User Guide

Catalog Number **RIP**

Imprint® RNA Immunoprecipitation (RIP) Kit

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RIP Kit Components and Storage

The RIP kits are shipped at two different temperatures. The wet ice shipment contains Part 1. The dry ice shipment contains Part 2. Storage temperatures are indicated below.

Catalog No.	Imprint RNA Immunoprecipitation Kit Part 1 – Catalog Number RIPPART1	2-8 °C
B0314	Mild Lysis Buffer	1 x 3 mL
B0439	Harsh Lysis Buffer	1 x 3 mL
B0564	RIP Wash Buffer	2 x 75 mL
B0689	Protein A Magnetic Beads*	1 x 300 µL
I5381	IgG from mouse serum	1 x 1 mg
I5006	IgG from rabbit serum	1 x 1 mg

Catalog No.	Imprint RNA Immunoprecipitation Kit Part 2 - Catalog Number RIPPART2	−20 °C
R1158	Ribonuclease Inhibitor	1 x 2.5 KU
P8340	Protease Inhibitor Cocktail	2 x 125 µL
M7023	Anti-Mouse IgG (whole molecule) antibody produced in rabbit	1 x 60 µL

Product Description

RNA immunoprecipitation (RIP) is a very powerful procedure for the study of RNA binding proteins (RBPs) and their RNA targets in ribonucleoprotein (RNP) complexes. Antibodies raised against specific RBPs are used to co-precipitate RNPs, i.e., the RBP along with its RNA partner. The RNA can then be identified by next generation sequencing, or if testing for a specific RNA, by RT-PCR.

The Sigma RIP kit provides all reagents needed for successful RIP except the user's specific antibody. The kit includes two lysis buffers, one mild for less tightly bound RNPs, the other harsh for strongly associated RNPs. Protein A magnetic beads are provided to collect immune precipitates and facilitate washing. The kit also includes both rabbit and mouse IgG for negative control RIPs, a rabbit anti-mouse bridging antibody to bind mouse monoclonal primary antibodies efficiently to protein A on the magnetic beads, as well as both protease and ribonuclease inhibitors to protect the RNPs from degradation during RIP.

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

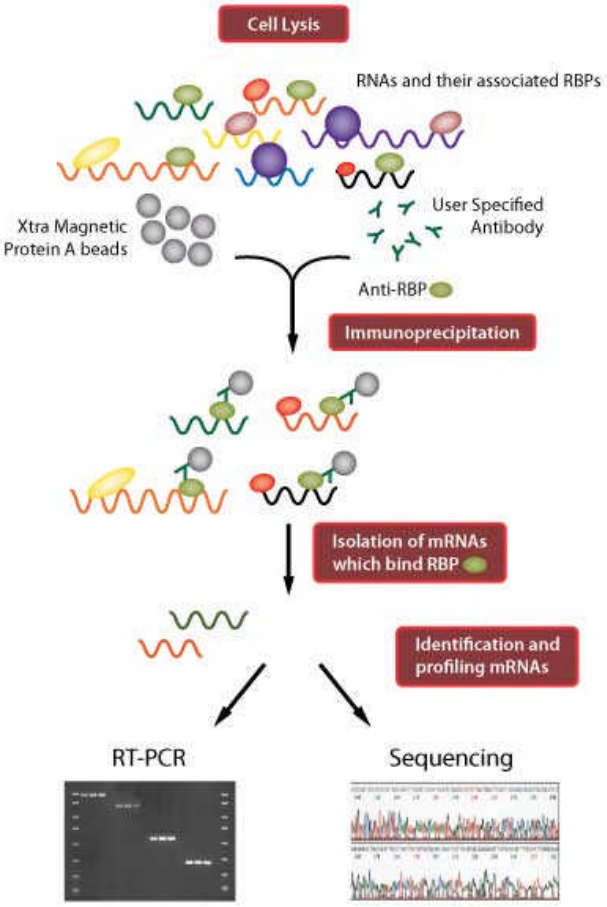
Consult individual component labels for correct storage temperatures.

Reagents and Equipment Required or Optional, but Not Provided

Other Materials (not provided) Reagent Description	Catalog Number
Ammonium Acetate 5 M	09691
10X PBS	P7059
TRI Reagent®	T9424
Linear Acrylamide (5 mg/ml), (available from Ambion®)	AM9520
1-Bromo-3-Chloropropane (or Chloroform Catalog No. C2432)	B9673
2-Propanol	190764
Specific Antibody	User
Positive Control Antibody Anti-SNRP70 produced in rabbit	User
Cells	User
Trypsin-EDTA Solution	T4049
DNase I	AMPD1
DL-Dithiothreitol (DTT) (optional)	D9779
Reverse Transcription Components (not provided)	
MMLV RT	M1302
Random Nonamers	R7647
Oligo dT Anchored	O4387
dNTPs	D7295
QPCR Components (not provided)	
SYBR® Green JumpStart™ Taq ReadyMix™ for Quantitative PCR	S4438
Specific Primers for Test RNA	User
Forward Specific Primer for U1 Positive Control RNA Target 5' TCCCAGGGCGAGGCTTATCCATT 3'	
Reverse Specific Primer for U1 Positive Control RNA Target 5' GAACGCAGTCCCCCACTACCACAAAT 3'	
JumpStart™Taq ReadyMix™ for Quantitative PCR	D7440
Hydrolysis probe (TaqMan® Probe)	
Equipment (not provided)	
Magnetic separator	
Vacuum aspirator	
Vortex mixer	
Rotating wheel/platform	
Centrifuge for cell culture	
Microfuge	
Ultra low temperature freezer (-80 °C)	
Timer	
Pipettes	
Nuclease-free filter pipette tips	
Cell Scraper	
Corning 15 ml and 50 ml tubes	
Nuclease free microfuge tubes 1.5 ml and 0.5 ml	
Real time thermal cycler	
PCR tubes 0.2 ml	

Procedure

Overview



Preparation for RIP Experiments

Any prior knowledge about the RBP and its RNA substrate is useful when using the Sigma RIP kit. For example, is the RBP of interest abundant? In which cellular compartment is it localized? What is the affinity of the RBP for its target[1]?

The total number of cells used per RIP must be optimized based on the abundance of the RNA-binding protein and RNA target being investigated. The volume of lysis buffer required for a RIP experiment is based upon the number of cells harvested. Cell number may vary based on prior knowledge of the RIP experiment to be performed. Typically one RIP reaction (i.e. one immunoprecipitation using one antibody) requires 100-200 µL of cell lysate from between 0.5 million and 10 million cells. Up to 20 million cells have, however, been used in successful RIP experiments.

Two different lysis buffers are supplied in the kit. One is a mild lysis buffer and the other is a harsh lysis buffer. The mild lysis buffer relies on a nonionic detergent. Use of this lysis buffer leaves the nuclei intact. The harsh lysis buffer includes two ionic detergents along with a nonionic detergent. This lysis buffer disrupts the nuclear membrane and also disrupts weak non-covalent protein-protein interactions. As a general rule of thumb, use the mild lysis buffer unless nonspecific background binding is too high or there is a known strong binding interaction between the RNA binding protein and its target RNA. Both buffers have been used in successful RIP experiments for both nuclear and cytoplasmic targets.

Reagents to cross-link are not provided with this kit. With many RIP targets, cross-linking is not necessary. Cross-linking may reduce the efficiency of cellular lysis, increase background signal, and is not entirely reversible[1].

Before using the lyophilized mouse or rabbit IgG negative control antibodies, vortex and gently tap the vial to make sure all of the lyophilization powder is at the bottom of the vial. Add 1.1 mL of 1X PBS buffer to each vial for a final concentration of 1 mg/mL. After addition of 1X PBS buffer, vortex gently to ensure proper mixing of reconstituted lyophilized antibody.

Throughout the entire RIP procedure, it is recommended to keep all samples on ice to minimize RNase activity. All of the RIP procedure steps are designed to be performed in order. Preparing the cellular lysate and antibody prebinding to the magnetic beads may, however, be performed in advance of the immunoprecipitation reaction and RNA precipitation.

Monolayer or Adherent Cells
Release cells by scraping

- Wash cells on flask or plate twice with ice-cold PBS that thoroughly covers the cells.
- Scrape off cells from each flask or plate in an appropriate amount of ice-cold PBS.
- Count cells.
- Collect cells by centrifugation at 200 x *g*, 5 minutes at 4 °C.

If releasing cells by trypsin digestion

- Rinse adherent cells once with enough ice-cold PBS to thoroughly cover the cells.
- Release cells in trypsin.
- Quench trypsin with 5-10X volume of complete cell culture media.
- Count cells.
- Pellet the cells by centrifugation at 200 x *g*, 5 minutes at 4 °C.
- Discard the supernatant and bring up the cells in a volume of ice-cold PBS equal to the cell culture media used after trypsin digestion.
- Repeat centrifugation, discarding the supernatant and resuspend pellet in ice-cold PBS once more.

Suspension Cells

- Harvest cells into a conical tube.
- Count cells.
- Collect cells by centrifugation at 200 x *g*, 5 minutes at 4 °C and discard the supernatant.
- Resuspend the cells in ice-cold PBS to wash.
- Repeat centrifugation, discarding the supernatant and resuspend pellet in ice-cold PBS once more.

Cell Lysis
Complete lysis buffer contains either mild or harsh lysis buffer with protease inhibitor cocktail, ribonuclease inhibitor and in some cases DTT or mercaptoethanol. Prepare the appropriate amount of complete RIP lysis buffer for the quantity of cells being harvested. If using an amount of cells at the lower end of the recommended lysis range (between 0.5 – 2 X 10^6), use 100 µL of complete lysis buffer per RIP reaction. If using an amount of cells at the upper end of the recommended lysis range (2 – 10 X 10^6), use 200 µL of complete lysis buffer per RIP reaction.

- Collect cells by centrifugation at 200 x *g* for 5 minutes at 4 °C and remove the supernatant.
- Add Protease inhibitor cocktail and Ribonuclease inhibitor to either mild or harsh lysis buffer to make complete lysis buffer. For cells with higher levels of RNases (such as monocytes), DTT (not supplied) may also be added to the lysis buffer at 0.5 mM to reduce disulfide bonds in RNases that are needed for stability.[2] Mercaptoethanol may be used in place of DTT. Additional Ribonuclease inhibitor may also be added if working with cell lines known to harbor higher levels of RNases (extra Ribonuclease inhibitor not supplied with the kit).

Complete RIP Lysis Buffer Preparation

Reagent	**Volume**	**Number of Reactions, N**	**Required volume,** (N x Volume)	**Volume**	**Number of Reactions, N**	**Required volume** (N x Volume)
Either mild or harsh lysis buffer	100 µL			200 µL		
Protease Inhibitor Cocktail	1 µL			2 µL		
1 M DTT (Optional; not supplied with kit)	0.05 µL			0.1 µL		
Ribonuclease Inhibitor 40 U/µL	0.4 µL			0.8 µL		

- After the addition of complete lysis buffer, incubate on ice for 15 minutes.
- After the 15 minute on ice incubation (cells in complete lysis buffer may be stored in −80 °C freezer overnight), centrifuge RIP lysis reactions in a microcentrifuge for 10 minutes at 16,000 x *g* at 4 °C.
- If frozen, remove cells in complete lysis buffer from −80 °C freezer and thaw on ice before centrifugation step.
- Collect supernatant and measure the volume.

Preparation of Magnetic Beads for Immunoprecipitation
RIP relies on the use of high quality antibodies to perform immunoprecipitation of RNA-binding protein - RNA complexes. The amount of antibody used will depend on whether it is purified or unpurified and the affinity of the antibody for immunoprecipitation. For purified antibodies, between 1-5 µg per immunoprecipitation is recommended as a guideline. If using an affinity isolated anti-SNRP70 polyclonal antibody produced in rabbit as a positive control, add 1 µg of this antibody directly to the immunoprecipitation reaction. Prebinding to the protein A magnetic beads is not necessary with a positive control anti-SNRP70 antibody. The amount of negative control antibody added to the separate negative control RIP reaction(s) should be equal to the amount of specific antibody added to the test RIP reactions.

Antibody prebinding to magnetic beads, with wash steps, removes antibody not bound to the beads, RNases and other contaminants that may be present in the antibody solution. This procedure is recommended for unpurified antibodies.

Warning: Protein A magnetic beads supplied in the kit settle extremely quickly. Resuspension of magnetic beads is critical to aliquoting the correct amount of beads per RIP reaction. After resuspension, it is recommended to immediately pipette out the required amount of magnetic beads from the middle of the magnetic bead suspension.

Polyclonal Antibody Prebinding
Washing Magnetic Beads

- Label an appropriate number of microfuge tubes for the number of immunoprecipitations. Samples will include antibodies of interest and negative control antibody (IgG) from the same species as antibodies of interest.
- Transfer 0.02 mL of magnetic beads suspension to each 1.5 mL microcentrifuge tube (0.5 mL microcentrifuge tubes are preferable if using a compatible magnetic separator), add 0.1 mL of RIP wash buffer to each tube and vortex briefly.
- Place tubes on a 1.5 mL microcentrifuge tube magnetic separator and discard the supernatant after beads are collected. Repeat wash step with 0.1 mL of RIP wash buffer.

Antibody Prebinding

- Centrifuge tubes briefly, resuspend beads in 0.1 mL of RIP wash buffer using a 0.5 mL microcentrifuge tube. Add 1-5 ug of antibody.
- Incubate with rotation for 30 minutes at room temperature.
- Centrifuge tubes briefly, resuspend magnetic bead-antibody complex and transfer entire volume to a 1.5 mL microcentrifuge tube.
- Place on a magnetic separator and pipette off the supernatant.
- Remove tubes from the magnet, add 0.5 mL of RIP wash buffer to each tube and vortex briefly.
- Place tubes on magnetic separator and pipette off the supernatant.
- Repeat the wash step.
- Remove tubes from the magnet, add 0.2 mL of RIP wash buffer to each tube and vortex briefly.
- Place tubes on ice.

Monoclonal Antibody Prebinding

Protein A does not bind mouse or rat antibodies efficiently. Therefore, if you are using a mouse or rat monoclonal antibody, a rabbit anti-mouse or rat bridging antibody must be prebound to the protein A magnetic beads first, followed by the monoclonal antibody of interest.

Washing Magnetic Beads

- Label an appropriate number of microfuge tubes for the number of immunoprecipitations. Samples will include antibodies of interest and negative control antibody of the same species as antibodies of interest.
- Transfer 0.02 mL of magnetic beads suspension to each 1.5 mL microcentrifuge tube (0.5 mL microcentrifuge tubes are preferable if using a compatible magnetic separator), add 0.1 mL of RIP wash buffer to each tube and vortex briefly.
- Place tubes on a 1.5 mL microcentrifuge tube magnetic separator and discard the supernatant after beads are collected. Repeat wash step with 0.1 mL of RIP wash buffer.

Bridging Antibody Prebinding

- Centrifuge tubes briefly, resuspend beads in 0.1 mL of RIP wash buffer using a 0.5 mL microcentrifuge tube. Add 1µL of bridging antibody (M7023).
- Incubate with rotation for 30 minutes at room temperature.
- Centrifuge tubes briefly, resuspend magnetic bead-bridging antibody complex and transfer entire volume to a 1.5 mL microcentrifuge tube.
- Place on a magnetic separator and pipette off the supernatant.
- Remove tubes from the magnet, add 0.5 mL of RIP wash buffer to each tube and vortex briefly.
- Place tubes on magnetic separator and pipette off the supernatant.
- Repeat the wash step.

Specific Antibody Prebinding

- Centrifuge tubes briefly, resuspend beads in 0.1 mL of RIP wash buffer and transfer to a 0.5 mL microcentrifuge tube. Add 1-5 ug of specific antibody.
- Incubate with rotation for 30 minutes at room temperature.
- Centrifuge tubes briefly, resuspend magnetic bead-specific antibody complex and transfer entire volume to a 1.5 mL microcentrifuge tube.
- Place on a magnetic separator and pipette off the supernatant.
- Remove tubes from the magnet, add 0.5 mL of RIP wash buffer to each tube and vortex briefly.
- Place tubes on magnetic separator and pipette off the supernatant.
- Repeat the wash step.
- Remove tubes from the magnet, add 0.2 mL of RIP wash buffer to each tube and vortex briefly.
- Place tubes on ice.

Direct IP (or IP without Prebinding)

It is not necessary to prebind magnetic beads with antibody if the antibody is purified (i.e., perform direct IP with the positive control anti-SNRP70 antibody). Antibody may be added directly to the IP reactions that are incubated for 3 hours to overnight at 4 °C. IP reactions are then pulled down with magnetic beads during a one hour incubation step at 4 °C. If adding antibody directly to IP reactions, wash 0.02 mL of magnetic beads per reaction with 0.1 mL of RIP wash buffer twice.

Immunoprecipitation of RNA-Binding Protein-RNA Complexes (RIP)

For each RIP reaction using mild lysis buffer, add IP buffer for a final volume of 0.5 mL. For each RIP reaction using harsh lysis buffer, add IP buffer for a final volume of 1 mL. Components in the IP buffer include RIP wash buffer, protease inhibitor cocktail and RNase inhibitor. Use the following table for either mild or harsh lysis buffer reactions.

IP Buffer Preparation

Reagent	**Volume**	**Number of Reactions, N**	**Required volume,** (N x Volume)
Wash buffer	1000 µL		
Protease Inhibitor Cocktail	10 µL		
RNase Inhibitor 40 U/µL	4 µL		

- Add IP buffer according to which lysis buffer was used and how many cells were lysed. IP reactions will have a final volume of 0.5 mL for mild lysis buffer reactions and 1 mL for harsh lysis buffer reactions.
- Before adding the IP buffer, remove 10% of the RIP lysate supernatant per RIP reaction and label ”10% Input”.
- Store this input sample at −80 °C until starting RNA purification. This 10% Input may be used for comparison in RT-PCR, and may also be used for an RNA quality assessment.

Antibody Prebound Magnetic Beads Immunoprecipitation

- Place washed antibody prebound magnetic beads on a magnetic separator and remove supernatant.
- Remove prebound magnetic beads from magnetic separator and resuspend in appropriate IP reaction.
- Incubate all tubes with rotation for 3 hours to overnight at 4 °C.
- Centrifuge the immunoprecipitation tubes briefly and place on a magnetic separator.
- 10 % Input samples may also be taken from the negative control reactions (optional). Remove 10% of the reaction volume supernatant after incubation with magnetic beads and keep on ice until RNA purification.
- Discard the remaining supernatant from the negative control reactions and the entire supernatant from the test reactions.

Antibody Only Immunoprecipitation

- Add antibody directly to the IP reaction.
- Incubate all tubes with rotation for 3 hours to overnight at 4 °C.
- Centrifuge the immunoprecipitation tubes briefly.
- Add 20 µL of washed magnetic beads to each RIP reaction and incubate at 4 °C for one hour. If using a mouse monoclonal antibody, add 1 µL of bridging antibody to each IP reaction and incubate for one hour at 4 °C before adding 20 µL of washed magnetic beads.
- 10% Input samples may also be taken from the negative control reactions (optional). Remove 10% of the reaction volume supernatant after incubation with magnetic beads and keep on ice until RNA purification.
- Discard the remaining supernatant from the negative control reactions and the entire supernatant from the test reactions.

Washing Steps

- Add 1 mL of wash buffer to all RIP reactions and vortex gently.
- Transfer the RIP reactions to fresh 1.5 mL microcentrifuge tubes.
- Spin tube briefly and place on magnetic separator.
- Remove supernatant and add 1 mL of wash buffer.
- Repeat wash step until magnetic beads have been washed a total of five times.
- After the last wash, resuspend the magnetic beads antibody-RNA binding protein complex in 200 µL of wash buffer. Bring the 10% Input samples up to a final volume of 200 µL using RIP wash buffer.

Purification of RNA (reagents not provided)

- Add 500 µL of TRI Reagent to each RIP reaction in 200 µL RIP wash buffer.
- Add 100 µL of 1-bromo-3-chloropropane to each TRI Reagent containing RIP reaction (Catalog No. C2432, chloroform, may be substituted).
- Vortex briefly and spin at 4 °C for 10 minutes at 16,000 x *g*.
- Remove aqueous phase being careful not to disturb the bottom organic phase, and place in a new tube.
- To the aqueous phase in a new tube, add 6 µL of linear acrylamide, 60 µL of 5 M ammonium acetate and 600 µL of 2-propanol and vortex briefly.
- Place at −80 °C for at least one hour to precipitate the RNA. Samples may be stored overnight at −80 °C before further processing.
- Thaw −80 °C reactions on ice.
- Centrifuge at 16,000 *x g* for 10 minutes at 4 °C and discard the supernatant carefully.
- Wash pellet once with 0. 5 mL of 80% ethanol solution.
- Centrifuge at 16,000 *x g* for 10 minutes at 4 °C.
- Discard the supernatant carefully and air dry the pellets in a laminar flow hood.
- Resuspend in 10-25 µL of RNase-free water, and place tubes on ice.

Assessment of Input RNA Quality

Nanodrop
Bioanalyzer

Analysis of Immunoprecipitated RNA

RNA may be analyzed using quantitative RT-PCR if binding targets of the RBP are known. If binding targets of the RBP are not known, or to discover new targets, RNA may be analyzed by next generation sequencing.

Note: We recommend WTA2 amplification if amounts of RNA recovered are insufficient for downstream applications.

DNase treatment prior to first strand cDNA synthesis is recommended to remove contaminating DNA. This will reduce the likelihood of genomic DNA affecting the analysis of RNA.

Presented below is an example of a reverse transcription reaction and two QPCR procedures. The positive control anti-SNRP70 antibody SYBR green quantitative PCR procedure may be used with the forward primer 5' TCCCAGGGCGAGGCTTATCCATT 3' and reverse primer 5' GAACGCAGTCCCCCACTACCACAAAT 3'. The hydrolysis probe procedure may be used with separately purchased hydrolysis probe assays, such as Life Technology's TaqMan.

For reverse transcription, any commercially available reverse transcription enzyme with random primers or oligo dT primers may be used.

Note: To make a 1% Input, prepare a 1:10 dilution of the 10% Input samples from Page 9 and 10 in RNase free water.

DNase treatment using DNase I, amplification grade AMPD1

Reagent	**Volume**
RNA	8 µL
10X reaction buffer	1 µL
DNase	1 µL
Total Volume	10 µL

Incubate for 15 minutes at room temperature.
Add 1 µL of stop solution to each DNase reaction.
Heat at 70 °C for 10 minutes.
Place on ice.

Add Reverse Transcriptase mixture

Reagent	**Volume**
10X RT Buffer	2 µL
10 mM dNTP mix	1 µL
Random primers or oligo dT primers	1 µL
0.25 µL 40 U/µL Ribonuclease Inhibitor	0.25 µL
1 µL MMLV (RT) or nuclease-free water for no RT controls	1 µL
Nuclease-free water	g.s.
Total Volume	10 µL

Cycling Parameters

Reverse Transcription	44 °C	50 minutes
Reverse Transcription	55 °C	10 minutes
Denaturation	92 °C	10 minutes
	4 °C	indefinite

Note: Random reverse transcription primers need to be used with anti-SNRP70 antibody RIP reactions, because the specific RNA target, U1 snRNA analyzed in these RIP reactions, is not polyadenylated. Oligo dT primers may be used with other polyadenylated RIP targets.

SYBR Green QPCR mix

Reagent	Volume
2X Ready Mix	12.5 µL
5 µM Forward Primer	1 µL
5 µM Reverse Primer	1 µL
cDNA	2 µL
100X ROX	0.25 µL
Nuclease-free Water	8.25 µL
Total Volume	25 µL

Cycling Parameters

Initial Denaturation	95 °C	1 cycle	3 minutes
Denaturation	95 °C		30 seconds
Annealing	60 °C		30 seconds
Extension	72 °C	>40 cycles, read	30 seconds

95 °C	1 minute	
55 °C	30 seconds	
95 °C	30 seconds	1 cycle read all melt curve

QPCR Hydrolysis probe mix

Reagent	Volume
2X Ready Mix	10 µL
25 mM $MgCl_2$	2.4 µL
100X ROX	0.02 µL
cDNA	2 µL
20X TaqMan probe	1 µL
Nuclease-free Water	4.58 µL
Total Volume	20 µL

Cycling Parameters

Initial Denaturation	94 °C	1 cycle	3 minutes
Denaturation	94 °C		15 seconds
Annealing/Extension	60 °C	>40 cycles, read	1 minute

Data Analysis
The qPCR run is deemed successful if no products or very high Ct's are observed in the "no reverse transcriptase" controls. Adjust the baseline to eliminate the minor background signal (if any) in these wells.

For SYBR green reactions, ensure that the melt curve indicates the production of a single PCR product. There should be a single peak in each well with each primer pair.

Calculate the yield (% Input) and specificity (Fold Enrichment) of your RIP reaction by plugging your average Ct values into the RIP-qPCR Data Analysis for % Input and Fold Enrichment Calculations shell (excel worksheet template) provided at our website.

RIP-qPCR Data Analysis (ΔΔCt method)[3, 4]

i. Normalize each RIP RNA fractions' Ct value to the Input RNA fraction Ct value for the same qPCR Assay (ΔCt) to account for RNA sample preparation differences.

   ΔCt [normalized RIP] = (Ct [RIP] – (Ct [Input] – Log_2 (Input Dilution Factor)))

   Where, Input Dilution Factor = (fraction of the input RNA saved)[1]

   The default Input fraction is 1% which is a dilution factor of 100 or 6.644 cycles (i.e. log_2 of 100). Thus, subtract 6.644 from the Ct value of the 1% Input sample as mentioned in the equation above.

   Average normalized RIP Ct values for replicate samples.

ii. Calculate the % Input for each RIP fraction (linear conversion of the normalized RIP ΔCt).

$$\textbf{\% Input} = 2^{(-\Delta Ct\ [\ normalized\ RIP])}$$

iii. Adjust the normalized RIP fraction Ct value for the normalized background [non-specific (NS) Ab] fraction Ct value (first ΔΔCt).

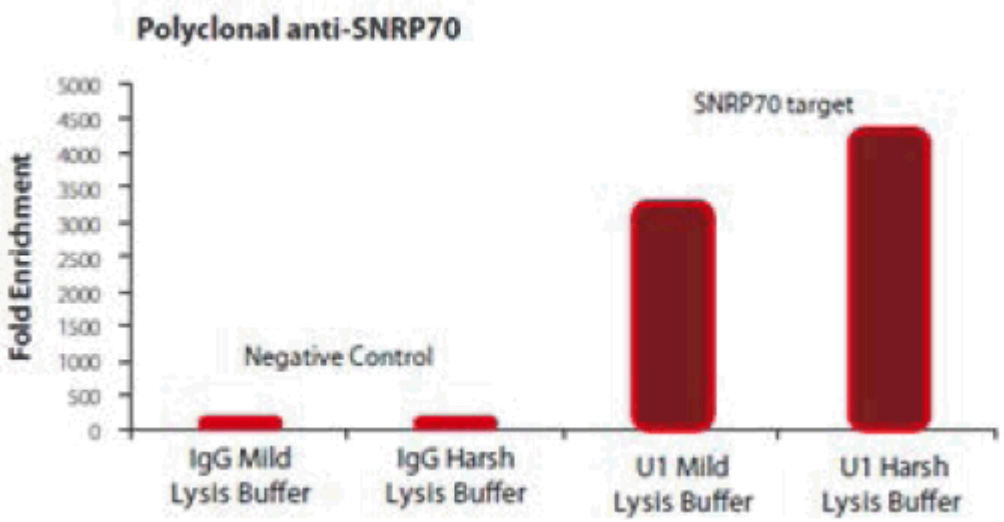
$$\Delta\Delta Ct\ [RIP/NS] = \Delta Ct\ [normalized\ RIP] - \Delta Ct\ [normalized\ NS]$$

iv. Calculate Assay Site IP Fold Enrichment above the sample specific background (linear conversion of the first ΔΔCt).

$$\textbf{Fold Enrichment} = 2^{(-\Delta\Delta Ct\ [RIP/NS])}$$

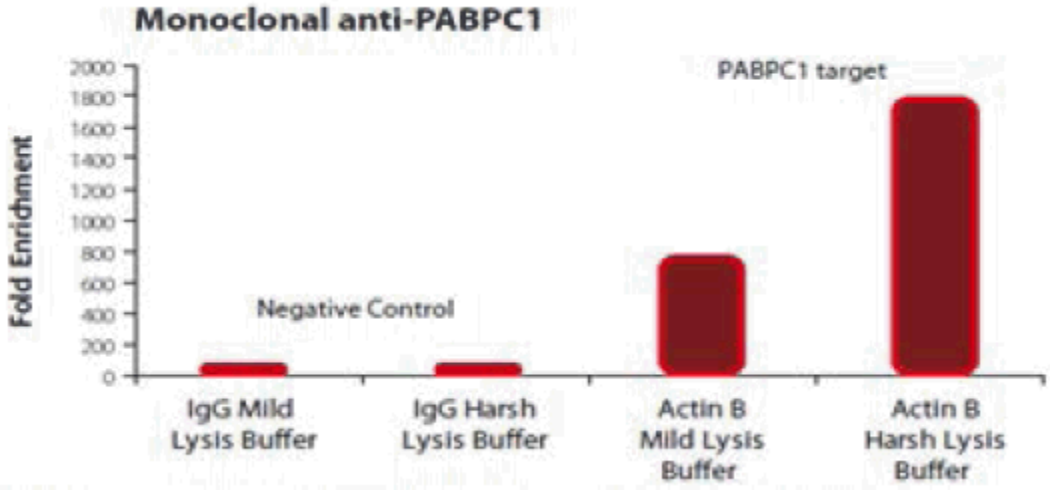
Expected Results

Figure 1.



RIP Lysate prepared from HeLa cells (0.75 x 10^6 cell equivalents per IP) were immunoprecipitated using 1 µg of either a normal Rabbit IgG, or Anti-SNRP70 antibody and the Imprint RNA Immunoprecipitation Kit. Immunoprecipitation of SNRP70-associated RNA was validated by qPCR using Control Primers, U1 snRNA.

Figure 2.



RIP Lysate prepared from HeLa cells (0.75 x 10^6 cell equivalents per IP) were immunoprecipitated using 1 µg of either a normal mouse IgG, or Anti-PABPC1 antibody along with Anti-mouse antibody produced in Rabbit and the Imprint RNA Immunoprecipitation Kit. Immunoprecipitation of PABP-associated RNA was validated by qPCR using Control Primers, Actin B.

Troubleshooting Guide

Observation	Cause	Recommended Solution
Little or no RT-PCR product	Insufficient cell number	Increase amount of cells used per RIP reaction.
	Insufficient cell lysis	Check lysis efficiency by observing a 5 µL aliquot of the lysate under a microscope.
	Antibody does not bind protein of interest	Check if the subclass or isotype of the antibody is correct. Choose an antibody that is qualified for use in RIP. Antibodies recognizing native protein conformations have the highest chance for success, i.e., immunoprecipitation qualified.
	RNA is not a target in the cell line or growth conditions used	Ensure RNA binding protein of interest is expressed and binding RNA target.
	RNA contains ethanol	Make sure RNA pellet is thoroughly dried after the wash step during RNA purification before adding RNase-free water.
	Incorrect reverse transcription primers	RNA transcripts that are not polyadenylated can not be reverse transcribed with oligo d(T) primer (i.e. U1 snRNA). Random primers are required for these types of RNA transcripts.
	Poor QPCR primer design	Ensure the primers are specific to the RNA target sequence and amplicon size is within 90-200 bp for qPCR.
	Incorrect PCR conditions	Optimize PCR conditions using purified total RNA from the same cell type
Little or no amplification difference between positive and negative controls	Insufficient washing	Resuspend protein A magnetic beads thoroughly by vortexing gently during the washing steps in the RIP protocol to ensure sufficient removal of nonspecific RNA-protein complexes.
	RNA contains contaminating DNA	Make sure RNA is DNase treated before reverse transcription step.
	Wash buffer not stringent enough	More stringent wash conditions may be used if the RBP-antibody affinity is strong. Adding urea between 0.5 M- 3 M, 0.1% or less SDS, deoxycholate or NP40 to the wash buffer may reduce unwanted background.
	Too few PCR cycles	Cycle numbers in the exponential phase will yield the best results.
	Antibody amount not optimized	Titrate the amount of specific and non-specific antibody used per RIP reaction. Determine the least amount of specific antibody needed for optimal signal to noise ratio. Due to a higher antibody binding capacity of the magnetic beads supplied in the kit, a lower amount of antibody may be required for optimal results compared to other magnetic bead protocols.

References:

1. Keene, J. D. et al., RIP-ChIP: the isolation and identification of mRNAs, microRNAs and protein components of ribonucleoprotein complexes from cell extracts. *Nature Protocols* **1**(1), 302-307 (2006).
2. Chen, Z. et al., RNase activity requires formation of disulfide bonds and is regulated by the redox stat. *Plant Mol. Biol.* May:**55**(1), 83-96 (2004).
3. Livak, K. J. & Schmittgen, T. D., Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**(4), 402-408 (2001).
4. Yuan, J. S. et al., Statistical analysis of real-time PCR data. *BMC Bioinformatics* **7**:85 (2006)

Legal Information

EE,SB,PHC 03/13-1