Chapter 4

Relative Quantification of siRNA Strand Loading into Ago2 for Design of Highly Active siRNAs

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Abstract

In RNA interference (RNAi), silencing is achieved through the interaction of double-stranded small interfering RNAs (siRNAs) with essential RNAi pathway proteins, including Argonaute 2 (Ago2). Based on these interactions, one strand of the siRNA is loaded into Ago2 forming the active RNA-induced silencing complex (RISC). Optimal siRNAs maximize RISC activity against the intended target and minimize off-target silencing. To achieve the desired activity and specificity, selection of the appropriate siRNA strand for loading into Ago2 is essential. Here, we provide a protocol to quantify the relative loading of individual siRNA strands into Ago2, one factor in determining the capacity of a siRNA to achieve silencing activity and target specificity.

Key words siRNA, Ago2, RT-qPCR, Small RNA, Transfection, Immunoprecipitation, Stem-loop, HeLa

1 Introduction

Short interfering RNA (siRNA)-based therapeutics has the potential to treat a variety of diseases, including cancer [1, 2]. Utilizing the native eukaryotic pathway of RNA interference (RNAi), siRNAs initiate posttranscriptional gene silencing through sequence-specific targeting of mRNAs [2]. Upon entering the cytoplasm, the siRNA duplex is recognized by RNAi pathway proteins that load one of the siRNA strands into Argonaute 2 (Ago2), an endoribonuclease [3, 4]. The selected strand, known as the guide strand, along with Ago2, forms the mature, active RNA-induced silencing complex (RISC) [5]. Active RISC targets and cleaves mRNA transcripts complementary to the guide strand [6, 7].

Proper guide strand loading is essential to achieve efficient knockdown of the intended target. Considerable effort has been invested in determining the mechanism of strand loading in a variety of organisms [8]. These studies have highlighted particular

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siRNA sequence features that are important for selection of the guide strand [9–12]. Loading of the unintended strand of the siRNA duplex (i.e., the passenger strand) can lead to significant non-specific, off-target effects [13, 14]. Additionally, loading of the passenger strand sequesters Ago2, preventing loading of the guide strand and limiting the magnitude of silencing of the intended target. Thus, to achieve maximal activity and specificity of silencing, it is essential to design siRNAs such that only the intended guide strand is loaded into Ago2.

Methods for quantifying the strand loading of siRNAs have included indirect reporter assays [7, 9], as well as direct detection of siRNA loading through Northern blotting or Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR) of siRNA from Ago2 immunoprecipitation [15]. Stem-loop RT-qPCR was developed to increase the length of small RNAs, like siRNAs, in order to make them more amenable as qPCR templates [16]; since then, several other articles have discussed the details and optimization of RT-qPCR on siRNAs [17–20]. Another option for lengthening small RNAs is to use enzymatic poly(A) tailing. Since this method indiscriminately amplifies all RNAs, a forward primer containing locked nucleic acids is preferred to increase qPCR specificity [21]. Among direct detection methods, RT-qPCR-based assays have higher sensitivity than Northern blots while maintaining high specificity [22]. However, RT-qPCR-based assays also require thoughtful assay design and selection of appropriate controls. Here, we describe a method and the controls to investigate relative strand loading for siRNAs into Ago2 in cultured mammalian cells.

2 Materials

2.1 Cell Culture

1. 0.25% trypsin (1×), phenol red.
2. Versene solution.
3. DMEM, high glucose.
4. FBS.
5. Penicillin-streptomycin.
6. 6-well plate.
7. Culture medium: DMEM, high glucose; 10% FBS; 1% Pen-Strep.
8. Culture medium without antibiotics: DMEM, high glucose; 10% FBS.

2.2 Transfection

1. Opti-MEM I Reduced Serum Medium.
2. Lipofectamine 2000 (LF2K).

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2.3 Cell Lysis

1. Complete Protease Inhibitor.
2. Lysis buffer: 0.5% Igepal CA630 (USB), 150 mM KCl, 25 mM Tris–HCl pH 7.5, 2 mM EDTA, 0.5 mM DTT (add immediately before use), 1× cOmplete Protease Inhibitor (add immediately before use).
3. 10× PBS pH 7.4, diluted to 1×.
4. Cell Lifters.
5. RNase-free water.

2.4 Bead Preparation and RNA Immunoprecipitation

1. Protein G Magnetic Beads.
2. Monoclonal anti-Ago2 antibody produced in rat, clone 11A9.
3. Wash buffer: 300 mM NaCl, 50 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 0.05% Igepal CA630.
4. Quick Start Bradford Assay.
5. BSA standard.

2.5 RNA Purification

1. Direct-zol RNA MiniPrep with TRI Reagent.
2. 100% absolute ethanol.
3. Proteinase K.
4. tRNA from *E. coli* MRE 600.
5. Proteinase K buffer: 300 mM NaCl, 200 mM Tris–HCl pH 7.5, 25 mM EDTA, 2% SDS.
6. RNaseZap.

2.6 Preparation of Oligonucleotides for RT-qPCR

1. 5 M NaCl.
2. TE, pH 8.0.

2.7 Reverse Transcription

1. dNTP Mix.
2. Stem-loop primer.
3. SuperScript III Reverse Transcriptase (includes Superscript, DTT, First-Strand Synthesis Buffer).
4. SUPERaseIn RNase Inhibitor.

2.8 qPCR

1. iQ SYBR Green Mix.
2. Forward Primer (Custom Synthesis).
3. Universal Reverse Primer (Custom Synthesis).
4. 96-well plate.
5. Plate Seal.

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3 Methods

The following methods detail the transfection of HeLa cells followed by the recovery and quantification of siRNA strands loaded into Ago2 (*see* **Note 1**). This protocol can be adapted to different siRNA sequences, delivery vehicles, or cell types. However, changing these variables may require optimization of RNA recovery conditions.

Assay outline:

Day 1: Plate cells.

Day 2: Transfect cells and conjugate antibody to magnetic beads.

Day 3: Lyse cells, immunoprecipitate Ago2, and perform organic extraction/ethanol precipitation.

Day 4: Suspend RNA in water and perform stem-loop RT-qPCR.

3.1 Assay Design (Samples, Standards, and Controls)

When comparing the two strands of one siRNA, no internal standard is needed; however, if there is a need to compare multiple strands from different siRNAs, an additional non-specific siRNA acting as an internal standard should be transfected, as presented here (*see* **Note 2**). The internal standard will account for variability in transfection, cell number, cell lysis, immunoprecipitation, and RNA recovery. The method presented here utilizes a set of standards for the quantification of siRNA; this method was chosen to account for the differences in reverse transcription and PCR amplification efficiencies (Fig. 1) (*see* **Note 3**). Standard curves are built by spiking in siRNA to a sample generated from mock-transfected cells, mimicking sample treatment conditions (Table 1). Additional controls were prepared to check for (1) non-specific amplification of contaminant DNA and (2) amplification of endogenous RNA.

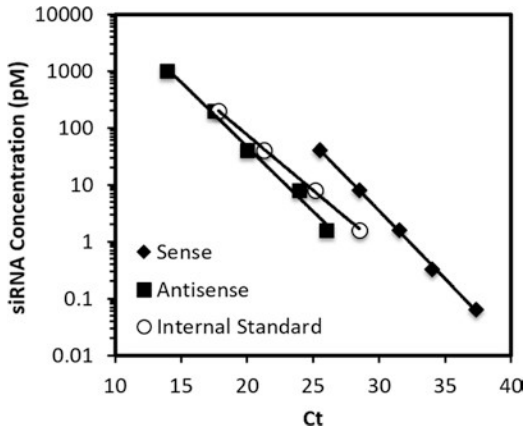

Fig. 1 Representative siRNA standard curves. Standards were generated by spiking in known quantities of siRNA into a mock-transfected lysate, followed by RT-qPCR. Amplification efficiencies were as follows: sense strand, 73.7%; antisense strand, 69.4%; and internal standard, 56.4%

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Table 1
Assay design

	1	2	3	4	5	6	7	8	9	10	11
	Sample	**IS**	**Sample standard curve**	**IS standard curve**	**Non-specific control**	**Non-specific control IS**	**–RT siRNA sample lysate**	**–RT IS sample lysate**	**–RT siRNA control lysate 1**	**–RT IS control lysate 1**	**–RT IS control lysate 2**
Sample primers	+	–	+	–	+	–	+	–	+	–	–
IS primers	–	+	–	+	–	+	–	+	–	+	+
Sample lysate +siRNA +IS	+	+	–	–	–	–	+	+	–	–	–
Control lysate 1 –siRNA + IS	–	–	+	–	+	–	–	–	+	+	–
Control lysate 2 –siRNA –IS	–	–	–	+	–	+	–	–	–	–	+
RT	+	+	+	+	+	+	–	–	–	–	–
Additional siRNA			15 amol – 250 fmol	15 amol – 250 fmol		250 fmol					

qPCR samples and controls for an siRNA strand (referred to as siRNA) and an siRNA used as an internal standard (referred to as IS). Sample lysates were co-transfected with 10 nM siRNA (Column 1) and 10 nM IS (Column 2). Control lysates were generated lacking the siRNA (Control Lysate 1) and lacking both the siRNA and IS (Control Lysate 2). These lysates were utilized to generate the standard curves (Columns 3 and 4), which also serve as positive controls, and negative controls (Columns 5–11). The negative controls included the verification of RT specificity for siRNA (Column 5) and IS (Column 6). The verification products generated are the result of transcription in the sample lysate (Columns 7 and 8), in Control Lysate 1 (Columns 9 and 10), and in Control Lysate 2 (Column 11)

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3.2 Stem-Loop RT-qPCR Primer Design

3.2.1 Outline for Primer Design

1. Determine the sequence of the siRNA strand. The passenger strand sequence for our siRNA will be used as an example: 5′-GGCCAGAAGGAUUUCAUUAUU-3′.
2. Find the reverse complement: 5′- AATAATGAAATCCTTC TGGCC-3′.
3. Generate the stem-loop primer sequence by combining the 44 nt stem-loop sequence, 5′-GTTGGCTCTGGTGCA GGGTCCGAGGTATTCGCACCAGAGCCAAC-3′ [17], with the first six nucleotides from **step 2**, 5′-GTTGGCTCT GGTGCAGGGTCCGAGGTATTCGCACCAGAG CCAAC***AATAAT***-3′.
4. Design the forward qPCR primer by taking the first 12–17 nucleotides of the siRNA sequence. Additional nucleotides can be added to the 5′ end, constituting a 5′ overhang, to raise the T_m to ~60 °C. We generated our forward primer by adding "GCGCG" to the first 15 nucleotides of our siRNA, resulting in the following sequence: 5′- **GCGCG**GGCCAGAAGGA TTTC-3′.
5. Design a universal reverse qPCR primer from sequences within the stem-loop. Primer T_m can be altered by changing the length of the primer from the 5′ terminus. We used the reverse primer: 5′-CCAGTGCAGGGTCCGAGGTA-3′ [19].
6. Confirm that qPCR primers do not form self-dimers, heterodimers, or stable hairpins and that the forward primer does not share complementarity with the stem-loop primer (*see* **Note 4**).

3.2.2 Expected Amplification Sequences (Fig. 2)

1. After reverse transcription, a 65 nt first strand cDNA results in the following sequence: 5′-GTTGGCTCTGGTGCAG GGTCCGAGGTATTCGCACCAGAGCCAACaata atgaaatccttctggcc-3′.

   The reverse complement of the siRNA sequence is in lowercase.
2. The second strand cDNA is expected to be 71 nt, consisting of the reverse complement of the first strand cDNA plus the additional six 5′ nt of the forward primer: 5′-**GCGCG ggccagaaggatttc**attattGTTGGCTCTGGTGC-GAATACCTCGGACCCTGCACCAGAGCCAAC-3′.

   The forward qPCR primer is in bold and siRNA sequence in lowercase.
3. The final PCR product is expected to be 63 nt with the following sequences:

   Forward strand:

   5′-**GCGCGggccagaaggatttc**attattGTTGGCTCTGGTGC GAATACCTCGGACCCTGCACTGG-3′

   Reverse strand:

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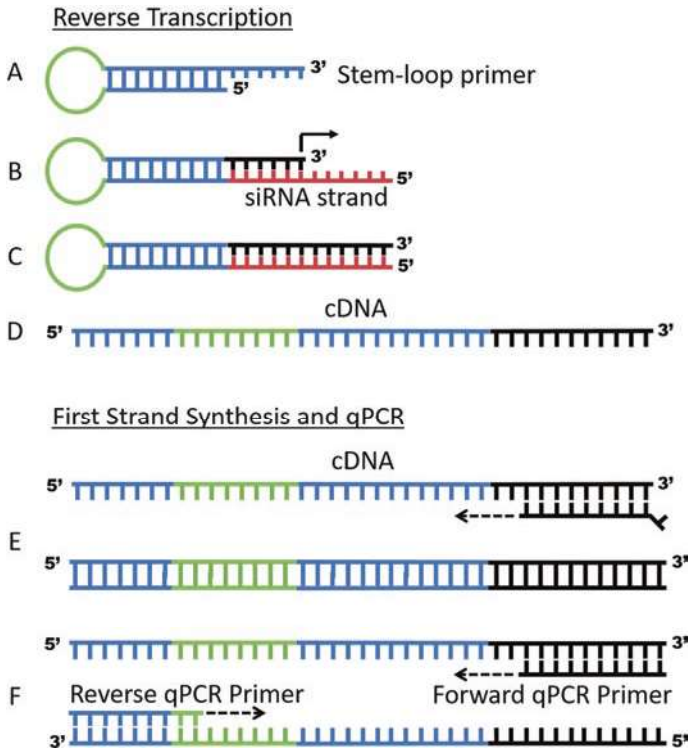

Fig. 2 Overview of stem-loop RT-qPCR amplification. The stem-loop primer is 44 nt with 6 nt complementary to the siRNA strand (**a**, **b**). Reverse transcription of the siRNA occurs from the 3′ end of the stem-loop primer adding 15 nt (**c**), making the final cDNA 65 nt (**d**). First strand synthesis occurs with the siRNA-specific primer (**e**), followed by PCR amplification (**f**)

5′-CCAGTGCAGGGTCCGAGGTATTCGCACC
AGAGCCAACaataat**gaaatccttctggccCGCGC**-3′.

The forward qPCR primer and its reverse complement are in bold in the forward strand and reverse strand, respectively. The siRNA sequence is in lowercase. The reverse primer and its reverse complement are underlined in the reverse strand and the forward strand, respectively.

3.3 HeLa Cell Transfection

For a 6-well plate, prepare a 500 μL transfection solution containing 5.75 μg (2.3 μg/mL based on final well volume) of LF2K, 10 nM final concentration of siRNA of interest, 10 nM internal standard siRNA, and Opti-MEM to volume (*see* **Note 5**).

1. Maintain HeLa cells in culture medium (*see* **Note 6**).
2. Trypsinize and then suspend cells in culture medium without antibiotics at 175,000 cells/mL, and plate 2 mL/well in a 6-well plate for 24 h.
3. Confirm that the cells are >90% confluent before proceeding with transfection.

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4. Verify siRNA concentration by measuring its absorbance at 260 nm by NanoDrop 2000 (*see* **Notes 7** and **8**).
5. Warm Opti-MEM to 37 °C.
6. Add 245 μL of Opti-MEM to a 1.7 mL centrifuge tube, and then add 2.5 μL of 10 μM siRNA and 2.5 μL of 10 μM internal standard.
7. Add 244.25 μL Opti-MEM to a new 1.7 mL centrifuge tube along with 5.75 μL of LF2K. Mix by gently pipetting.
8. Wait for 5 min, then add the diluted siRNA in the first centrifuge tube to the diluted LF2K solution in the second tube, mix by pipetting, and incubate for 20 min.
9. While the transfection solution is incubating, replace the medium in the 6-well plate with fresh culture medium without antibiotics.
10. After 20 min, mix the transfection solutions by pipetting several times before adding dropwise to each respective plate/well. Gently rock plates back and forth and side to side, avoiding circular motions. Return plates to incubator for 24 h.

3.4 Conjugation of Ago2 Antibody to Magnetic Protein G Beads

1. Transfer 25 μL of Protein G Magnetic Beads to a 1.7 mL microcentrifuge tube, making sure to achieve a homogeneous suspension prior to transfer (*see* **Note 9**).
2. Add 475 μL of 1× PBS. Place the tube on a magnetic separation rack for 1 min and then remove and discard the supernatant (*see* **Note 10**).
3. Suspend the beads in 1.5 mL of 1× PBS, and add 2.25 μL (3.375 μg) of Ago2 antibody.
4. Incubate overnight at 4 °C with end-over-end rotation.
5. Wash the beads 1× in 250 μL of wash buffer. Remove and discard the supernatant.
6. Resuspend the beads 1× in 250 μL of 1× PBS, and transfer them to a new 1.7 mL microcentrifuge tube.

3.5 Cell Lysis

1. Aspirate the medium, and wash the plate two times with a volume of ice-cold PBS equal to the volume of medium removed (2 mL per well for a 6-well plate), working with the plate on ice.
2. Add 500 μL of ice-cold lysis buffer, and rock to make sure the buffer wets the entire well.
3. Incubate on ice for 30 min.
4. Scrape cells and transfer lysate to a centrifuge tube.
5. Pellet debris at 16,000 × *g* for 30 min at 4 °C, and transfer to a fresh microcentrifuge tube (*see* **Note 11**).

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6. Measure protein concentration by Quick Start Bradford Assay using a BSA standard. Lysates may need to be diluted to yield protein concentrations in the linear range of the standard curve and to minimize interference of the lysis buffer with quantification (*see* **Note 12**).

3.6 RNA Immunoprecipitation (RIP)

1. Remove 1× PBS from the prepared magnetic beads, and load 400 µg of lysate, diluted to 0.5 mL with lysis buffer, into the 1.7 mL centrifuge containing the beads.
2. Incubate at 4 °C for 3 h with end-over-end rotation.
3. Briefly centrifuge the tube, place it on a magnetic separator for 1 min, and remove the supernatant (*see* **Note 11**).
4. Wash the beads 3× in 250 µL of wash buffer. Each time, mix by pipetting up and down, and then place tube on magnetic separator for 1 min before removing and discarding the supernatant.
5. Wash the beads 1× in 250 µL 1× PBS, and transfer them to a new microcentrifuge tube (*see* **Note 11**).

3.7 RNA Purification

1. Place tube on magnetic separator.
2. Remove and discard the supernatant.
3. Suspend the beads in 140 µL Proteinase K Buffer with 6 µL of 20 mg/mL Proteinase K and 4 µL of 390 µg/mL tRNA (*see* **Note 13**).
4. Incubate beads at 60 °C for 15 min, mixing occasionally to bring the beads back into suspension.
5. Using the Zymo Research Direct-zol RNA Purification kit (*see* **Note 14**), add 3 volumes of TRI Reagent (450 µL), mix thoroughly, and freeze if not immediately performing reverse transcription (RT).
6. Add 1 volume of absolute ethanol and vortex.
7. Apply entire volume (600 µL) to Zymo-Spin Column, and centrifuge at 16,000 × *g* for 30 s.
8. Discard flow-through, apply remaining sample to column, and centrifuge again.
9. Wash spin column with 400 µL of RNA wash buffer.
10. Mix 75 µL DNA Digestion Buffer and 5 µL DNase I, then add to spin column, and incubate for 15 min.
11. Wash column with 400 µL RNA PreWash solution, and centrifuge for 30 s, discarding flow-through.
12. Wash column with 700 µL RNA wash buffer and centrifuge for 2 min.

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13. Transfer column to a new centrifuge tube, and elute in 50 μL of RNase-free water preheated to 60 °C.
14. Proceed immediately to reverse transcription reaction or freeze recovered sample at −80 °C.

3.8 RT-qPCR Preparation

Suspend oligonucleotides in TE to a final concentration of 100 μM. Verify concentration using the NanoDrop, and adjust the concentration accordingly while making the first primer dilution.

3.8.1 Preparation of Stem-Loop Primer for RT

1. Fold the stem-loop primer by adjusting NaCl concentration to 50 mM (add 0.3 μL of 5 M NaCl to 30 μL of ~100 μM primer); then heat primer to 95 °C for 10 min; decrease to 75 °C for over 30 min; hold temperature at 75 °C, 68 °C, 65 °C, and 62 °C for 1 h at each temperature; hold at 60 °C for 2 h; and then decrease to 4 °C and hold.
2. Make a 200 nM stem-loop primer working stock by diluting 1:10 to 10 μM, verifying the primer concentration by measuring the absorbance at 260 nm (*see* **Note 7**), and then by diluting (1:10) to 1 μM, making adjustments to the dilution depending upon the measured concentration. Working primer stocks can be stored at −20 °C.

3.8.2 Preparation of qPCR Primers

Make a 10 μM working stock of each primer by first diluting primer 1:5 in TE, measuring the absorbance at 260 nm (*see* **Note 7**), and then diluting the primer ~1:2 (adjusted based on absorbance) to 10 μM. Store working stocks at −20 °C.

3.9 Reverse Transcription Reaction

1. A single reaction mix is prepared by adding the following components to a nuclease-free microcentrifuge tube:
   (a) 0.5 μL 10 mM dNTP mix.
   (b) 1 μL of 1 μM stem-loop RT primer (50 nM final concentration).
   (c) 4 μL 5× First-Strand Synthesis Buffer.
   (d) 2 μL 0.1 M DTT.
   (e) 0.2 μL SUPERaseIn RNase Inhibitor (20 U/μL).
   (f) 0.25 μL SuperScript III RT (200 U/μL).
   (g) 7.05 μL water.
   (h) 5 μL RNA sample (dilute 1:5 from purified RNA).
2. Prepare +RT and −RT master mixes without the RNA sample by scaling the volumes for a single qPCR reaction to the desired number of qPCR reactions, omitting the stem-loop primer, RNA sample, and 5 μL of water.
3. Prepare a second set of master mixes by adding the specific stem-loop primer. This will give four master mixes (+RT-IS, +RT-siRNA, −RT-IS, −RT-siRNA).

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4. Assemble the RT reactions by adding 10 µL of the appropriate master mix to 5 µL of the corresponding 1:5 diluted RNA, according to Table 1, and 5 µL of water. For making standard curves or control samples requiring the addition of siRNA, replace the 5 µL of water with 5 µL of siRNA at the appropriate concentration (Table 1; Columns 3, 4, and 6).
5. Load thermal cycler and incubate as follows (*see* **Note 15**):
   (a) 1 Cycle:
      - 16 °C for 30 min.

   (b) 60 Cycles:
      - 30 °C for 30 s.
      - 42 °C for 30 s.
      - 50 °C for 1 s.

   (c) 1 Cycle:
      - 85 °C for 5 min.
      - 4 °C Forever.

6. Proceed immediately to qPCR or freeze at −20 °C.

3.10 qPCR Reaction

1. A single reaction mix is prepared by adding the following components:
   (a) 12.5 µL 2× iQ SYBR Green Mix.
   (b) 0.75 µL 10 µM Forward Primer.
   (c) 0.75 µL 10 µM Universal Reverse Primer.
   (d) 2 µL water.
   (e) 9 µL RT sample (cDNA diluted 1:5).
2. Prepare master mixes (without cDNA) by scaling the volumes for a single qPCR reaction to the desired number of qPCR reactions. Mix gently and spin down master mix.
3. Assemble the qPCR reaction by combining 11 µL of master mix with 9 µL of RT product.
4. Load real-time qPCR thermal cycler (BioRAD MyiQ) as follows:
   (a) 95 °C for 10 min.
   (b) 50 Cycles:
      - 95 °C for 15 s.
      - 60 °C for 1 min.

   (c) 72 °C for 5 min.
   (d) 95 °C for 1 min.
   (e) Melting curve: 0.5 °C per 10 s from 55 °C to 95 °C.

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5. Verify the formation of a product with a T_m of ~84 °C. In addition, the C_t values of all samples and standards should be lower than those for every negative control (*see* **Note 16**).

4 Notes

1. The methods for cell culture can be scaled to increase signal by RT-qPCR as needed. Transfection of 250 pmol siRNA into 1 well from a 6-well plate of HeLa cells (350,000 cells/well plated 24 h prior to transfection) generated sufficient signal by RT-qPCR.
2. When investigating endogenous small RNAs (i.e., a miRNA), a miRNA with consistent expression in the tissue of interest is a suitable internal standard [23].
3. Analysis of qPCR data can be performed using either the $\Delta\Delta C_t$ method [24] or with the use of a known set of standards. Standards were used here because of the difference in reverse transcription and PCR efficiencies for different siRNA strands.
4. The OligoAnalyzer tool (https://www.idtdna.com/calc/analyzer) is useful for quickly checking primer hairpins, self-dimers, and heterodimers. If such structures do exist, depending on the issue, the reverse primer can be shifted or stem-loop primer sequence and reverse primer altered. If the forward primer forms a self-dimer or hairpin, accurate quantification may require increasing the qPCR annealing temperature or performing a pre-amplification step at a higher T_m followed by the normal qPCR reaction.
5. An siRNA concentration of 10 nM was chosen for these experiments, because it is the highest concentration we could confidently use without knowingly having any adverse effects on cell function [25].
6. Transfection efficiency can be highly variable. Maintaining cells in a metabolically active state, not exceeding a ~90% confluence, and maintaining a consistent LF2K:cell ratio will minimize transfection variance.
7. Because siRNAs are less than 100 nt, siRNA-specific extinction coefficients are recommended when measuring siRNA concentrations. Theoretical extinction coefficients can be calculated at https://www.idtdna.com/calc/analyzer.

$$\text{Concentration} = \left(\text{A260} \times \text{extinction coefficient}\right) / \text{path length}$$

8. Practice RNase-free technique, and use certified RNase-free reagents and consumables. This includes establishing a separate workspace that is cleaned with an RNase inhibitor (e.g., RNaseZap) prior to use and being cognizant of potential sources of RNase contamination. One common source of

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contamination is the touching of the inside of a microcentrifuge cap when opening and closing microcentrifuge tubes; this can be avoided by using one hand to hold the tube and the second hand to open the cap. RNase-free technique is not critical for the manipulation of cells; however, RNase-free technique should be practiced for all lysis steps. It is recommended not to use DEPC-treated water as residual DEPC can interfere with enzymatic assays; suitable RNase-free water is available from commercial sources.

9. 25 μL of magnetic beads is more than enough to complex the amount of Ago2 antibody in our assay. 25 μL was chosen because it is the minimum quantity of beads that can be confidently manipulated throughout the immunoprecipitation.
10. When using a magnet for the manipulation of magnetic beads, ensure even suspension before applying samples. It is also good practice to briefly centrifuge the tube to remove any solution from the cap and then to resuspend the solution by pipetting.
11. Western blot analysis can be useful to verify the immunoprecipitation of Ago2 during assay development and troubleshooting. However, it is not practical to run a Western blot for every sample. Therefore, it is recommended to take samples of the diluted lysate, the Ago2-depleted lysate, and a fraction of the immunoprecipitated Ago2-Protein G Magnetic Beads (Fig. 3), and analyze these samples as necessary.

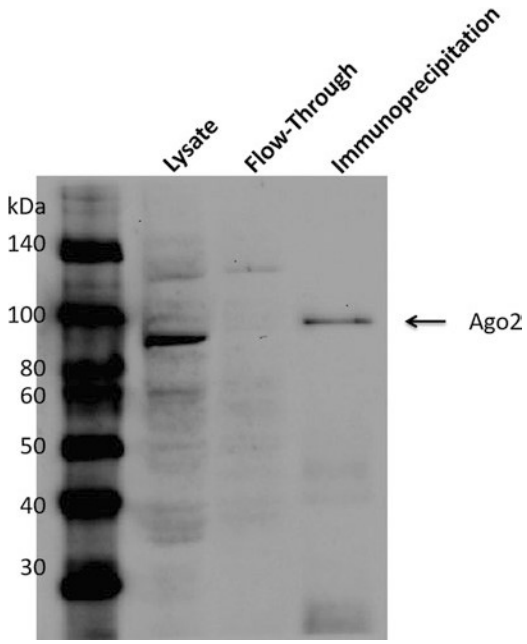

Fig. 3 Verification of Ago2 immunoprecipitation. Confirmation of Ago2 immunoprecipitation from HeLa cell lysates with an anti-Ago2 (11A9) antibody followed by Western blotting with an anti-Ago2 (C34C6) antibody

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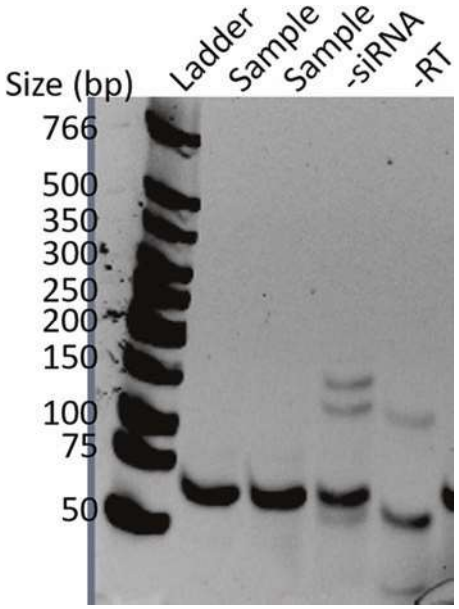

Fig. 4 Verification of PCR products. Representative qPCR products from Columns 1 (sample), 5 (siRNA), and 7 (RT), resolved on a 4–20% TBE gel and stained with SYBR Gold. Ladder is a low MW DNA ladder from NEB

12. A 1:5 dilution is a good starting dilution; for our experiments, protein concentrations after dilution were ~200 μg/mL.
13. Proteinase K and tRNA mixture should be added to extraction buffer immediately before use. The addition of tRNA to samples before RNA extraction from Ago2 improved the sensitivity of the detection, presumably by facilitating the efficiency of purification or RT.
14. The method of small RNA purification is important as some methods can lead to different precipitation efficiencies. In 2011, an article was retracted after the authors discovered bias in small RNA purification [26]. Multiple manufacturers have validated silica-based spin column purification methods as suitable for the unbiased purification of small RNAs.
15. A pulsed RT protocol [18] was chosen because greater separation was observed between sample and negative control C_t values; similar C_t values were observed for both pulsed RT and standard RT sample C_t values [16].
16. When using a new set of primers, it is useful to verify product formation by native gel electrophoresis. The hallmark of a specific product is a single band on a gel at the anticipated size. Negative control wells typically have several bands produced from non-specific amplification or primer dimers, possibly including a band of the anticipated size (Fig. 4).

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Limiting the number of amplification cycles can also maximize the discrimination between specific and non-specific product formations.

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