Magna RIP™
RNA-Binding Protein Immunoprecipitation Kit

Magna RIP Kit (Catalog No. 17-700)
EZ-Magna RIP Kit (Catalog No. 17-701)
Magna RIP Quad (Catalog No. 17-704)

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

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Introduction

Gene regulation plays a critical role in complex cellular processes such as development, differentiation, and cellular response to environmental changes. In addition to transcriptional regulation of gene expression by transcription factors, cells utilize post-transcriptional regulatory mechanisms. One such mechanism involves use of certain RNA-binding proteins (RBPs) to temporally and coordinately regulate the rate of mRNA translation of functionally related gene products. While the regulation of gene expression by transcription factors has been well studied over time, the post-transcriptional regulation of mRNAs by RBPs and the role of non-coding RNAs in this process is a relatively nascent field that remains to be thoroughly explored.

RNA-binding protein immunoprecipitation (RIP) is the RNA analog of the more well-known ChIP application (chromatin immunoprecipitation), which identifies DNA targets of DNA-binding proteins in an *in-vivo* cellular context. RIP can be used to identify specific RNA molecules (of many types) associated with specific nuclear or cytoplasmic binding proteins. These experiments involve immunoprecipitation of endogenously formed complexes of RNA-binding proteins and co-isolation of any RNA species associated with that RNA-binding protein. Purification of these RNA species allows interrogation and identification of mRNAs (and potentially non-coding RNAs associated with them) and can be directly measured using down stream applications including quantitative reverse transcription polymerase chain reaction (RT-PCR), microarray analysis (RIP-chip) and "deep-sequencing" or 2nd-generation sequencing based platforms (RIP-Seq).

The Millipore universal RIP immunoprecipitation kit allows researchers to perform RIP experiments using antibodies directed against RNA-binding proteins or epitope tags. Additionally, we offer RIP validated antibodies available separately in kit type format including controls useful for validation of RIP experiments, designated RIPAb+ kits (see www.millipore.com).

Magna RIP™ & EZ-Magna RIP™ Component Boxes		
Magna RIP (Cat. # 17-700)	**EZ-Magna RIP (Cat. # 17-701)**	**Magna RIP Quad (Cat. # 17-704)**
MAGNARIP01 (Store at 4°C)	MAGNARIP01 (Store at 4°C)	MAGNARIP01 (Qty. 4) (Store at 4°C)
MAGNARIP02 (Store at -20°C)	MAGNARIP02 (Store at -20°C)	MAGNARIP02 (Qty. 4) (Store at -20°C)
	MAGNARIP03 (Store at -20°C)	

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Kit Components

MAGNARIP01 (Component box of all RIP Kits) (Store at 4°C)		
Component	**Catalog #**	**Quantity**
Magnetic Beads Protein A/G	CS203178	0.66 mL
RIP Wash Buffer	CS203177	100 mL
RIP Lysis Buffer	CS203176	2.4 mL
0.5 M EDTA	CS203175	0.5 mL
10% SDS	CS203174	0.3 mL
Salt Solution I	CS203173	1.0 mL
Salt Solution II	CS203185	0.3 mL

MAGNARIP02 (Component box of all RIP kits) (Store at -20°C)		
Precipitate Enhancer	CS203208	0.1 mL
Normal Mouse IgG	CS200621	125 µg
Rabbit IgG Purified	PP64B	125 µg
Protease Inhibitor Cocktail 200X**	CS203220	20 µL
RNase Inhibitor	CS203219	75 µL
Proteinase K (10 mg/mL)	CS203218	0.36 mL
Nuclease free water	CS203217	0.3 mL

MAGNARIP03 (Component box of 17-701 only) (Store at -20°C)		
Positive Control Antibody (Anti-SNRNP70)**	CS203216	23 µL (0.5 µg/ µL) Sufficient for Two Immunoprecipitations
RIP Primers U1 snRNA** FOR: 5'-GGG AGA TAC CAT GAT CAC GAA GGT-3' REV: 5'-CCA CAA ATT ATG CAG TCG AGT TTC CC-3'	CS203215	75 µL (5 µM of each control primer specific for the human U1 snRNA)

Note: Kit component volumes include sufficient reagents for 12 immunoprecipitations with additional overfill volume.

**The Anti-SNRNP70 rabbit polyclonal antibody is expected by sequence similarity of the immunogen to cross react with SNRNP70 of human, mouse, rat and canine origins. The U1 snRNA RIP primers should also be able to amplify U1 snRNA converted cDNA in those species.

Storage

Upon receipt, store components at the temperatures indicated on the labels.
Kit components are stable for 6 months from date of shipment when stored as directed.

Materials Not Supplied

Reagents

- Cells, stimulated or treated as needed for the experimental system
- Antibody of interest for RNA-binding protein immunoprecipitation (RIP)
- PBS (RNase free) (e.g. Fisher, Cat. # BP2438-4)
- Phenol:chloroform:isoamyl alcohol (125:24:1 pH = 4.3)*** (e.g. Fisher, Cat. # BP1754I)
- Chloroform (e.g. Fisher, Cat. # BP1145)
- 100% Ethanol (molecular biology grade)

For Standard End-Point RT-PCR

- cDNA Synthesis kit or Reverse Transcriptase (e.g. ABI Cat. # PN 4387406)
- *Taq* DNA polymerase and Buffer for standard PCR
- dNTPs, 2.5 mM each

For qRT-PCR

- cDNA Synthesis kit or Reverse Transcriptase (e.g. ABI Cat. # PN 4387406)
- SYBR Green Master Mix for qPCR (e.g. ABI Cat. # PN 4367659)

Equipment

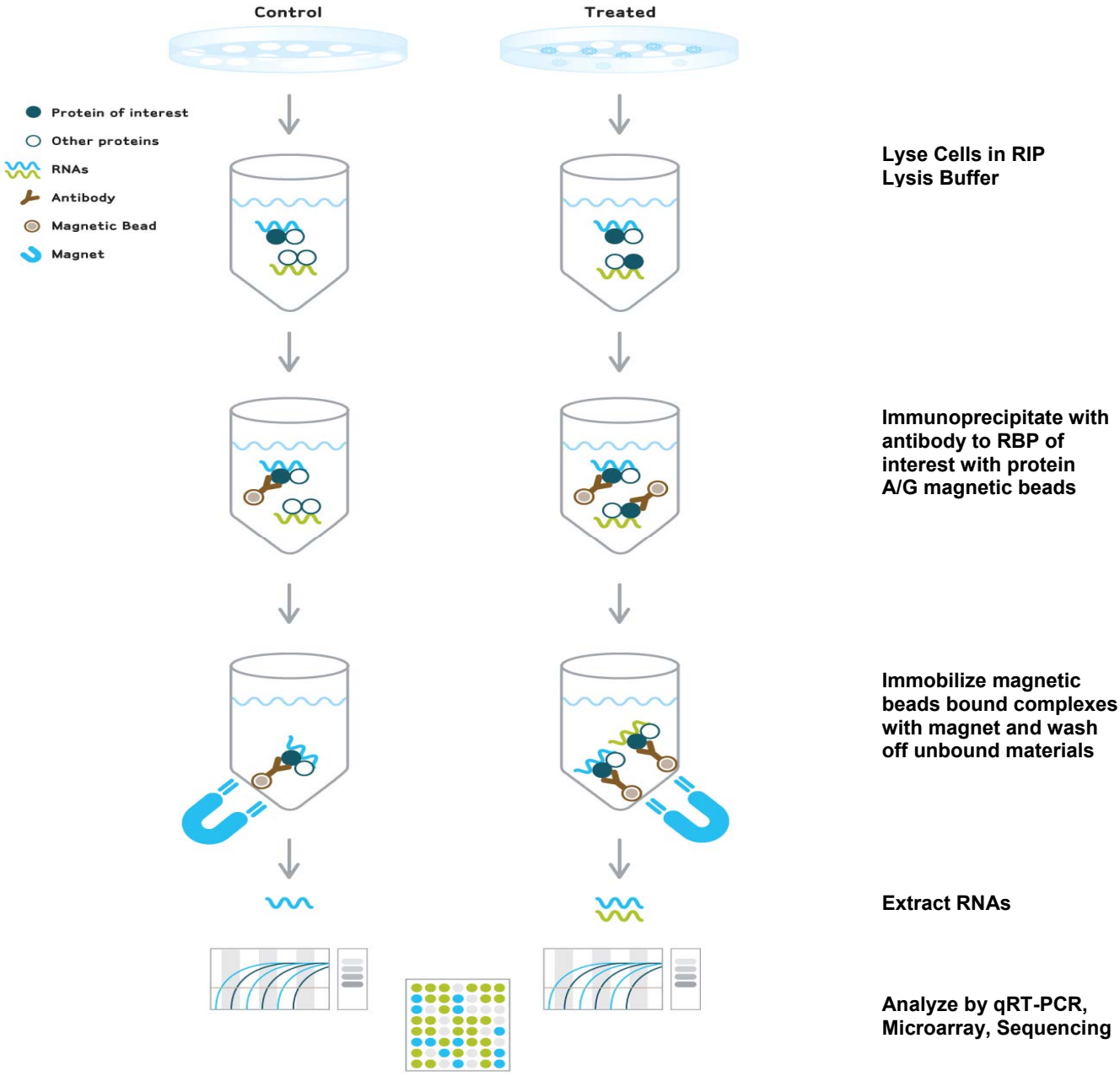
- Magnetic Separator (Millipore Cat. # 20-400)
- Vacuum Aspirator
- Vortex mixer
- Rotating wheel/platform
- Centrifuge for cell culture
- Microfuge
- Ultra low temperature freezer (below -80°C)
- Thermomixer (55°C capable)
- Variable temperature water bath or incubator
- Rotator
- Timer
- Pipette (2 mL, 5 mL, 10 mL, 25 mL)
- Variable volume (5-1000 µL) pipettes
- Nuclease-free filter pipette tips
- Cell scraper
- Centrifuge tube (15 mL and 50 mL)
- Nuclease-free Microfuge tubes, 1.5 mL
- Thermal cycler (end point or real time)
- PCR tubes, 0.2 mL

Hazardous Information

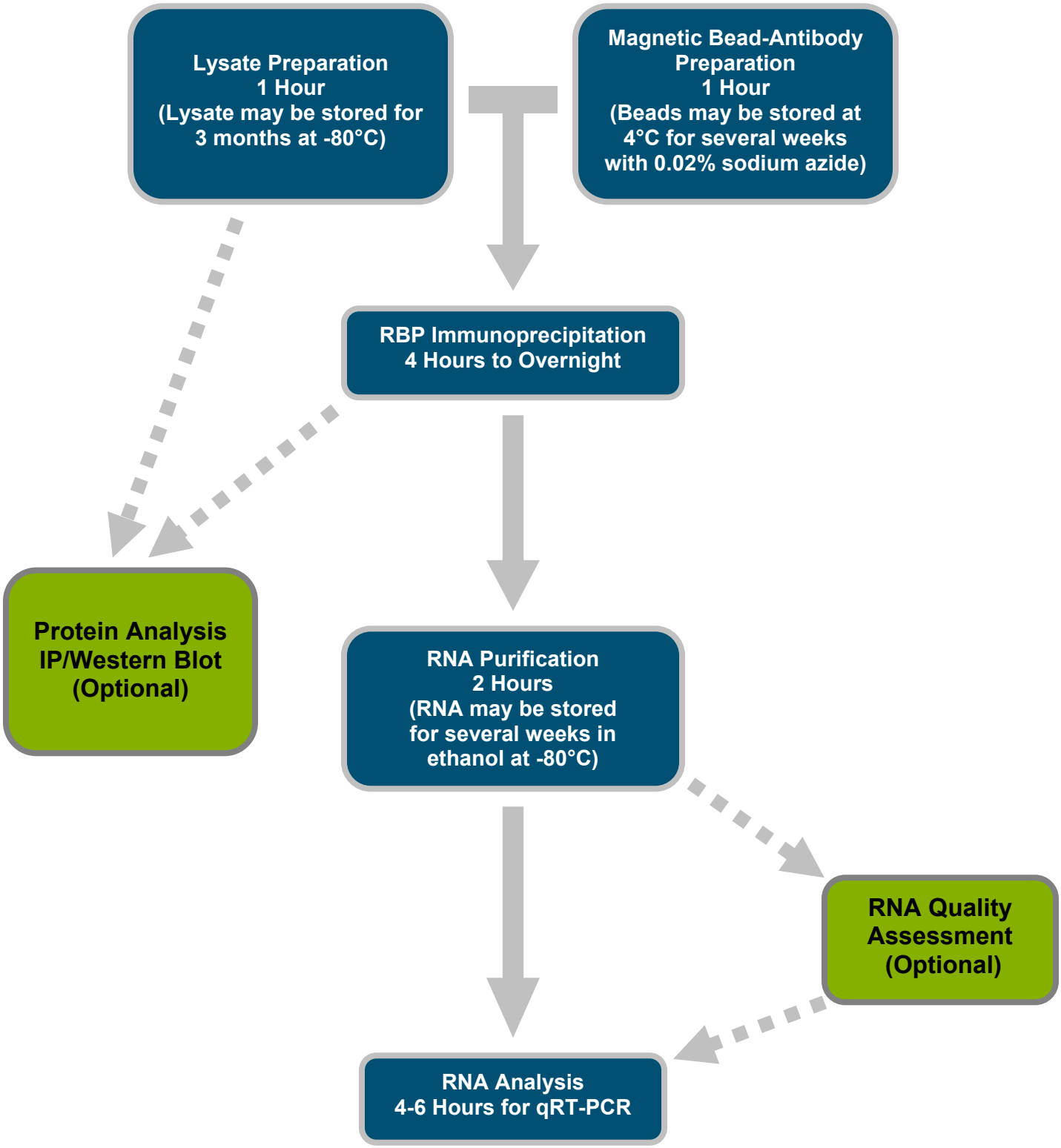
***This protocol utilizes organic extraction, in which phenol is used. Contact with phenol causes burns and can be fatal. Use gloves and other personal protective equipment when working with phenol.

**The Protease Inhibitor Cocktail 200X contains dimethyl sulfoxide (DMSO) which can penetrate skin and mucous membranes upon contact. Use gloves and other personal protective equipment when working with the protease inhibitor cocktail.

RIP Workflow



RIP Time Management



Detailed Protocol

Planning lysate requirements for RIP experiments

- Calculate the number of desired immunoprecipitations. Samples include the antibodies of interest (user supplied) and a negative control IgG of the same species as the antibody of interest. Anti-SNRNP70 (Cat. # CS203216) and negative control Normal Rabbit IgG (Cat. # PP64B) can be used as controls for the RIP procedure, and both components are included in the EZ-Magna RIP kit (Cat. # 17-701).
- It is important to get a concentrated cell lysate for the immunoprecipitation to be successful. Typically one RIP reaction (i.e. one immunoprecipitation using one antibody) requires 100 µL of cell lysate from ~2.0 x 10^7 cells or one 15 cm plate. The calculation for the volume of RIP Lysis Buffer required for a RIP experiment is based upon the volume of the cell pellet harvested. This volume may vary based on the type of cells utilized. An example of the size of HeLa cell culture and required RIP Lysis Buffer are shown below (Table 1). Bear in mind that once you've demonstrated successful RIP with a candidate antibody in a certain cellular context, the quantity of lysate per RIP may be reduced or further optimized as necessary.
- The total number of the cells or total amount of protein used per RIP must be optimized based upon the abundance of the RNA-binding protein being investigated as well as the planned method of RNA detection.

Table 1. Approximate volumes of RIP lysis buffer per cell culture vessel (HeLa cells)

Type of vessel	Surface Area (cm^2)	Cell Number	Volume of RIP Lysis Buffer (µL)
T-75	75	~1.0 x 10^7	50
T-225	225	~3.0 x 10^7	150
10 cm plate	78.5	~1.0 x 10^7	50
15 cm plate	176.6	~2.3 x 10^7	115

RNase control

Throughout this method, all standard precautions should be taken to minimize RNase contamination. Gloves should be worn at all steps of the procedure to minimize introduction of RNAses. All instruments, glassware and plastic-ware that touch cells or cell lysates should be certified Nuclease-free or should be pretreated using DEPC or other RNase inactivation reagents according to established protocols for working with RNA. RNAse inhibitor (Cat. # CS203219) is included as a component in this kit. All solutions utilized that are not kit components should be certified DNase-free and RNase-free from the manufacturer wherever possible.

I. Lysate preparation

- Stimulate or treat, if necessary, adherent mammalian cells at ~80-90% confluency in flasks or culture dishes

Complete RIP Lysis Buffer preparation

- Prepare the appropriate amount of complete RIP Lysis Buffer for the quantity of cells being harvested (see Table 2). For 100 µL of RIP Lysis Buffer, add 0.5 µL of protease inhibitor cocktail and 0.25 µL of RNase inhibitor and keep it on ice.

Table 2. Complete RIP Lysis Buffer

		x 1	x N
1	RIP Lysis Buffer	100 µL	100 µL x_____=_____
2	Protease Inhibitor Cocktail	0.5 µL	0.5 µL x_____=_____
3	RNase Inhibitor	0.25 µL	0.25 µL x_____=_____

For monolayer or adherent cells:

1. Wash the cells on the flasks or plates twice with 10 mL of ice-cold PBS.
2. Add 10 mL of ice cold PBS. Scrape cells off from each flask or plate and transfer to a centrifuge tube. Count cells using a hemacytometer if desired.
3. Collect cells by centrifugation at 1500 rpm for 5 minutes at 4°C and discard the supernatant.
4. Re-suspend the cell pellet in an equal pellet volume of complete RIP Lysis Buffer. Mix by pipetting up and down until the cells have been dispersed and the mixture appears homogeneous. Incubate the lysate on ice for 5 min. This step allows the hypotonic RIP buffer to swell the cells. Dispense ~200 µL each of the lysate into nuclease-free microcentrifuge tubes and store at -80°C.

While the volume of the amount dispensed is not critical, the volume of lysate per antibody is generally 100 µL per RIP and often a positive and negative antibody are utilized in each experiment, so 200 µL of cell lysate correlates to a single use aliquot. Aliquoting of lysate should be adjusted accordingly to avoid multiple freeze-thaws for large scale lysate preparations.

For suspension cells:

1. Harvest cells into a 15 mL conical tube. Count cells using a hemacytometer.
2. Collect cells by centrifugation at 1500 rpm for 5 minutes at 4°C and discard the supernatant.
3. Re-suspend the cells in 10 mL of ice cold PBS to wash. Collect cells by centrifugation at 1500 rpm for 5 minutes at 4°C and discard the supernatant.
4. Repeat the step 3 for one additional wash.
5. Re-suspend equal pellet volume of complete RIP Lysis Buffer. Mix by pipetting up and down until the mixture looks homogeneous. Incubate the lysate on ice for 5 min. Dispense ~200 µL each of the lysate into nuclease-free microcentrifuge tubes and store at -80°C.

While the volume of the amount dispensed is not critical, the volume of lysate per antibody is generally 100 µL per RIP and often a positive and negative antibody are utilized in each experiment, so 200 µL of cell lysate correlates to a single use aliquot. Aliquoting of lysate

should be adjusted accordingly to avoid multiple freeze-thaws for large scale lysate preparations.

For Tissue sample:

1. Wash freshly resected whole tissue three times with ice-cold PBS.
2. Tease apart the tissue in ice-cold PBS using a Dounce homogenizer, or some other cell separation device, until a single-cell suspension is obtained.
3. Collect cells by centrifugation at 1500 rpm for 5 minutes at 4°C and discard the supernatant.
4. Re-suspend equal pellet volume of complete RIP Lysis Buffer. Mix by pipetting up and down until the mixture looks homogeneous. Incubate the lysate on ice for 5 min. Dispense ~200 µL each of the lysate into nuclease-free microcentrifuge tubes and store at -80°C.

While the volume of the amount dispensed is not critical, the volume of lysate per antibody is generally 100 µL per RIP and often a positive and negative antibody are utilized in each experiment, so 200 µL of cell lysate correlates to a single use aliquot. Aliquoting of lysate should be adjusted accordingly to avoid multiple freeze-thaws for large scale lysate preparations.

Notes

- This method employs a single freeze-thaw to gently lyse the cells.
- Immediate initial freezing of the lysate is essential to complete the lysis process.
- Lysate may be stored up to 3 months at -80°C.
- Avoid additional freeze-thaw cycles to prevent protein and RNA degradation.
- Freshly isolated cells are essential for the success of the RIP reaction.

II. Preparation of magnetic beads for immunoprecipitation

The RIP procedure relies on the use of high quality antibodies to perform immunoprecipitation of RNA-binding protein/RNA complexes. The amount of antibody used for immunoprecipitation will depend on the presentation (e.g. purified or unpurified) and effective affinity of the candidate antibody when used for immunoprecipitation. For purified antibodies, 5 µg per immunoprecipitation is suggested as a guideline, but the quantity may need to be optimized when not using a Millipore RIPAb+ validated antibody, and quantity used should reflect that used in successful immunoprecipitation reactions performed by the end user.

When performing wash steps with magnetic beads, the use of a vacuum aspirator is recommended. To avoid introduction of RNases, use an aspirator pipette with the addition of RNase free sterile microtips where possible.

1. Completely disperse and re-suspend magnetic beads by end over end rotation or by pipetting.
2. Label the appropriate number of microfuge tubes for the number of desired immunoprecipitations.
   - Samples include antibodies of interest (user supplied) and negative control antibody of the same species as the antibody.

- Anti-SNRNP70 (Cat. # CS203216) and negative control Normal Rabbit IgG (Cat. # PP64B) serve as controls for the RIP assay when using EZ-Magna RIP (Cat. # 17-701).

3. Transfer 50 µL of magnetic beads suspension to each tube.
4. Add 0.5 mL of RIP Wash Buffer to each tube and vortex briefly.
5. Place the tubes on a magnetic separator (i.e. Millipore Cat. # 20-400) and discard the supernatant after bead aggregation.
6. Remove the tubes from the magnet. Add 0.5 mL of RIP Wash Buffer to each tube and vortex briefly.
7. Place the tubes on the magnetic separator and discard the supernatant.
8. Remove the tubes from the magnet and re-suspend the beads in 100 µL of the RIP Wash Buffer. Add ~5 µg of the antibody of interest to the tube.
9. Incubate with rotation for 30 minutes at room temperature.
10. Centrifuge the tubes briefly and place on the magnetic separator and remove the supernatant.
11. Remove the tubes from the magnet. Add 0.5 mL of RIP Wash Buffer to each tube and vortex briefly.
12. Place the tubes on the magnetic separator and discard the supernatant.
13. Repeat step 11 to 12 for one additional wash.
14. Remove the tubes from the magnet. Add 0.5 mL of RIP Wash Buffer to each tube and vortex briefly. Place the tubes on ice.

Supernatant can be removed gently and easily with no sample loss

III. Immunoprecipitation of RNA-binding Protein-RNA complexes (RIP)

1. Prepare the RIP Immunoprecipitation Buffer. Each immunoprecipitation requires 900 µL of RIP Immunoprecipitation Buffer. Add 35 µL of 0.5 M EDTA, and 5 µL RNase inhibitor to 860 µL of RIP Wash Buffer for each reaction. (Table 3.)

Table 3. RIP Immunoprecipitation Buffer

		x 1	x N
1	RIP Wash Buffer	860 µL	860 µL x_____=_____
2	0.5 M EDTA	35 µL	35 µL x_____=_____
4	RNase Inhibitor	5 µL	5 µL x_____=_____
	Total	900 µL	900 µL x_____=_____

2. Place the tubes from Step 14 of section **II** on the magnetic separator and discard the supernatant. Add 900 µL of RIP Immunoprecipitation Buffer to each tube.
3. Thaw the RIP lysate (From section **I**) quickly and centrifuge at 14,000 rpm for 10 minutes at 4°C. Remove 100 µL of the supernatant and add to each beads-antibody complex in RIP Immunoprecipitation Buffer. The final volume of the immunoprecipitation reaction will be 1.0 mL.
4. Remove 10 µL of the supernatant of RIP lysate and place it into a new tube and label "**input**". Store this input sample at -80°C until starting RNA purification (section **IV**). This represents '10% input' which will be used to generate a standard curve or for comparison in RT-PCR methods (real-time or end-point). This input RNA sample can also be used for the optional RNA quality assessment (section **V**).

- (Optional) Remove 10 µL of the supernatant of RIP lysate to test the expression of RNA-binding protein of interest by western blotting. Add 10 µL of 2 X SDS-PAGE loading buffer to the 10 µL of RIP lysate followed by heating at 95°C. The RIP lysate can be directly applied on SDS-PAGE.

5. Incubate all the tubes with rotating for 3 hours to overnight at 4°C.
6. Centrifuge the immunoprecipitation tubes briefly and place on the magnetic separator and discard the supernatant.
7. Remove the tubes from the magnet. Add 0.5 mL of RIP Wash Buffer to each tube and vortex briefly.
8. Place the tubes on the magnetic separator and discard supernatant.
9. Repeat step 7 to step 8 five times to wash the beads total six times with 500 µL of cold RIP Wash Buffer.
   - (Option) Remove 50 µL each out of 500 µL of the beads suspension during the last wash to test the efficiency of immunoprecipitation by western blotting. The proteins can be eluted off the beads by re-suspending the beads in 1X SDS-PAGE loading buffer followed by heating at 95°C. The beads can then be centrifuged down and the supernatant directly applied on SDS-PAGE.

IV. Purification of RNA

1. Prepare the proteinase K buffer. Each immunoprecipitate requires 150 µL of proteinase K buffer containing 117 µL of RIP Wash Buffer, 15 µL of 10% SDS, 18 µL of 10 mg/mL proteinase K. Order of addition is intended to reduce risk of denaturation of proteinase K by addition of concentrated SDS.

Table 4 Proteinase K Buffer

	x 1	x N
RIP Wash Buffer	117 µL	117 µL x_____=_____
10 % SDS	15 µL	15 µL x_____=_____
Proteinase K	18 µL	18 µL x_____=_____
Total	150 µL	150 µL x_____=_____

2. Re-suspend each immunoprecipitate from step 9 of section **III** in 150 µL of proteinase K buffer.
3. Thaw the input sample from the step 4 of section **III** and add 107 µL of RIP Wash Buffer, 15 µL of 10% SDS, and 18 µL of proteinase K to the tubes and bring up the volume to 150 µL.
4. Incubate all tubes at 55°C for 30 minutes with shaking to digest the protein.
5. After the incubation, centrifuge the tubes briefly and place the tubes on the magnetic separator. Transfer the supernatant into a new tube.
6. Add 250 µL of RIP Wash Buffer to each tube contacting the supernatant.

Note: Make sure caps are tightly secured prior to vortexing solution in steps 7 & 8 below.

7. Add 400 µL of phenol:chloroform:isoamyl alcohol to each tubes. Vortex for 15 seconds and centrifuge at 14000 rpm for 10 minutes at room temperature to separate the phases.
8. Remove 350 µL of the aqueous phase carefully and place it in a new tube. Add 400 µL of chloroform. Vortex for 15 seconds and centrifuge at 14000 rpm for 10 minutes at room temperature to separate the phases.
9. Remove 300 µL of the aqueous phase carefully and place it in a new tube.
10. To each tube add 50 µL of Salt Solution I, 15 µL of Salt Solution II, 5 µL of Precipitate Enhancer and then 850 µL of absolute ethanol. Mix and keep at -80°C for one hour to overnight to precipitate the RNA.
11. Centrifuge at 14,000 rpm for 30 minutes at 4°C and discard the supernatant carefully.

12. Wash the pellet once with 80% ethanol. Centrifuge at 14,000 rpm for 15 minutes at 4°C. Discard the supernatant carefully and air dry the pellets.
13. Re-suspend in 10 to 20 µL of RNase-free water, and place the tubes on ice.

V. Assessment of Input RNA Quality (Optional)

1. NanoDrop®
   - Typically, optical absorbance of the total RNA (or 'input') can be measured using a NanoDrop spectrophotometer. Ideally we expect both the A_{260}/A_{280} ratios to be close to 2.0, implying the purity of RNA and the absence of any contaminating proteins or chemicals. If this ratio is less than 1.8, there may be problems with further downstream applications. Because the quantities of RNA present in the immunoprecipitate are generally low, measurement of the samples by NanoDrop is not recommended, but may be used to assess quality of the RNA from the input sample if desired.

2. Bioanalyzer or Experion™
   - The molecular weight profile of the subset of RNAs immunoprecipitated can be analyzed on a nano chip using Agilent's Bioanalyzer or RNA HighSense chip using Bio-Rad's Experion. Bioanalyzer or Experion is a convenient and more sensitive alternative to using formaldehyde-agarose gels. For the total RNA (or 'input') it is helpful to evaluate the 18s/28s rRNA ratio. A ratio between 1.6 and 2.0 indicates RNA of good integrity. However for the immunoprecipitated material such a ratio can not be obtained unless rRNAs are known to be targets of the immunoprecipitated RNA-binding protein in question. And so, a Bioanalyzer profile of immunoprecipitated RNA only serves to provide information about any extensive degradation of RNA.

VI. Analysis of immunoprecipitated RNA

RNAs isolated using the Magna RIP kit can be analyzed by several molecular methods including end-point RT-PCR and quantitative RT-PCR (if binding targets of the RBP are known), or by microarray or deep sequencing methods. Given RNA targets of known sequence, gene specific primers can be designed that allow validation (and quantification) of the RNA immunoprecipitated by the antibodies used. Once successful RIP can be confirmed, further interrogation of the population of RNAs in an immunoprecipitation may be pursued by population based methods such as comparative microarray hybridization of resulting cDNAs or by deep sequencing of molecularly adapted products of the RIP reaction (see Baroni, T.E. *et al*. (2008). *Methods Mol Biol.* 419:93-108).

Presented below are illustrative methods for performing end-point or real time quantitative measurement of RIP experiments using the control antibody supplied in the EZ-Magna RIP kit (Cat. # 17-701, Anti-SNRNP70 Cat. # CS203216).

For reverse transcription, the user for EZ-Magna RIP kit can use any commercially available reverse transcription enzymes and kit systems that use random hexamers for priming. Since the mature U1 snRNA co-precipitated with U1 spliceosomal SNRNP70 protein is not polyadenylated, oligo d(T) priming is not recommended.

Example of 1st strand cDNA synthesis (e.g. High Capacity RNA-to-cDNA Kit, ABI Cat. # 4387406)

Note: RNase-free aerosol resistant tips are recommended for use in this section to minimize risk of contamination.

1. Label the appropriate number of 0.2 mL PCR tubes for the number of samples to be analyzed and place on ice.
2. Add 9 µL (up to 2 µg of RNA) of the appropriate sample to the PCR tube and return on ice.
3. Add the appropriate amount of reagents to each PCR reaction tube on ice as indicated in Table 5.

Table 5. Reverse Transcription reagent volumes

Reagent	Volume for 1 reaction (µL)
RNA	9.0
2X RT Buffer	10.0
20X Enzyme Mix	1.0
Total per reaction	**20.0**

4. Place the PCR reaction tubes in a thermal cycler.
5. Start the following RT reaction program:

RT Reaction	37°C	60 min
Stop the reaction	95°C	5 min
Hold	4°C	Hold

6. Remove the PCR tubes. Dilute the reaction with 180 µL of Nuclease-free water (10X dilution). Reactions can be stored at -20°C.

Standard end-point RT-PCR

1. Label the appropriate number of 0.2 mL PCR tubes for the number of samples to be analyzed and place on ice.
   - At a minimum, there will be 4 samples to undergo PCR using the RIP Primers included in this kit: cDNA from positive and negative control antibody immunoprecipitations, Input and a no template tube as a control for DNA contamination.
   - The RIP Primers are specific for the human U1 snRNA gene. It is recommended that the user design appropriate specific primers for cDNA from other species and determine the PCR reaction conditions empirically.
2. Add 2 µL of the appropriate sample to the PCR tube and return to ice.
3. Add the appropriate amount of reagents to each PCR reaction tube on ice, adding the H_2O first and the Taq polymerase last, as indicated in Table 6.
   - It is recommended that the user employ a Hot-Start Taq polymerase. If user is not employing a Hot-Start Taq polymerase, Taq must be added to each tube after the initial denaturation step.
   - If a master reaction mix is desired, dispense enough reagents for one extra tube to account for loss of volume.

Table 6. PCR reagent volumes

Reagent	Volume for 1 reaction (µL)
DNA	2.0
H_2O	12.6
10X PCR Buffer(-$MgCl_2$)	2.0
$MgCl_2$ (50mM)	0.6
2.5 mM dNTP	1.6
RIP Primers U1 snRNA	0.8
Taq (5 U/µL)	0.4

4. Place the PCR reaction tubes in a thermal cycler.
5. Start the following PCR reaction program:

Initial Denaturation	94°C	3 min	
Denature	94°C	20 sec	} repeat for a total of 20 times
Anneal	60°C	30 sec	
Extension	72°C	30 sec	
Final Extension	72°C	2 min	

6. Remove the PCR tubes. Reactions can be stored at -20°C.
7. Remove 10 µL of each PCR reaction for analysis by 2% agarose gel electrophoresis with a 100 bp DNA marker. For U1 snRNA included in Cat. # 17-701, the expected size of the PCR product is 100 base pairs. See Figure A. (pg. 15) for an example.

Real-time Quantitative PCR

1. Add 2 µL of the cDNA sample to the PCR plate suitable for your real time instrument of choice (Performing a triplicate of qPCR reactions per RIP sample is recommended).
2. Prepare a master reaction mix as shown in Table 7. Dispense enough reagents for one extra tube to account for loss of volume.
3. Add 23 µL of qPCR mix to the 2 µL of the sample.
4. Use caps or an optical tape to seal the plate and start the qPCR reactions.

Table 7. qPCR reagent setup and running parameters

qPCR reagent assembly for 1 reaction:

ddH_2O	9.5 µL
Sybr-Green Master Mix	12.5 µL
Primer mix	1 µL
Total	23 µL

qPCR parameters:

Initial Denaturation	95°C 10 min	
Denature	95°C 15 sec	} 40 times
Anneal and Extension:	60°C 1 min	

Figure A: PCR Analysis of RIP

RIP was performed using HeLa cell lysate and either anti-SNRNP70 (Cat. # CS203216) or Normal Rabbit IgG (Cat. # PP64B) as the immunoprecipitating antibody. Purified RNA was then analyzed by RT-PCR using RIP Primers specific for the U1 snRNA (Cat. # CS203215). PCR product was observed in the anti-SNRNP70 RIP (lane 3) and substantially less was detected in the Normal rabbit IgG RIP (lane 2). U1 snRNA specific cDNA was also observed in the 10% Input (lane 4) and not in the "No template" PCR control (lane 1).

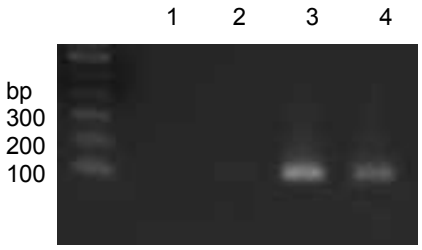
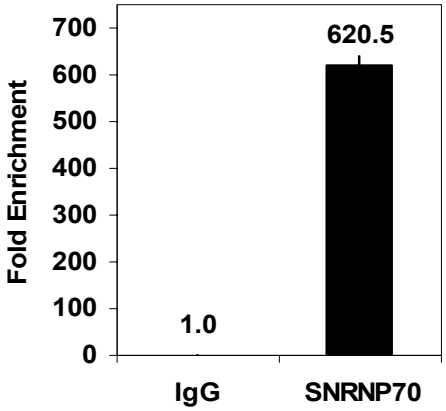


Figure B: PCR Analysis of RIP

RIP was performed using HeLa cell lysate and either anti-SNRNP70 (Cat. # CS203216) or Normal Rabbit IgG (Cat. # PP64B) as the immunoprecipitating antibody. Purified RNA was then analyzed by qRT-PCR using RIP Primers specific for the U1 snRNA (Cat. # CS203215).



RIP OPTIMIZATION AND TROUBLESHOOTING

Step	Potential Problems	Experimental Suggestions
Immuno-precipitation	Antibody doesn't immunoprecipitate protein in the RIP lysate	• Confirm the antibody can immunoprecipitate the RBP of interest by IP Western prior to RIP analysis • Choose an antibody directed to a different epitope of the antigen. • Use Millipore RIPAb+ validated antibodies where possible • Perform IP from a dilution series of antibody with a fixed amount of RIP lysate or vice versa. • Increase incubation time of the antibody of interest with the RIP lysate to overnight at 4°C. • Confirm antibody isotype is compatible with immunoprecipitation by Protein A or G. This kit is not recommended for use of IgM or chicken IgY antibodies.
	Insufficient quantity of magnetic beads in immunoprecipitation	• The magnetic beads settle to the bottom of the tube over time. Make sure the magnetic beads are well mixed prior to removing the appropriate volume for IP. • Carefully aspirate beads when using vacuum aspirator and use a high strength neodymium magnetic rack such as the Millipore Cat. # 20-400 MagnaGrIP Rack.
RNA Purification	Incomplete Proteinase K digestion	When performing proteinase K digestion, make sure that the temperature is set at around 55°C. Proteinase K will be inactivated by prolonged incubation at temperatures above 65°C.
	Poor A_{260}/A_{280} ratios	• Avoid the interphase when extracting RNA using phenol:chloroform and choloroform extractions. • Assess presence of RNAses by evaluating RNA extracted from lysate inputs on a Bioanalyzer or Experion instrument.
	Low RNA yield	• Most RNA-binding protein immunoprecipitations do not yield measureable amounts of RNA. Sub nanogram quantities of RNAs can however be detected by RT-PCR. • If RNAs are not detectable following cDNA synthesis, consider Immunoprecipitation troubleshooting above.
	RNA degraded	• Use RNAse inhibitor in solutions as recommended in this protocol. Make certain RNAse free work conditions exist and RNAses are not being introduced. • Follow the guideline for the RNase control at the beginning of the protocol • Use RNAse inactivating reagents to ensure work area and materials are RNAse free

RNA Purification	No RNA detected	• Increase incubation time for the ethanol precipitation at -80°C. • The RNA ethanol precipitates are sometimes very small. Be sure not to suck up RNA precipitate when removing the supernatant. • Confirm the antibody can immunoprecipitate the RBP of interest by IP Western prior to RIP analysis.

Step	Potential Problems	Experimental Suggestions
RT-PCR	No PCR product	• Increase in varying amounts the cDNA added to the PCR reaction. • Increase the number of cycles for the amplification reaction. • Ensure amplification reaction program is correctly set on thermal cycler. • Re-examine primers for correct T_m. • Perform PCR on cDNA from total RNA (or Input RNA) to confirm amplification conditions and ability of primers to generate a single DNA product of the expected size. • Confirm the antibody can immunoprecipitate the RBP of interest by IP Western prior to RIP analysis
	High background level	Insufficient wash after immunoprecipitation. Increase the times to wash the beads. More stringent washing may be achieved by adding optimally determined concentration of sodium chloride, SDS, deoxycholate or chaotropic agents like urea (1~3M). However further caution should be taken to make sure that the antibody or target RBP are not affected by harsh washing conditions.
	PCR product is a smear	• Decrease in varying amounts the cDNA added to the PCR reaction. • Use HotStart *Taq* polymerase to avoid non-specific annealing of primers.
	No difference in quantity between PCR product from SNRNP70 and Normal Rabbit IgG IPs	• Ensure correct mass of antibody and the correct RIP lysate are used for IP as indicated in protocol. • Decrease amount of cDNA added to the PCR reaction. • Decrease the cycle number at which the cDNA is analyzed. It is important that the PCR products are analyzed within the linear amplification phase of PCR, in which differences between quantities of starting DNA can be measured. • Confirm the antibody successfully immunoprecipitate the SNRP70 by IP Western.

References

1. Baroni, T.E., Chittur, S.V., George, A.D., Tenenbaum, S.A. (2008). *Methods Mol Biol.* 419:93-108.

2. Keene, J.D., Komisarow, J.M., Friedersdorf, M.B. (2006). *Nat Protoc.* 1(1):302-7.

3. Baroni, T.E., Lastro, M.T., Ranganathan, A.C., Tenenbaum, S.A., Conklin, D.S., Aguirre-Ghiso, J.A. (2007). *Methods Mol Biol.* 383:227-44.

4. Penalva, L.O., Tenenbaum, S.A., Keene, J.D. (2004). *Methods Mol Biol*. 257:125-34.

5. Tenenbaum, S.A., Lager, P.J., Carson, C.C., Keene, J.D. (2002). *Methods.* 26(2):191-8.

6. Tenenbaum, S.A., Carson, C.C., Lager, P.J., Keene, J.D. (2000). *Proc Natl Acad Sci U S A.* 19;97(26):14085-90.

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