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Label-Free Electrophoretic Mobility Shift Assay (EMSA) for Measuring Dissociation Constants of Protein-RNA Complexes

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Abstract

The electrophoretic mobility shift assay (EMSA) is a well-established method to detect formation of complexes between proteins and nucleic acids and to determine, among other parameters, equilibrium constants for the interaction. Mixtures of protein and nucleic acid solutions of various ratios are analyzed via polyacrylamide gel electrophoresis (PAGE) under native conditions. In general, protein-nucleic acid complexes will migrate more slowly than the free nucleic acid. From the distributions of the nucleic acid components in the observed bands in individual gel lanes, quantitative parameters such as the dissociation constant (K_d) of the interaction can be measured. This unit describes a simple and rapid EMSA that relies either on precast commercial or handcast polyacrylamide gels and uses unlabeled protein and nucleic acid. Nucleic acids are instead detected with SYBR Gold stain and band intensities established with a standard gel imaging system. We used this protocol specifically to determine K_d values for complexes between the PAZ domain of Argonaute 2 (Ago2) enzyme and native and chemically modified RNA oligonucleotides. EMSA-based equilibrium constants are compared to those determined with isothermal titration calorimetry (ITC, *UNIT 7.4*). Advantages and limitations of this simple EMSA are discussed by comparing it to other techniques used for determination of equilibrium constants of protein-RNA interactions and a troubleshooting guide is provided.

Keywords

Ago2; electrophoresis; equilibrium constant; protein-nucleic acid interaction; RNA

INTRODUCTION

Nucleic acid-protein interactions are ubiquitous and absolutely essential in biological information transfer, including replication, transcription, repair, and RNA metabolism (Cusack et al., 2017). Gel electrophoresis using polyacrylamide gels (PAGE, *UNIT 10.4*) and the electrophoretic mobility shift assay (EMSA) remain important and are widely used approaches for detecting nucleic-acid protein interactions and determining the stability of individual complexes (Fried, 1989; Hellman and Fried, 2007; Chen, 2011; Alves and Cunha, 2012). This protocol details the steps for establishing the equilibrium dissociation constant K_d of an RNA-protein complex by EMSA. Solutions of RNA and protein are mixed in different ratios, and the binding reactions then separated by non-denaturing PAGE (<https://>

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tools.thermofisher.com/content/sfs/brochures/1601945-Protein-Interactions-Handbook.pdf).

In general, a protein-RNA complex will migrate more slowly through the gel matrix relative to the RNA alone, thus causing a shift on the gel, e.g. (Yang et al., 1999). Individual bands can be visualized by end-labeling the RNA radioactively (^{32}P), or using fluorescent or chemiluminescent probes in combination with a gel imager. However, in our assay, we have used non-labeled RNA together with the SYBR Gold stain (Tuma et al., 1999) for detecting and quantifying bands. From the distribution of the RNA component among the protein-RNA and RNA bands in individual lanes on the gel, one can determine the equilibrium dissociation constant K_d of the complex in a straightforward manner. An approximate value for the equilibrium constant can be obtained by finding the concentration of protein at which roughly half the nucleic acid component is bound and half remains free. A more precise way to determine K_d is to plot individual fractions of the nucleic acid bound in each reaction versus the concentration of protein and then perform a non-linear regression (Heffler et al., 2012) using free or commercially available software.

Human Argonaute 2 (Ago2) is an 859 amino acid protein that lies at the heart of the RNA-induced silencing complex (RISC) (Sheu-Gruttadauria and MacRae, 2017). The protein binds small interfering RNA (siRNA) duplexes consisting of 21mer guide (antisense) and passenger (sense) strands with 3'-terminal dinucleotide overhangs. Ago2 contains multiple domains, including the MID (binds 5'-end of guide siRNA), PIWI (harbors the endonuclease active site), PAZ (binds 3'-end of guide siRNA) and two linkage domains. The passenger strand is eventually cleaved or discarded and the target RNA loaded into Ago2 opposite the guide strand, resulting in cleavage of the former by the PIWI domain via a dual-metal ion mechanism (Watts and Corey, 2012; Wilson and Doudna, 2013). RISC contains other Ago proteins besides Ago2, i.e. Ago1, Ago3 and Ago4, and while all are associated with micro RNAs (miRNAs), only Ago2 exhibits endonuclease activity (Meister et al., 2004). Chemically modified siRNAs are widely explored as therapeutics for treatment of a range of diseases (Crooke et al., 2018; Shen and Corey 2018). ONPATPRO (Patisiran) is a modified siRNA formulated in lipids and manufactured by Alnylam Pharmaceuticals Inc. (Cambridge, MA) that was approved by the US FDA in August of 2018 for treatment of polyneuropathy of hereditary transthyretin-mediated amyloidosis in adults (Sheridan, 2017). The PAZ domain of Ago2 binds the last two nucleotides of guide siRNA and assists in separating guide and passenger siRNA (Ma et al., 2004; Elkayam et al., 2012; Schirle and MacRae, 2012) (Figure 1). The identities of the 3'-overhanging nucleotides in the siRNA guide strand and their chemical modification affect the interaction with Ago2 PAZ and silencing activity (Kandeel et al., 2014; Alagia et al., 2018). We relied on the EMSA protocol presented here to determine equilibrium dissociation constants K_d of the interactions between native and chemically modified RNAs and the human Ago2 PAZ domain.

BASIC PROTOCOL

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA) USING LABEL-FREE, NATIVE AND CHEMICALLY MODIFIED OLIGO-RIBONUCLEOTIDES AND HUMAN ARGONAUTE 2 PAZ

DOMAIN PROTEIN (AGO2-PAZ) TO DETERMINE EQUILIBRIUM DISSOCIATION CONSTANTS K_d OF PROTEIN-RNA COMPLEXES

The following procedures outline the mixing of RNA and protein solutions of defined concentrations in various ratios to establish the binding reactions, the preparation of polyacrylamide gels to run EMSAs for analyzing protein-RNA binding, staining and imaging of gels to measure the intensities of individual bands and the distribution of RNA between the free and protein-bound forms, and the determination of K_d values using standard software to compute non-linear regressions. However, neither the synthesis of RNA oligonucleotides nor the expression and purification of the Ago2 PAZ domain are described in detail. Briefly, RNAs were provided by Alnylam Pharmaceuticals Inc. (Cambridge, MA; native oligoribonucleotides) or AM Biotechnologies LLC (Houston, TX; 2'-OMe/3'-phosphorodithioate-(PS2; both non-bridging phosphate oxygen atoms replaced with sulfur) oligoribonucleotide, here referred to as MS2-modified RNA). Native RNAs were synthesized by standard or adapted solid phase phosphoramidite synthesis, purified (UNIT 10.3) by high performance liquid chromatography (HPLC, UNIT 10.5) and desalted (UNIT 10.7). Identities and purities of all oligonucleotides were confirmed by electrospray ionization mass spectrometry (ESI-MS) and ion-exchange HPLC (IEX-HPLC), respectively. The MS2-modified RNA was synthesized and purified as reported in (Yang et al., 2017; UNIT 4.77). An expression system for human Ago2 PAZ (amino acids 227–371, Figure 1B) was generated by gene synthesis (GenScript Inc.) and subcloning into the pHD116 plasmid. The plasmid was transformed into *E. coli* BL21 (DE3) Gold cells using standard procedures. After expression the protein was first purified by Ni affinity chromatography and, following cleavage of the His₆ tag by PreScission protease (GE Healthcare), by ion exchange and gel filtration chromatography. The identity of the Ago2 PAZ domain was established by tryptic digestion in combination with MS analysis.

Materials—Human Ago2 PAZ domain protein (180 μ M in 5 mM HEPES, 100 mM KCl, 10 mM DTT)

RNA(s) of interest (500 nM); see Figure 2 for sequences.

NaCl (Acros, CAS number: 7647-14-5)

EDTA (Sigma, CAS number: 60-00-4)

DTT (Sigma, CAS number: 3483-12-3)

Tris base (RPI Corp., CAS number: 77-86-1)

Boric acid (RPI Corp., CAS number: 10043-35-3)

APS (Biorad catalog number: 161–0700)

Bromophenol blue dye

10,000x Sybr Gold (Invitrogen catalog number: S11494)

Nuclease free water (Promega P119E-C)

Agarose (see recipes)

Glycerol (RPI, CAS number: 56-81-5)

Ficoll (25mg/ml) (Sigma, CAS number: 26873-85-8)

Gel Materials

30% acrylamide/bis solution 37.5:1 (Bio-Rad catalog number: 161-0158)

Sterile water

Tetramethylethylenediamine (TEMED, Bio-Rad catalog number: 161-0800)

10% alkaline phosphatase buffer (APS, see recipes)

5x Tris/borate/EDTA buffer (TBE, see recipes)

10x binding buffer

5x TBE buffer (1 L)

10% APS (1 mL)

5% polyacrylamide gel solution (see Reagents and Solutions)

Microcentrifuge tubes

Equipment: 1.5 mm Mini gel plates

Gel electrophoresis chamber, e.g. Bio-Rad Mini-PROTEAN Tetra Cell System

Black cassettes

Shaker

Microcentrifuge tubes

ChemiDoc MP imaging system (Bio-Rad)

1. Make 5% native TBE polyacrylamide gel by first assembling the two 1.5 mm-thick glass gel plates (i.e. with clips) and set into the gel apparatus.
2. Then seal the bottom of the gel plates with 1% agarose with a 1.0 mL micropipette.
Make sure to pipette agarose on both sides of the gel plates to seal them as well.
3. Prepare 10 mL of 5% (0.5x TBE) polyacrylamide gel solution (see reagents and solutions).
4. Using a 1.0 mL micropipette, inject the polyacrylamide solution into the gel cast. Stick the comb into the assembled gel sandwich. Wait 1 hour to let the gel polymerize.

5. Prepare electrophoresis running buffer by diluting 100 mL of 5x TBE buffer with 900 mL of sterile water to create 1 L of 0.5x TBE.
6. Pre-run the polyacrylamide gel by submerging the cast gel in the running buffer from step 5 and run at 45V-60V for 1 hour or until the current is stable.
7. Prepare 100 μ L of RNA stock (500 nM) per gel in nuclease free water and dilute PAZ protein with 1x binding buffer to create two protein stock solutions, one of 10 μ M concentration and the other of 40 μ M concentration.
8. Allow the stock solutions prepared in the previous step to warm to room temperature and then prepare nine human Ago2 PAZ protein and RNA binding mixtures in separate microcentrifuge tubes according to Table 1. Create control sample by mixing 10 μ L of RNA and 10 μ L of bromophenol blue dye. Incubate at room temperature for 30 minutes.
9. After the incubation is complete, add 2 μ L of ficoll solution to each tube, including the control.
10. Load 20 μ L of each sample into a well with the gel; the control sample should be loaded into the first lane.
11. Run the gel at 40–70V, 6–15mA for 1 hour or until the samples are roughly 2/3 down the gel (Figure 2).
12. Add 5 μ L of 10,000x Sybr Gold to 50 mL of 0.5x TBE buffer to create 50 mL of 1x Sybr Gold solution.
13. Stain each gel by submerging it in 50 mL of 1x Sybr gold in a black cassette for 30 minutes.
Note: the Sybr Gold stain does not bind to the Ago2 PAZ domain.
14. Rinse the gels with deionized water (optional).
15. Image gel using ChemiDoc MP Imaging System with a Blot/UV/Stain-Free Sample Tray (<http://www.bio-rad.com/en-us/product/chemidoc-mp-imaging-system?ID=NINJ8ZE8Z>) (Figure 2).
16. Calculate the binding affinity between the RNA and the protein by analyzing the gel images with ImageJ. See the anticipated results section below for more details.

Reagents and Solutions

10x binding buffer

0.1 mM HEPES

1.5 M NaCl

30 mM EDTA

10 mM DTT

1x binding buffer (10 mL)

10x binding buffer: 1 mL

50% glycerol: 2 mL

Sterile water: 7 mL

5x TBE buffer (1L)

54 g Tris base

27.5 g boric acid

20 mL of 0.5M EDTA pH 8.0

10% APS (1 mL)

0.1 g APS

1 mL of sterile water

1% agarose

0.5 g of agarose

50 mL of 0.5x TBE buffer

5% polyacrylamide gel solution

Sterile H₂O: 7.27 mL

30% acrylamide: 1.66 mL

5x TBE: 1.00 mL

10% APS: 70 mL

TEMED: 3.5 mL

COMMENTARY

Background Information

There are multiple techniques for determining the equilibrium dissociation constant K_d of a protein-nucleic acid binding interaction besides the EMSA (Fried, 1989; Hellman and Fried, 2007; Chen, 2011; Alves and Cunha, 2012; Heffler et al., 2012). These include the filter-binding assay (Woodbury and von Hippel, 1983), fluorescence intensity (Teplova et al., 2000), chemical shift differences using ¹H-¹⁵N heteronuclear single quantum coherence (HSQC) NMR titration experiments (Frank et al., 2012), surface plasmon resonance (SPR; McDonnell, 2001; Ma et al., 2004), acoustic measurements (Cooper and Whalen, 2005; Godber et al., 2005), biolayer interferometry (BLI; Lou et al., 2016; *UNIT 7.25*), microscale thermophoresis (MST; Mueller et al., 2017), and isothermal titration calorimetry (ITC; Rozners et al., 2015; *UNIT 7.4*), among others.

The choice to use one method versus another may depend on the amounts of material available, the particular environment in which the experiment is to be conducted (e.g. solution, chip, microfluidics, cell, etc.), whether labeling can be accomplished relatively easily and/or cheaply, or whether equipment that is needed with some of the above

techniques is accessible to the investigator. Thus, EMSAs, spectroscopic approaches and SPR require less material than filter-binding assays and ITC. An important consideration concerns the K_d limit, that is the upper end of the stability range for the complex between protein and nucleic acid of interest at which a particular technique still affords precise data. In this regard, EMSA, SPR and BLI allow reliable measurements of interactions with K_d values of as low as 10^{-12} M (Jing and Bowser, 2011). This contrasts with the K_d limit for ITC that is around 10^{-8} to 10^{-9} M. However, ITC, despite this limitation and the need for relatively large amounts of material, offers the benefits of a solution environment and label-free binding partners; it is also the method of choice to establish precise thermodynamic parameters of a binding interaction (H , S and G ; *UNIT 7.4*). Most of the alternative approaches that yield the K_d of an interaction require labeling of either protein or nucleic acid, e.g. ^{15}N (protein; NMR), ^{32}P (DNA or RNA; filter-binding assay, EMSA), biotinylation (DNA or RNA; SPR, BLI *UNIT 7.25*), or fluorescent probes (protein or nucleic acid; MST, EMSA) (Jiang and Egli, 2011; *UNIT 7.15*).

We opted to use EMSA with label-free oligoribonucleotides to determine the K_d of the interaction between human Ago2 PAZ and RNA. Advantages of this approach are speed, low cost and the small amounts of material needed. A familiar limitation lies in the environment, i.e. a gel matrix and not solution, that can potentially affect the binding reaction. The complex between PAZ domain and RNA is known to be of a 1:1 stoichiometry (Figure 1A), but published data reveal somewhat divergent values for the stability of the complex. Thus, the K_d values for the interactions between Ago2 PAZ and mono- (e.g. UMP) and dinucleotides (e.g. UpUMP) varied between 10 and 60 μM based on ITC (Kandeel et al., 2014). In contrast, the K_d of the interaction between Ago1 PAZ and an 11mer/13mer RNA duplex with a 3'-terminal dinucleotide overhang (Figure 2A) was reported to be 2.2 nM as determined by SPR (Ma et al. 2004; Ago1 and Ago2 PAZ have highly similar sequences, Figure 1B). In order to verify the EMSA-based K_d values, we therefore decided to also conduct independent ITC experiments. The outcomes of the two approaches are discussed in the section 'Anticipated Results'.

Critical Parameters and Troubleshooting

We tested two types of polyacrylamide gels and varied their percentages.

1. TBE polyacrylamide gel. We started from 15% and then continuously lowered the percentage to 12%, 10%, 8%, 5%, and finally to 3.5%, as gels of higher % polyacrylamide prevented the complex from getting into and migrating through the gel. However, a 3.5% gel turned out to be too frail to handle and it also showed no significant improvement compared to the 5% gel in terms of the migration of the complex. Thus, a 5% gel was judged to be optimal in terms of structural integrity and migration of the complex, even at higher concentrations of PAZ protein.
2. Tris-glycine polyacrylamide gel. We assayed binding reactions on 4%–20%, 12%, 10%, and 7.5% gels. Although the complex band migrated through the gel, increasing the protein:RNA ratio did not result in reduced and increased intensity

of the RNA and complex band, respectively and such gels do apparently not constitute a suitable environment for the PAZ-RNA complex.

We also examined the effects of varying the pH, e.g. 1% TBE pH 8.0 and 0.5% TBE pH 7.6, and the addition of detergents (sodium cholate 0.04%, 1/10 CMC, and 0.008% Triton X-100, ½ CMC). However, detergents proved to be detrimental to the complex. Thus, a 0.5x TBE buffer for both gel and running buffer yielded much better results. We did not use Mg^{2+} or Ca^{2+} in the buffer and neither was used for annealing the RNAs. We don't anticipate that the presence of these ions would have a significant effect. Moreover, neither ion is present in or near the binding pocket of the PAZ domain.

We observed that gel bands representing the PAZ-RNA complex tended to smear and that migration was limited at the highest ratio between protein and RNA. This issue may become exacerbated by the use of higher concentrations of protein and RNA owing to the lack of labels on the latter. However, the RNA bands were typically quite sharp, allowing reasonably precise density measurements even in cases where migration of the complex was limited or bands representing the complex were spread out.

For PAGE experiments one can of course use either pre- or handcast gels. We relied on commercial precast gels in some cases but also poured our own gels. There are various systems on the market that facilitate handpouring gels, e.g. Sure Cast Gel Handcast System offered by ThermoFisher (<https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-gel-electrophoresis/protein-gels/surecast-gel-handcast-system.html>).

Anticipated Results

To analyze the gel image obtained, the NIH program ImageJ was used (Schneider et al., 2012). The program can be downloaded from the NIH website for free (<https://imagej.nih.gov/ij>). The image shown in Figure 2 was first inverted and then the raw integrated density (RID) of each band was measured with ImageJ. ImageJ may not have the RID as a default setting. If that is the case, go to the "Analyze" tab, click "Set Measurements", and then check the "Integrated Density" box and click "OK". The results were then transferred into an Excel spreadsheet to perform the following calculations. Each RID was subtracted from the lowest RID, with the lowest RID being subtracted from itself resulting in zero. These values are the relative RIDs. Each relative RID was divided by the largest relative RID and then multiplied by 100, with the largest relative RID being divided by itself and then multiplied by 100, resulting in 100. These are the percentage RIDs. Finally, each percentage RID was subtracted from 100, resulting in the percentage of binding, i.e. complex formation, for each protein concentration.

These results then served as input for the program GraphPad Prism (version 5.00 for Mac; GraphPad Software, La Jolla CA; <https://www.graphpad.com/>). Select the option that plots a single "Y" value for each data point and press create. Enter the protein concentrations in the x-column and enter the percent binding in the y-column. Label both columns. Next, click on the results tab and select nonlinear regression (curve fit) under "XY analyses". Then select the equation that is labeled "one site – specific binding", which computes the K_d value (Figure 3).

The K_d values obtained for the PAZ complexes with native and modified RNA are shown in Table 2.

Our EMSA is relatively simple, cheap and quick. But does it also provide reasonably accurate data, keeping in mind the usual limitations of a gel-based assay? We decided to compare the EMSA-based data to K_d values obtained from isothermal titration calorimetry (ITC) experiments. These K_d s were 0.6 μM (PAZ complex with native RNA; Figure 4A) and 1.9 μM (PAZ complex with modified RNA; Figure 4B) and therefore quite similar to the EMSA data. Typically, one would carry out the EMSA in triplicate and then report average values.

An earlier publication had reported a 2.2 nM K_d for the Ago1 PAZ complex with the same native RNA construct that was employed here and using SPR (Ma et al., 2004). However, we believe that this may be an overestimation of the tightness of binding between PAZ domain and RNA. For one, Ago1 and Ago2 PAZ have similar sequences (Figure 1B) and it is unlikely that the former should exceed the latter in terms of its affinity for RNA by three orders of magnitude. In addition, the buried surface (binding interface, calculated with the program PRince (Barik et al., 2012); <http://www.facweb.iitkgp.ac.in/~rbahadur/prince/home.html>) in a complex between thrombin and an anti-thrombin RNA aptamer (Long et al., 2008; PDB ID 3DD2) that exhibits a K_d of 1 nM (Abeydeera et al., 2016), protein 719 \AA^2 and RNA 790 \AA^2 , is much larger than that between Ago2 PAZ and the 3'-end of an RNA (complex with PDB ID 4F3T, Figure 1A): protein 344 \AA^2 and RNA 465 \AA^2 . However, it is of note that complexes can exhibit dramatically different stabilities as a consequence of a key interaction that leaves the buried surface essentially unchanged. Thus, replacement of a single phosphate (PO2) by a phosphorodithioate (PS2) moiety in RNA aptamers was reported to trigger a dramatic change in the dissociation constant of the complexes with their respective targets from ca. 1 nM to ca. 1 pM (anti-thrombin and anti-VEGF aptamers; Abeydeera et al., 2016). Finally, the K_d values for complexes between Piwi protein PAZ domains and various RNAs based on ITC were recently reported to be between 2 and 34 μM (Tian et al., 2011).

Time Considerations

The entire experiment, from pouring the gels to imaging and calculating the K_d value takes around 6 hours. Pouring, prepping, waiting for the gel to polymerize, and pre-running it can be accomplished in around 2 hours. Doing the calculations to prepare the individual samples, preparing them, loading the samples and running the gel takes 2–3 hours. Finally, gel imaging, measuring band densities with ImageJ and obtaining the K_d value using Prism took another hour. With one gel apparatus, two gels can be run simultaneously and thus K_d values for two complexes can be obtained on the same day.

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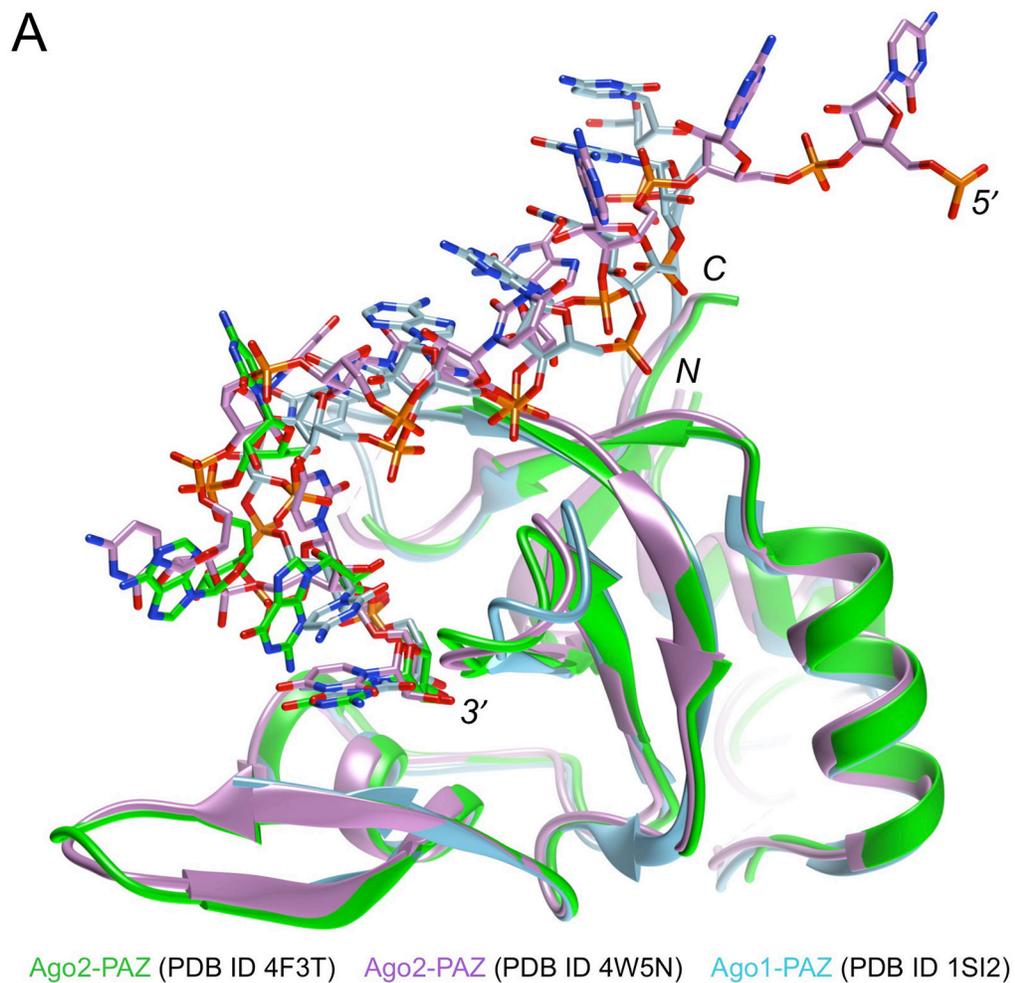
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B

200	FHQSVRPAMWKMLNIDVSATAFYKAQPVIEFMCEVLDIRNIDEQPKPLTDSQRVRFKE	259
	FHQSVRP++WKMLNIDVSATAFYKAQPVIEF+CEVLD ++I+EQ KPLTDSQRV+FTKE	
202	FHQSVRPSLWKMLNIDVSATAFYKAQPVIEFVCEVLDFKSIEEQKPLTDSQRVKFTKE	261
260	IKGLKVEVTHCGQMKRKYRVCNVTRRPASHQTFPLQLESGQTVECTVAQYFKQKYNLQLK	319
	IKGLKVE+THCGQMKRKYRVCNVTRRPASHQTFPLQ ESGQTVECTVAQYFK ++ L L+	
262	IKGLKVEITHCGQMKRKYRVCNVTRRPASHQTFPLQESGQTVECTVAQYFKDRHKLVLK	321
320	YPHLPCLQVGQEQQKHTYLPLEVCNIVAGQRCIKKLTDNQTSTMIKATARSAPDRQEEISR	379
	YPHLPCLQVGQEQQKHTYLPLEVCNIVAGQRCIKKLTDNQTSTMI+ATARSAPDRQEEIS+	
322	YPHLPCLQVGQEQQKHTYLPLEVCNIVAGQRCIKKLTDNQTSTMIRATARSAPDRQEEISK	381

Figure 1.

Overlay of crystal structures of human Ago1- and Ago2-PAZ in complex with RNA oligonucleotides (A) and alignment of the human Ago1 (top line) and Ago2 (bottom line) sequences (B). Only a portion of the 859 amino acids is included; the consensus sequence is shown in the middle line and PAZ domain residues are highlighted: 225–369 (Ago1) and 227–371 (Ago2). The PAZ domain binds the 3'-end of guide siRNA. The models shown are from crystal structures of full-length Ago2 bound either to duplex RNA (Schirle & MacRae, 2012) or an miRNA single strand (Elkayam et al., 2012). In both cases only PAZ domain

residues 226–351 were included in the illustration. In both crystal structures, only portions of the 3′-half of the RNA guide strand were visible in the electron density. The third structure depicted is of a complex between a separately expressed human Ago1 PAZ domain (residues 224–349 were resolved in the electron density) and an RNA nonamer (Ma et al., 2004). Coordinates were retrieved from the Protein Data Bank (<https://www.rcsb.org/>) (Berman et al., 2000) and the structural figure in panel A was generated with the program UCSF Chimera (Pettersen et al., 2004).

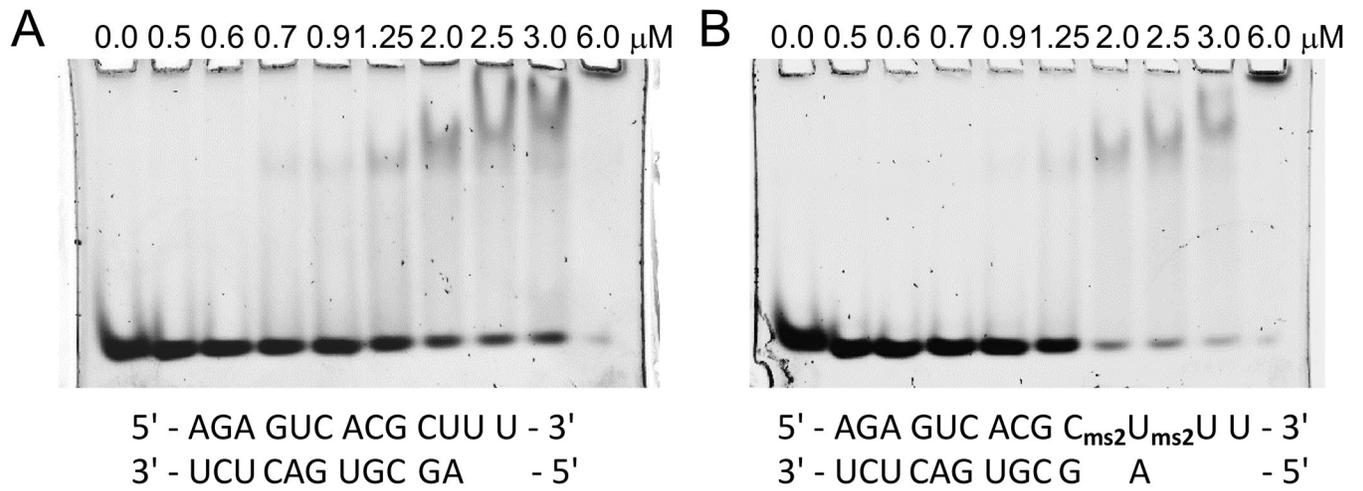
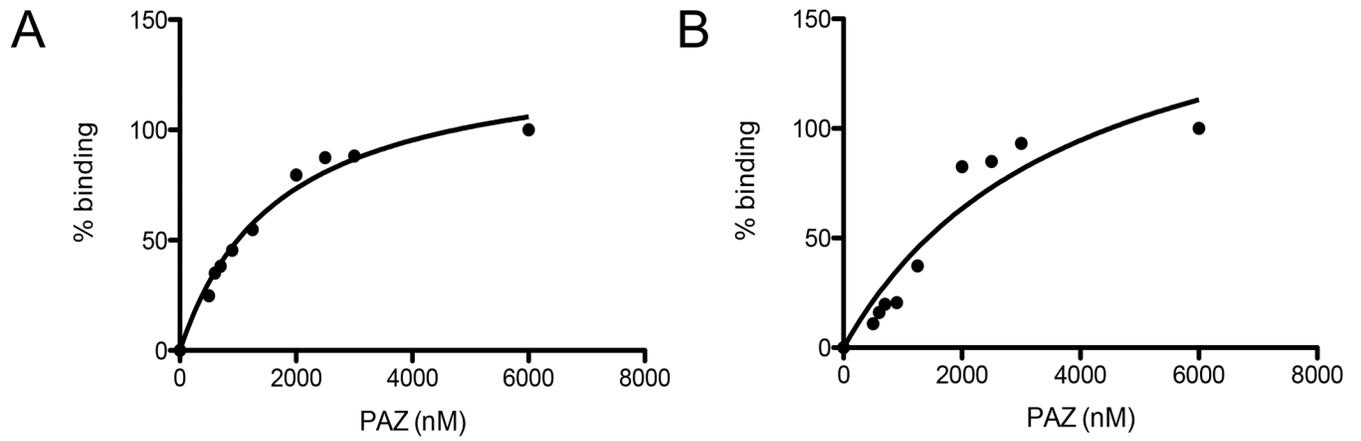


Figure 2.

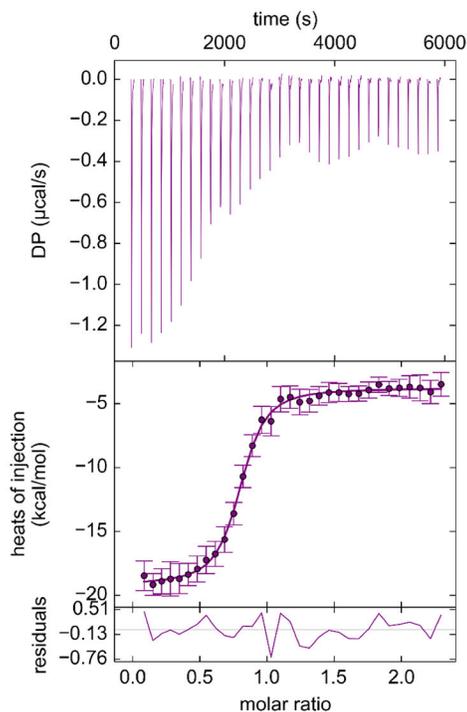
Gel images of binding reactions with various ratios between Ago2 PAZ and (A) native RNA and (B) MS2-modified RNA. RNA sequences are shown below the gels, protein concentrations are shown above individual lanes, and the RNA concentration was 250 nM.



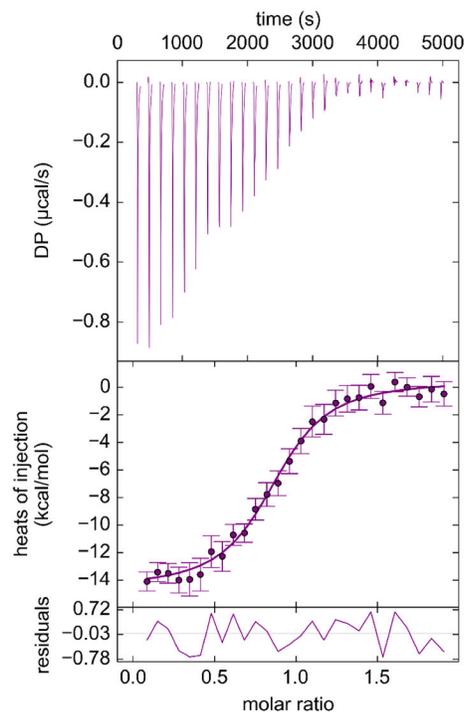
5' - AGA GUC ACG CUU U - 3'
 3' - UCU CAG UGC GA - 5'

5' - AGA GUC ACG C_{ms2}U_{ms2}U U - 3'
 3' - UCU CAG UGC G A - 5'

Figure 3.
 Non-linear regressions for the PAZ complex with (A) native RNA, and (B) modified RNA based on the gels depicted in Figure 2.



$$K_d = 0.63 \mu\text{M} / \Delta H = -15.4 \text{ kcal/mol}$$



$$K_d = 1.93 \mu\text{M} / \Delta H = -15.0 \text{ kcal/mol}$$

Figure 4.

Results of isothermal titration calorimetry experiments for the PAZ complex with (A) native RNA and (B) modified RNA. The experiments were conducted under the following conditions: 56 μM Ago2 PAZ and 600 μM RNA in a buffer composed of 5 mM HEPES pH 7.5, 150 mM KCl, 10% glycerol and 10 mM DTT. RNA was added to the protein solution by 28 to 33 1.2 μL injections at 25°C. Note the 1:1 stoichiometry of the complexes, consistent with the structural models depicted in Figure 1A.

Table 1.

Sample Volumes and Concentrations of PAZ and RNA needed for ESMA.

Protein concentration (nM)	PAZ protein added (μ L)	1x binding buffer added (μ L)	RNA(s) of interest added (μ L)
500	1.0 (10 μ M stock)	9.0	10.0 (500 nM stock)
600	1.2	8.8	10.0
700	1.4	8.6	10.0
900	1.6	8.2	10.0
1250	2.5	7.5	10.0
2000	4.0	6.0	10.0
2500	5.0	5.0	10.0
3000	6.0	4.0	10.0
6000	3.0 (40 μ M stock)	7.0	10.0

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Table 2:

K_d values obtained for PAZ complexes with native and modified RNA.

RNA	K_d [μ M], nonlinear fit
5' - AGA GUC ACG CUU U - 3' 3' - UCU CAG UGC GA - 5'	1.7
5' - AGA GUC ACG C _{ms2} U _{ms2} U U - 3' 3' - UCU CAG UGC G A - 5'	3.9

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