Chapter 2

Quantification of miRNAs Co-Immunoprecipitated with Argonaute Proteins Using SYBR Green-Based qRT-PCR

Hong-Duc Phan, Junan Li, Ming Poi, and Kotaro Nakanishi

Abstract

MicroRNAs (miRNAs) are small non-coding RNAs that trigger post-transcriptional gene silencing. These RNAs need to be associated with the Argonaute proteins to be functional. This assembly begins with loading of a miRNA duplex, followed by the ejection of one of the strands (passenger). The remaining strand (guide) together with the Argonaute protein forms a ribonucleoprotein effector complex (the RNA-induced silencing complex, RISC). Mutation on the Argonaute protein, if affecting either step of the RISC assembly, impacts the function of miRNAs. Therefore, any observation of decreased miRNA level of mutants will provide insights into the role of those amino acid residues in the mechanical function of the Argonaute protein. In this chapter, we introduce a method to relatively quantify a specific miRNA co-immunoprecipitated with wild type and mutant Argonaute proteins from HEK293T cells, using Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR). Spiking a synthetic exogenous miRNA as an internal control with RNA extraction prior to cDNA synthesis will normalize the C_t values obtained from the qRT-PCR assays and enable us to quantify the relative level of Argonaute-bound miRNA.

Key words qRT-PCR, miRNA, Argonaute, Immunoprecipitation, SYBR Green

1 Introduction

miRNAs are single-stranded RNAs of about 22 nucleotides (nt) that are generated from the endogenous hairpin-shaped transcript [1, 2]. In human, miRNA duplexes are loaded into four Argonaute paralogs (Ago1-4) [1, 3]. After passenger-strand ejection, the remaining guide and the Argonaute protein form the RNA-induced silencing complex (RISC) [4, 5]. This ribonucleoprotein complex also provides a scaffold for the components essential for translational repression and deadenylation [3, 4, 6]. To understand the versatile functions of Argonaute proteins, previous studies compared the physiological activities between wild type (WT) and the mutant proteins. For example, FLAG-tagged human Argonaute (FLAG-Ago) proteins were expressed in HEK293T cells and

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purified by immunoprecipitation with anti-FLAG antibody from the cell lysate [7]. The miRNA-binding activities of Argonaute mutants were evaluated by Northern blot using a probe for a particular miRNA [7–9]. This method has been routinely used to identify residues on Argonaute proteins playing important role(s) during loading of miRNA and/or passenger-strand ejection [10–12]. Despite its high specificity, Northern blot has some disadvantages, such as being time-consuming, low throughput, and low sensitivity for small RNA detection [13–15].

Recently, Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) is becoming more popular as an alternative method to Northern blot. However, there are two major obstacles preventing the use of qRT-PCR in quantifying miRNA co-immunoprecipitated with FLAG-Ago. First is the primer design for qRT-PCR. Historically, qRT-PCR has been developed for detection of mRNA; therefore, the techniques and knowledge based on mRNA detection may not be applicable for miRNA, primarily due to the much shorter length. The use of stem-loop qRT-PCR may partially solve the problems, but this method requires the use of stem-loop reverse transcription primers (also known as TaqMan® MicroRNA assay), which has been licensed to Applied Biosystems™ [16, 17]. Another obstacle is that a reference gene such as a housekeeping gene needs to be used as an internal control to normalize variances between samples [18]. Previous studies often used fortuitously bound RNA such as GAPDH as a reference gene for the normalization of the C_t value when employing qRT-PCR to detect a specific mRNA co-immunoprecipitated with Argonaute proteins [19–21]. This approach may introduce considerable variance in the quantification of miRNA co-immunoprecipitated with Argonaute mutants when the mutation affects the binding of such RNAs.

To address the issue of primer design, we employed SYBR Green-based qRT-PCR with polyT adaptor primers (Fig. 1) [22]. This approach is cost-effective, especially in the case of systematic quantification of different miRNAs co-immunoprecipitated with a large number of Argonaute protein mutants. In this method, miRNAs will be first polyadenylated by poly(A) polymerase (Fig. 1, step 1), prior to the complementary DNA (cDNA) synthesis by reverse transcription using an oligo-dT adaptor primer composed of a polyT primer and a universal tag on its 5′ side (Fig. 1, step 2). Then, an equal amount of an exogenous miRNA, cel-miR-39, was spiked as the internal control into the immunoprecipitated Argonaute proteins whose amount was adjusted based on western blot analysis before RNA extraction (Fig. 2) [23]. The difference in threshold cycles for the miRNA of interest and the spiked cel-miR-39 enables us to normalize RNA extraction from the different immunoprecipitated proteins.

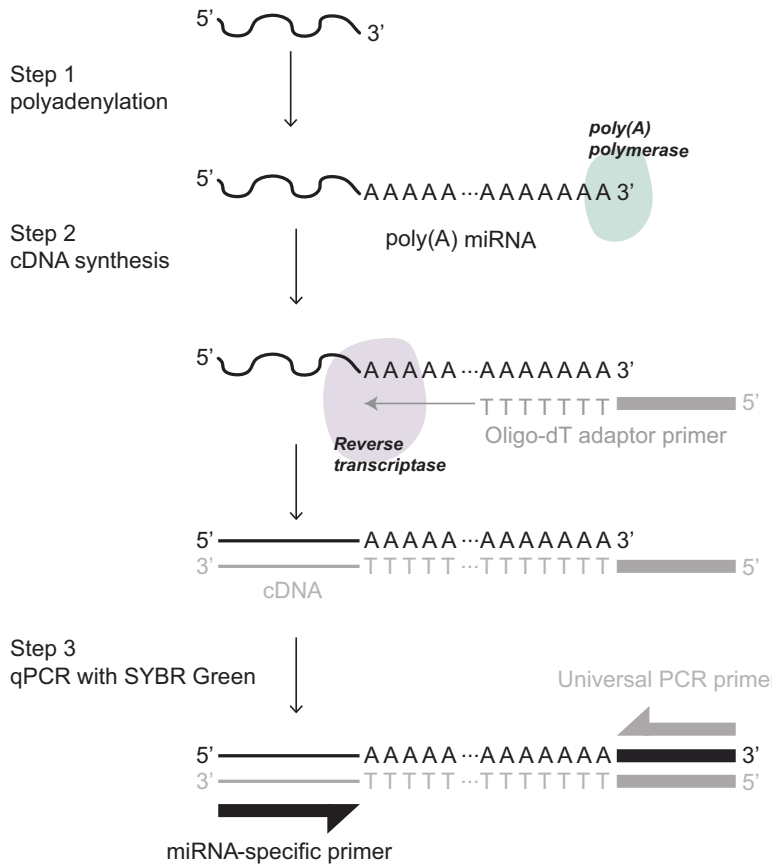

Fig. 1 Schematic representation of polyT adaptor qRT-PCR method. Step 1: poly(A) tail is added to miRNA by poly(A) polymerase. Step 2: cDNA is synthesized by reverse transcriptase using oligo-dT adaptor primer. Step 3: cDNA is amplified by SYBR Green-based qPCR with miRNA-specific and universal PCR primers

2 Materials

1. Dynabeads Protein G (Thermo Fisher Scientific).
2. Magnetic rack, DynaMag-2 Magnet (Thermo Fisher Scientific) or equivalent.
3. Ultrasonic processor.
4. Bench top tube rotator.
5. Vortex mixer.
6. Anti-FLAG M2 antibody, F1804 (Sigma). The antibody is diluted 1000 times with the blotting buffer containing 5% of skim milk and 1% of sodium azide as working solution for Western blot.
7. Lysis buffer: 30 mM HEPES-KOH (pH 7.4), 100 mM KOAc, 2 mM $Mg(OAc)_2$, 10 μg/μl Aprotinin*, 10 μM Leupeptin*,

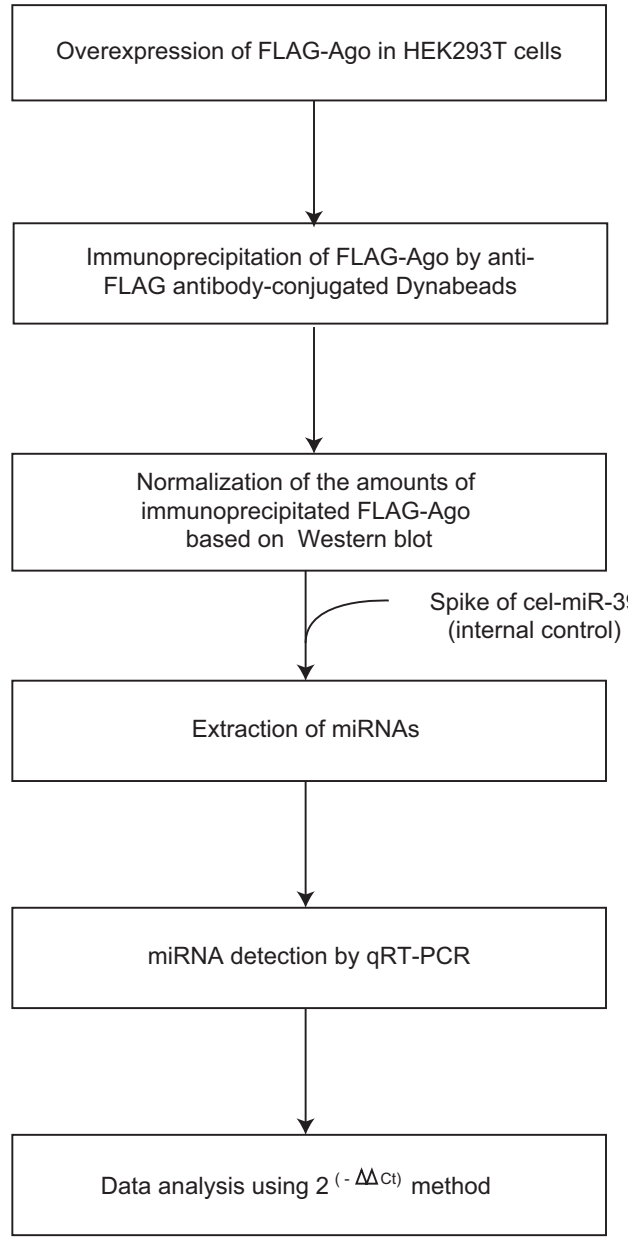

Fig. 2 Workflow of SYBR Green-based qRT-PCR of miRNAs using an exogenous miRNA as an internal control

1 μM Pepstatin*****, and 1 mM phenylmethylsulfonyl fluoride (PMSF)***** (*****: freshly prepared).

8. Wash buffer: 30 mM HEPES-KOH (pH 7.4), 100 mM KOAc, 2 mM $Mg(OAc)_2$, 800 mM NaCl, and 1% Triton X-100.
9. Cel-miR-39 (GE Dharmacon): 5′ UCACCGGGUGUAAAU CAGCUUG 3′.

10. Proteinase K reaction buffer: 200 mM Tris–HCl (pH 7.5), 25 mM EDTA (pH 8.0), 300 mM NaCl, and 2% sodium dodecyl sulfate (SDS) (w/v).
11. Proteinase K, 20 mg/ml.
12. TRIzol LS reagent (Thermo Fisher Scientific).
13. Direct-zol RNA kits (Zymo Research).
14. TransIT-X2 Dynamic Delivery System (Mirus Bio).
15. qScript microRNA cDNA Synthesis Kit (Quanta Bio).
16. PerfeCTa SYBR Green SuperMix (Quanta Bio)
17. Universal PCR primer (Quanta Bio).
18. Forward primer to detect miR-19b and cel-miR-39 (ordered from Quanta Bio) (*see* **Note 1**).

Cel-miR-39	GCAGCTGATTTCGTCTTGGTAA
miR-19b	GTGCAAATCCATGCAAAACTG

19. 2× SDS-loading dye: 125 mM Tris–HCl (pH 6.8), 4% Sodium dodecyl sulfate (SDS), 20% Glycerol, 0.04% Bromophenol blue, and 100 mM beta-mercaptoethanol* (*freshly prepared).
20. Opti-MEM reduced serum media (Gibco).
21. Nitrocellulose blotting membrane (GE Healthcare).
22. Electrophoresis chamber (Bio-RAD).
23. Blotting buffer: 20 mM Tris-base, 150 mM NaCl, and 0.001% Tween 20.
24. Blocking buffer: 20 mM Tris-base, 150 mM NaCl, 0.001% Tween with 10% (w/v) skim milk.
25. Novex NuPAGE SDS-PAGE gel (Invitrogen).
26. Image StudioLite (Li-cor).
27. Odyssey® Imaging Systems (Li-cor).
28. IRDye® 800CW Goat anti-Mouse IgG (H + L), 0.1 mg (fluorescent-labeled secondary antibody, Li-cor). The antibody is diluted at 1:16,000 with dilution buffer containing 50% PBS (pH 7.4), 50% Odyssey buffer and 0.01% Tween as working solution for Western blot.
29. pCAGEN-FLAG-Ago2 WT [24].
30. pCAGEN-FLAG-Ago2 Y529E is made using the following forward and reverse site-directed mutagenesis primers: Forward: 5′-CGGCAAGACGCCCGTGGAAGCCGAG-3′, Reverse: 5′-ACGCGCTTGACCTCGGCTTCCACGGGC-3′.
31. The gene of MBP is amplified from pMAL vector (Novagen) with the following forward and reverse primers and processed with EcoRI and NotI. Then, the fragment is cloned into

pCAGEN vector using the same restriction enzyme sites. Forward: 5′-GAGTGAATTCATGAAAATCGAAGAAGGTAAACTGGTAAT-3′, Reverse: 5′-CACTGCGGCCGCTCAATTAGTCTGCGCGGCTGC-3′ (EcoRI and NotI sites are underscored).

3 Methods

We quantified miR-19b, one of the most abundant miRNAs in HEK293T cells [25] and compare the miRNA-binding activities between the FLAG-Ago2 WT and Y529E mutant. The latter was previously reported as a mutant deficient in miRNA binding by Northern blot analysis [12]. Non-tagged MBP was used as a negative control.

3.1 Overexpression of MBP, FLAG-Ago2 WT, and FLAG-Ago2Y529E Proteins in HEK293T Cells

1. Transfect the mixture of 10 μg plasmids, 45 μl of TransIT-X2 reagent, and 1.5 ml OMEM into the 10 cm plates of 80% confluent HEK293T cells, according to the manufacturer manual's instruction. Cultivate the cells for 48–50 h.
2. After rinsing the cells once with 10 ml cold PBS, detach the cell off the plates by vigorously pipetting the cells with PBS. Harvest the cell by centrifugation at 1000 × *g* for 10 min at 4 °C. Measure the weight of the cell pellet (*x* mg). Hereafter, handle the samples at 4 °C through the whole procedure.
3. Resuspend the cell pellet with five times of the pellet weight (5× μl) of lysis buffer in a 1.7 ml microcentrifuge tube. Place the tube in an ice bucket and sonicate at 30% amplitude using Microtip for 20 s in 4 s burst with at least 60-s intervals. Incubate on ice for 5 min, and centrifuge at 17,000 × *g* for 20 min at 4 °C. Collect the supernatant and aliquot into 100 μl fraction in microcentrifuge tubes. Then, flash-freeze the lysate in liquid nitrogen and store them at −80 °C.

3.2 FLAG-Ago Immunoprecipitation Using Anti-FLAG M2 Antibody

1. For each FLAG-Ago sample, aliquot 60 μl of Dynabeads Protein G into a 1.7 ml microcentrifuge tube and resuspend with 1 ml of Lysis buffer. Set the tubes on the magnetic rack and remove the solution. Repeat this wash process three times.
2. Resuspend the beads in 60 μl of Lysis buffer, followed by mixing with 2 μl of 1 mg/ml anti-FLAG M2 antibody. Shake the tube on the vortex mixer at room temperature for 1 h.
3. Resuspend the antibody-conjugated beads with 1 ml of Lysis buffer. Set the tube on the magnetic rack and remove the solution. After repeating this wash process three times, discard the supernatant.

4. Quickly thaw the frozen 100 μl-cell lysate aliquot from **step 3** of Subheading 3.1, and incubate with the antibody-conjugated beads at 4 °C for 2 h on a benchtop tube rotator.
5. Add 1 ml of Wash buffer to the beads and resuspend the mixture. Set the tubes on the magnetic rack and discard the supernatant. Repeat this wash process five times.

3.3 Semi-quantification of Immunoprecipitated Protein by Western Blot

We followed the standard western blot protocol [26] with some modifications.

1. Take 3 μl of the beads from **step 5** in Subheading 3.2 and mix with 2 μl of 2× SDS-loading dye and 7 μl of water to the final volume of 12 μl. Heat the sample at 90 °C for 5 min.
2. After spinning down the sample, load the supernatant on the SDS-PAGE gel. Run the gel at a constant voltage of 180 V until the bromophenol blue reaches to the bottom.
3. Transfer the sample from the gel to the nitrocellulose blotting membrane in the electrophoresis chamber at a constant voltage of 80 V for 1.5 h at 4 °C.
4. Block the membrane with 20 ml of blocking buffer for 1 h.
5. Incubate the membrane for 2 h at room temperature with 10 ml of anti-FLAG M2 antibody working solution.
6. After discarding the antibody, add 20 ml of blotting buffer to the membrane and incubate them for 10 min. Repeat this wash process three times.
7. Incubate the membrane for 1 h with 40 ml of secondary antibody working solution.
8. After discarding the second antibody, add 20 ml of Blotting buffer to the membrane, and incubate them for 10 min. Repeat this wash process three times.
9. Fluorescence from the fluorescent-labeled secondary antibody is detected in the 800 channel of Odyssey Imaging System.
10. Quantify the fluorescent intensity from the immunoprecipitated Ago protein using Image StudioLite software (Fig. 3).

3.4 Extraction of RNA from Immunoprecipitated FLAG-Ago (see Note 2)

1. Adjust the amount of beads based on the intensities of immunoprecipitated Ago of the Western blot data from **step 10** in Subheading 3.3.
2. Add 250 μl of proteinase K buffer and 10 μl of 20 mg/ml proteinase K to the protein immunoprecipitated beads. Incubate the mixture at 50 °C for 30 min.
3. Spike 1 μl of 10 nM of cel-miR-39 into each sample as an internal control (*see* **Note 3**).

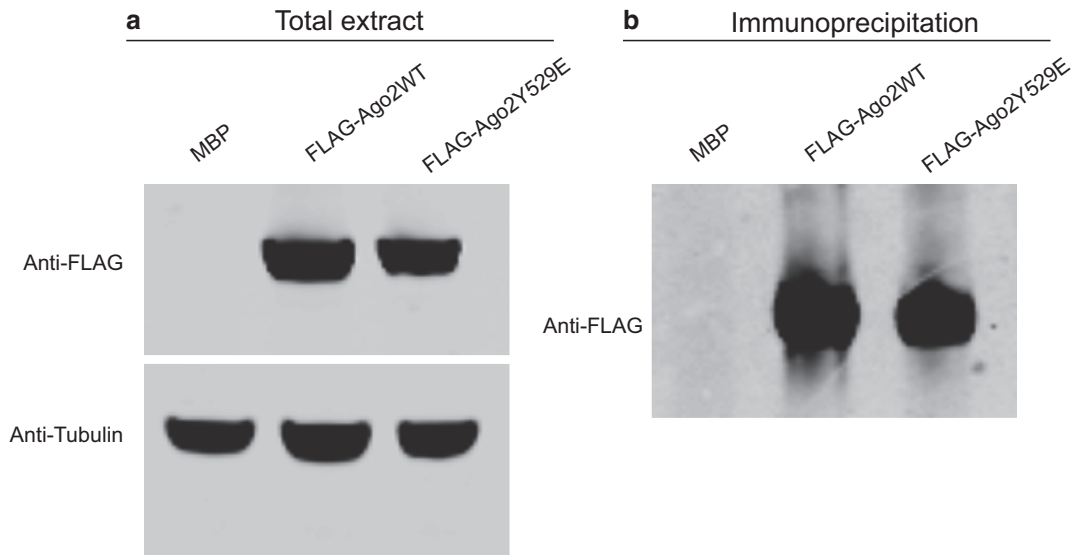

Fig. 3 Expression and immunoprecipitation of FLAG-Ago2 WT and Y529E. (**a**) Expression levels of FLAG-Ago2 WT and Y529E were detected by anti-FLAG antibody. Anti-tubulin antibody was used to detect tubulin as an internal control. Non-tagged MBP was used as a negative control. Western blot analysis was triplicated. (**b**) Immunoprecipitated FLAG-Ago2 WT and Y529E were detected by anti-FLAG antibody

4. Add 200 μl of TRIzol LS reagent to each sample and vortex the mixture for 30 s, followed by incubation at room temperature for 5 min. Centrifuge at 17,000 × *g* for 10 min to remove the beads and debris. Transfer the supernatant to a new tube.
5. Mix the supernatant with an equal volume of 100% ethanol and transfer the mixture to the spin column of Direct-zol RNA kits. Hereafter, follow the instruction of the kit to extract the total RNA.
6. Elute the total RNA from the spin column with 20 μl of RNase-free water. The RNA eluents can be stored at −80 °C until they are used for cDNA synthesis step.

3.5 cDNA Preparation

1. Take 7 μl of RNA for the poly(A) tailing reaction, following the qScript microRNA cDNA Synthesis Kit's instructions: incubate the reaction at 37 °C for 1 h, followed by another incubation at 70 °C for 5 min.

Reagents	Volume (μl)
5× Poly(A) tailing buffer	2
RNA	7
Poly(A) polymerase	1

2. Set up the cDNA synthesis reaction, following the qScript microRNA cDNA Synthesis Kit's instructions: incubate the reaction at 42 °C for 20 min, followed by another incubation at 85 °C for 5 min.

Reagents	Volume (μl)
Poly(A) tailing reaction	10
microRNA cDNA reaction mix	9
qScript reverse transcriptase	1

3. Transfer 4 μl of the cDNA product successively to 16 μl of RNase-free water to generate fivefold serial dilution samples and store them at −20 °C.

3.6 cDNA Amplification by SYBR Green qPCR

1. Set up the qPCR reaction to amplify miR-19b as follows:

Reagents	Volume (μl)
2× SYBR Green premix	5
10 μM miR-19b primer	0.2
10 μM Universal PCR primer	0.2
cDNA (from **step 3** in Subheading 3.5)	4
Double distilled water	0.6

2. Set up the qPCR reaction to amplify cel-miR-39 as follows:

Reagents	Volume (μl)
2× SYBR Green premix	5
10 μM cel-miR-39 primer	0.2
10 μM Universal PCR primer	0.2
cDNA (from **step 3** in Subheading 3.5)	4
Double distilled water	0.6

3. Run the PCR as follows:
   Pre-incubation (1 cycle): 95 °C for 2 min.
   PCR (40 cycles):
   Denaturation: 95 °C for 5 s.
   Annealing: 60 °C for 30 s (to collect fluorescence signals).

3.7 Data Analysis

The miRNA-binding activity of Ago2 Y529E mutant was compared to that of WT by quantifying their bound miR-19b (*see* **Note 4**).

1. The threshold cycle for miR-19b co-immunoprecipitated with FLAG-Ago2 WT is normalized to the spiked cel-miR-39 using the following equation:

$$\Delta C_{\mathrm{t,WT}} = C_{\mathrm{t,WT}}\left(\text{miR-19b}\right) - C_{\mathrm{t,WT}}\left(\text{cel-miR-39}\right)$$

   where $C_{\mathrm{t,WT}}$ (miR-19b) and $C_{\mathrm{t,WT}}$ (cel-miR-39) are the threshold cycles for miR-19b and cel-miR-39 extracted from FLAG-Ago2WT sample, respectively.

2. The threshold cycle for miR-19b co-immunoprecipitated with FLAG-Ago2 Y529E is normalized to the spiked cel-miR-39 using the following equation:

$$\Delta C_{\mathrm{t,Y529E}} = C_{\mathrm{t,Y529E}}\left(\text{miR-19b}\right) - C_{\mathrm{t,Y529E}}\left(\text{cel-miR-39}\right)$$

   where $C_{\mathrm{t,\ Y529E}}$ (miR-19b) and $C_{\mathrm{t,\ Y529E}}$ (cel-miR-39) are the threshold cycles for miR-19b and cel-miR-39 extracted from FLAG-Ago2 Y529E sample, respectively.

3. The relative amount of miR-19b co-immunoprecipitated with FLAG-Ago2 Y529E to that with FLAG-Ago2 WT is given by

$$\frac{X_{\mathrm{N,Y529E}}}{X_{\mathrm{N,WT}}} = \frac{2^{-\Delta C_{\mathrm{t,Y529E}}}}{2^{-\Delta C_{\mathrm{t,WT}}}} = 2^{-\left(\Delta C_{\mathrm{t,Y529E}} - \Delta C_{\mathrm{t,WT}}\right)} = 2^{-\Delta\Delta C_{\mathrm{t}}}$$

   where $X_{\mathrm{N,WT}}$ and $X_{\mathrm{N,Y529E}}$ are the normalized initial amounts of miR-19b co-immunoprecipitated with FLAG-Ago2 WT and Y529E mutant, respectively, and $\Delta\Delta C_{\mathrm{t}} = \Delta C_{\mathrm{t,Y529E}} - \Delta C_{\mathrm{t,WT}}$ [27].

4. $X_{\mathrm{N,Y529E}}/X_{\mathrm{N,WT}}$ is shown in bar graph with two-sided Student's *t*-test values calculated (Fig. 4). The relative amount of miR-19 co-immunoprecipitated with MBP to that with FLAG-Ago2 WT (i.e. $X_{\mathrm{N,MBP}}/X_{\mathrm{N,WT}}$) is calculated in the same way.

4 Notes

1. A list of pre-designed primers to detect miRNAs has been validated and published on the website of the manufacturer (http://www.quantabio.com/products/microrna-profiling). For miRNAs without pre-validated primers, a guide note for primer design has also been published (http://www.quantabio.com/products/microrna-profiling).
2. It is important to use RNase-free materials in Subheadings 3.4–3.6.

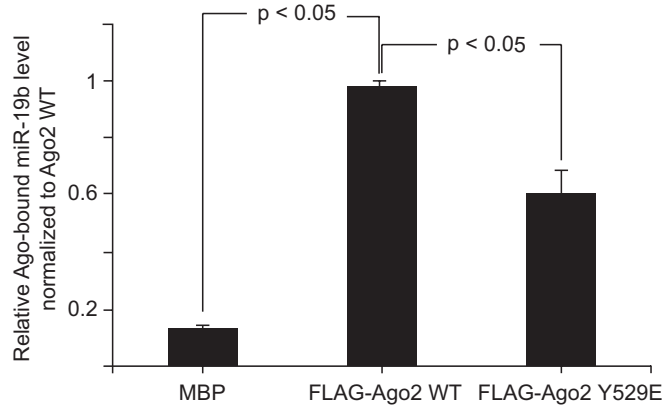

Fig. 4 The amount of miR-19b bound to FLAG-Ago2 Y529E and MBP relative to FLAG-Ago2 WT. Relative quantification of miR-19b level in FLAG-AGo2 Y529E, WT and MBP. FLAG-Ago2 Y529E bound less amount of miR-19b than FLAG-Ago2 WT ($p < 0.05$), which is consistent with the previous report [12]. Experiments were conducted three times

3. Alternative exogenous miRNAs, which are not expressed in the sample, can be spiked in the sample as the internal control. The level of exogenous spiked miRNAs should be in the linear range of C_t value of the miRNA of interest.
4. This method is applicable for detecting any miRNAs by changing the specific miRNA primers.

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