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MicroRNAs (miRNAs) are small molecules of noncoding RNA that range between ~19-22 nucleotides in length. In recent years, scientists have observed that these small RNA molecules exist in the extracellular environment within eukaryotic organisms. Furthermore, these microRNA molecules are known to associate with a family of proteins named Argonaute proteins. These microRNA/Argonaute protein complexes are the core of a larger assembly of proteins that compose the RNA Induced Silencing Complex (RISC). The RISC has exhibited the ability to inhibit the translation of, or cleave, its target messenger RNA (mRNA), the latter of which being exclusive only to Argonaute 2 protein (Ago2). It has also been observed in the literature that these Argonaute/miRNA complexes often target genomic regions associated with various cancers in humans and, currently, more than 2000 miRNAs have been discovered and published in the literature.

Current methods for analyzing miRNA expression involve total RNA extraction using methods such as ethanol precipitation. However, total RNA extraction does not take into consideration that the major functional component of post-transcriptional inhibition is indeed that Argonaute protein/miRNA complex and not the miRNA alone. In this study, we investigate a novel method to capture and detect the active Ago2/miRNA (miRNP) complex and quantitate associated miRNAs by utilizing an

antibody against Ago2 and subsequent application of real-time PCR to successfully capture the active miRNP complex and quantitate the associated miRNAs.

DETECTION OF ARGONAUTE (AGO) PROTEIN ASSOCIATED MIRNA BY COMBINING ANTI-AGO ANTIBODY RECOGNITION WITH REAL-TIME PCR

by

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Approved by

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To My Mother

APPROVAL PAGE

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CHAPTER I

INTRODUCTION

Statement of Problem

MicroRNAs (miRNAs) are small RNA molecules that consist of ~19-25 nucleotides. It has been discovered that mature miRNAs circulate in the body in complex with a family of proteins known as Argonaute (Ago) proteins. This microRNA-Ago2 complex is a major component of the RNA-induced silencing complex (RISC) and has exhibited the ability to silence the translation of, or cleave, target mRNA complementary to the sequence of miRNA. Furthermore, the cleaving ability of RISC is specific to Ago2 protein binding with its guide miRNA strand.

It is important to understand that miRNAs alone are not capable of silencing target genes. It is only by incorporation into the RISC that gene silencing is possible. Therefore, the miRNA/Ago2 complex is the active and essential component for gene silencing/mRNA cleavage activity. However, the ratio of free miRNA compared to Ago2 associated miRNA circulating the body at a given time is uncertain. The concentration of Ago2 bound miRNA circulating within the body is expected to be higher than that of free miRNA.

Current methods to analyze miRNA expression in organisms rely on total RNA extraction and isolation from the cellular lysate followed by either quantitative PCR or gel electrophoresis. However, looking at the entire RNA profile of the organism is not indicative of RISC activity because it is impossible determine whether the precipitated miRNA was free or bound by Ago. It is for this reason that other methods of isolating Ago associated miRNAs need to be investigated.

Specific Aims

The specific aim of this research project is to investigate an alternative method for RISC isolation and quantitation of associated miRNAs. In a prior research project, our group was able to develop a qualitative assay for the identification of miRNAs using Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) Spectrometry. While this presents an effective and efficient method to determine the specific identity of miRNA, it is subject to the same limitations as total RNA extraction when the target is RISC associated miRNA. We seek to:

1. <u>Investigate the use of antibodies raised against Argonaute 2 protein as a way to isolate Argonaute proteins in complex with miRNA</u>.

   The function of miRNA in gene silencing is directly dependent on its partnership with Ago proteins. Furthermore, the ability to cleave mRNA is exhibited only in association with Ago2. Because of this, it is reasonable for us to investigate a method that first isolates the protein. Our ability to quantitate the miRNA involved in translational inhibition is directly dependent on our ability to isolate

the Ago2 protein which binds with the miRNA. To accomplish this, we seek to utilize antibodies to isolate Ago2.

2. Capture the Ago2-miRNA complex using the specific antibodies, elute the bound miRNA and quantitate using qRT-PCR.

   After successful isolation of the Ago2 protein, we will apply the same principle to isolation of the Ago2/miRNA complex. The binding of Ago2 with its target miRNA will be completed, in vitro, by a reaction between Ago2 and the miRNA Let-7a. After isolating the Ago2/miRNA complex we will elute the bound miRNA and perform quantitation using qRT-PCR.

Hypothesis

We hypothesize that given an antibody specific for Ago2, it should be possible to isolate the Ago2-miRNA component of the RISC and subsequently elute the miRNA for quantitation using real-time PCR. This approach would allow a more accurate approach for assessing translational inhibition due to the Ago2-miRNA complex.

CHAPTER II

REVIEW OF THE LITERATURE

MicroRNA

MicroRNAs are small RNA molecules consisting of anywhere from ~19-25 nucleotides. Post-transcriptional gene silencing, also known as RNA interference (RNAi) is the only known function of these small RNA molecules. MiRNAs function by binding to partially complementary sequences in the 3'-untranslated region of messenger RNA (mRNA) [7]. Binding of these untranslated sequences either suppresses translation of, or causes degradation of the target mRNA.

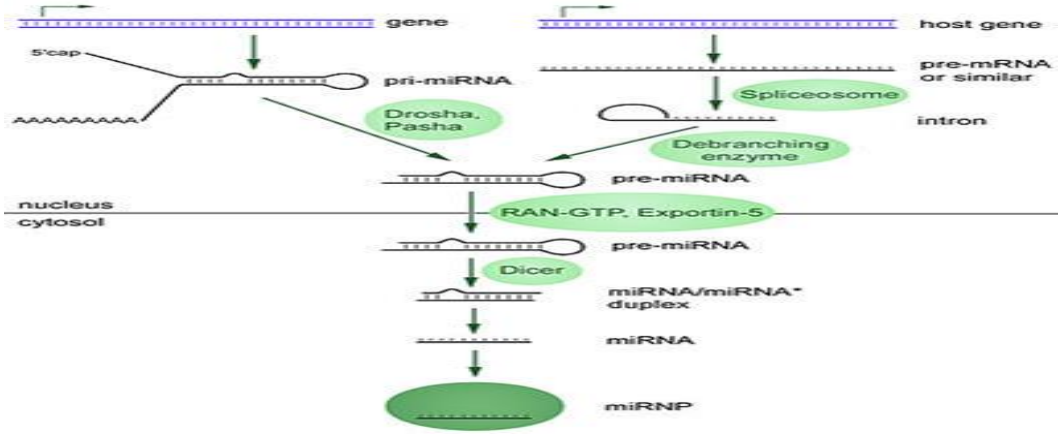
The translational inhibition activity of miRNAs has made them ideal candidates as possible blood-based biomarkers for cancer and disease. About 50% of all known human miRNA genes are located in genomic regions involved with cancers [1,2,20]. Furthermore, some hypothesize that miRNAs are passively released from the cell during tissue injury, such as after a heart attack [2].

MicroRNA Biogenesis

MiRNA biogenesis can be accomplished by either transcription of the host DNA or from the splicing of primary mRNA. In the first pathway, miRNA is transcribed from the non-coding intergenic regions of the host DNA by RNA polymerase II into primary

miRNA (pri-miRNA) molecules. These pri-miRNA molecules contain a stem and terminal loop structure with flanking segments[2]. The pri-miRNA molecules are then further processed by the RNase III enzyme Drosha in complex with partner protein DGCR8[2,7,17]. Drosha works with DGCR8 to cleave the pri-miRNA molecule into smaller precursor (pre-miRNA) molecules with a stem-loop hairpin structure. These pre-miRNAs are then exported out of the nucleus to the cytoplasm by Exportin-5[2,7,17]. Upon entry into the cytoplasm, the pre-miRNA is cleaved by RNase III enzyme Dicer along with transactivator RNA-binding protein (TBRP). This produces a 19 to 25 nucleotide RNA duplex containing the mature strand and its complementary strand[2]. It is the mature strand that is bound by the Ago protein while the complementary strand is degraded. The mature miRNA is the guide that leads the Ago protein to the complementary mRNA sequence and initiates the cleavage or silencing of the target mRNA.

Figure 1. MiRNA Biogenesis in the Cell



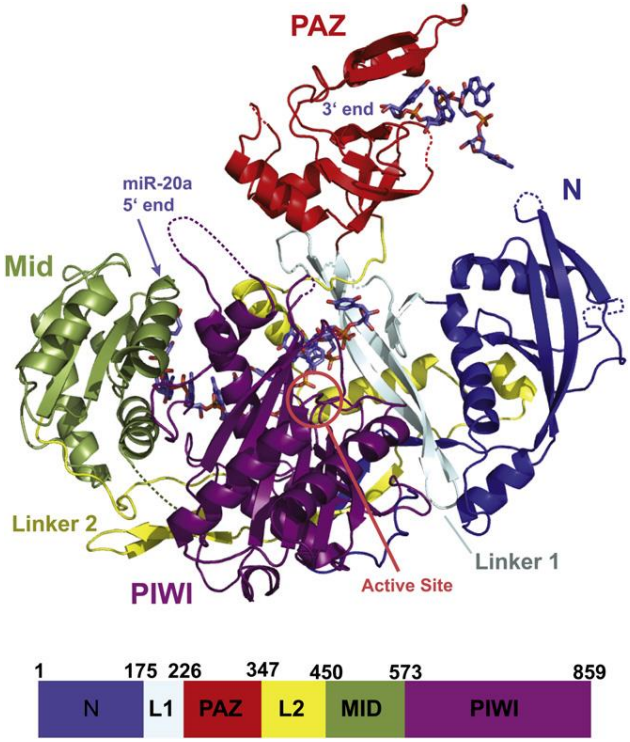
Source: **http://en.wikipedia.org/wiki/MicroRNA**

Argonaute Proteins

The Argonaute (Ago) family of proteins is a major component of the RNA-induced silencing complex (RISC). The Ago family is composed of four members, Ago 1-4. Ago proteins bind together with miRNA molecules to either inhibit the translation of or cleave target mRNA molecules, the latter of which is specifically Ago2 associated.

Ago proteins contain a PAZ and PIWI (P-element induced wimpy testes (PIWI), Argonaute and Zwille), and Mid domain[3]. The PAZ and PIWI domain contain an oligonucleotide-binding fold that engages the 3' end of the miRNA guide strand. The 5' end of the strand is confined within a pocket of the Mid domain[3]. This confinement acts to envelope the miRNA, thus preventing its degradation by nucleases in the extracellular environment. Also, the majority of the interactions between the Ago protein and the guide strand are through the RNA backbone, which explains the ability of Ago proteins to bind miRNAs of essentially any sequence[3].

Figure 2. Structure of Human Argonaute 2 Protein in Complex with miRNA-20



Structure of hAgo2 in complex with miRNA-20. Shown are the PAZ, PIWI, and Mid domains, as well as the N domain. Note that miRNA is bound by the active site. The 5' end is bound to the Mid domain while the 3' end interacts with the PAZ domain. Source: Elad Elkayam, et. al, The Structure of Human Argonaute-2 in complex with miR-20a, Cell, 150,100-110, July 6, 2012.

RNA-Induced Silencing Complex (RISC), Translational Inhibition, and Messenger RNA (mRNA) Cleavage

The RISC mediates the degradation of mRNAs that are complementary to the miRNA guide which is loaded into the complex. In particular, the miRNA guide strand is loaded into Ago2 protein which is a major component of the RISC. However, while Ago2 is the major player in the RISC other proteins have been identified as essential

components of RNA interference (RNAi) and the RISC, one of which is the protein Dicer.

Dicer proteins, which are ~200 kDa, contain ATPase, RNA helicase, and PAZ domains. They also contain two catalytic RNase III domains and a C-terminal dsRNA binding domain (dsRBD)[5]. The primary role of these proteins involves the processing of precursor miRNA (pre-miRNA) into mature miRNA by cleaving of the loop from the duplex hairpin structure of the precursor miRNA. However, these enzymes also play a role in the downstream steps of RNAi[5]. Specifically, after the precursor miRNA has been processed into double-stranded miRNA, a heterodimer of Dicer, Dcr-2 and R2D2, differentiates between the stability of the 5' termini of the RNA duplex. Dcr2 binds to the 5' end of the RNA duplex which is thermodynamically less stable while R2D2 binds to the 5' end of the more stable strand and facilitates its loading into Ago2[5]. The strand bound by Dcr2, the least thermodynamically stable strand, is then degraded.

In order for the bound miRNA to be loaded into Ago2, unwinding must first occur. This process requires additional proteins and ATP however the function of these proteins is not fully understood. While Dicer and other protein represent the "complete RISC", the minimal requirement for RISC activity is Ago2 and the miRNA guide strand.

Enzyme-Linked Immunosorbent Assay (ELISA)

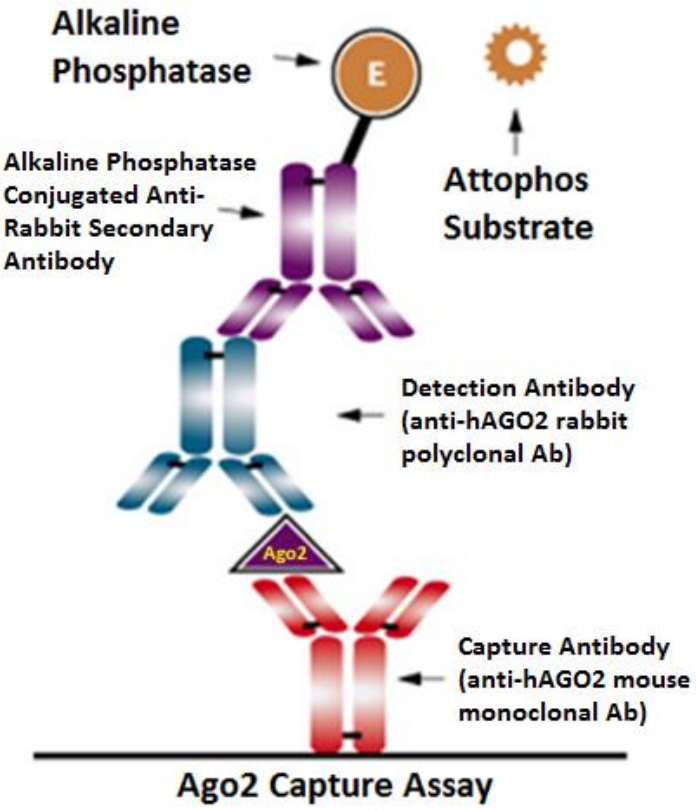
Enzyme-Linked Immunosorbent Assay (ELISA) is a bioanalytical technique that utilizes antigen-specific antibodies to detect the presence of a specific biological antigen. The assay is carried out on a solid-phase surface by adsorbing either the antigen or

antibody to the surface, hence the term immunosorbent. Typically, immunosorbent assays are performed in the wells of a polystyrene microtiter plate, however, immunosorbent assay applications have extended to platforms such as microarray, microfluidic chip technology, and magnetic beads. ELISAs can be performed in a variety of configurations using either fluorescent or chemiluminescent techniques as end-point detection methods. ELISAs can be competitive or non-competitive and configurations for the assay can be direct, indirect, or sandwich configurations.

In our study, we utilize the non-competitive sandwich ELISA configuration. In the sandwich configuration the capture antibody is first adsorbed to the plate wells. The antigen is then added to the wells and incubated in the presence of the capture antibody to allow binding. Then, the detection antibody is added to the wells and allowed to bind the antigen. It is essential that the two antibodies bind at different epitopes on the antigen, and that the epitope regions do not overlap. Binding of the antigen by the two antibodies effectively completes the "sandwich". The method of detection at this point can be either direct or indirect. Using direct detection requires that the detection antibody be conjugated to an enzyme such as alkaline phosphatase or horse-radish peroxidase. Indirect methods utilize another secondary antibody that recognizes an epitope on the detection antibody. This secondary antibody is conjugated with the enzyme instead of the detection antibody. The last step involves addition of a fluorescent or chemiluminescent substrate that reacts with the enzyme conjugate to produce a detectable signal. It is also important to note that a washing step is incorporated between each step

in order to reduce background noise in the assay due to non-specific binding between reagents.

Figure 3. Sandwich Configuration for the AGO2 Capture Assay



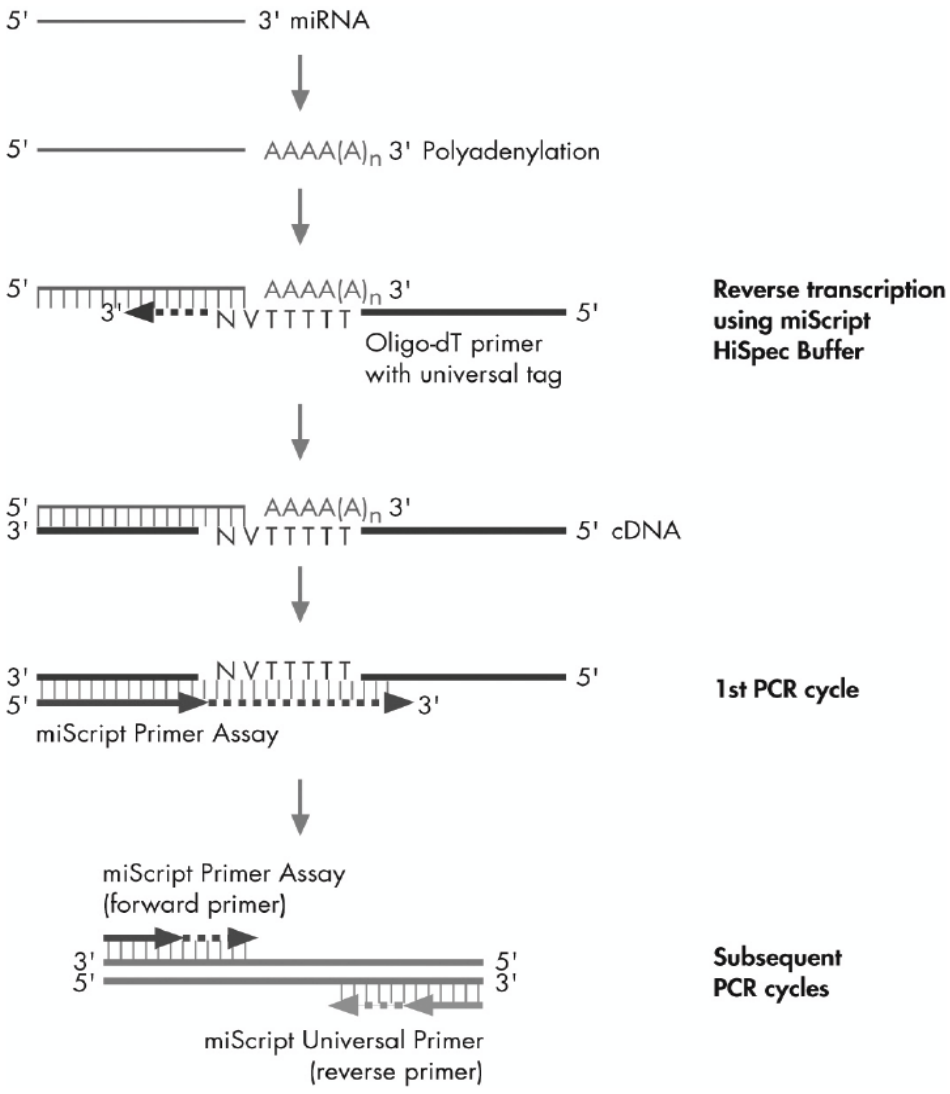
Real-Time Polymerase Chain Reaction

Real-Time Polymerase Chain Reaction (RT-PCR) is an analytical technique based on general PCR methodology. The term "Real-Time" comes from the fact that the end-point detections are carried out in real-time as the nucleic acid is amplified. The technique uses sequence specific primers, along with DNA polymerase to amplify a target sequence of nucleic acid. The nucleic acid of interest may be DNA or RNA, with the latter requiring a reverse-transcription step to convert the RNA sequence into a complimentary DNA (cDNA) sequence prior to performing RT-PCR.

Detection of the amplified product can be achieved using fluorescent dyes that intercalate in between the grooves of DNA, such as SYBR Green, or fluorescent probes that are complimentary regions of the target strand. PCR amplification is exponential; therefore, detection plots exhibit an exponential curve. This allows RT-PCR to be quantitative when comparing the plot an unknown quantity of nucleic acid to that of a known quantity or control.

RT-PCR techniques have been helpful in evaluating the level of gene expression in cells. For our purposes, it presents a suitable method for quantitation of miRNA associated with Ago2. As has been previously stated, gene silencing via translational inhibition is RISC dependent. With Ago2 being the major component of the RISC, gene regulation via translational inhibition can be evaluated by quantitating the amount of Ago2 bound miRNA using RT-PCR. Determining the amount of silencing activity and target gene region can give a lot of insight into the epigenetics of post-transcriptional gene silencing.

Figure 4. Flow Diagram of Reverse Transcription and Real-Time PCR using the Qiagen miScript Real-Time PCR Kit.



Source: Qiagen miScript User Handbook

MicroRNA and Gene Therapy

It has been observed that miRNAs play a role in several biological processes such as development, differentiation, apoptosis, and proliferation[2]. Furthermore, it has been observed that almost 50% of genes targeted by miRNA are located in genomic regions associated with cancers. Many of the genomic targets of miRNA are involved in tumor initiation and progression indicating that miRNAs may possess oncogenic or tumor-suppressive activities[2].

It is has been proposed that miRNAs could be released within small exosomes into the peripheral blood during tissue injury, such as during a heart-attack, and therefore may also be released during cell lysis due to rapid cell growth during tumor metastasis[2]. Therefore, the expression of miRNA in the blood could serve as potential biomarkers for cancers. With the number of oncogenic genomic targets associated with miRNAs, the therapeutic factor of miRNAs is one of great interest in the treatment of cancers and genetic disease.

The ability of miRNA to bind its target mRNA without being fully complimentary to the target allows miRNA to bind numerous target mRNAs. Therefore, one miRNA could potentially inhibit many target genes at once and subsequently suppressing several genetic pathways in the process.

CHAPTER III

CAPTURE AND DETECTION OF AGO2 USING A SPECIFIC ANTIBODY FOR RECOMBINANT HUMAN ARGONAUTE 2 PROTEIN

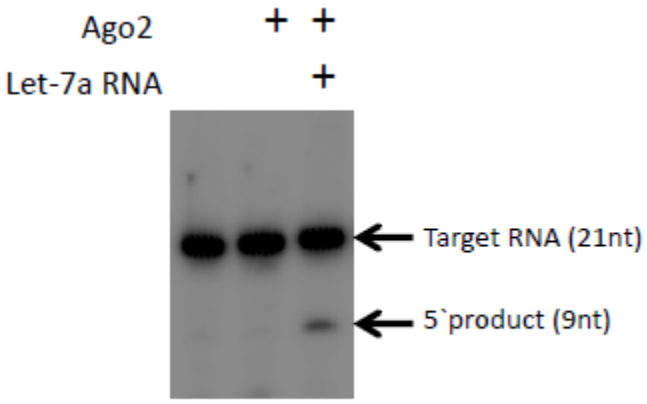
Introduction

Our efforts to capture the miRNA-Ago2 complex target the Ago2 component of the complex. An ELISA is ideally suited to accomplish this task. However, an ELISA depends on the harmonious gelling of several factors such as configuration, antigen, antibody affinity and epitope binding, antibodies that can work as a matched pair, reduction of non-specific binding, reagent concentrations, and method of end-point detection. With so many factors involved, it is necessary to determine the optimal parameters at necessary to detect Ago2.

Materials and Methods

Recombinant human Argonaute 2 protein was purchased from Sino Biological. Mouse anti-human Ago2 monoclonal and rabbit anti-human Ago2 polyclonal antibodies were purchased from Abova. An anti-rabbit alkaline phosphatase conjugated antibody was purchased from Jackson ImmunoResearch. AttoPhos fluorescent substrate was purchased from Promega. Tris-buffered saline with 0.2% Tween was purchased from Sigma Aldrich. Costar 96 well flat bottom polystyrene plates were used for the assay.

Figure 5. Results from Sino Biological's In Vitro RISC Assay with Recombinant Ago2 Protein.



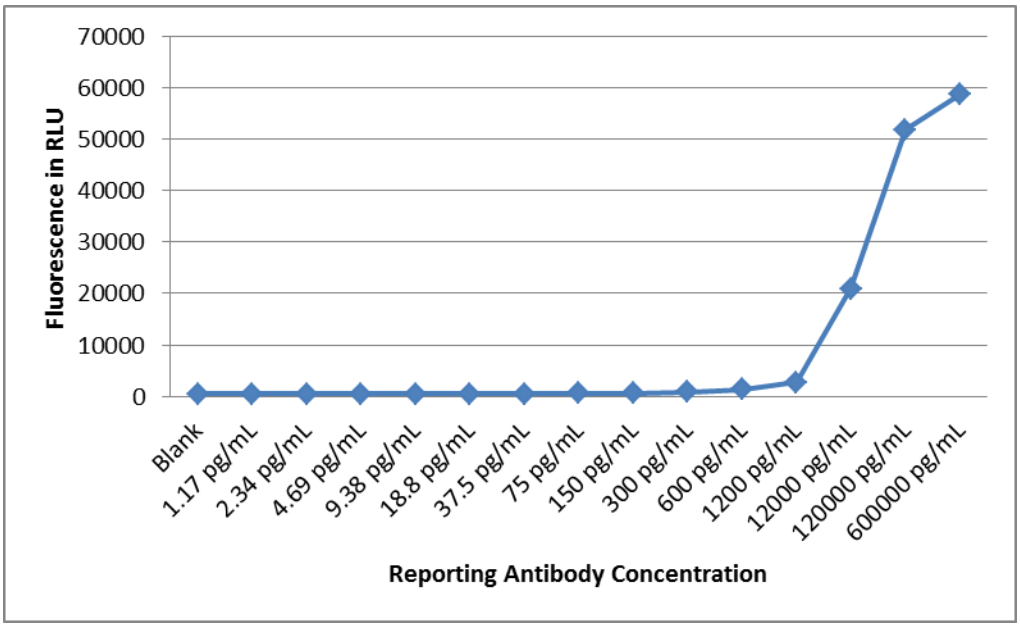
Credit: Sino Biological; Ago2 is enzymatically active in our conditions. The activity is guide RNA (let-7a) specific. The shown conditions are the rate limiting conditions (the cleavage activity increases significantly when reactions are incubated at 37C). 20ng per reaction seems to be already enough to saturate the cleavage activity in our experimental system. Ago2 (Cat.nr. 11079-H07B) was dissolved in H20 to concentration of 100ng/ul. Protein was stored at 4C and was still active after 2 weeks kept at 4C.

Evaluation of the AttoPhos Fluorescent Substrate

The fluorescent limit of detection (LOD) for using the PolarSTAR Optima plate reader was determined. Serial dilutions of the alkaline phosphatase conjugated anti-rabbit secondary antibody were performed. The dilution range for the conjugated antibody was from 0.6 µg/mL to 1.17 pg/mL for a total of 14 samples.

To the wells of a microtiter plate 2 µL of each sample was pipetted in triplicate. 25 µL of the 1 mM AttoPhos substrate was then added to each sample well and the plate was incubated in the dark, while shaking, for 15 minutes. The fluorescence of each sample was then measured using the Optima FLUORstar fluorometer. The incubation was repeated for 15 and 30 minutes respectively and re-measured. The gain for the measurement was adjusted from the three blank wells which contained only 25 µL of 1X PBS.

Figure 6. Limit of Detection for AttoPhos Fluorescent Substrate



From the data, the limit of detection for the AttoPhos fluorescent substrate was determined to be 1.2 x 10^{-4} pg/mL with the linear dynamic range falling between 1.2 x 10^{-4} pg/mL and 6.0 x 10^{-5} pg/mL.

Evaluation of the Blocking Solution

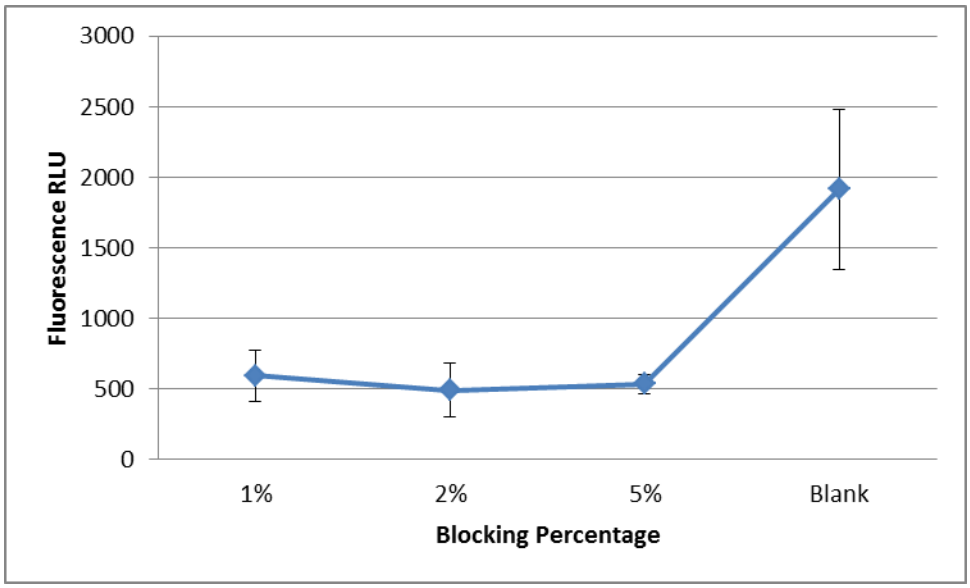
Following adsorption of the antibody to the microtiter plate wells, it is necessary to block the remaining spaces on the well surface, where no antibody adsorption occurred, in order to prevent nonspecific binding of a reagent following its addition to the wells. Nonspecific binding of reagents to the well surface would result in inaccuracies, when detecting Ago2, which could result in misinterpretations of the data. To alleviate this problem a blocking solution containing a protein, such as bovine serum albumin (BSA), is used to fill the empty spaces remaining on the bottom surface of the well. The

blocking protein adsorbs to the remaining spaces on the well's surface, and has no specificity toward the antibodies being used in the assay. While the antibodies are not specific for the blocking proteins, high concentrations of blocking protein may potentially for a monolayer over the antibodies which have already been adsorbed to the well surface, effectively blocking the available binding sites. Therefore, it is necessary to optimize the blocking process by determining the efficiency of the blocking solution at lower concentrations.

We decided to determine the blocking efficiency at 1, 2, and 5 percent blocking solution. The 1 and 2 percent concentrations were prepared from dilutions of the initial 5% stock concentration. 50µL of 1, 2, and 5% blocking solution were added to the plate wells, in triplicate, and incubated for one hour at room temperature to allow sufficient adsorption. Following incubation, the plate was washed 4X with TBST to remove any excess blocking solution. 50 µL of secondary antibody, conjugated with alkaline phosphatase, diluted 5000X (from a stock concentration of 0.6 mg/mL) in 1, 2, and 5% blocking buffer respectively was added to the wells corresponding the blocking percentages of the diluent.

The plate was incubated at room temperature for one hour while shaking. The plate was then washed 3X with TBST to remove any excess. 50 µL of 1 mM AttoPhos fluorescent substrate was added to each well and incubated in the dark for 15 minutes while shaking. The fluorescence from each sample was measured using the POLARstar Optima plate reader.

Figure 7. Evaluation of Blocking Efficiency at Varying Percentages of Blocking Solution



After plotting the data, it was observed that the reduction of non-specific binding by each percentage was comparably equal. Using this observation, it was determined that the best percentage for our use was one percent. The logic behind this was due to several considerations. The first being using a lower percentage of blocking solution in the assay allows faster diffusion to the unoccupied spaces. Also, less blocking protein in the blocking solution would lower the possibility of monolayer formation. Monolayer formation of the blocking protein would potential cover the capture antibody thereby inhibiting binding of the antibody to its target antigen, Ago2.

Evaluation of Incubation Times and Concentrations for Anti-hAGO2 Rabbit Polyclonal and Anti-Rabbit Secondary Antibodies

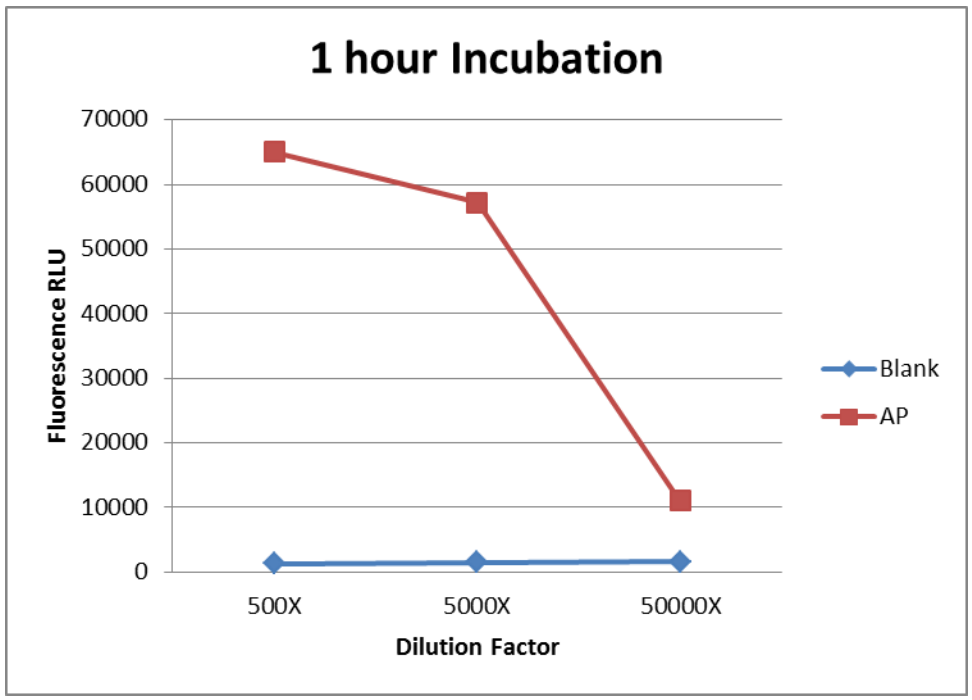
The incubation time necessary for optimal binding between the rabbit anti-hAGO2 polyclonal antibody and the anti-rabbit secondary antibody was determined. 50 µL of the anti-hAGO2 polyclonal antibody was added to 24 wells of two microtiter plates, at a concentration of 62.5 ng/mL, and as a negative control 1X PBS was added to the negative wells. The plates were incubated overnight at room temperature to allow antibody adsorption.

The plates were then washed two times with 1X TBST and blotted on a paper towel to remove excess liquid. 200 µL of blocking solution (1% non-fat dry milk in PBS) was added to the wells and incubated for one hour at room temperature. The plates were then washed three times with 1X TBST and blotted on a paper towel to remove excess liquid.

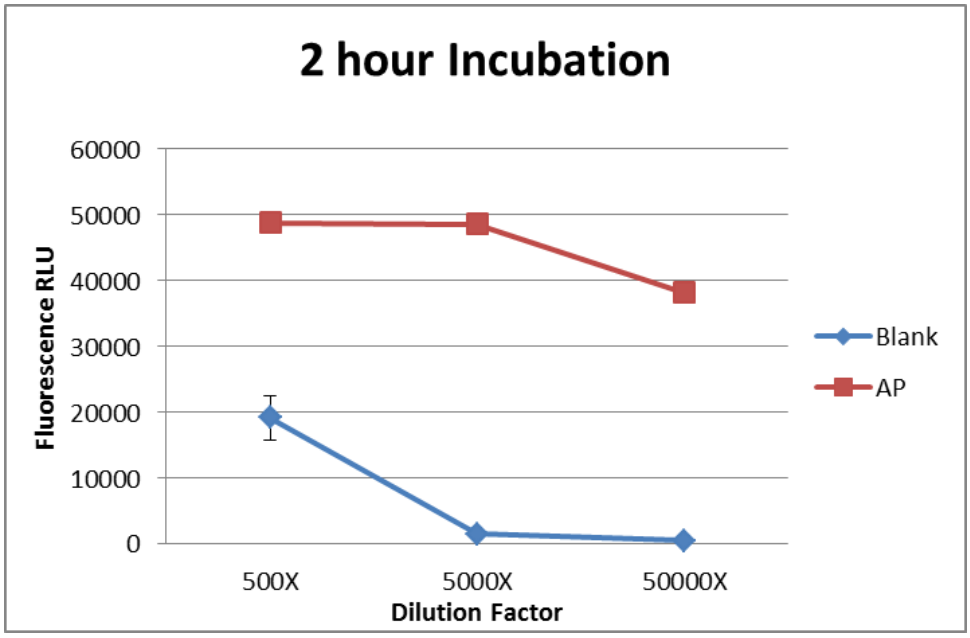
The anti-rabbit secondary antibody was then added to the wells, in triplicate, at concentrations of 1.2 µg/mL, 120 ng/mL, and 12 ng/mL. The first plate was then incubated for one hour while shaking. The other plate was incubated for two hours while shaking. After each incubation, the plate was washed five times with 1X TBST and blotted on a paper towel to remove excess liquid. 50 µL of 1 mM AttoPhos fluorescent substrate was added to the wells and incubated in the dark, while shaking, for 15 minutes. The fluorescence was then measured using the POLARstar Optima plate reader.

Figure 8. Plotted Data from the Evaluation of the Anti-Rabbit Secondary Antibody

A.



B.



A plot of the data suggests that a 5000X dilution (120 ng/mL) of the secondary antibody was sufficient enough to produce a strong detectable signal. This was also in compliance with the suggested dilution factor from the manufacturer.

Detection of Recombinant Human Ago2 Protein Using Sandwich ELISA

The complete sandwich assay was performed to determine whether the recombinant human Ago2 protein could be detected using the antibodies we acquired. 50 µL of the mouse monoclonal antibody was added to 18 wells of a polystyrene microtiter plate and allowed to adsorb by incubating overnight at room temperature. Following the incubation, the plate was washed two times with 1X TBST and blotted on a paper towel to remove excess liquid. To the sample wells, 300 µL of 1% blocking solution was added to the wells and allowed to incubate for one hour at room temperature. Following the incubation, the plate was washed two times with 1X TBST and blotted on a paper towel to remove excess liquid.

To nine of the plate wells, 50 µL of recombinant Ago2 protein was added at a concentration of 2 µg/mL using 1% blocking solution as the diluent. To the remaining nine wells, 50 µL of 1X PBS was added as a negative control. The plate was sealed and incubated for one hour while shaking. Following the incubation, the plate was washed three times with 1X TBST and blotted on a paper towel to remove excess liquid.

To the first 6 wells (3 negatives, 3 samples) 50 µL of rabbit anti-human Ago2 polyclonal antibody was added at a concentration of 2.5 µg/mL using 1% blocking solution as the diluent. To the following 6 wells, polyclonal antibody was added at a

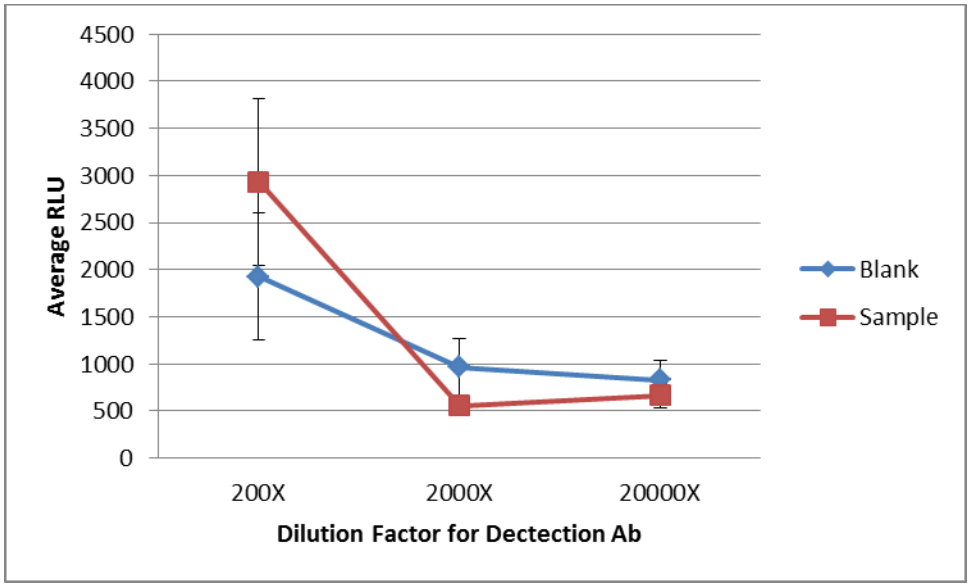
concentration of 250 ng/mL, and the remaining 6 wells at a concentration of 25 ng/mL. The plate was then sealed and incubated for one hour while shaking. Following the incubation, the plate was washed three times with 1X TBST and blotted on a paper towel to remove excess liquid.

To all wells, 50 µL of the anti-rabbit alkaline phosphatase conjugated secondary antibody was added at a concentration of 120 ng/mL. The plate was sealed and incubated for one hour while shaking. Following the incubation, the wells were washed five times with 1X TBST and blotted on a paper towel to remove excess liquid.

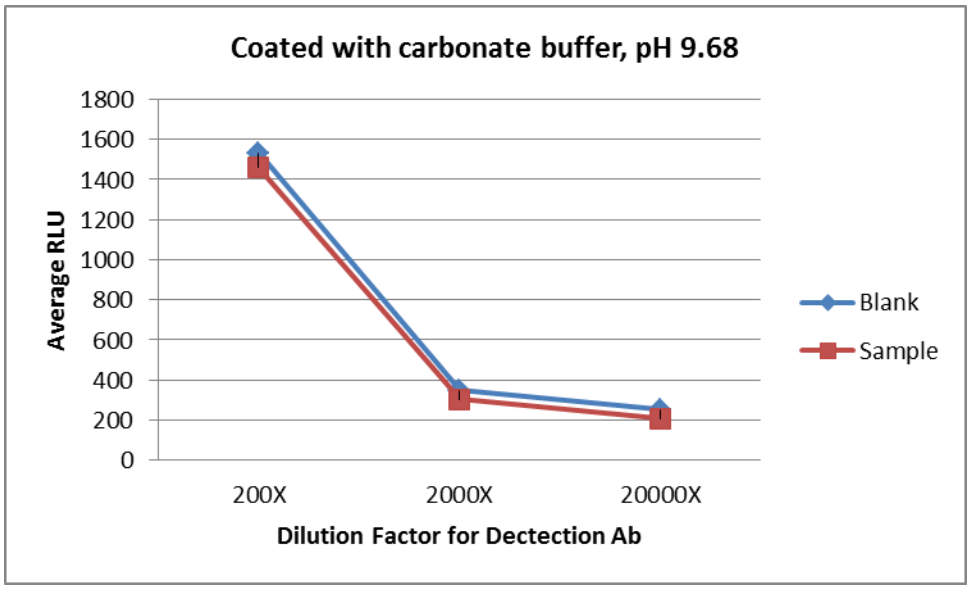
To all wells, 50 µL of 1 mM AttoPhos fluorescent substrate was added. The plate was sealed and incubated for 15 minutes in the dark while shaking. Following the incubation, the fluorescence was measured using the POLARstar Optima plate reader. The experiment was then repeated following the exact steps while using carbonate buffer as the coating buffer. The data from each experiment is below.

Figure 9. Detection of Recombinant Human Argonaute 2 Protein using Sandwich ELISA

A.



B.



The data plot suggests that recombinant Ago2 protein is slightly detectable as antibody concentrations increase. However, the signal to noise ratio could be improved.

The data suggests a low binding affinity by the selected antibodies and an assay that will require further optimization. The second data plot represents the repeated experiment using carbonate buffer as the coating buffer. The graph suggests that detectability of human Ago2 is lower when using carbonate buffer most likely due to decreased antibody adsorption in the buffer conditions. Therefore, we decided that using 1X PBS as the coating buffer would be our choice buffer moving forward.

Evaluation of Diluents

Due to the low binding affinity of the antibodies, we looked at the options of using 1X PBS vs 1% blocking solutions as diluents. The option to use 1X PBS as a diluent for during certain binding steps in the assay could possibly help improve binding between antibody and antigen but it comes at the risk of increased non-specific binding throughout the assay which would lead to higher background noise.

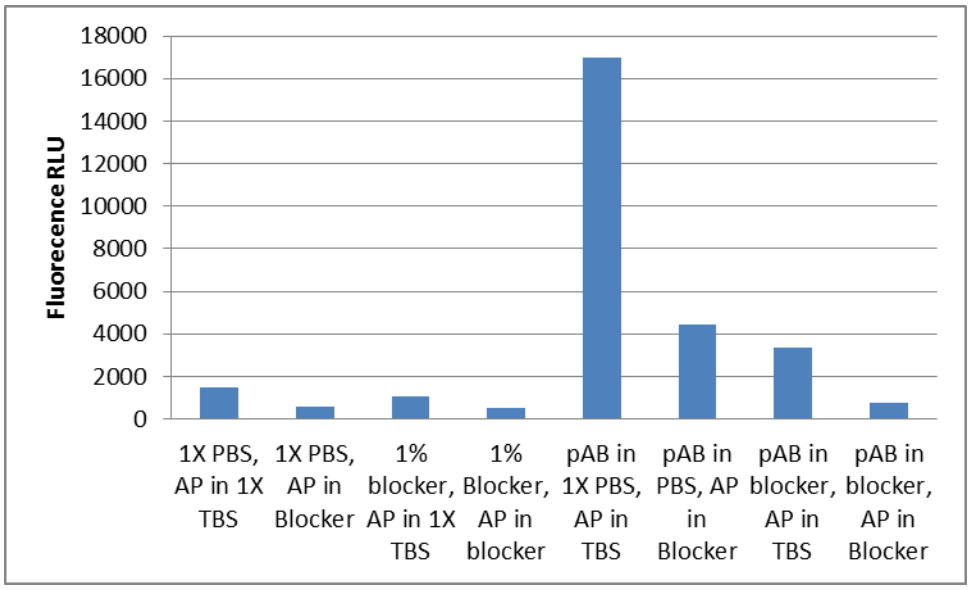
To determine the efficiency of the diluents, 8 wells of a polystyrene microtiter plate were blocked by adding 200 µL of 1% blocking solution and allowing to incubate for one hour at room temperature. Following the incubation, the wells were washed with 1X TBST and blotted on a paper towel to remove excess liquid. The first four wells received diluents only with two receiving 50 µL of 1X PBS and the other two receiving 50 µL of 1% blocking solution. To the remaining four wells, 50 µL of the rabbit anti-Ago2 polyclonal antibody was added to each well at a concentration of 2.5 µg/mL, with the diluent for two of the wells being 1X PBS and the diluent for the other two being 1% blocking solution. The plate was sealed and allowed to incubate for one hour while

shaking. Following the incubation, the wells were washed three times with 1X TBST and blotted on a paper towel to remove excess liquid.

To the 8 wells, 50 µL of the anti-rabbit alkaline phosphatase conjugated secondary antibody was added. For four of the wells the diluent for the secondary antibody was 1X TBS. For the remaining four, the diluent was 1% blocking solution. The reasoning behind the change in diluent for the alkaline phosphatase was due to observations in the literature that suggested that PBS diluents can inhibit the enzyme activity of alkaline phosphatases. To insure that this was not the case, TBS was used as a diluent along with the 1% blocking solution which contains PBS. The plate was sealed and allowed to incubate for one hour while shaking. Following the incubation the wells were washed five times with 1X TBST and blotted on a paper towel to remove excess liquid.

Following the incubation, 50 µL of 1 mM AttoPhos Substrate was added to the wells and allowed to incubate for 15 minutes in the dark while shaking. The fluorescence was then measured using the POLARstar Optima plate reader.

Figure 10. Evaluation of Diluents



The above graph shows the efficiency of each diluent during various steps of the assay. As expected, using diluents that contained no blocking proteins resulted in higher levels of non-specific binding due to diffusion of blocking protein off the well surface. Therefore, while there is the possibility of increased antigen binding in using 1X PBS as a diluent, the increased background noise would negate any increased detectability do to non-specific binding and possibly result in false positives.

In analyzing the data from the graph, it would appear that the most favorable use of diluents with respect to lowest background noise is using the 1% blocking solution. Also, while there seems to be increased signal when using TBS as the diluent for the secondary antibody, the increase in signal is most likely due to non-specific binding by the secondary antibody and not lack of inhibition by PBS.

Results of Study

In the RNA-induced silencing complex, the Ago2-miRNA complex is a major component and plays a critical role with regard to translational inhibition and mRNA cleavage. Our initial approach has been to utilize the bioanalytical technique of ELISA as a method to capture and detect the Ago2 component on a solid phase. After several modifications to the assay parameters, we were able to successfully obtain a signal from the detection of Ago2 protein but further optimization is still required.

However, our ability to quantitate Ago2 bound miRNAs does not strictly depend on the sensitivity of the capture assay and can simply rely on specificity and sensitivity of the PCR itself. For quantitative PCR analysis of the bound miRNA, we need only a relatively small amount of the bound miRNA. Therefore, a small amount of the captured miRNA-Ago2 complex should be more than enough to proceed with detection of the Ago2 associated miRNA by qRT-PCR.

We have determined that ELISA is a practical method to capture and detect Ago2 component of the miRNA-Ago2 complex. We now seek to utilize this technique to successfully immobilize the miRNA-Ago2 complex from a sample. The binding reaction between Ago2 and miRNA has been reported in the literature and can easily be accomplished in vitro. Our biggest challenge may be posed by the possible affect that conformational change upon binding of the target miRNA could have on the binding affinity of the antibody. However, given an antibody with a greater binding affinity for human Ago2 protein, there is great potential in the capabilities of using ELISA in tandem with real-time PCR to detect and quantitate Ago2 associated miRNAs.

CHAPTER IV

ASSAY DEVELOPMENT FOR DETECTION OF AGO2 ASSOCIATED MICRO-RNAs BY REAL-TIME PCR

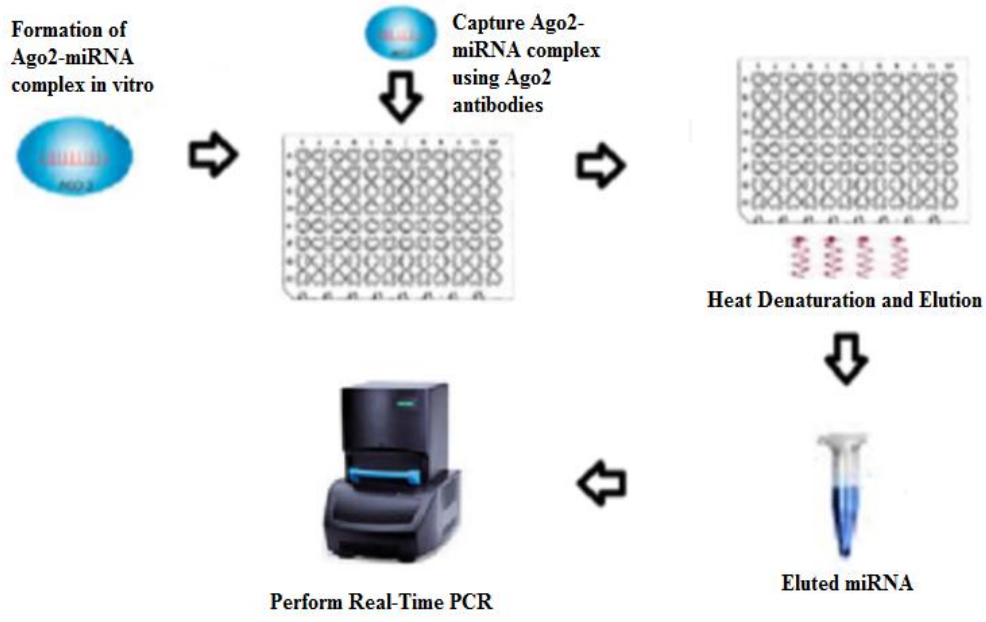
Introduction

The cleaving of mRNA by the RISC is specific only to Ago2 protein once it has bound a miRNA guide strand. This characteristic ability of Ago2 led us to investigate a method to detect Ago2 associated miRNAs. We first sought to capture the Ago2 component using an antibody specific for Ago2. What follows details a practical method for detecting Ago2 bound miRNA.

Coupling the immunoassay technique of ELISA with quantitative real-time PCR could serve as a novel high-throughput method for detecting Ago2 associated miRNAs. We decided to use a polyclonal antibody specific for human Ago2, adsorbed on a well surface, as a way to capture Ago2. As was previously observed, the binding affinity of this particular antibody for Ago2 was weaker than expected using the ELISA "sandwich" configuration. However, we hypothesize that the polyclonal antibody would offer a greater probability of capture due to the antibody being specific for several epitopes on Ago2.

There are already several protocols published in the literature that detail the steps for in vitro formation of the miRNA-Ago2 complex. We decided to follow a protocol which was published by the vendor from which we acquired the recombinant human Ago2 protein. While formation of the miRNA-Ago2 complex has been accomplished, no other group has published work involving the capture of the miRNA-Ago2 complex using a solid phase ELISA. It must also be noted that while the polyclonal antibody may present a greater chance of capturing the complex, the low binding affinity may be lowered once the Ago2 has bound to its miRNA guide strand, due to conformational changes that occur upon binding.

Figure 11. Flow Diagram for miRNA Detection Assay



Background Due to Non-Specific Binding by miRNA Let-7a

PCR is a technique that has the capability to detect nucleic acid down to the level of one copy. This is due to the ability of PCR to amplify nuclei acid exponentially. Because of the sensitivity of PCR, it was important that our first step was to determine the affinity of our miRNA for the polyclonal antibody which is being used to immobilize miRNA-Ago2 complex. MiRNA with a non-specific affinity for the antibody could result in skewed results and errors in quantitation.

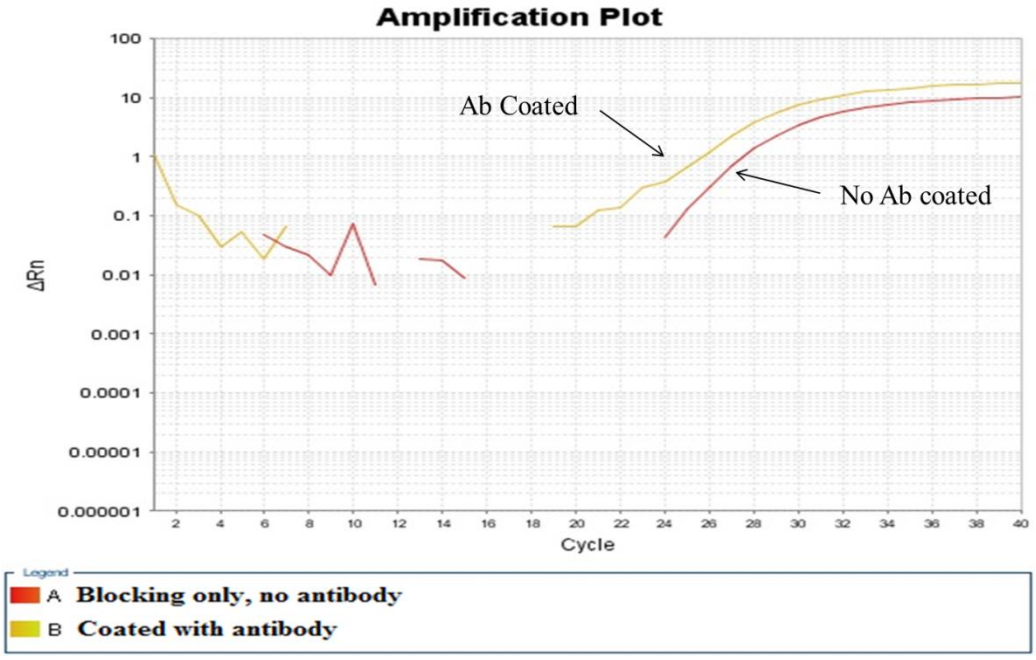
In order to determine the affinity of Let-7a for the polyclonal antibody, a well of a polystyrene plate was coated with 50 µL of the polyclonal antibody at a concentration of 2.5 µg/mL. Another well served as a negative control and was not coated with the antibody, but rather 1X PBS only. The wells were left to incubate overnight at room temperature to allow adsorption of the antibody. Following the incubation, the wells were washed two times with 1X TBST and blotted on a paper towel to remove excess liquid. 100 µL of 1% blocking solution was then added to the wells and allowed to incubate for one hour at room temperature. Following the incubation, the plate was then washed three times with 1X TBST and blotted on a paper towel to remove excess liquid.

Two each well, 50 µL of miRNA Let-7a complex mixture without the Ago2 protein was added to each well. The complex reaction mixture was made up in a microfuge tube by adding 2 µL of Let-7a at a concentration of 3.2 µM in 1X PBS, TM buffer (40 U/mL RNasin, 25 mM $MgCl_2$, 1 M KCl, 16.7 mM DTT), with 1X PBS to a volume of 50 µL. The plate was sealed and incubated for one hour while shaking. Following the incubation, the plate was washed five times with 1X TBST and blotted on

a paper towel to remove excess liquid. To each well, 50 µL of Tris-EDTA buffer, with RNasin was added to the wells. The plate was sealed and incubated at 65 °C for 15 minutes. The sample was then transferred from each well to clean RNase free microfuge tubes.

Reverse transcription was then performed on the samples using the Qiagen miScript Reverse Transcription (RT) kit. 2 µL of each sample was used for the reverse transcription reactions and the total volume was 20 µL. Following reverse transcription reaction, the RT sample was diluted according to the suggested kit protocol. The sample was then used for real-time PCR using the Qiagen miScript SYBR Green PCR kit. For the PCR reaction, 2 µL of the cDNA was used per 20 µL reaction. The forward primer for the reaction was designed by Qiagen Geneglobe using the mature Let-7a sequence 5`-p-UGAGGUAGUAGGUUGUAUAGUU-3'. Activation of the HotStarTaq DNA polymerase was achieved by an initial incubation at 95 °C for a time of 15 minutes. Denaturation was achieved by incubating at 94 °C for 15 seconds. Annealing was achieved by incubating at 55 °C for 30 seconds and extension was accomplished by incubation at 70 °C for 30 seconds. The cycle was repeated for a total of 40 PCR cycles.

Figure 12. Background PCR Noise Due to Non-Specific Binding by miRNA Let-7a



The data revealed a background that was higher than expected for the assay. It would appear that the non-specific binding between miRNA Let-7a with the rabbit anti-Ago2 polyclonal antibody was rather significant when comparing the two amplification plots. Having such a high background due to PCR amplification of the non-specifically bound miRNA poses an issue for the detectability of Ago2 associated Let-7a.

In order to address the high background, we took into consideration the introduction of an exoribonuclease as a way to digest all unbound miRNA in the complex reaction prior to adding the reaction mix to the coated microtiter wells.

Reduction of Background Noise Using Exoribonuclease XRN-1

We acquired an exoribonuclease, XRN-1, from New England Biolabs with the intention of using it to digest the excess miRNA present in the Ago2/Let-7a complex reaction. The hypothesis was that digesting the excess miRNA would reduce the PCR background significantly enough to make the Ago2 associated miRNA amplification plot distinguishable. Our first step was to determine the necessary amount of XRN-1 needed to digest the amount of excess Let-7a in the Ago2/Let-7a complex reaction.

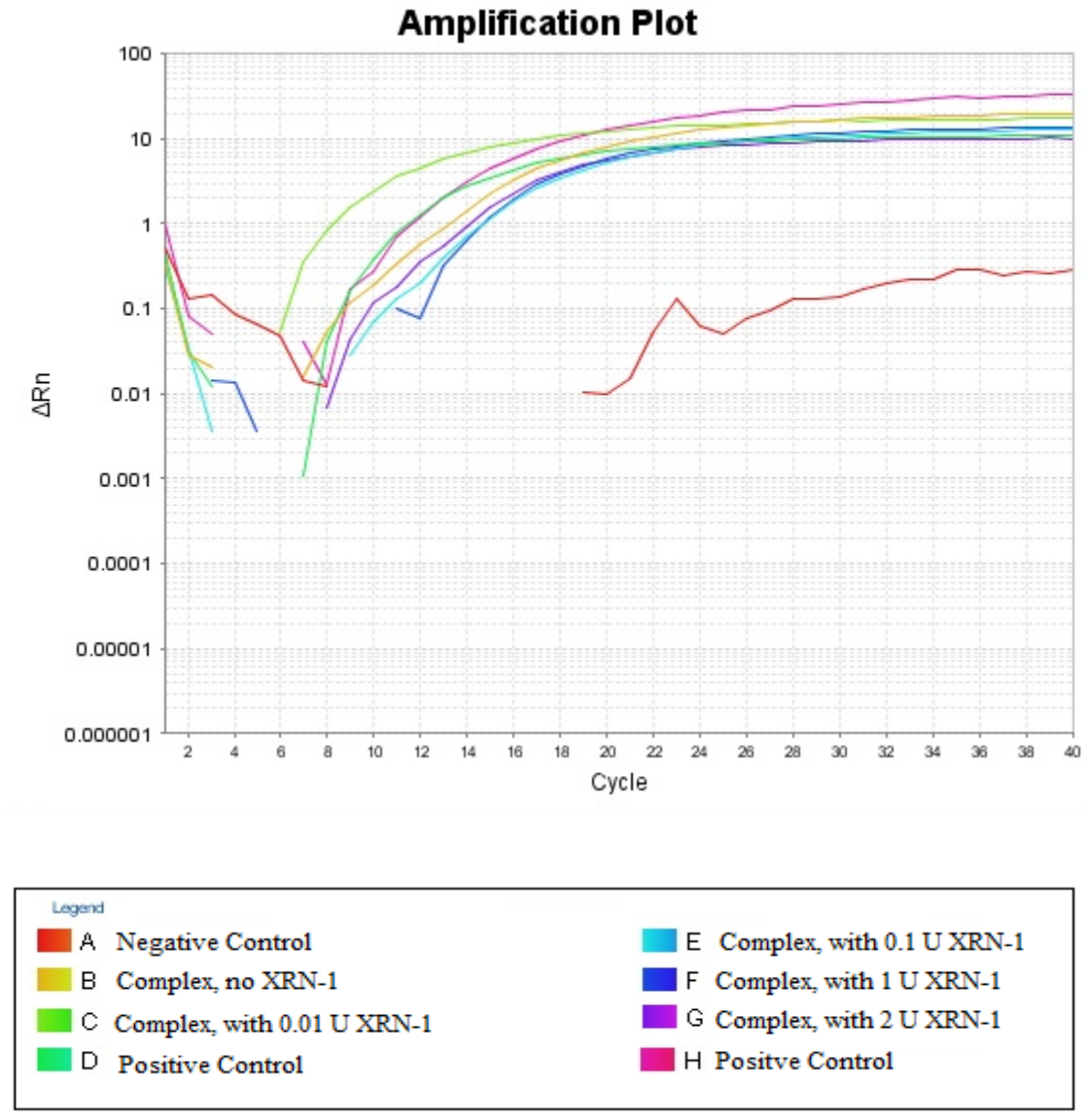
XRN-1 is an exoribonuclease that specifically recognizes the 5' phosphate group of RNA. We wanted an exoribonuclease that specifically targeted the 5' phosphate group because the 5' phosphate is engaged by the Mid domain of the Ago2 protein and, thereby, protected from degradation by XRN-1. Therefore, Let-7a miRNA that is not bound by Ago2 would be degraded by XRN-1. Also, any accessible 3' end nucleotides of the Ago2 bound miRNA would not be susceptible to degradation by XRN-1. In order to determine the amount of XRN-1 necessary to digest the amount of XRN-1 we performed a titration with the XRN-1 and the amount of miRNA present in the Ago2/Let-7a complex reaction.

We numbered seven microfuge tubes. A master mix was made up for the Ago2/Let-7a complex reaction; 11 µL of 800 nM Ago2, 41.25 µL of TM buffer, 11 µL of 3.2 µM phosphorylated Let-7a miRNA, and 46.75 µL of deionized water. To tubes 2-6, 20 µL of the Ago2/Let-7a complex master mix was added. To tube one, only one fourth of the amount of miRNA, 0.8 µM, was added and the volume was adjusted to 20 µL with deionized water. To tube seven the full amount of miRNA in the complex reaction, 3.2

μM, was added and the volume was adjusted to 20 μL with deionized water. Neither tubes one or seven received XRN-1. These tubes served as positive controls with tube one representing the amount of miRNA that should remain if only Ago2 bound miRNA remained, and tube seven representing the full amount of miRNA in the reaction. The one fourth amount was determined from the ratio of Ago2 to Let-7a in the reaction because that was the amount of Let-7a used in the reaction is four times the amount of Ago2 in order to facilitate incorporation of Let-7a into every Ago2 protein.

To tubes two through six, 0.01, 0.1, 1, and 2U (U = 1 μL) of XRN-1 was added respectively in that order to each reaction tube. Each tube was vortexed gently and briefly centrifuged. The tubes were then incubated at 30 °C for 15 minutes followed by a 10 minute incubation at 70 °C to deactivate the XRN-1. Following the incubation, 2 μL of each sample was used in seven reverse transcription reactions using the Qiagen miScript reverse transcription kit. Following the reverse transcription reaction, real-time PCR was performed using the Qiagen miScript Real-Time PCR kit with SYBR Green.

Figure 13. Data from XRN-1 Titration



The results of the titration experiment were rather surprising yet not unexplainable. From the amplification plot, you will observe that there was little to no digestion of miRNA Let-7a by XRN-1 and that the levels are essentially the same. The conditions of the Ago2/Let-7a complex reaction are not the optimal conditions by which the XRN-1 operates. Also, the free ATP that is present in the reaction mix for the Ago2/Let-7a complex may be acting to quench the activity of the exoribonuclease which specifically targets the 5' phosphate of ribonucleic acids. This is a detail that we overlooked in utilizing this exoribonuclease. Also, the optimal temperature for XRN-1 is 37 °C while the optimal temperature for the Ago2/Let-7a complex formation is 30 °C. In order to maintain the integrity of the protein-miRNA complex we decided to carry out the titration at 30 °C.

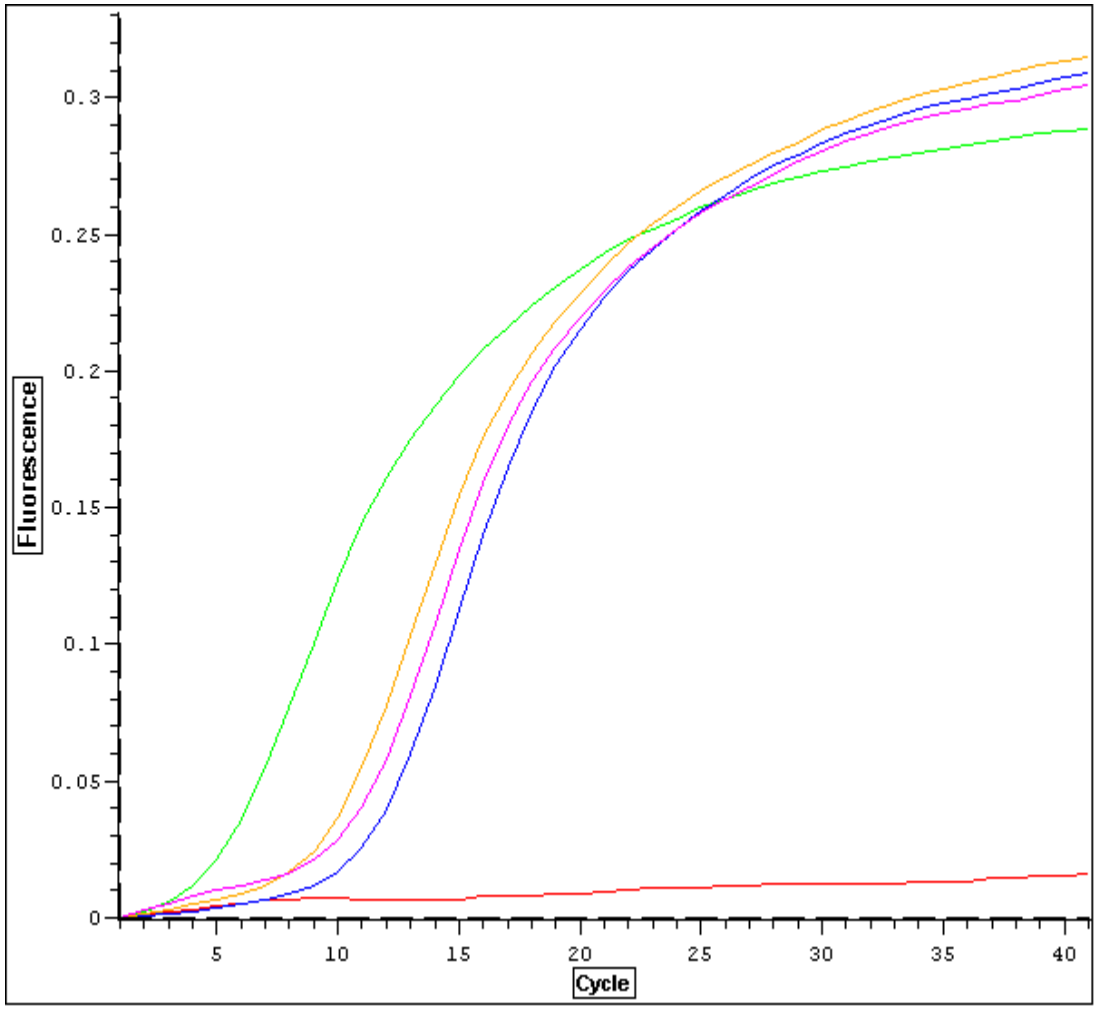
XRN-1 Time Study

Following the past titration experiment two problems were noted. The ability of the XRN-1 to digest Let-7a was inhibited by the presence of ATP. And, the efficiency of XRN-1 was reduced when the digestion was carried out at 30 °C. To alleviate the inhibition of miRNA digestion, ATP was excluded from the TM buffer. Also, the reaction was again carried out at 30 °C.

To further optimize the conditions of digestion, a time study was performed. In a microfuge tube, a master mix containing all the components of the Ago2/Let-7a reaction without Ago2 protein or ATP for a total volume of 20 µL. To the tube, 1 µL of 0.5 U XRN-1 was added and the tube was allowed to incubate at 30 °C. At the time points of

15, 30, 45, and 1 hour, 5 µL aliquots were taken from the reaction tube using a micropipette and transferred to a clean microfuge tube. The aliquot was then incubated at 70 °C to deactivate the XRN-1. The four samples corresponding to the four time points were then used for the reverse transcription reactions. For each reaction, 2 µL of each sample was used. Following reverse transcription, the sample was diluted according to the Qiagen miScript RT kit protocol and used in the real-time PCR reaction using the Qiagen miScript SYBR Green PCR kit.

Figure 14. XRN-1 Time Study at 30 °C



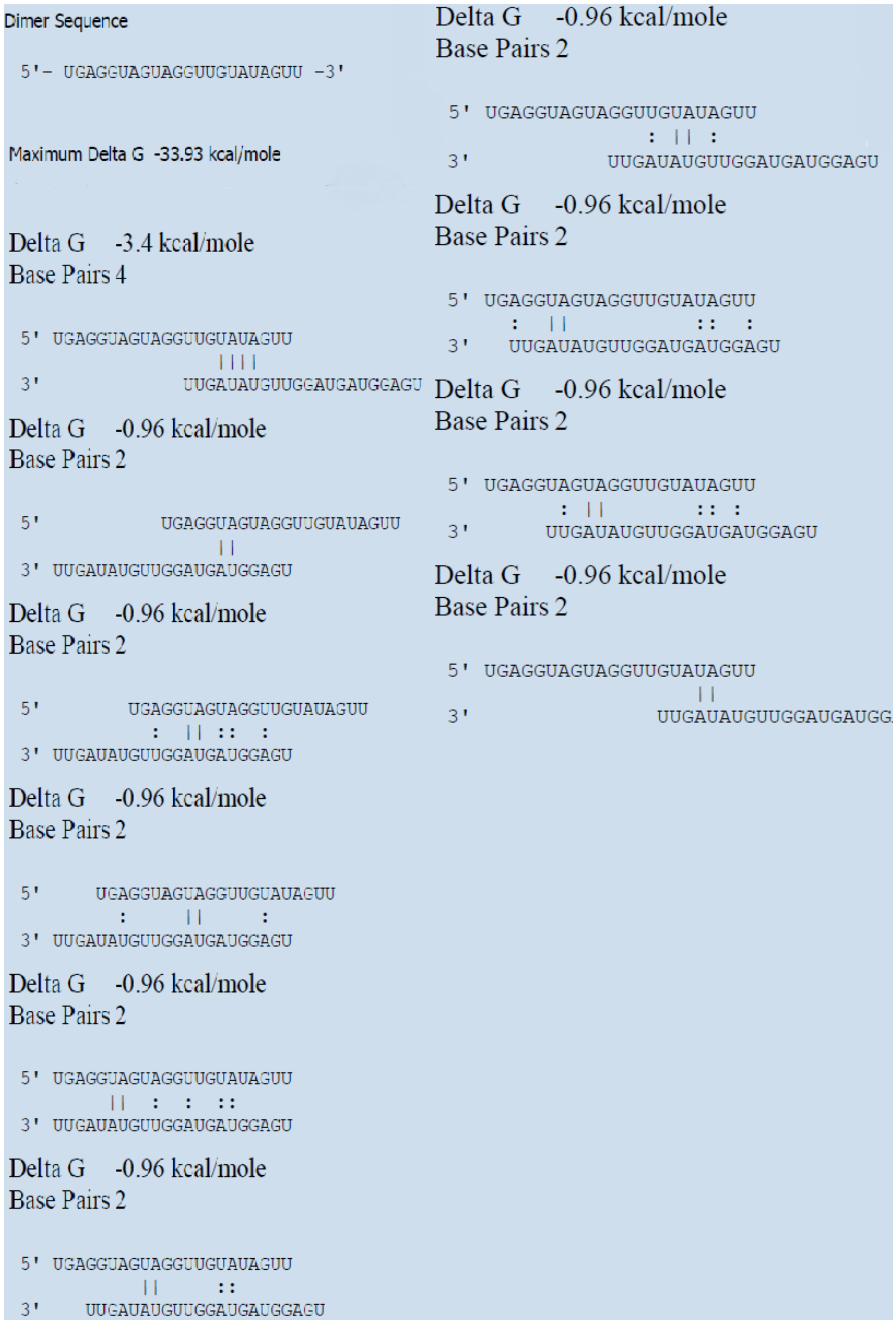
Legend
Red – Negative Control
Green – Positive Control (1.6 uM Let-7a, no XRN-1)
Blue – 1.6 uM Let-7a, 5U XRN-1, 15 minute incubation, 30ºC
Yellow – 1.6 uM Let-7a, 5U XRN-1, 30 minute incubation, 30ºC
Magenta – 1.6 uM Let-7a, 5U XRN-1, 1 hour incubation, 30ºC

The results from the XRN-1 time study indicated a less than optimal conditions for maximum nuclease activity. After fifteen minutes, the activity seemed to decline and indicated that the XRN-1 was no longer functional and was therefore dead. For the time study, the free ATP had been removed from the reaction mixture eliminating the possibility of quenching due to the presence of free ATP. I was then decided that, at 30 °C, the efficiency of the XRN-1 was limited or the enzyme had died.

We determined that there was a possibility that at 30 °C the XRN-1 may not have sufficient energy to properly digest miRNA Let-7a due to the secondary structures of the miRNA being able to inhibit complete digestion of the miRNA. Incompletely digested Let-7a molecules would still be able to be reverse-transcribed, amplified, and detected via real-time PCR. In order to determine the prospective of this hypothesis we decided to briefly look at the possible secondary structures of Let-7a and the binding energy associated with each structure.

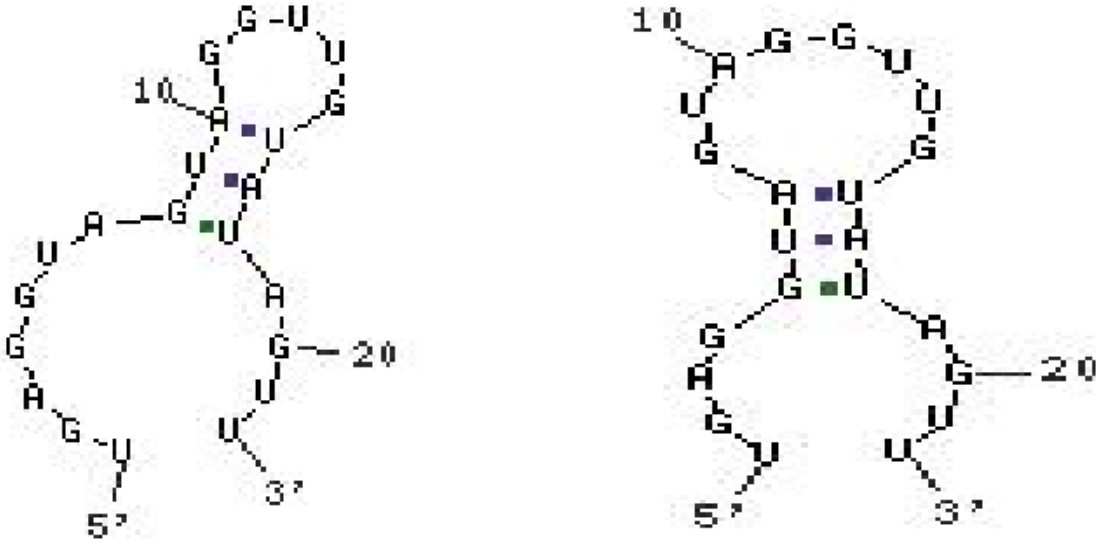
There are many software programs that have been developed to determine the secondary structures of nucleic acids, their probability of formation, and the binding energies between each structure. Secondary structures of single stranded nucleic acids include homo dimers, heterodimers, twists and loops. Depending on the number of bases involved in hydrogen bonding within, or between, the nucleic acid molecules the amount of energy required to break these hydrogen bonds varies. Therefore, at lower temperatures, digestion of the miRNA by XRN-1 may be incomplete. With this understanding, the XRN-1 time study was repeated using the same protocol and parameters with the incubation temperature at 37 °C.

Figure 15. Homo Dimer Structures and ΔG for Human miRNA Let-7a



Data generated using Integrated DNA Technologies OligoAnalyzer 3.1 Software

Figure 16. Secondary Structures and ΔG for Human Let-7a miRNA

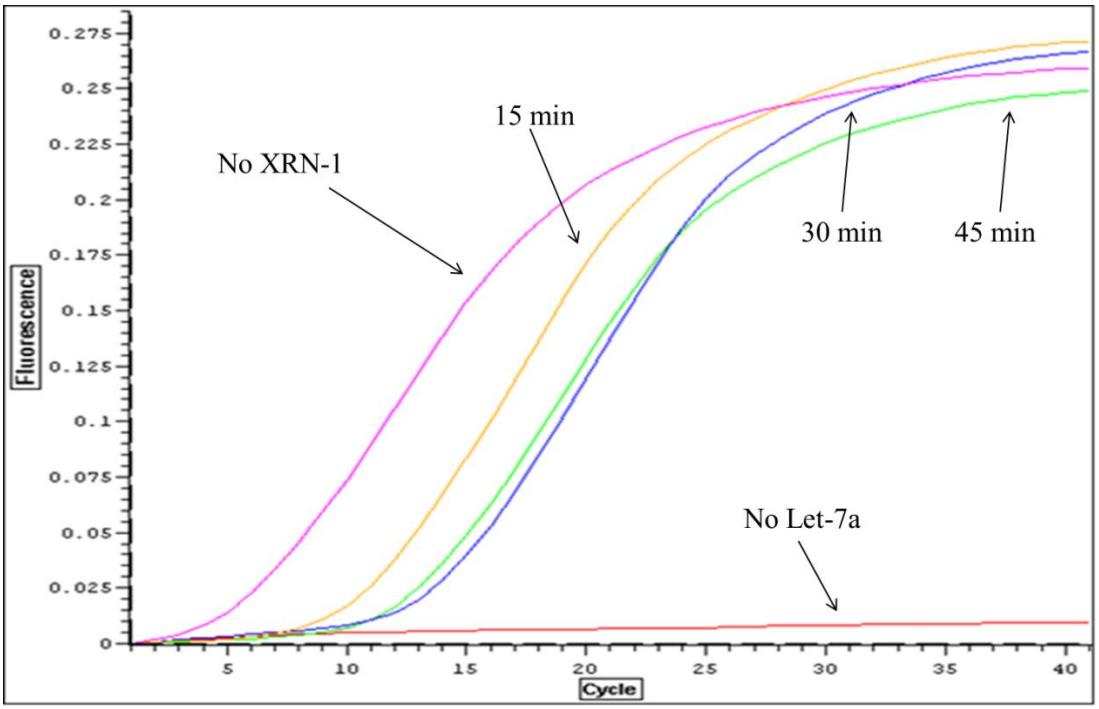


Delta G = -1.06 kcal/mole

Delta G = -0.42 kcal/mole

Data generated using Integrated DNA Technologies OligoAnalyzer 3.1 Software

Figure 17. XRN-1 Time Study at 37 °C



The PCR data from the time study still indicates incomplete digestion by XRN-1 and lose of full enzymatic activity after fifteen minutes. While more digestion was observed at a temperature of 37 °C, the efficiency was still low and activity seemed to halt. The conditions for the complex seem to be incompatible with the conditions XRN-1 needs to be fully efficient at cutting the excess miRNA. Nevertheless, the conditions necessary to form the Ago2/Let-7a complex must not be altered. We would continue to utilize the XRN-1 as a means to lower the background for the PCR detection but understand that, due to the reduced activity of the XRN-1, the background may only be slightly lowered.

Detection of AGO2 Associated Let-7a Using Antibody Capture and Real-Time PCR

We are now ready to proceed with the detection of Ago2 associated miRNAs by combining ELISA based capture with quantitative real-time PCR. Using the rabbit anti-human Ago2 polyclonal antibody to pull down the Ago2 protein, bound to miRNA Let-7a, and later detecting the Let-7a we hope to distinguish Let-7a bound by Ago2 from free Let-7a. We hypothesize that Ago2 bound Let-7a would produce a shift left in the amplification plot away from the background signal for the assay. This shift would indicate a greater presence of miRNA due to its binding with the Ago2 that was pulled down onto the solid phase by the Ago2 specific antibodies.

First, 50 µL of the rabbit anti-hAgo2 polyclonal antibody was added to the wells of a polystyrene microtiter plate and incubated overnight at room temperature to allow antibody adsorption. Following the incubation, the wells were washed two times with 1X

TBST and blotted on a paper towel to remove excess liquid. To the wells, 200 µL of 1% blocking solution was added to the wells and incubated for one hour at room temperature. Following the incubation, the wells were washed two times with 1X TBST and blotted on a paper towel to remove excess liquid.

During the incubation with the blocking solution, the Ago2/Let-7a complex reaction was set up and carried out. To a nuclease free 0.2 mL PCR tube 2 µL of recombinant human Ago2 was added at a concentration of 10 ng/µL, 7.5 µL of TM buffer (333 mM KCl, 5 mM $MgCl_2$, 1.67 mM DTT), 2 µL of phosphorylated Let-7a miRNA, and 8.5 µL of nuclease free water for a total volume of 20 µL. Another reaction tube was set up with the same reagents but without recombinant Ago2 protein and used as a control. The tubes were closed and incubated for 30 minutes at 30 °C using a thermocycler.

Following the incubation 5 µL of XRN-1, the equivalent of 5 units, was added to each reaction tube. The tubes were closed and allowed to incubate for an additional 30 minutes at 37 °C in the thermocycler in order to allow digestion of the free Let-7a. Following the incubation, 25 µL of 1% diluent was added to the reaction tubes to bring the final volume to 50 µL. The entire contents of each reaction tube were then transferred to the antibody coated microtiter wells.

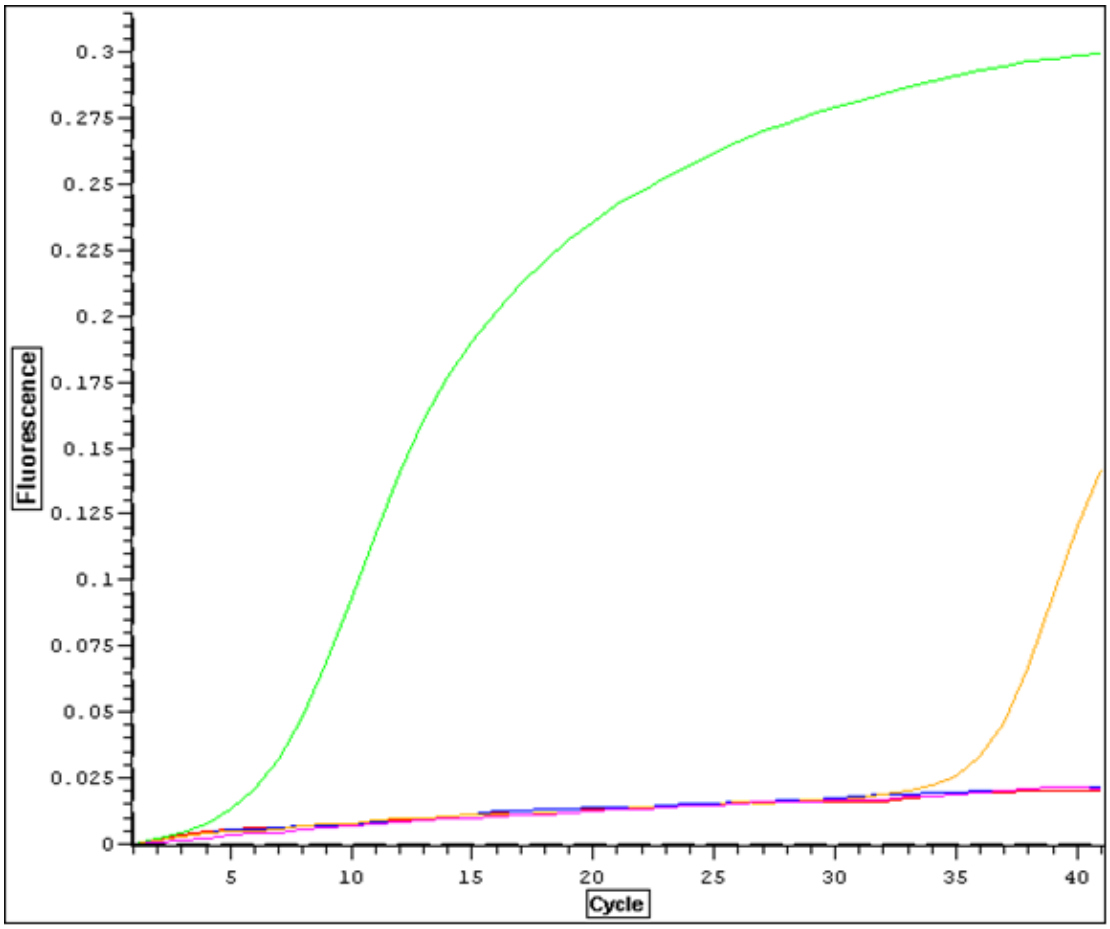
After the reaction mix was added to the wells, the plate was incubated for one hour at room temperature while gently shaking on a plate shaker. Following the incubation, the plate was washed five times with 1X TBST and blotted on a paper towel to remove excess liquid. To each sample well, 50 µL of Tris-EDTA buffer was added

and the plate was placed in an incubator set at 65 °C for 15 minutes to allow denaturation of the Ago2/Let-7a complex. Following the incubation, the contents of each well was quickly transferred to individual nuclease free microfuge tubes using a microtiter pipette. Each sample was then reverse transcribed using the Qiagen miScript Reverse Transcription Kit. For each reverse transcription reaction, 2 µL of each sample was used along with 4 µL of the 5X miScript HiSpec Buffer, 2 µL of the 10X nucleics mix, 2 µL of the miScript reverse transcriptase mix, and RNase-free water for a final volume of 20 µL. As a positive control 3.2 µM Let-7a miRNA was used, with 3.2 µM corresponding to the total amount of Let-7a miRNA present in the reaction. The reverse transcription reactions were incubated for 60 minutes at 37 °C followed by a five minute incubation at 95 °C to inactivate the reverse transcriptase.

After completion of the reverse transcription reaction, the cDNA products were diluted according to the kit protocol by addition of at least 200 µL of nuclease-free water. The diluted cDNA products were then used in the real-time PCR reaction. Nuclease-free water was used as a PCR negative. The Qiagen miScript SYBR Green PCR Kit was used to perform the real-time PCR. For the PCR setup, white 0.2 mL PCR strip tubes were used with clear strip caps. To each tube, 10 µL of 2X QuantiTect SYBR Green PCR Master Mix, 2 µL of 10X miScript Universal Primer, 2 µL of the 10X miScript Primer Assay (Let-7a forward primer), 2 µL of the diluted cDNA product, and RNase-free water for a total volume of 20 µL. The reaction tubes were then mixed and briefly centrifuged. The PCR cycle consisted of an initial incubation step of 15 minutes at 95 °C to activate the HotStarTaq DNA polymerase. The cycle was then 15 seconds at 94 °C to allow

denaturation, 30 seconds at 55 °C for annealing, and 30 seconds at 70 °C to allow extension. The complete PCR ran for 40 cycles.

Figure 18. Real-Time PCR Amplification Plot from Let-7a Detection Assay

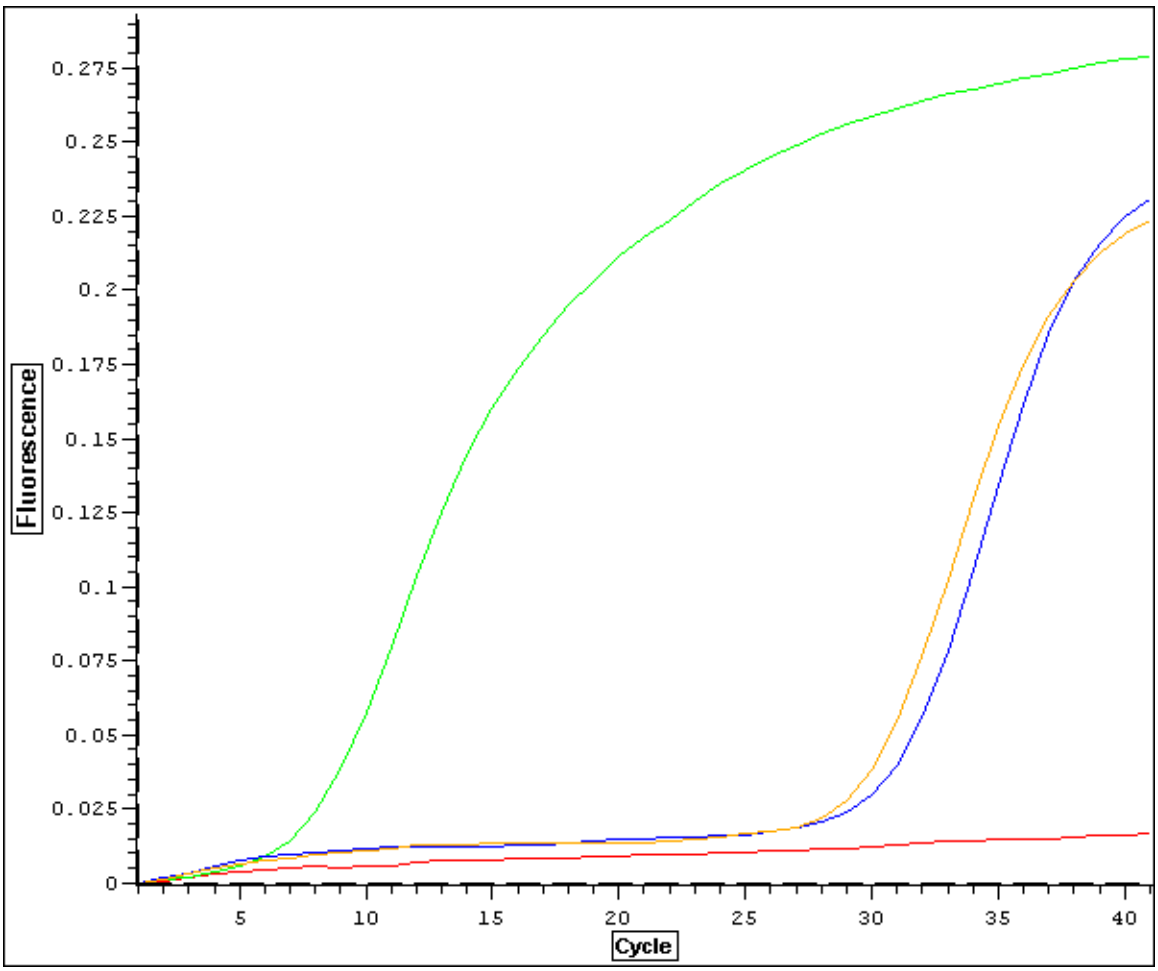


Legend
Red = PCR Negative Control
Green = Positive Control
Blue = Complex Reaction w/ Ago2, coated well
Yellow = Complex Reaction no Ago2, coated well
Magenta = Complex Reaction w/ Ago2, uncoated well

The results from our first attempt at detecting the Ago2 associated Let-7a yielded unexpected results. However, these results further support data that was observed in earlier stages of our study. From the observed data, all reactions that contained Ago2 failed to produce an amplified signal for the PCR reaction within 40 cycles. If you will recall from the experimental procedure, the diluent used prior to adding the complex reaction to the coated wells was 1% blocking solution. The decision to use the blocking solution as the diluent was made because of the need to block non-specific binding between the free Let-7a and the adsorbed antibodies and well surfaces. Also, the lack of a signal from reactions that contained Ago2 would further support the low binding affinity of the antibody for the recombinant Ago2 protein when adsorbed on a solid phase.

In order to determine whether our hypothesis was correct, we repeated the experiment using 1X PBS as a diluent instead of 1% blocking solution. By using a diluent that did not possess excess proteins, we predicted that the probability of the antibody binding the protein complex would be increased. However, in not using the blocking solution as a diluent, we risk further increasing the background of the assay.

Figure 19. Real-Time PCR Amplification Plot from Let-7a Detection Assay Using 1X PBS as Diluent



Legend
Red = PCR Negative Control
Green = PCR Positive Control
Yellow = Complex Reaction w/ Ago2, coated well
Blue = Complex Reaction no Ago2, coated well

As seen in figure 19 above, the use of 1X PBS as a diluent for the complex reaction mix as opposed to using 1% blocking solution resulted in a generated signal from the PCR reaction. We observed amplification from the wells containing the Ago2/Let-7a complex. However, as expected, the high background signal was generated as well due to the strong affinity between the polyclonal antibody and miRNA Let-7a. In order for the detectability of the complex to increase it is necessary that the resultant background noise be reduced.

In earlier experiments we attempted to preemptively reduce the background noise produced by the affinity of the polyclonal antibody to the miRNA by using an exoribonuclease to digest the free miRNA that existed in the reaction. This however proved to be not as effective as we had hoped and is observable in the results obtained in this previous experiment. As you may recall, the ratio of Ago2 to Let-7a in our complex reaction is 1:4 with the purpose ensuring that every Ago2 protein in the reaction has bound a miRNA. Due to the affinity of the antibody for the miRNA, we have inadvertently pushed the assay background noise to higher levels. As is often the case in scientific studies and experimental design, compromises have to be made as the data sees fit. We moved forward in our study with a new mind set, that being a modification of the Ago2 and Let-7a ratios for the complex reaction. We decided to invert the ratio for the complex reaction and used 4:1 Ago2 to Let-7a with the hope that it would help eliminate the problem of the background noise from the free Let-7a.

For our next experiment, we not only inverted the ratios for the reaction we also used lower molar amounts of Ago2 and Let-7a. As with the past experiment the two

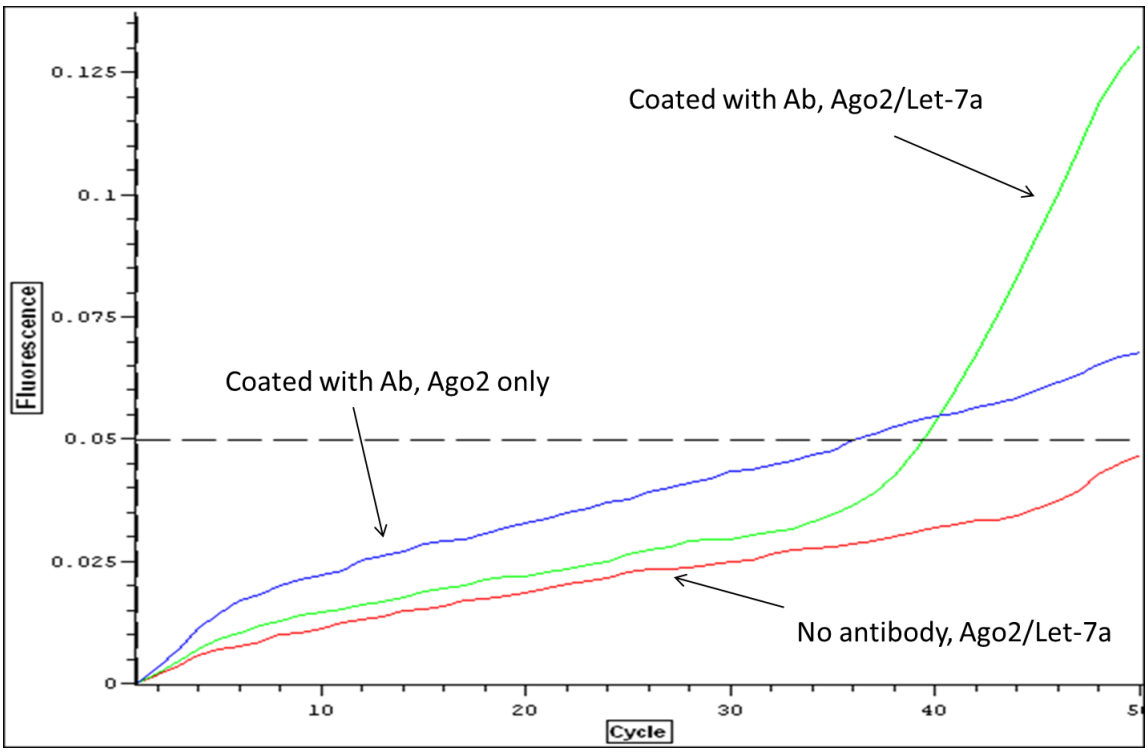
wells of a polystyrene microtiter plate were coated by adding 50 µL of the polyclonal antibody at a concentration of 5 µg/mL and allowed to incubate overnight at room temperature. 50 µL of 1X PBS was used for the uncoated well. Following the incubation, the wells were washed two times with 1X TBST and gently blotted on a paper towel to remove excess liquid. To each well, 200 µL of 1% blocking solution was added and allowed to incubate for one hour at room temperature. While the incubation with the blocking solution was going on the Ago2/Let-7a complex reaction was carried out.

Three clean, nuclease free, 0.2 mL microtubes were used for the complex reactions. In the first tube, 2 µL of [10 ng/µL] Ago2, 7.5 µL of TM buffer (3.33 mM ATP, 333 mM KCl, 5 mM $MgCl_2$, 1.67 mM DTT), 2 µL of [25 nM] phosphorylated Let-7a, and nuclease free water were added for a total reaction volume of 20 µL. To the second tube, the same reagents were added in the same amounts but no Let-7a was added to this reaction, only Ago2. The reaction tubes were then closed and incubated for 30 minutes at 30 °C using a PCR thermocycler.

The timing for the microtiter plate incubation and reaction incubations were staggered. Prior to the completion of the complex reaction incubation, the wells of the microtiter plate were washed two times with 1X TBST and gently blotted on a paper towel to remove excess liquid. To each well, 45 µL of 1X PBS was then added. Then, to the first coated well, 5 µL of the complex reaction was added. To the other coated well, 5 µL of the reaction that lacked Let-7a was added, and to the uncoated well 5 µL of the complex reaction was added. The plate was then sealed and allowed to incubate for one hour while shaking.

Following the incubation, the plate wells were washed three times with 1X TBST and gently blotted on a paper towel to remove excess liquid. To each well, 50 µL of TE (Tris-EDTA) buffer was added and the plate was allowed to incubate for 15 minutes at 65 °C to denature that protein content of the well and allow elution of the Let-7a. The liquid was then removed from each well and respectively transferred to a labelled, clean, microtube. 2 µL of each elution sample was then used for 10 µL reverse transcription reactions according to the protocol for the Qiagen miScript Reverse Transcription Kit. Following the reverse transcription reaction, 5 µL of undiluted cDNA was used in the 20 µL real-time PCR reaction according to the protocol for the Qiagen miScript SYBR Green PCR Kit.

Figure 20. Real-Time PCR Amplification Plot for the Modified Let-7a Detection Assay



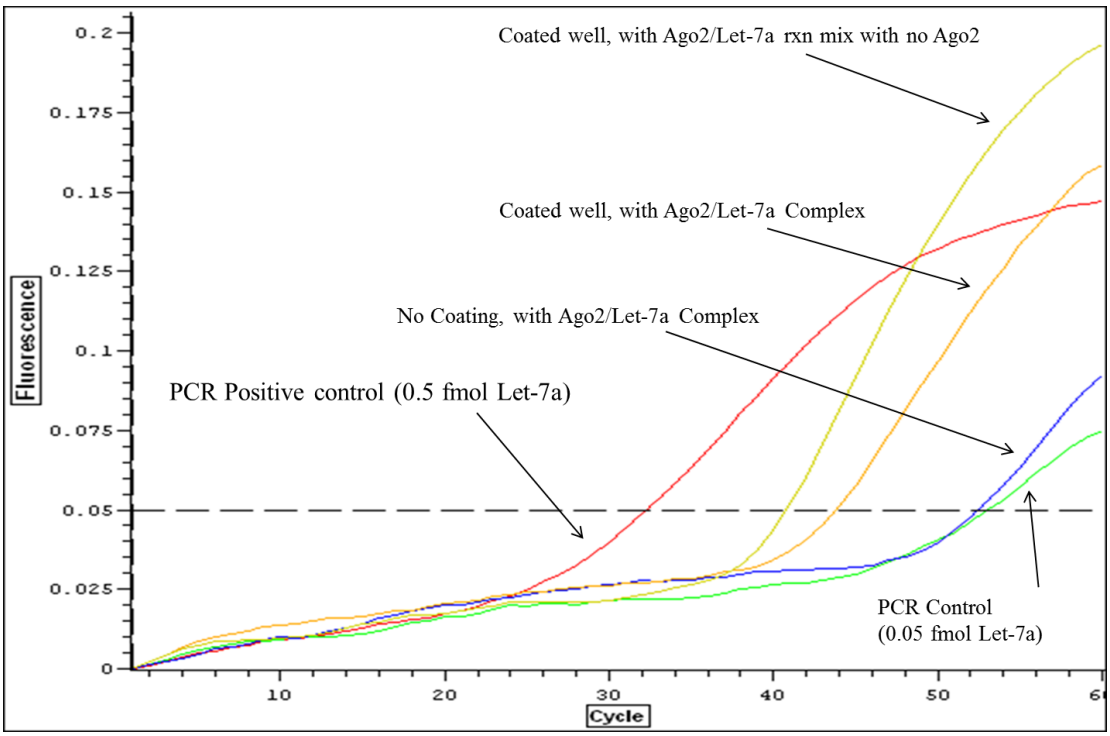
Legend
Red = no antibody coated, Ago2/Let-7a complex
Green = polyclonal antibody coated, Ago2/Let-7a complex
Blue = polyclonal antibody coated, complex reaction mix with Ago2 only, no Let-7a

From figure 20, a signal is observed from the sample eluted from the coated well incubated with the Ago2/Let-7a complex but not from the well that is uncoated but incubated with the Ago2/Let-7a complex. This is most certainly a positive development in our study. The data demonstrates the necessity of the antibody and its ability to isolate the active complex from a sample. The question now lies with the position of the background noise for the assay. The current experiment supports the use of the antibody as a viable means of isolation but we now need to compare the amount of complex

associated isolated from the sample with the amount of background noise due to the attraction between the miRNA and the antibody.

Therefore, the previous experiment was repeated in the same manner but with the addition of a few other controls. In the repeat of the experiment, we coated three wells with the polyclonal antibody, and left one uncoated. One of the coated wells, and the uncoated well were incubated with the Ago2/Let-7a complex reaction. The remaining coated well was incubated with the Ago2/Let-7a complex reaction but the Ago2 was excluded from the reaction. PCR controls containing 0.5 fmol and 0.05 fmol Let-7a were used. These controls were to correspond to the estimated amount of free Let-7a remaining after the dilution of the complex reaction mix and to estimated removal of free Let-7a during the washing steps. All steps of the experiment were the same as in the previous experiment.

Figure 21. Real-Time PCR Amplification Plot for the Detection of Ago2 Associated Let-7a



Legend
Red = PCR positive control (0.5 fmol Let-7a)
Green = PCR control (0.05 fmol Let-7a)
Dark Blue = No Coating, with Ago2/Let-7a Complex
Gold = Coated well, with Ago2/Let-7a Complex
Yellow = Coated well, with Ago2/Let-7a rxn mix with no Ago2

Results of Study

As can be seen from the amplification plot in figure 21, there is a clear distinction between the PCR amplification signal from the coated well incubated with the Ago2/Let-7a and the coated well incubated with just Let-7a. The plot shows that the well incubated with Let-7a only contained a higher number of copies and thus became detectable at a lower cycle number, however this outcome is not surprising. Given the size of the

miRNA-Ago2 complex, compared to the free miRNA, we would expect that the free miRNA would diffuse to the well surface faster than the complex and block some of the available binding sites. As a result, we would expect the shift in the amplification signal for the free miRNA when the miRNA-Ago2 complex is not present in the sample. This difference in the two amplification signals shows proof of concept that isolation and quantitation of Ago2 associated miRNAs can be achieved using this analytical method.

CHAPTER V

CONCLUSION AND FUTURE WORK

Conclusion

The role of miRNA is translational inhibition has been widely studied and it along with its binding partner Argonaute 2 protein play a key role in the functionality of the RNA-Induced Silencing Complex (RISC). Conventional methods for studying the RISC involve molecular techniques that rely on the total extraction of the cellular RNA from the lysate. However, this method of extracting all of the cellular RNA for analysis does not take into account the partnership with the Ago2 for functionality in the matter of translational inhibition.

In this study we investigated a novel solution to this problem. We utilized an antibody raised against the Ago2 protein as a tool that we could use to isolate the functional miRNA from a sample by targeting the miRNA-Ago2 complex. While there is still work to be done, preliminary results show promise. Although the affinity of our selected antibody against Ago2 was not optimal, we were able to demonstrate the presence of Ago2 associated miRNAs and distinguish the Ago2 associated miRNA from the non-Ago2 associated miRNA using real-time PCR.

There is great potential in the optimization of the method we have investigated. Most significantly, our investigation provides the foundation for development of an analytical method that could detect the functional miRNA directly from samples such as

blood. This could potentially lead to new breakthroughs in how the RISC and the regulation of translational inhibition are studied.

Future Work

In our future work, we seek to investigate the use of different antibodies to go after the Ago2 component of the miRNA-Ago2 complex. The use of a different antibody could alleviate the issues we encountered by offering a higher binding affinity and possibly a lower affinity for the free miRNA. However, each is a case by case issue and further optimization will need to be performed with the new antibody, from the parameters we have already established. After successfully completing the optimization of the assay, we then will seek to quantitate the active miRNA using qRT-PCR. The last phase will involve quantitation of the active miRNA from biological samples.

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