning activity of Hsp90 is important for efficient RNAi [33]. In addition to Hsp90, other factors are also implicated in Ago loading [30, 32, 34]. Currently, it is unclear whether all of the factors involved in Ago loading activity are known [6, 32, 34–36]. In addition, production of active, recombinant proteins is resource-intensive. Thus, reconstitution of efficient loading activity of Ago proteins in vitro using purified components is challenging [30, 33, 34].

Experimental approaches which assay the binding of targeted RNA by Ago complexes [6], the cleavage of the targeted RNA [12], or the reduced translation of targeted mRNAs [8], are not direct assays for Ago loading. While connected, the efficiency of the execution phase of RNAi does not necessarily correlate with efficiency of the programming phase of RNAi [33]. These distinct phases must be separated and studied individually to understand the function and regulation of RNAi and to further improve its biotechnology applications.

To facilitate investigation of Ago loading activity and discovery of Ago loading factors in the absence of a defined biochemical system with purified components, a protocol using cellular extracts and synthetic small RNAs was developed (Fig. 2a) [37, 38]. This protocol provides an activity assay for small duplex RNA loading of Ago proteins. The Ago2 loading assay presented here was originally developed to compare loading activities in nuclear and cytoplasmic extracts (Fig. 2b) [37]. No loading activity was observed in nuclear extracts, which do not contain Hsp90 protein [37]. Thus, the Ago2 loading assay enabled the discovery of an unexpected layer of RNAi regulation, which appears to restrict Ago2 loading to the cytoplasmic compartment.

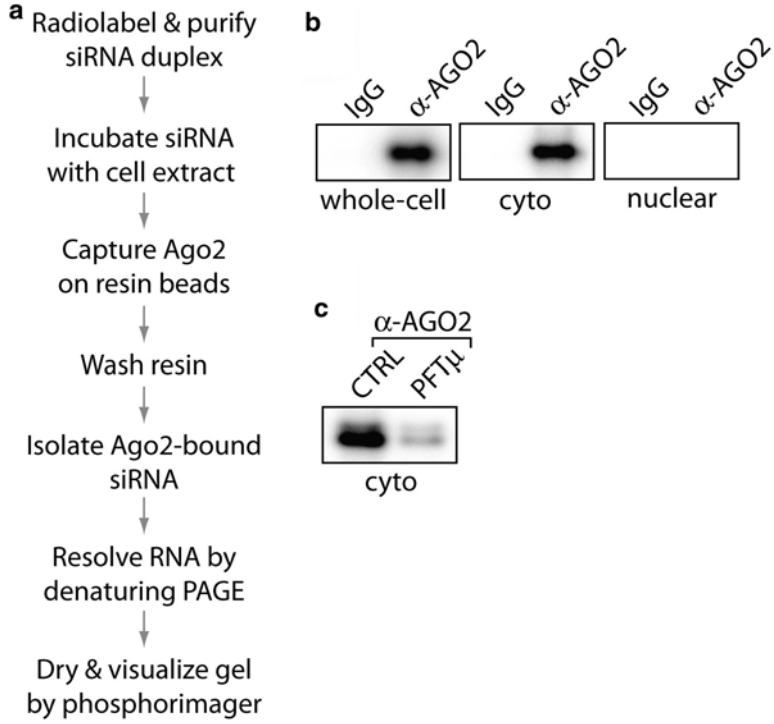

Fig. 2 In vitro Ago2 loading assay. (**a**) Flowchart for performing in vitro Ago2 siRNA loading assay with human cell extracts. (**b**) Typical results from Ago2 loading assays using whole-cell, cytoplasmic (cyto) or nuclear extracts prepared from HeLa cells. The antibody used for immunoprecipitation is indicated above the gel. (**c**) Treatment of HeLa cells with pifithrin-μ (PFTμ), a known Hsp70/90 inhibitor, prior to extract preparation results in reduced Ago2 loading activity in vitro

2 Materials

2.1 Chemicals and Reagents

2.1.1 Preparation of Radiolabeled RNA

1. RNase-free distilled and deionized water (ddH_2O).
2. siRNA strands dissolved in ddH_2O at 100 μM:
   (a) siLuc guide (siLuc_as): rUrGrUrUrCrArCrCrUrCrGrArUrArUrGrUrGrCTT.
   (b) siLuc passenger (siLuc_ss): rGrCrArCrArUrArUrCrGrArGrGrUrGrArArCrATT.
3. [γ]-^{32}P-ATP (7000 Ci/mmol) (MP Biomedicals) (*see* **Note 1**).
4. SUPERase-In RNase inhibitor (Ambion) (*see* **Note 1**).
5. T4 polynucleotide kinase (PNK) and 10× PNK buffer: 0.7 M Tris–HCl, pH 7.6, 100 mM $MgCl_2$, 50 mM dithiothreitol.
6. Redistilled phenol, water-saturated.
7. Chloroform:isoamyl alcohol (24:1).
8. Acetone.

9. Black India ink (local art supply store).
10. Yeast transfer RNA (tRNA) at 10 mg/mL in ddH_2O.
11. Acetone.
12. Lithium perchlorate ($LiClO_4$).
13. Urea, molecular biology grade.
14. 10 % ammonium persulfate (APS), prepared fresh.
15. *N,N,N,N′*-Tetramethyl-ethylenediamine (TEMED).
16. 10× TBE: 0.89 M Tris base, 0.89 M boric acid, 20 mM EDTA.
17. 4× Native loading buffer: 4× TBE, 40 % glycerol (v/v), 0.2 mg/mL xylene cyanol, 0.2 mg/mL bromophenol blue.

2.1.2 Ago Loading Assay

HeLa cell extracts (*see* **Note 2**).

1. 40 % acrylamide solution, 19:1 (acrylamide:bisacrylamide) crosslinking.
2. Protein G Plus/Protein A agarose (EMD Millipore, IP05-1.5ML) (*see* **Note 1**).
3. Anti-Ago2 antibody (Abcam, ab57113) (*see* **Note 1**).
4. Mouse IgG antibody (Millipore, 12-371) (*see* **Note 1**).
5. 100 mM adenosine triphosphate (ATP).
6. 1 M phosphocreatine.
7. Creatine kinase at 4U/μL.
8. 0.5 M ethylendiaminetetraacetic acid (EDTA).
9. Denaturing loading buffer: 90 % formamide, 1× TBE, 5 mM EDTA, 0.2 mg/mL bromophenol blue, 0.2 mg/mL xylene cyanol.
10. IP Equilibration Buffer (IPEB): 20 mM Tris, pH 7.4, 0.15 M NaCl, 2 mM $MgCl_2$, 0.05 % NP-40 (Igepal CA-630) (v/v), 0.1 % Polyvinylpyrrolidone (PVP) (w/v).
11. IP Binding Buffer (IPBB): 20 mM Tris, pH 7.4, 0.15 M NaCl, 3 mM $MgCl_2$.
12. IP Wash Buffer (IPWB): 20 mM Tris, pH 7.4, 0.5 M NaCl, 4 mM $MgCl_2$, 0.05 % NP-40 (Igepal CA-630) (v/v).

2.2 Equipment and Supplies

1. Temperature-controlled heating block.
2. Automatic pipettor and pipettes.
3. Clear plastic wrap (Saran™ wrap or Glad™ wrap).
4. 3MM Whatman filter paper.
5. Temperature-controlled vacuum gel drier.
6. Phosphorimager and phosphorimager screen.
7. 1.5 mL microfuge tubes.

8. Room temperature bench-top centrifuge (1.5 mL tube rotor).
9. Liquid and solid ^{32}P radioactive waste containers.
10. Geiger counter.
11. Scintillation counter.
12. Electrophoresis power supply (with constant current setting).
13. Denaturing polyacrylamide gel electrophoresis apparatus.
14. Water-cooled native polyacrylamide gel electrophoresis apparatus.
15. Glass plates ($19 \times 20 \times 0.3$ and $16 \times 20 \times 0.3$ cm) and gel spacers and combs (0.75 cm).
16. Large binder clips (2 in wide) (local business supply store).
17. Clear scotch tape.
18. Razor blades.
19. Bunsen burner.
20. Microcentrifuge tube rotator.
21. RNase-free mini-spin filtration columns (<10 μm) (Pierce® Spin Cups-Paper Filter) (*see* **Note 1**).
22. Vortex mixer.

3 Methods

3.1 General Methods

3.1.1 Tissue Culture and Cell Extracts

It is expected that the user will have some experience with tissue culture and existing protocols for how to grow and maintain healthy cell cultures. For the protocol described here, we recommend HeLa cells as they are straightforward to culture. HeLa cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5 % fetal bovine serum (FBS) and 0.5 % non-essential amino acids (NEAA). Cells were grown in sterile incubators at 37 °C in 5 % CO_2. Extracts were prepared according to methods described previously (*see* **Note 2**) [37, 38].

3.1.2 Handling RNA

General guidelines for working with RNA solutions include the use of baked glassware or DEPC-treated plastic-ware, wearing of gloves, practicing single-use of pipette tips, keeping work surfaces clean and free of dust, and using reagents that are certified RNase-free by the manufacturer. RNA should generally be kept cold in neutral buffers that lack divalent metal ions and stored long-term at −80 °C, preferably in a dry form when not being actively used.

3.2 Preparation of Radiolabeled siRNA for the Ago Loading Assay

RNA is commonly "end-labeled," where a radioactive nucleotide or phosphate is placed at the terminal end of the RNA sequence. Here we use 5′-end labeling, which requires that the RNA not have a 5′-phosphate (*see* **Note 3**). Although 5′-end radiolabeling of RNA is a standard procedure, it is described here because of

special considerations, including differential labeling of the siRNA guide strand, purification on a native gel, high specific activity labeling, and precipitation with an uncommon reagent. The success of the subsequent Ago2 loading assay in cell extracts requires purified siRNA of very high specific radioactivity. The siRNA used in this protocol targets a sequence of the *Luciferase* mRNA [18]. There are no special considerations when choosing an siRNA or duplex RNA for this method. However, standard siRNA designs should be used initially or as controls, such as a duplex RNA of 19 nucleotides that contains 3′ dTdT overhangs [18, 20].

3.3 5′-End Labeling With T4 Polynucleotide Kinase (PNK)

1. Mix the reaction components below in a 1.5 mL microcentrifuge tube:
   (a) 1 μL of siLuc_as (guide strand) (100 μM stock).
   (b) 2 μL 10× PNK buffer.
   (c) 2.5 μL [γ]-^{32}P-ATP (~0.3 mCi).
   (d) 2 μL PNK (10 U/μL).
   (e) 1 μL SUPERase-In (40 U/μL).
   (f) ddH_2O to 20 μL.
2. Incubate at 37 °C for 2.5 h. Phenol/chloroform extract (*see* **Note 4**). Keep on ice or store at
   (a) −20 °C until ready to proceed.

CAUTION: Work behind a shield and follow safety regulations when handling radioactivity.

3.4 Preparing and Purifying Radiolabeled Duplex siRNA

Prior to use in the Ago loading assay, the radiolabeled guide strand must be annealed to a complementary passenger strand and gel-purified to remove unincorporated [γ]-^{32}P-ATP, single-stranded RNAs that did not anneal, and RNA that may have partially degraded during labeling.

3.4.1 Annealing Radiolabeled siRNA Guide and Passenger Strands

1. Add 1.2 μL (120 pmol) of siLuc_ss (passenger strand) RNA to the radiolabeled siLuc_as guide strand phenol extracted from above.
2. Incubate at 90 °C for 3 min. Remove from heating block and let cool at room temperature (RT) for 15 min.
3. Mix 8 μL of Native loading buffer with siRNA sample. Place siRNA sample on ice or freeze at −20 °C until ready for gel-purification.

3.4.2 Casting a Native Polyacrylamide Gel for Purification of Radiolabeled siRNA

1. Prepare a 40 mL solution containing 15 % acrylamide (19:1 acrylamide:bisacrylamide), 1× TBE buffer.
2. Add 10 % APS (6 μL/mL) and TEMED (1 μL/mL) and mix by inverting or stirring.

3. Pour into an assembled glass sandwich (19 × 20 × 0.75 cm) and position a 15-well comb in the top of the gel (*see* **Note 5**).
4. Allow the gel to polymerize for 30 min. Remove the comb and place the gel in a water-cooled gel electrophoresis apparatus. Add 1× TBE buffer to anode and cathode tanks. Be sure to cover the electrodes and gel wells with sufficient buffer. Rinse the wells with 1× TBE buffer.
5. Pre-run the gel at 30–35 mA for 15 min. Make sure water is turned on and circulating to keep the gel cool.

3.4.3 Resolving the Radiolabeled siRNA by Native Gel Electrophoresis

1. Turn off the current to the gel. Load siRNA sample into the wells. One sample should fit into a single well if possible. Otherwise split the sample equally into multiple wells.
2. Turn current to the gel back on and run at 30–35 mA until the bromophenol blue band has migrated about 2/3 of the way through the gel (1.5–2 h). Make sure water circulation is turned on (*see* **Note 6**). The glass plates should remain cool to the touch and not exceed 30–35 °C.
3. Turn off the current to the gel and carefully drain the anode tank (typically the lower tank) buffer into a radioactive liquid waste container (*see* **Note 7**). Rinse the gel apparatus to remove trace radioactivity from the surface of the lower buffer chamber.

CAUTION: Work behind a shield and follow safety regulations when handling radioactivity.

3.4.4 Purifying Radiolabeled siRNA by Native Polyacrylamide Gel Electrophoresis

1. Remove the gel cassette from the apparatus, rinse the glass plates to remove trace radioactivity from the external surface of the gel cassette (*see* **Note 8**), then place the gel cassette behind a shield.
2. Separate the glass plates with a plastic wedge tool so that the gel sticks to one plate. Cover the exposed gel with clear plastic wrap (Saran™ wrap).
3. Place three small drops of radioactive dye (1 μL [γ]-^{32}P-ATP in 30 μL black India ink) on the Saran™ wrap at three corners of the gel and allow them to air dry. Cover them with clear tape.
4. Expose in darkroom to autoradiography film placed on top of the gel. Expose for 15–60 s and develop.
5. Slide the film underneath the glass that the gel is on. Orient and align the spots at the three corners of the gel to locate the RNA bands. Carefully peel back the Saran wrap and cut out the band with a flamed (RNase-free) razor.
6. Crush the gel slice into a paste with a 1 mL plastic pipette tip (RNase-free and flame-sealed at the small end) in a 1.5 mL microcentrifuge tube.

7. Pipette 300 μL of nuclease-free water down the side of the tip and into the microcentrifuge tube so as to collect all the gel bits. Rotate at 4 °C for 4–16 h to elute RNA.
8. Spin down sample to pellet elution and gel bits. Cut ~2 mm off the end of a 1 mL pipette tip with flamed scissors or a flamed razor and use the tip to move the entire sample, gel bits and elution, to an RNase-free filter spin column (~10 μm cutoff) (*see* **Note 9**). Spin at 2000 × *g* for 2 min to collect eluted RNA. Discard gel bits.
9. Add 10 μg of tRNA to the elution and split the sample evenly into two microcentrifuge tubes to accommodate the total volume needed for precipitation. Precipitate RNA from solution by adding 9 vol of 2 % lithium perchlorate ($LiClO_4$) in acetone and incubating at −20 °C for >15 min (*see* **Note 10**).
10. Spin at 12,000 × *g* for 10 min, wash pellet with ice-cold acetone, and spin again at 12,000 × *g* for 2 min. Let the pellet air dry at room temperature.
11. Resuspend RNA pellet in 30 μL of RNase-free water. Measure the radioactivity by scintillation counting of 1 μL (do not use scintillation fluid). Label the date and CPM/μL on the side of the tube and store RNA frozen at −20 °C or −80 °C (*see* **Note 11**).

3.5 In Vitro Ago Loading Assay

3.5.1 Loading Ago2 and Capturing on Resin

1. Mix the reaction components in the order shown below in a 1.5 mL microcentrifuge tube and rotate at RT for 1 h (*see* **Note 12**).
   - 250 μL extract.
   - 1 μL siRNA (1–2 × 10^6 CPMs/uL).
   - 2.5 μL ATP (100 mM).
   - 2.5 μL phosphocreatine (1 M).
   - 2.5 μL creatine kinase (4 U/μL).
2. While loading reactions rotate, equilibrate resin for immunoprecipitation. Resuspend Protein G Plus/Protein A resin, aliquot 40 μL into two 1.5 mL microcentrifuge tubes, and add 1 mL of IPEQ buffer. Rotate for 5 min at RT.
3. Pellet resin by centrifugation at 2000 × *g* for 1 min at RT. Remove buffer from resin with a pipet. Repeat wash step with IPBB buffer.
4. Spin down loading reaction at 12,000 × *g* for 5 min at RT to remove precipitation (*see* **Note 13**). Move supernatant to equilibrated resin. Add 2 μg of IgG to one tube and 2 μg of anti-Ago2 to the other tube. Rotate at RT for 1 h.

3.5.2 Isolating Ago2-Bound siRNA

1. Spin down loading reaction and resin mixtures at 2000 × *g* for 1 min. Discard supernatant.

2. Wash the resin 3 times by adding 0.5 mL of IPWB, rotating at RT for 5 min, then spinning at 2000 × *g* for 1 min. Perform a final wash with 0.5 mL of IPBB. On the final wash, move resin and buffer to a new tube before centrifugation (*see* **Note 14**).
3. Add 40 μL of ddH_2O, 0.5 μL of EDTA (0.5 M), 0.5 μL of tRNA (10 mg/mL), and 100 μL of phenol to the resin in each tube (*see* **Note 15**). Vortex for 2 min.
4. Spin down sample at 12,000 × g for 5 min. Carefully collect the top aqueous layer by pipetting and move to a new tube. Add 700 μL of 2 % $LiClO_4$ in acetone, vortex briefly, and precipitate at 4 °C for >15 min.
5. Centrifuge samples at 12,000 × g for 10 min, remove supernatant and wash pellet by adding 0.5 mL of acetone, inverting to mix, and spinning at 12,000 × *g* for 2 min. Remove acetone and allow pellet to air dry on bench top.

3.5.3 Visualizing Ago2-Bound siRNA by Resolving Complexes on a Denaturing Gel

1. Prepare a 40 mL solution containing 15 % acrylamide (19:1 acrylamide:bisacrylamide), 7 M urea, 1× TBE buffer, and 2 % glycerol (*see* **Note 16**).
2. Add 10 % APS (6 μL/mL) and TEMED (1 μL/mL) and mix by inverting.
3. Pour into an assembled glass sandwich (19 × 20 × 0.75 cm) and position a 15-well comb in the top of the gel.
4. Allow the gel to polymerize for 30 min. Remove the comb and place in a denaturing gel electrophoresis apparatus. Add 1× TBE buffer to the anode and cathode tanks. Be sure to cover the electrodes and gel wells with sufficient buffer. Rinse the wells with 1× TBE buffer.
5. Pre-run the gel at 40 mA for 30 min. The outer glass plate of the gel should become very warm to the touch (*see* **Note 17**).
6. Add 6–10 μL of formamide loading buffer to the dried RNA samples. Heat to 90 °C for 3 min. Let cool to RT on bench top. Vortex briefly and centrifuge at 2000 × *g* for 30 s.
7. Turn off the current to the gel. Load RNA sample into individual wells.
8. Turn current to the gel back on and run at 40 mA until the bromophenol blue band has migrated about 1/2 of the way through the gel (1 h). Monitor the gel so that it does not get too hot (*see* **Note 18**). The RNA will run between the two blue dye bands on the gel.
9. Stop the gel, separate the glass plates, and lay Saran™ wrap over the top of the gel. Flip the gel over and peel the glass plate off so that the gel is now adhered to the Saran™ wrap. Press a sheet of Whatman 3 M paper to the gel and dry in a vacuum gel dryer at 80 °C for 2 h.

10. Expose the dried gel to a phosphorimager screen overnight. Develop the screen to visualize radioactive RNA that co-eluted with Ago2 in the in vitro loading assay. Typical results for different HeLa cell extracts are shown in Fig. 2b.

3.6 Analysis and Application of the Ago2 Loading Assay

Ago 2 loading assay results should show a single band on a denaturing gel after immunoprecipitation of Ago2 from HeLa whole-cell or cytoplasmic extracts incubated with radiolabeled duplex (Fig. 2b). No band or a very faint band should appear in the samples immunoprecipitated with non-specific IgG antibody when used. For HeLa nuclear extracts, either no band or very faint bands are detected for Ago2 loading (Fig. 2b). This observation reflects the fact that known loading factors have been reported as absent from HeLa nuclear extracts, as well as nuclear extracts from other tested cell lines [37]. This assay can be used to test different loading conditions and the effect of compounds on loading activity. For example, the Hsp90 inhibitor pifithrin-μ has been reported to inhibit Ago2 loading and RNAi activity [33]. Here, we demonstrate that pifithrin-μ does inhibit Ago2 loading of duplex siRNA from a HeLa cytoplasmic extract in vitro (Fig. 2c). These results suggest that the Ago2 loading assay presented here depends on Hsp90 activity and recapitulates the loading mechanism of Ago2 inside of cells.

The Ago2 loading assay can be modified to investigate other aspects of loading. Whether or not an Ago protein is accessible for loading can be tested by first immunoprecipitating Ago protein, then incubating with a radiolabeled single-strand guide RNA [37, 38]. Another modification is to run the eluted RNA on a native, non-denaturing gel. Native gel electrophoresis might be helpful in discriminating between duplex loading and passenger strand removal. If the passenger strand has not been completely removed two distinct bands will be observed. The faster migrating band represents RNA that was bound to Ago2 whereas the slower migrating band represents duplex RNA containing both guide and passenger strands [37, 38].

When considering the use of other cell types or extracts for Ago2 loading, or investigating the loading of other Ago proteins, several factors should be considered. HeLa cells appear to have a robust RNAi system and other cell lines, such as primary fibroblasts, may not produce comparable Ago loading activities. While many cell types and extract will need to be individually optimized, general rules would include increasing the amount of extract used, increasing the concentration of the extract, and increasing the amount of radioactive siRNA used [38]. For various Ago proteins, the specificity and performance of the antibody in immunoprecipitation applications can affect the results. Antibodies would therefore need to be tested. A FLAG-HA Ago2 fusion has been successfully used in this assay before [37]. Therefore, affinity tagged versions of Ago proteins may provide a more universal and predictable immunoprecipitation step in this assay.

4 Notes

1. Vendor names are provided for convenience. The chemicals from the specific commercial suppliers named have been tested to work in this assay, the same reagents from other vendors may also be suitable.
2. HeLa cell extracts were prepared as in Gagnon et al. [37, 38]. For whole-cell extracts, cells were sonicated (same sonication conditions used for nuclear extract preparations) in ice-cold nuclear lysis buffer [20 mM Tris, pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, 0.05 % NP-40 (Igepal CA-630) (v/v), 10 % glycerol (v/v)] containing phosphatase and protease inhibitors. Insoluble cell debris was removed by centrifugation at 10,000 × *g* at 4 °C and extracts were flash frozen in liquid nitrogen and stored at −80 °C for later use.
3. The siRNA strands used in this protocol are chemically synthesized and by default do not have 5′ phosphate groups. Thus, they are immediately ready for 5′ radiolabeling.
4. Phenol extraction is a standard method. Briefly, add 1 volume of redistilled water-saturated phenol to the sample. Vortex for 30 s to mix, then spin at top speed to separate phenol and aqueous phases. Collect the top aqueous phase by pipetting. Add 1 volume of chloroform:isoamyl alcohol (24:1), vortex for 30 s to mix, then spin at top speed to separate chloroform and aqueous phases. Collect top aqueous phase by pipetting.
5. Prior to assembling glass plates with spacers for pouring the gel, the plates should be clean and free of detergent. Glass plates can be wiped or rinsed with ethanol before assembly to remove dust. Glass plates can also be baked, although a thorough washing is often sufficient to remove most contaminating RNases.
6. The glass plates should remain cool to the touch and not get above 30–35 °C. If gel running temperatures exceed 40 °C, the integrity of the siRNA duplex may become compromised, depending on its GC content and thermal stability.
7. The anode tank buffer where the positive electrode is located is contaminated with high levels of radioactive ^{32}P. Transfer from the tank to an approved liquid waste container should be performed in a sink or over a large bucket to prevent lab contamination on bench surfaces or floors. Monitor this process carefully with a Geiger counter. Afterward, decontaminate the area and electrophoresis apparatus by washing with soapy water.
8. Radioactivity can adhere to the outside of the plates that contain the gel. Since the gel between the glass plates is radioactive, the surface of the gel plates cannot be checked for contamination directly. After rinsing, wipe the outside of the glass plates with a paper towel and use a Geiger counter to check the paper towel.

9. Spin column filters are useful for removing gel bits and allowing the eluted RNA in solution to flow through. Avoid filter units with low molecular weight cutoffs as these tend to get clogged by the gel bits. Prepare spin columns for RNA work by filtering RNase-free water or buffer over the filter prior to adding sample.
10. The radiolabeled RNA is extremely dilute and will not precipitate well by standard ethanol precipitation. Addition of tRNA acts as a co-precipitant and helps keep the precipitated RNA insoluble during the acetone wash step. $LiClO_4$ in acetone efficiently precipitates very dilute and small RNAs. $LiClO_4$ is a dangerous oxidizer—handle with caution.
11. Radiolabeled RNA-specific activity should range from several hundred thousand to a few million CPMs/μL. The half-life of ^{32}P is 14.2 d.
12. For in vitro Ago2 loading assays, loading needs to be performed in the extract before Ago2 is subsequently captured with immuno-affinity resin. If Ago2 is bound to the resin first, duplex siRNA loading is inefficient. Thus, resin and antibody should not be mixed with the siRNA and extract together, but only added after an initial time of Ago2 loading in solution. No buffer is added to the extract since the extract preparation already contains the necessary buffer and salt components.
13. It is important to centrifuge samples prior to addition of resin to remove any precipitation that has occurred during incubation. Otherwise, the precipitated material will co-pellet with the resin in later steps and increase contaminating background levels during analysis.
14. Moving resin to a new tube on the final wash can help reduce background levels, which are important for obtaining clean results for IgG controls.
15. Binding of Ago2 to small RNA is Mg^{2+}-dependent. Addition of excess EDTA helps disrupt this interaction. In addition, EDTA is not soluble in acetone, so it also acts as a co-precipitant to help visualize the precipitated RNA. Addition of tRNA also acts as a co-precipitant and helps keep the precipitated RNA insoluble during the acetone wash step.
16. Glycerol is an important component of the gel. Do not omit. During drying of the gel prior to visualization, glycerol prevents the gel from shrinking and cracking, which can make a gel unusable for publication.
17. Pre-running the gel is necessary to heat it up. It should be very warm or hot to the touch. The heat and the urea in the gel help to keep the RNA denatured.
18. The glass plates should be warm or hot to the touch during the run, but not unbearable. If the plates get too hot they will

crack. If samples "smile," where the middle samples run faster than the outer samples, this indicates uneven heating of the gel. Too much smiling should be avoided by reducing the current passing through the gel.

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