



# miRNA Target IP Kit

Catalog No. 25500

(Version B1)

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Revision	Date	Description of Change
B1	August, 2024	BSA is no longer provided as a powder. It is provided suspended in Wash Buffer AM7. See instructions for storing after first thaw.
		Updated formatting.

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# Contents

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<b>Overview</b> .....	1
<b>Flow Chart of Process</b> .....	2
<b>Principle of the Method and Data</b> .....	3
<b>Kit Components and Storage</b> .....	4
Additional Materials Required .....	5
<b>Experimental Set Up</b> .....	6
Experimental Design .....	6
Growth of Cells .....	7
Buffer Preparation .....	8
Quick Chart for Preparing Buffers .....	10
<b>Protocols</b> .....	11
Section A: miRNA Transfection .....	11
Section B: Lysate Preparation .....	12
Section C: Antibody Conjugation to Protein G Beads .....	12
Section D: RNA Complex Immunoprecipitation .....	13
Section E: Proteinase K Digestion and RNA Purification .....	14
Section F: Analysis of Samples by qRT-PCR .....	17
<b>References</b> .....	19
<b>Appendix</b> .....	20
Section G: Troubleshooting Guide .....	20
Section H: Recommendations for Primer Design .....	22
Section I: Data Analysis .....	23
Section J: Use of Magnetic Beads and Included Bar Magnet .....	23
<b>Technical Services</b> .....	27

## Overview

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MicroRNAs (miRNAs) are a class of small, non-coding RNA molecules that act as post-transcriptional regulators of gene expression by binding to the 3-prime Untranslated Region (3'UTR) of messenger RNA transcripts (mRNAs). Most commonly, the interaction of a miRNA with a 3'UTR results in either transcript degradation or inhibition of translation.

miRNA regulation plays a large role in many different biological processes, including development, proliferation and differentiation. Changes in miRNA expression levels have been found in a variety of cancers, as well as in cardiac disease and neurological disorders, making miRNA-mediated regulation an important topic in biomedical research.

Over 1,000 different miRNAs have been found in the human genome. Because they bind with only partial complementarity, most miRNAs target multiple mRNA transcripts (up to 100 in some cases). In addition, individual mRNAs frequently contain binding sites for more than one miRNA, and miRNA families that differ by as little as a single base pair have been found. This creates a complex regulatory network that can act in a tissue and cell-state dependent manner. As a result, it has been estimated that the expression of up to 60% of mammalian proteins may be regulated by miRNAs.

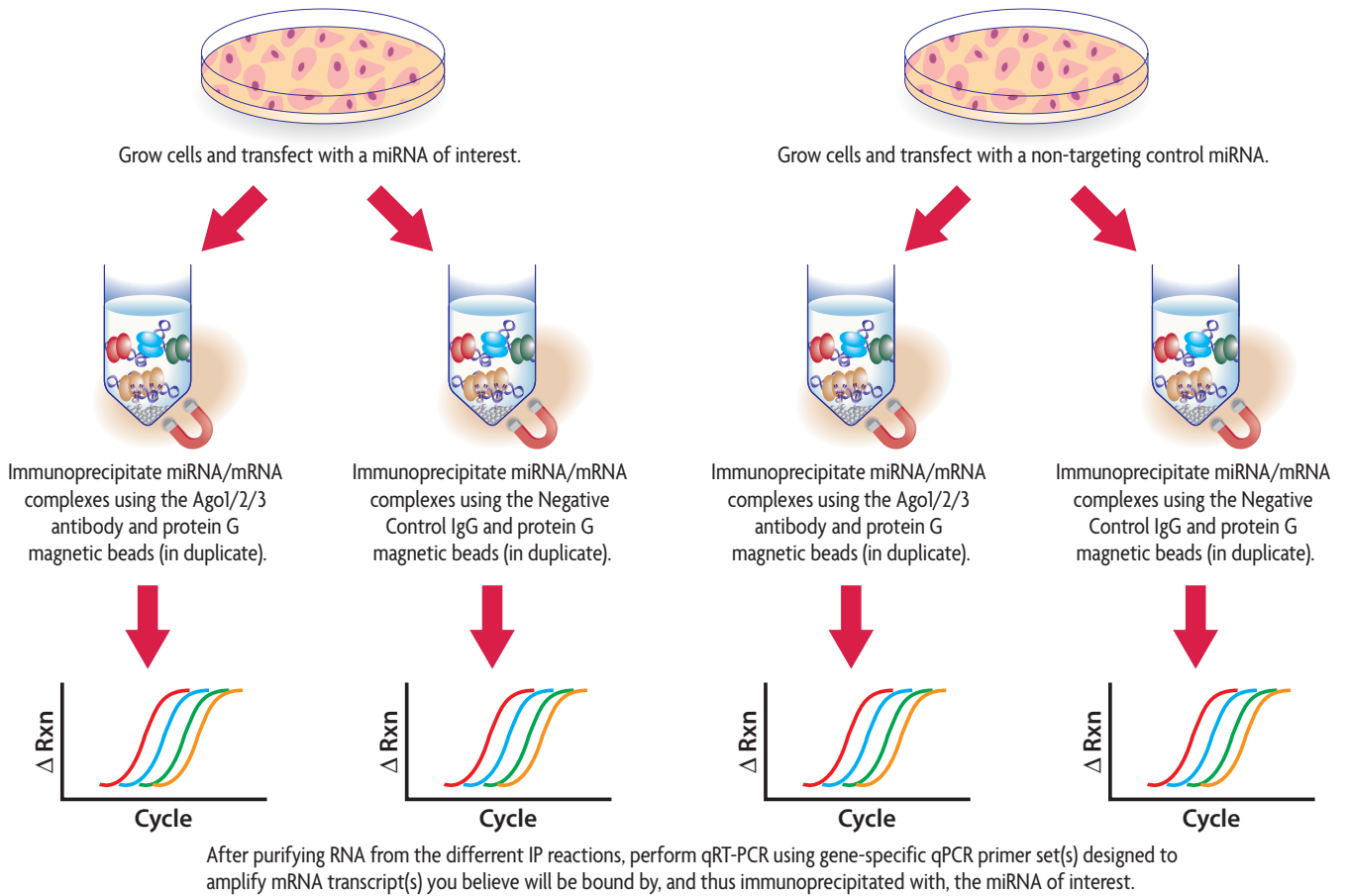
The targeting of a miRNA molecule to a specific mRNA is mediated through the formation of an RNA Induced Silencing Complex (RISC). Key components in the RISC complex are an Argonaute protein (Ago) and a miRNA. The Ago protein binds the miRNA, positioning it in a conformation that enables the RISC complex to base-pair in a Watson-Crick manner with a mRNA transcript. The sequence of the miRNA molecule contained in a RISC complex enables the precise targeting of specific mRNA transcripts.

Active Motif's miRNA Target IP Kit utilizes protein G magnetic beads and a pan-Ago antibody that recognizes Ago 1, Ago 2 and Ago 3 proteins to validate the actual physical interactions of a miRNA with endogenous mRNA transcripts. It has been validated in both human and mouse cell lines.

The kit supplies sufficient reagents to perform 10 miRNA Target IP reactions using the included Ago1/2/3 Antibody and 10 negative control IP reactions using the included Negative Control IgG. Following their immunoprecipitation (IP) as part of the RISC complex, the mRNA transcripts are purified and amplified by qRT-PCR using gene-specific primers. A fold induction is then calculated using the Ago1/2/3 Antibody and Negative Control IgG data, which will indicate if the mRNA transcripts that were amplified were bound by the miRNA of interest. It should also be possible to profile the immunoprecipitated mRNA molecules using conventional expression microarrays or RNA-Seq.

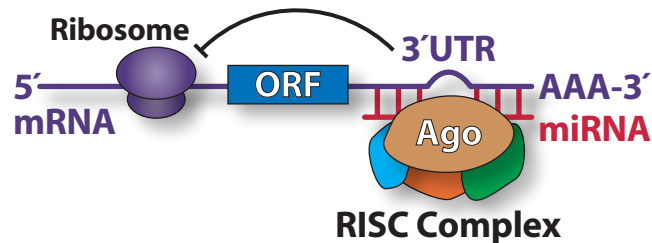
Product	Format	Catalog No.
miRNA Target IP Kit	10 rxns	25500

# Flow Chart of Process



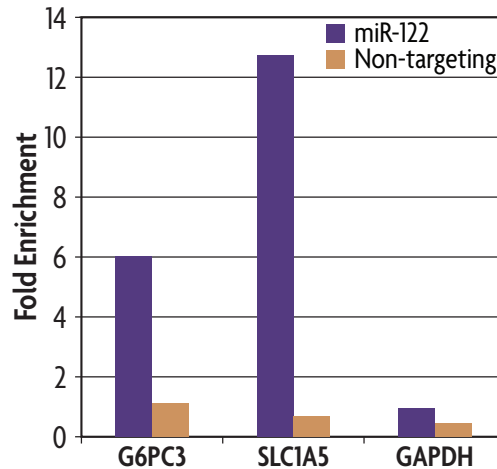
## Principle of the Method and Data

The targeting of a miRNA to a specific mRNA is mediated through the formation of an RNA Induced Silencing Complex (RISC). While RISCs can contain a combination of different RNA-binding proteins, at a minimum a RISC is comprised of an Argonaute protein (Ago) and a miRNA. The Ago protein binds the miRNA in a manner that enables the miRNA-loaded RISC complex to base-pair with a mRNA transcript. The miRNA Target IP Kit utilizes a pan-Ago antibody and protein G-coupled magnetic beads to immunoprecipitate miRNA/mRNA complexes that have associated with Ago1, Ago2, or Ago3. This enables one to validate which mRNA transcripts were bound by a specific miRNA.



**Figure 1: Translational repression by a miRNA-directed, RISC Complex.**

The key components in a RISC Complex are an Argonaute protein (Ago) and a miRNA. The Ago protein binds the miRNA, positioning it in a conformation that enables the RISC to base-pair in a Watson-Crick manner with a mRNA transcript. This leads to either inhibition of translation (shown) or increased degradation of the targeted transcript.



**Figure 2: miR-122 targets G6PC3 and SLC1A5.**

The miRNA Target IP Kit was used on samples of HT1080 cells that had been transfected with a miR-122 mimic or a non-targeting miRNA control for 8 hours. Following IP using the Ago1/2/3 antibody or Negative Control IgG included in the kit, qRT-PCR was performed on the samples using primers for G6PC3 and SLC1A5, which are known targets of miR-122, and for GAPDH, a common housekeeping gene that is not known to be targeted by miR-122

## Kit Components and Storage

Please store each component at the temperature indicated in the table below. Do not re-freeze the Protein G Magnetic Beads. Once thawed, Protein G beads should be stored at 4°C

Reagents	Quantity	Storage
Ago1/2/3 antibody (mAb) (1.0 µg/µL)	50 µL	-20°C
1 M DTT	100 µL	-20°C
RNase Inhibitors	2 x 60 µL	-20°C
Protease Inhibitor Cocktail (PIC)	2 x 100 µL	-20°C
Ribonucleoside Vanadyl Complex	55 µL	-20°C
BSA Solution (50 mg/mL)	4 mL	-20°C
Proteinase K (10 µg/µL)	560 µL	-20°C
Precipitation Buffer	1.5 mL	-20°C
Glycogen (20 µg/µL)	40 µL	-20°C
IGF2 Primer pair (2.5 µM each)	90 µL	-20°C
Protein G Magnetic Beads*	1 mL	4°C
10X PBS	100 mL	4°C
Negative Control Mouse IgG (0.2 µg/µL)	250 µL	4°C
Lysis Buffer AM6	5 mL	4°C
10X Wash Buffer AM7	20 mL	4°C
0.5 M EDTA	1 mL	RT
10% SDS	500 µL	RT
Salt Solution	500 µL	RT
Bar magnet	1 magnet	RT
Glue dots	2 sheets	RT

\* The Protein G Magnetic Beads are shipped on dry ice, and can be stored frozen until their first use. However, Protein G beads should not be re-frozen by the customer. After they have been thawed the Protein G Magnetic Beads should be stored at 4°C.

## Additional Materials Required

- RNase-free tubes (PCR and microcentrifuge)
- RNase-free filtered pipette tips and pipettor
- RNase-free water
- Rocking platform for culture plates
- Cell scraper (rubber policeman)
- 15 and 50 mL conical tubes
- Microcentrifuge (table top centrifuge 4°C)
- Freezer capable of -80°C storage
- Apparatus to rotate tubes end-over-end at 4°C (e.g. a Labquake from Barnstead/Thermolyne with a tube holder for microcentrifuge tubes)
- Vortex
- Heat block or thermo-mixer
- Phenol:chloroform:isoamyl alcohol (25:24:1) (e.g. Fisher Scientific AC327115000)
- Chloroform
- 100% ethanol (absolute)
- 80% ethanol
- Thermal cycler
- miRNA Mimics and non-targeting miRNA Mimics Active Motif offers over 800 different human miRNA Mimics and non-targeting miRNA Mimics. Please go to [activemotif.com/mimics](http://activemotif.com/mimics) for more information.
- DharmaFECT 4 Transfection Reagent (e.g. Dharmacon Catalog No. T-2004-01), or a reagent known to work well for miRNA transfection in your specific cell type
- (Optional) 2-Step Reverse Transcription Kit (e.g. Applied Biosystems High-Capacity RNA-to-cDNA Kit, Applied Biosystems Catalog No. 4387406)
- (Optional) Gene-specific qPCR primer pairs for enrichment analysis; see Sections F & H
- (Optional) SYBR Green qPCR master mix (e.g. Bio-Rad Catalog No. 170-8882)



## Experimental Set Up

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING!

### Experimental Design

This kit was designed to validate if specific mRNA transcripts are bound by a particular miRNA. Therefore, you will transfect cells with your miRNA of interest and then perform immunoprecipitation (IP) using a pan-Ago antibody that recognizes Ago1, Ago2 and Ago3. This will pull down miRNA/mRNA complexes that have associated as part of a RISC complex; IP will also be performed with a Negative Control IgG.

After purifying the RNA and making cDNA, you will use gene-specific qPCR primer set(s) designed to amplify mRNA transcript(s) you believe will be bound by, and thus immunoprecipitated with, the transfected miRNA of interest. The Ago1/2/3 Antibody and Negative Control IgG data will then be used to calculate a Fold Enrichment; if a specific mRNA was bound by the miRNA, more copies of that transcript will be pulled down by the pan-Ago IP relative to the Negative Control IgG. As a negative control you should also perform pan-Ago and Negative Control IgG IP experiments on cells that have been transfected with a non-targeting control miRNA to be certain the effect seen with the miRNA of interest is not due to non-specific binding.

When planning your experiment, first determine the number of immunoprecipitation reactions you plan to perform. Enough reagents are supplied in this kit to perform 20 total IP reactions: sufficient Ago1/2/3 Antibody is included to perform 10 miRNA Target IP reactions while Negative Control IgG is included for 10 negative control IP reactions.

We recommended using 10 million cells per IP, and setting up reactions in duplicate. Please refer to the descriptions included in the Buffer Preparation section for complete details on buffer preparations, and to the Quick Chart for Preparing Buffers to determine the necessary volumes based on the number of IP reactions.

Performing IP experiments in duplicate using both the Ago1/2/3 Antibody and the Negative Control IgG will require 40 million cells that have been transfected with a miRNA of interest and 40 million cells that have been transfected with a non-targeting control miRNA.

Sample Type	miRNA Target IP	Negative IgG IP	Total
Cells transfected with miRNA mimic	2 x 10 <sup>7</sup> cells	2 x 10 <sup>7</sup> cells	4 x 10 <sup>7</sup> million
Cells transfected with non-targeting miRNA	2 x 10 <sup>7</sup> cells	2 x 10 <sup>7</sup> cells	4 x 10 <sup>7</sup> million
Number of IP reactions	4	4	8

Please see page 2 for a flow chart of the process.

## Growth of Cells

Prior to performing the immunoprecipitation reactions using cell lysates, growing cells will be transfected with either a miRNA of interest or with a non-targeting control miRNA. We recommended that you seed your cells to yield 70-80% confluence at the time you perform your transfections, though the optimal amount may vary by cell line. Following transfection, the cells will then be incubated for 8-24 hours before beginning the IP process by preparing cell lysates.

The table below provides information for some commonly used cell culture dishes so you can estimate how many you will need for your experiment. Keep in mind that the cells will continue to grow after the miRNA transfection. The number of cells present when you harvest them to prepare lysates will depend upon a variety of factors including the cell type, their health, the amount of time that elapses following the miRNA transfection and any effects that the miRNA may have upon cell growth.

As we recommend using 10 million cells per IP, and setting up reactions in duplicate, you will need 20 million cells for each type of IP being performed. You may therefore need to grow, transfect and pool multiple dishes in order to have enough cells for your IP reactions.

Sample Type	6-well plate	60 mm dish	10 cm dish	15 cm dish
Surface Area (cm <sup>2</sup> )	9	21	55	152
Seeding Density	0.3 x 10 <sup>6</sup>	0.8 x 10 <sup>6</sup>	2.25 x 10 <sup>6</sup>	5.0 x 10 <sup>6</sup>
Cells at Confluency*	1.2 x 10 <sup>6</sup>	3.2 x 10 <sup>6</sup>	8.8 x 10 <sup>6</sup>	2.0 x 10 <sup>7</sup>
Growth Medium Volume	3 mL	5 mL	10 mL	20 mL
1X PBS (used per wash)	1 mL	2 mL	5 mL	10 mL
Complete Lysis Buffer (used per cell pellet)	100 µL	100 µL	150 µL	150 µL

\* The number of cells on a confluent plate or dish will vary with cell type. For this table, HeLa cells were used. Please adjust the cell number as needed based on your particular cell type.

## Buffer Preparation

**Note:** RNA is highly susceptible to RNase degradation. Ensure all ancillary reagents and tubes are RNase-free. Wear gloves at every step of the protocol (including buffer preparation) to avoid RNase contamination from hands.

### 1X PBS

Prepare 31 mL of 1X PBS for every 15 cm dish for washing the cells prior to preparing the lysates. To a 50 mL conical tube add 27.9 mL sterile water and 3.1 mL 10X PBS. Mix by inverting. Place 1X PBS on ice to chill. Use the Quick Chart for Preparing Buffers to adjust the volumes based on the number of dishes used. The volumes listed are based on the use of one 15 cm dish per IP reaction. 1X PBS can be prepared in large quantities and stored at 4°C for 6 months.

### 1 M DTT

DTT is provided as a 1 M stock solution. It is used as provided to prepare Complete Lysis Buffer. In addition, a 100 mM DTT solution will be prepared in RNase-free water by adding 25  $\mu$ L 1 M DTT to 225  $\mu$ L RNase-free water. The 100 mM DTT solution is used to prepare the Immunoprecipitation Buffer. Any unused 100 mM DTT solution should be stored at -20°C for future use.

### RNase Inhibitors

The RNase Inhibitors are supplied ready to use, and will be used to prepare Complete Lysis Buffer.

### Protease Inhibitor Cocktail (PIC)

Thaw the PIC at room temperature until fully dissolved, which takes about 30 minutes. Vortex the PIC gently, spin down briefly, then add it to the Complete Lysis Buffer immediately before use.

### Ribonucleoside Vanadyl Complex

Ribonucleoside Vanadyl Complex is supplied ready to use. Thaw at room temperature and vortex briefly before use. It will be used to prepare Complete Lysis Buffer.

### Complete Lysis Buffer

Complete Lysis Buffer should be prepared fresh before each experiment and discarded at the end of the day. We recommend using a minimum of 150  $\mu$ L Complete Lysis Buffer per pellet for cell numbers ranging from 5 - 20 million cells. For samples containing 1 - 5 million cells, use 100  $\mu$ L Complete Lysis Buffer per pellet. It is not advisable to use less than 100  $\mu$ L. See the Quick Chart for Preparing Buffers to prepare the amount of buffer needed for each experiment.

## **Protein G Magnetic Beads**

Follow the instructions in Section C to conjugate the Ago1/2/3 antibody and the Negative Control IgG to the beads for use in the IP reactions. For best results, gently shake and invert the tube to resuspend the magnetic beads. The beads settle quickly, and therefore should be resuspended just before pipetting. We recommend cutting 2 mm from the end of a pipet tip prior to pipetting to prevent the tip from becoming clogged. The Protein G Magnetic Beads are shipped on dry ice, and can be stored frozen until their first use. However, the beads should not be re-frozen. After they have been thawed the Protein G Magnetic Beads should be stored at 4°C.

## **10X Wash Buffer AM7**

Prepare a 1X Wash Buffer AM7 solution in RNase-free water. Use the Quick Chart for Preparing Buffers to determine the amount of Wash Buffer needed for each experiment.

## **BSA Solution (50 mg/mL)**

BSA is now provided ready to use in Wash Buffer AM7. Use 200  $\mu$ L of the BSA Solution (50 mg/mL) per 50  $\mu$ L Protein G Magnetic Beads. Any unused solution should be dispensed into aliquots and stored at -20°C for future use.

## **Ago1/2/3 Antibody**

The supplied Ago1/2/3 antibody is a pan-Ago mouse monoclonal antibody that recognizes Argonaute proteins Ago 1, Ago 2 and Ago 3. Use 5  $\mu$ L (5  $\mu$ g) antibody per IP reaction.

## **Negative Control IgG**

The Negative Control IgG is used to determine the background in the immunoprecipitation reactions. Use 25  $\mu$ L (5  $\mu$ g) Negative Control IgG per IP reaction.

## **0.5 M EDTA**

The 0.5 M EDTA is supplied ready to use. It is used to prepare the Immunoprecipitation Buffer.

## **10% SDS**

The 10% SDS is supplied ready to use. However, SDS may precipitate upon storage. If needed, warm the bottle to 37°C for 5 minutes and vortex to dissolve any precipitates. It is used in Section E during the Proteinase K digestion.

## **Proteinase K (10 mg/mL)**

The Proteinase K is supplied ready to use. It will be used to make fresh Proteinase K Digestion Buffer in Section E.

## **Precipitation Buffer**

The Precipitation Buffer is supplied ready to use. It is used in Section E to precipitate the RNA. Take care to not confuse this buffer with Immunoprecipitation Buffer, which you will prepare fresh by following the Quick Chart for Preparing Buffers for use in Section D.

## Salt Solution

The Salt Solution is supplied ready to use. It is used in Section E to precipitate the RNA.

## Glycogen

The Glycogen provided is at a concentration of 20 mg/mL. Make a 4-fold dilution of the Glycogen stock by adding 120  $\mu$ L RNase-free water directly to the provided tube to make a 5 mg/mL solution. Mark the tube as 5 mg/mL for future use. Use 5  $\mu$ L of the diluted stock per reaction.

## Quick Chart for Preparing Buffers

Reagents to prepare	Components	1 IP	2 IPs	10 IPs	20 IPs
1X PBS	10X PBS	3.1 mL	6.5 mL	32 mL	65 mL
	Sterile water	27.9 mL	58.5 mL	288 mL	585 mL
	<b>TOTAL REQUIRED</b>	<b>31 mL</b>	<b>65 mL</b>	<b>320 mL</b>	<b>650 mL</b>
Complete Lysis Buffer	1 M DTT	1.5 $\mu$ L	3.5 $\mu$ L	17 $\mu$ L	33 $\mu$ L
	RNase Inhibitors	0.4 $\mu$ L	0.9 $\mu$ L	4.3 $\mu$ L	8.3 $\mu$ L
	Protease Inhibitor Cocktail	6 $\mu$ L	14 $\mu$ L	68 $\mu$ L	132 $\mu$ L
	Ribonucleoside Vanadyl Complex	0.3 $\mu$ L	0.7 $\mu$ L	3.4 $\mu$ L	6.6 $\mu$ L
	Lysis Buffer AM6	142 $\mu$ L	331 $\mu$ L	1.61 mL	3.12 mL
	<b>TOTAL REQUIRED</b>	<b>150 <math>\mu</math>L</b>	<b>350 <math>\mu</math>L</b>	<b>1.7 mL</b>	<b>3.3 mL</b>
1X Wash Buffer AM7	RNase-free water	6.7 mL	18 mL	81 mL	144 mL
	10X Wash Buffer AM7	740 $\mu$ L	2 mL	9 mL	16 mL
	<b>TOTAL REQUIRED</b>	<b>7.4 mL</b>	<b>20 mL</b>	<b>90 mL</b>	<b>160 mL</b>
Immunoprecipitation Buffer	1X Wash Buffer AM7	850 $\mu$ L	1.89 mL	9.43 mL	18.9 mL
	RNase Inhibitors	5 $\mu$ L	11 $\mu$ L	56 $\mu$ L	111 $\mu$ L
	Ribonucleoside Vanadyl Complex	2 $\mu$ L	4.5 $\mu$ L	22 $\mu$ L	45 $\mu$ L
	100 mM DTT	10 $\mu$ L	22 $\mu$ L	111 $\mu$ L	222 $\mu$ L
	0.5 M EDTA	34 $\mu$ L	75 $\mu$ L	378 $\mu$ L	756 $\mu$ L
	<b>TOTAL REQUIRED</b>	<b>900 <math>\mu</math>L</b>	<b>2 mL</b>	<b>10 mL</b>	<b>20 mL</b>

### Section A: miRNA Transfection

The protocol below is for transfection of miRNAs into HT1080 cells growing in 10 cm dishes using DharmaFECT 4 Transfection Reagent, and is meant as a general guideline. You can scale the protocol as needed if you wish to grow your cells and transfect them in different sizes of plates.

Moreover, the optimal transfection reagent and conditions will vary depending on what cells are being transfected, what type of molecule is being transfected and what type of plate the cells are growing in. Dharmacon offers a Cell Type Guide on its website that details which of its reagents have performed best for miRNA transfections into a variety of commonly used cell lines. Please see <http://dharmacon.gelifesciences.com/rnai-and-custom-rna-synthesis/transfection/sirna-and-microrna-transfection> for more information.

Be sure to set up enough plates so that you can perform transfection of your miRNA of interest as well as a non-targeting control miRNA, and so that there will be sufficient lysate from each of the transfections to perform IP in duplicate using both the Ago1/2/3 Antibody and the Negative Control IgG.

On Day 1, you will seed your cells in plates so that they grow to a confluency of 70-80% the following day. You will then perform miRNA transfections on the plates and wait for 8-24 hours before preparing cell lysates. So, try to time when you seed your cells so that they will be ready for transfection and lysate preparation at the desired timepoints.

1. Trypsinize and count your cells, then seed 10 cm dishes with  $2.25 \times 10^6$  cells in a final volume of 15 mL antibiotic-free complete media per plate, which is a dilution of 150,000 cells/mL. (Complete media is the “standard” media your cells are maintained in; it may contain serum.)
2. Grow cells overnight at 37°C with 5% CO<sub>2</sub>.

On Day 2, you will perform miRNA transfections on your cells after they have reached a confluency of 70-80%. You will make up dilutions of each miRNA and the transfection reagent in separate tubes using serum-free media, incubate, combine each diluted miRNA with the diluted transfection reagent and incubate again, add complete media to each mix, then remove the media from the cells and add the appropriate transfection medium to each plate.

3. For each miRNA transfection, prepare Mix 1 by adding 150 µL of miRNA (5 µM) to a microcentrifuge tube followed by 1,850 µL of serum-free media.
4. At the same time, prepare Mix 2 by adding 35 µL of DharmaFECT 4 to a microcentrifuge tube followed by 1,965 µL of serum-free media.
5. Gently mix the contents of each tube by pipetting up and down gently, then incubate the mixes at room temperature for 5 minutes.
6. Add the contents of Mix 2 to a 15 mL conical tube, then add Mix 1 to Mix 2 in the same tube. Mix gently and incubate at room temperature for 20 minutes.

7. Add 11 mL of antibiotic-free complete media to the mixture made in step 6, then mix gently.
8. Remove the media from the dish of cells, then add the 15 mL of transfection mix to the dish.
9. Grow the cells at 37°C with 5% CO<sub>2</sub> for 8-24 hours before preparing the cell lysate.

## Section B: Lysate Preparation

This protocol describes cell lysate preparation from one 15 cm dish (approximately  $1.5 \times 10^7$  cells). If you have grown your cells and transfected the miRNAs in plates that are smaller than 15 cm, you can pool multiple plates of cells when the cells are scraped from the plates (Step 3 below) to ensure that you have at least  $1.0 \times 10^7$  cells for each immunoprecipitation reaction.

1. When cells are ready to harvest, prepare fresh Complete Lysis Buffer for each cell pellet. The volumes listed below are sufficient to process one 15 cm plate.
2. Wash the plate twice with 10 mL ice-cold 1X PBS. (5 mL is sufficient for 10 cm plates.)
3. Add 10 mL ice-cold 1X PBS to each plate. Hold the plate at an angle and use a rubber policeman to scrape the cells down to collect them at the bottom edge of the plate. Use a pipette to transfer the cells to a 50 mL conical tube on ice.
4. Pellet the cells by centrifugation for 5 minutes at 1,500 rpm ( $453 \times g$ ) at 4°C.
5. Remove the supernatant and discard. Resuspend each pellet in 1 mL ice-cold 1X PBS by pipetting up and down. Keep samples on ice for the remainder of the procedure.
6. Take a 10  $\mu$ L aliquot of each cell pellet and determine the cell number using a hemocytometer. It is important to use equivalent cell numbers for each immunoprecipitation reaction.
7. Transfer 10 million cells ( $1.0 \times 10^7$ ) of each sample to an RNase-free microcentrifuge tube. We recommend setting up each IP reaction in duplicate.
8. Centrifuge for 5 minutes at 1,500 rpm ( $453 \times g$ ) at 4°C. Remove the supernatant and discard. Resuspend each pellet in 150  $\mu$ L ice-cold Complete Lysis Buffer by pipetting up and down. (If using a different volume of cells, please refer to the chart on page 7.)
9. Incubate lysate on ice for 5 minutes, then place tubes at -80°C. Keep the lysate at -80°C for at least 1 hour while preparing the Protein G Magnetic Beads, as this freezing step helps to improve cell lysis. If desired, the lysate may be stored at -80°C for up to 3 months.

## Section C. Antibody Conjugation to Protein G Beads

The Protein G Magnetic Beads require blocking and washing prior to antibody conjugation. To begin, determine the total number of immunoprecipitation reactions to be performed, including both Argonaute (Ago) and negative IgG reactions, then label a separate RNase-free microcentrifuge tube for each.

For information regarding the use of the magnetic beads and the included bar magnet and glue dots, please refer to Appendix Section J.

**Note:** RNA is highly susceptible to RNase degradation. Ensure all ancillary reagents and tubes used throughout the remainder of the protocol are RNase-free. Wear gloves at every step of the protocol (including buffer preparation) to avoid RNase contamination from hands.

1. Resuspend the Protein G Magnetic Beads by end-over-end rotation of the vial for 5 minutes at room temperature. As magnetic beads have a tendency to settle to the bottom of the tube, it is important to resuspend the contents completely before use. Following resuspension, use a wrist flick to collect beads out of the cap prior to opening the vial.
2. Add 50  $\mu$ L Protein G Magnetic Beads to each labeled tube.
3. Add 200  $\mu$ L BSA Solution (50 mg/mL) to each tube. Vortex 5 seconds. Allow tubes to incubate with end-over-end rotation for 10 minutes at room temperature.
4. Quick spin the tubes to collect the contents to the bottom. Place the tubes on the magnet to pellet the beads. Remove and discard the supernatant.
5. Add 500  $\mu$ L 1X Wash Buffer AM7 to each tube. Vortex 5 seconds. Place the tubes on the magnet to pellet the beads. Remove and discard the supernatant.
6. Wash a second time with 500  $\mu$ L 1X Wash Buffer AM7. Vortex 5 seconds. Place the tubes on the magnet to pellet the beads. Remove and discard the supernatant, then remove the tubes from the magnet.
7. Add 100  $\mu$ L 1X Wash Buffer AM7 to each tube and 5  $\mu$ g of the appropriate antibody to each of the labeled tubes.

Ago1/2/3 antibody: Use 5  $\mu$ L antibody per Argonaute IP reaction

Negative Control IgG: Use 25  $\mu$ L antibody per negative control IP reaction

8. Incubate with end-over-end rotation for 30 minutes at room temperature.
9. Centrifuge the tubes for 20 seconds at 9,000 rpm (8,150  $\times$  *g*) at room temperature to collect the contents at the bottom of each tube.
10. Place the tubes on the magnet to pellet the beads. Remove and discard the supernatant. Add 500  $\mu$ L 1X Wash Buffer AM7 to each tube and vortex 5 seconds to mix.
11. Repeat step 10 two additional times. Leaving the beads in the final wash solution, place the tubes on ice.

## Section D. RNA Complex Immunoprecipitation

In this section the antibody-conjugated beads will be combined with the lysate to immunoprecipitate miRNA/mRNA complexes associated with Argonaute proteins (Ago1, Ago2 or Ago3). Background levels can be determined using Negative IgG-conjugated beads.

1. Remove the lysate prepared in Section B from the freezer and thaw on ice.
2. Freshly prepare Immunoprecipitation Buffer as described in the Buffer Preparation section of the manual. Place the buffer on ice.
3. Centrifuge the thawed lysate for 10 minutes at 14,000 rpm (20,817  $\times$  *g*) at 4°C.



4. During the lysate centrifugation, place the tubes of antibody-conjugated Protein G Magnetic Beads from Section C, Step 11 on the magnet to pellet the beads. Remove and discard the supernatant.
5. Remove the tubes from the magnet and add 900  $\mu\text{L}$  of Immunoprecipitation Buffer to each tube of beads. Vortex 5 seconds to mix.
6. Following lysate centrifugation, transfer 10  $\mu\text{L}$  of each supernatant to a new RNase-free tube and label as Input. Store the Input samples at  $-80^{\circ}\text{C}$  for use in Section E.
7. Transfer the remaining supernatant ( $\sim 100 \mu\text{L}$ ) to the appropriate tube of antibody-conjugated beads. Discard the pellet.
8. Incubate the IP reactions for 4 hours to overnight with end-over-end rotation at  $4^{\circ}\text{C}$ .
9. Centrifuge the tubes for 20 seconds at 9,000 rpm ( $8,150 \times g$ ) at room temperature to collect the contents at the bottom of each tube.
10. Place the tubes on the magnet to pellet the beads. Remove and discard the supernatant. Add 500  $\mu\text{L}$  ice-cold 1X Wash Buffer AM7 to each tube and vortex 5 seconds to mix.
11. Repeat step 10 five additional times for a total of 6 washes.
12. Place the tubes on the magnet to pellet the beads. Remove and discard the supernatant. Add 10  $\mu\text{L}$  1X Wash Buffer AM7 to each tube to resuspend the beads. Place the tubes on ice.

## Section E. Proteinase K Digestion and RNA Purification

In this section the samples will be treated with Proteinase K and SDS to degrade the Argonaute proteins, disrupt the antibody binding, and elute the RNA from the beads.

1. Freshly prepare Proteinase K Digestion Buffer. Calculate the amount of Buffer needed to process both the IP reactions and the Input samples.

**Note:** It is important to add the chemicals in the order listed below to prevent denaturation of Proteinase K by the concentrated SDS solution.

Reagent	1 reaction	No. of rxns	Volume Needed
1X Wash Buffer AM7	107 $\mu\text{L}$	x _____	= _____
10% SDS	15 $\mu\text{L}$	x _____	= _____
Proteinase K (10 mg/mL)	18 $\mu\text{L}$	x _____	= _____
<b>Total Volume</b>	<b>140 <math>\mu\text{L}</math></b>	x _____	= _____

2. Preheat a thermo-mixer or heat block to  $55^{\circ}\text{C}$ .
3. Thaw the Input samples from Section D, Step 6 on ice.
4. Add 140  $\mu\text{L}$  of the freshly prepared Proteinase K Digestion Buffer to all tubes. This includes the Input samples and the IP reactions from Section D, Step 12.

5. Incubate the samples at 55°C for 30 minutes with shaking. If a thermo-mixer is not accessible, flick each tube by hand every 5 minutes and vortex once halfway through the incubation to resuspend the magnetic beads and ensure complete digestion.
6. Centrifuge the tubes for 20 seconds at 9,000 rpm (8,150 x *g*) at room temperature to collect the contents at the bottom of each tube.
7. Place the tubes on the magnet to pellet the beads, then transfer the eluate to a new RNase-free tube.
8. Add 250 µL 1X Wash Buffer AM7 to each tube.
9. Add 400 µL phenol:chloroform:isoamyl alcohol (25:24:1) to each tube.

**Note:** We highly recommend purchasing phenol:chloroform:isoamyl alcohol rather than mixing it yourself. This mixture can be difficult to homogenize, and if it is not made properly, assay performance can suffer.

10. Vortex for 20 seconds, then centrifuge for 10 minutes at 14,000 rpm (19,722 x *g*) at room temperature.
11. Transfer the aqueous (top) phase into a new RNase-free tube. Try to ensure consistency in recovery for all samples. Add 400 µL chloroform to each tube.
12. Vortex for 20 seconds, then centrifuge for 10 minutes at 14,000 rpm (19,722 x *g*) at room temperature.
13. Transfer the aqueous (top) phase into a new RNase-free tube. The volume will be approximately 350 µL. Try to ensure consistency in recovery for all samples.
14. To prepare the samples for precipitation, add the following to each tube:

Reagent	1 reaction	No. of rxns	Volume Needed
Precipitation Buffer	50 µL	x _____	= _____
Salt Solution	15 µL	x _____	= _____
Glycogen (5 mg/mL)	5 µL	x _____	= _____
Absolute ethanol	875 µL	x _____	= _____
<b>Total Volume</b>	<b>945 µL</b>	x _____	= _____

**Note:** If you collect more than 350 µL of the aqueous phase, or if you notice that your samples are freezing while stored at -80°C, add 900 µL of 100% ethanol instead of 875 µL. If the samples freeze, poor signal will likely be seen.

15. Vortex the tubes for 20 seconds. Place tubes at -80°C for 1 hour to 24 hours to precipitate the RNA.
16. Centrifuge for 30 minutes at 14,000 rpm (20,817 x *g*) at 4°C. Carefully discard the supernatant without disturbing the pellet. If the pellet is not visible, leave ~100 µL of the supernatant in the tube.
17. Add 500 µL of 80% ethanol to each tube. Centrifuge for 15 minutes at 14,000 rpm (20,817 x *g*) at 4°C. Carefully discard the supernatant without disturbing the pellet.

**Note:** If you do not plan to convert the RNA to cDNA immediately, the pellets may be stored in 100  $\mu$ L absolute ethanol for 2 weeks at  $-80^{\circ}\text{C}$ . When ready to use, centrifuge for 15 minutes at 14,000 rpm (20,817  $\times g$ ) at  $4^{\circ}\text{C}$ , then carefully discard the supernatant and proceed with step 18.

Input samples will be used only for troubleshooting to verify that an mRNA transcript was present in the original lysate prior to IP. Therefore, they can either be converted to cDNA along with the other samples or stored as RNA. Because they will be more stable and you will be able to store them for more than 2 weeks, we recommend to convert them to cDNA with the other samples.

18. Allow the pellets to air dry for 10 minutes at room temperature.
19. Resuspend pellets in 20  $\mu$ L RNase-free water and place on ice. Proceed immediately with Section F. Analysis of Samples by RT-PCR.

## Section F. Analysis of Samples by qRT-PCR

The precipitated RNA can be analyzed by Reverse Transcription (RT) followed by real-time PCR. We recommend using a 2-step RT-PCR system to first convert the RNA to cDNA. This cDNA is then analyzed by qPCR. Follow the instructions that came with your specific RT-PCR Kit to prepare your samples.

You will need to have already generated gene-specific qPCR primer set(s) that were designed to amplify the cDNA that will be made from the mRNA transcript(s) you believe will be bound by, and thus immunoprecipitated with, the miRNA of interest that was transfected into your cells.

The IGF2 Primer pair included in this kit can be used in qRT-PCR as a positive control to validate that your IP was successful. It shows strong enrichment following IP with our pan-Ago antibody.

Please consult Appendix Section H for information on designing qPCR primers as well as validating their function by determining their Primer Efficiency through the generation of a standard curve. Use only those primer pairs with an Primer Efficiency between 80-110%.

The following is an example preparation of cDNA using Applied Biosystems High-Capacity RNA-to-cDNA Kit (Applied Biosystems, Catalog No. 4387406). Follow the instructions specific for your Reverse Transcription Kit of choice.

1. In a microcentrifuge tube on ice, prepare the RT reactions as follows.
2. Vortex the samples for 5 seconds, then centrifuge for 1 minute at 1,500 rpm (453  $\times g$ ) at  $4^{\circ}\text{C}$ .

Reagent	Sample / Input	No RT Control
2X RT Buffer	10 $\mu$ L	10 $\mu$ L
RNA (Sample or Input)	9 $\mu$ L	9 $\mu$ L
RNase-free water	–	1 $\mu$ L
Reverse Transcriptase	1 $\mu$ L	–
<b>Total Volume</b>	<b>20 <math>\mu</math>L</b>	<b>20 <math>\mu</math>L</b>

3. To generate cDNA, place tubes in a thermal cycler at 37°C for 60 minutes, followed by 95°C for 5 minutes, then hold the samples at 4°C until you are ready to perform qPCR.
4. Prepare the primers for qPCR analysis. See the Recommendations for Primer Design in the Appendix. For the IGF2 control primer pair, use 1  $\mu$ L of provided mix per PCR.

Below is an example qPCR reaction. Please follow the instructions for your specific qPCR instrument. We recommend using a commercially available SYBR Green qPCR master mix (e.g. Bio-Rad Cat # 170-8882) and preparing triplicate reactions.

5. Prepare a dilution of cDNA for use in qPCR. The dilution factor required may vary depending on the abundance of the transcript of interest. We suggest a 2- to 5-fold dilution of cDNA in RNase-free water as a starting point.
6. In a microcentrifuge tube on ice, prepare a qPCR mix as follows. If you are preparing a master mix, do not add the cDNA. Add each cDNA sample directly to the wells of the plate or strip.

Reagent	Sample / Input
2X SYBR Green master mix	5 $\mu$ L
Primer mix (2.5 $\mu$ M each primer)	1 $\mu$ L
RNase-free water	2 $\mu$ L
cDNA (from Step 5 above)	2 $\mu$ L
<b>Total volume</b>	<b>10 <math>\mu</math>L</b>

7. Pipette the reactions into the wells of a reaction plate or strip appropriate for your real-time PCR system. Seal with optical adhesive film or optical caps, vortex then centrifuge the reaction plate briefly. Use the run specifications dictated by your machine and reverse transcription kit. The example below applies when using the Applied Biosystems RNA-to-cDNA Kit and the Bio-Rad SYBR Green qPCR master mix and qPCR machine.
8. Place tubes in a real-time PCR instrument and program as below:  
95°C for 2 minutes  
(95°C for 15 seconds, 58°C for 20 seconds, 72°C for 20 seconds) for 40 cycles  
65°C for 5 seconds
9. Analyze the results. Suggestions for data analysis can be found in Appendix Section I of the manual.

## References

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1. Bomsztyk, K., *et al.* (2019) *Nucleic Acids Research* 47(12): e69.
2. Kanter, J.E., *et al.* (2019) *J. Clin. Invest.* 129(10):4165-4179
3. Levy, S., *et al.* (2022) *Cell Reports* 38, 110457
4. Chapin, N., *et al.* (2022) *Communications Biology* 5:596
5. Hopf, A., *et al.* (2022) *Bioengineering (Basel)* 9(9):412

### Section G. Troubleshooting Guide

Problem/question	Recommendation
What is the fewest number of cells that can be used for IP?	We recommend using 10 million cells in order to obtain the most consistent results; the enclosed protocols were optimized using that amount of cells. However, we have had success using 5 million cells, and have even been able to go down to as few as 1 million cells in some instances. Your results will vary depending on the cell type and how the cells respond to the miRNA transfection.
Can I use any cell type? What about cells from different species?	You may use any cell type derived from humans or mice. The kit was validated using 293, MCF-7, HT1080 and HeLa cells from humans, as well as with 3T3 mouse cells. If you wish to work with cell types other than those just listed, it is suggested to use 10 million cells to ensure good enrichment. Then, if the fold enrichment values are high, you can use 5 million cells for subsequent reactions. This assay was not tested in any species other than human and mouse, but it may work in other species. It is up to the discretion of the researcher as to whether the miRNA Target IP Kit is appropriate for their cell type.
Can I use an antibody other than the supplied Ago 1/2/3 antibody?	Yes. If you have antibodies that have been validated to successfully immunoprecipitate other RNA-binding proteins, then this kit should function for use in RIP (RNA-binding Protein Immunoprecipitation). However, Active Motif has not tested other antibodies, so results with other antibodies are not guaranteed.
Can I use the immunoprecipitated/purified mRNA molecules with conventional expression microarrays or RNA-Seq?	Yes, the mRNA produced should be appropriate for these uses. However, Active Motif has not tested these other applications, so cannot guarantee that they will work. Please consult the manufacturer's protocols of these alternate methods.

Problem/question	Recommendation
I don't see any pellets after the RNA Precipitation step. Why?	It is important to use enough cells and/or antibody (preferably 5 million and 5 µg) per reaction. It is crucial to include all of the listed components when preparing buffers, such as RNase Inhibitors. It is necessary to have frequent/continual mixing of the tubes during the proteinase K digestion step. Collection of enough of the aqueous layer is necessary when performing the phenol:chloroform and chloroform extractions. Also, ensure that the mixture does not freeze during RNA precipitation. If any of these guidelines are not followed, you may not see a pellet. If all were followed, it is likely that RNases were somehow introduced into your sample at some point during the procedure. Ensure all ancillary reagents and tubes are RNase-free. Wear gloves at every step of the protocol (including during buffer preparation) to avoid RNase contamination from hands.
I saw a pellet after the RNA precipitation step, but I'm not seeing the fold enrichment I expected in my qRT-PCR. Why?	It is possible that RNases could have degraded the RNA after step E-16, before it was converted to cDNA. But, if you completed the RT reaction fairly quickly after resuspending the pellets in water, this is unlikely. Ensure that your qPCR primer sets have an efficiency of 80-110%. Run your qRT-PCR again. If you still do not get the results you expect, run qRT-PCR on the Input samples to determine if the mRNA was present in the lysate prior to performing IP. If it is, then either your RNA degraded or else the biology of your system may work differently than you thought. That is, the miRNA of interest may not bind to the mRNA transcripts you expected.



## Section H: Recommendations for Primer Design

The following are a list of recommendations to consider when designing qPCR primers. We recommend designing primers with the same annealing temperature to enable the use of different primer sets within the same experiment.

- Design and analyze your potential primer pairs using an in silico PCR program (i.e. Primer3 at <http://frodo.wi.mit.edu/> or the UCSC Genome Browser at <http://genome.ucsc.edu/cgi-bin/hgPcr>).
- Primers that dimerize should be avoided as they will be bound by SYBR Green, which will compromise accurate quantitation. You can test your primers for self-complementarity and secondary structure at [http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).
- Ideally, the amplicons should be 50-100 bp in length with a GC content of 30-80%.
- Avoid runs of identical nucleotides. If repeats are present, there must be fewer than 4 consecutive G residues. Make sure that the last 5 nucleotides at the 3' end contain no more than 2 G and/or C residues.
- Primers should be designed to anneal optimally between 58°C to 60°C with a recommended length of 18-22 bp.
- Use only primer sets that have an Primer Efficiency between 80-110%.

### Determination of Primer Efficiency

Your qPCR primer set will be used to produce a standard curve that will enable you to determine if the Primer Efficiency of your primers is sufficient. The slope of the standard curve will also be used when calculating the Fold Enrichment of the IP performed using the Ago1/2/3 Antibody

relative to the IP performed using the Negative Control IgG

1. Produce a standard curve by performing qPCR with your primer set on known genomic or cDNA quantities in triplicate. Run five samples that are 10-fold dilutions, e.g. 0.005 ng, 0.05 ng, 0.5 ng, 5 ng and 50 ng.
2. The efficiency of the primers can be calculated using the slope of the standard curve and the formula below. Many qPCR instrument programs will automatically calculate the average of each of the triplicate reactions as well, as the Primer Efficiency.

$$\text{Primer Efficiency} = [10^{(-1/\text{slope})} - 1] \times 100\%$$

For example, if the slope = -3.508

$$\text{Primer Efficiency} = [10^{(-1/\text{slope})} - 1] \times 100\% = [10^{(-1/-3.508)} - 1] \times 100\%$$

$$\text{Primer Efficiency} = [1.928 - 1] \times 100\% = 0.928 \times 100\%$$

$$\text{Primer Efficiency} = 92.8\%$$

The ideal efficiency is 100% ±10% (i.e. 90-110%, where -3.32 = 100% efficiency).

## Section I: Data Analysis

Below is a strategy that can be used to determine the Fold Enrichment of each Argonaute IP reaction as compared to its equivalent Negative Control IgG IP reaction. You will need to use the slope of the standard curve that was generated when you determined the Primer Efficiency for each qPCR primer set.

### Calculate Fold Enrichment

1. Average the triplicate CT values for each Argonaute and Negative Control IgG reaction. Some machines may average your triplicate reactions automatically.
2. Using the slope of the standard curve that was already determined in Section H and the formula below, calculate the Fold Enrichment of each Argonaute IP in relation to the corresponding Negative control IP.

$$\text{Fold Enrichment} = \text{AE}(\text{Neg IgG CT} - \text{Ago CT})$$

$$\text{where AE} = \text{Amplification Efficiency} = 10^{(-1/\text{slope})}$$

For example, if the slope = -3.508, the Neg IgG  $C_T$  = 32.04, and the Ago  $C_T$  = 29.19

$$\text{Amplification Efficiency} = 10^{(-1/\text{slope})} = 10^{(-1/-3.508)} = 1.928$$

$$\text{Fold Enrichment} = 1.928^{(32.04 - 29.19)} = 1.928^{(2.85)} = 6.5$$

## Section J. Use of Magnetic Beads and Included Bar Magnet

**Caution:** The included neodymium bar magnet is extremely powerful and is easily broken if handled incorrectly.

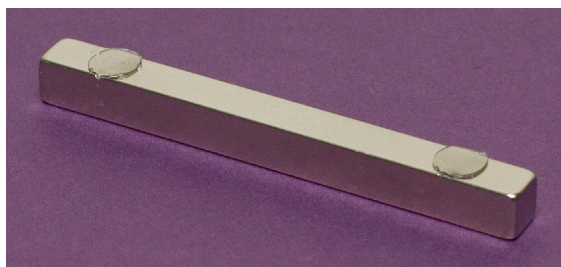
- The magnet should be stored in the provided tube.
- Be careful when working near metal objects or surfaces. A free magnet will jump great distances onto nearby metal surfaces with surprising speed. This can break the magnet.
- Use the provided Mini Glue Dots to attach the bar magnet to an empty pipette tip box to create an effective magnetic stand for use with either PCR strips or microcentrifuge tubes.
- If the magnet becomes attached to a flat metal surface, it should be removed by sliding it off the edge of the surface. The magnet may break if you attempt to pull one end or pry it away from the metal.

### Creating a magnetic stand for 8-well PCR strips:

**Note:** 8-well strip tubes for use with standard 96-well PCR cyclers are recommended (e.g. Thermo Fisher AB-0451).

1. Place a strip of PCR tubes in the wells of an empty tip box (200  $\mu$ L tips) and place the magnet directly against the tubes. This is the way the magnet will be positioned when the glue dots are used to affix it to the box.

2. Remove the covering tape from one side of two glue dots and attach the glue dots on the bar magnet (the uncovered face of the dot is placed on the magnet) as shown below.

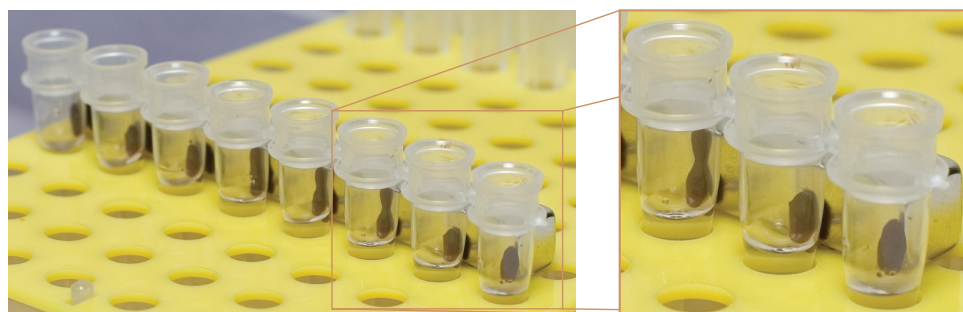


3. Remove the covering tape from the exposed side of the glue dots. Fix the magnet to the tip box so that it is against the PCR tubes. The magnetic stand is now ready for use.

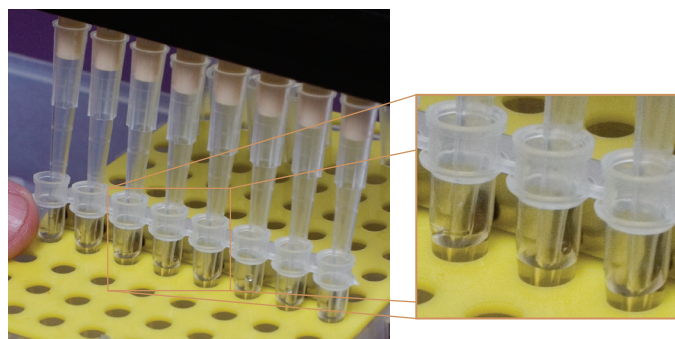
**Note:** Familiarize yourself with using the magnetic stand before performing with PCR tubes for the first time. Add 5  $\mu\text{L}$  of magnetic beads to 100  $\mu\text{L}$  Wash Buffer in one tube of an 8-well strip of PCR tubes. Use this tube with the assembled bar magnet stand to become familiar with use of the beads and magnet. It is difficult to re-suspend the beads if the tubes are directly adjacent to the magnet, so it is best to move the tubes away from the magnet for resuspension steps.

#### Washing should be performed as follows:

1. Place the tubes in the rack against the magnet and allow the beads to be pinned to the side of the tube, as shown below.



2. Remove supernatant with a 200  $\mu\text{L}$  pipette or a 200  $\mu\text{L}$  eight-channel pipette.



3. Move the tube strip into a row that is not adjacent to the magnet.
4. Add wash buffer and pipet up and down to fully re-suspend the beads. Ensure that a minimal amount of beads cling to the tips when the re-suspension is complete.
5. Repeat steps 1-4 until desired washing steps are complete.

### Centrifugation of 8-well PCR strip tubes:

When working with 8-well PCR strip tubes, it may be desirable to centrifuge the tubes to collect the liquid and beads from the insides of the caps. This is easily accomplished in a centrifuge fitted with adaptors for spinning microtiter plates. Place a standard 96-well plate in the adaptor to hold the tubes in place. Be sure to balance the rotor (i.e. place a microtiter plate and tubes of appropriate mass in the rotor's opposing 96-well plate adaptor). Spin the plates briefly to let the rotor reach a speed of 1000 x g before allowing the rotor to stop.

### Creating a magnetic stand for microcentrifuge tubes:

1. Remove the covering tape from one side of two glue dots.
2. Place two microcentrifuge tubes in the wells of an empty tip box (1000  $\mu$ L) and place the magnet directly against the tubes. This is the way the magnet will be positioned when the glue dots are used to affix it to the box.
3. Attach the glue dots on the bar magnet (the uncovered face of the dot is placed on the magnet) as shown above.
4. Remove the covering tape from the exposed side of the glue dot. Fix the magnet to the tip box so that it is against the tubes. The magnetic stand is now ready for use.

**Note:** Microcentrifuge tubes are held less securely in this assembled tube stand than in a typical commercial magnetic stand. This is not a problem if the below washing protocol is followed. Work with 1 tube at a time, and keep the tubes in the standard tube rack unless you are holding the tube next to the magnet.

### Washing is best performed one tube at a time, as follows:

1. Place the tube in a standard microcentrifuge tube rack and open the cap.
2. Place the opened tube in the assembled magnetic stand. The beads will pellet more rapidly if the bottom of the tube is held against the magnet, as shown below, and then slowly lowered into the well. This will pellet the beads up onto the side of the tube.



3. Allow the beads to pellet completely and remove supernatant with a 1000  $\mu\text{L}$  pipette. You can either leave the tube in the rack or pull it out when you remove the buffer. The beads will remain on the side of the tube, even when not next to the magnet.
4. Return the tube to the standard microcentrifuge tube rack, add 800  $\mu\text{L}$  wash buffer and fully resuspend the beads by pipetting up and down.
5. Repeat steps 2-4 until desired washing steps are complete. After the final wash has been removed, the last traces of wash buffer should be removed with a 200  $\mu\text{L}$  pipette.

## Technical Services

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If you need assistance at any time, please call or send an e-mail to Active Motif Technical Service at one of the locations listed below.

North America

Toll free: 877 222 9543  
Direct: 760 431 1263  
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