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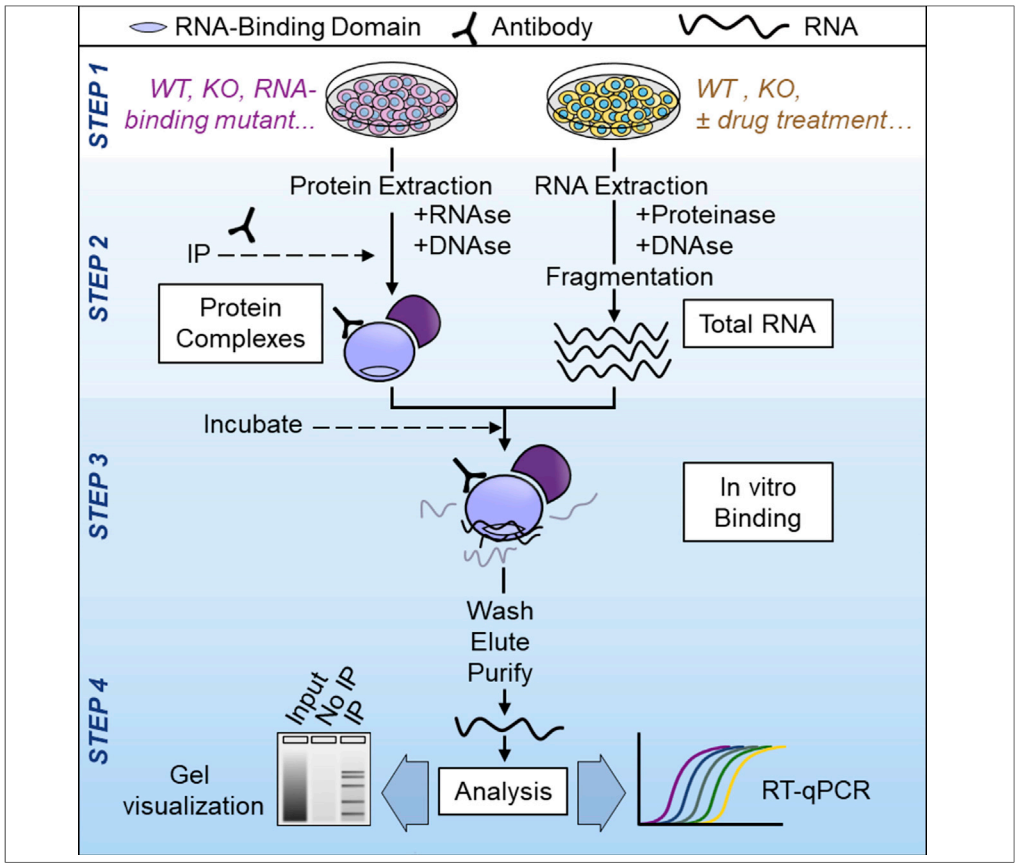
Protocol

An Optimized Immunoprecipitation Protocol for Assessing Protein-RNA Interactions *In Vitro*



RNA-binding proteins are key regulators of cell identity and function, which underscores the need for unbiased and versatile protocols to identify and characterize novel protein-RNA interactions. Here, we describe a simple and cost-effective *in vitro* RNA immunoprecipitation (iv-RIP) method to assess the direct or indirect RNA-binding ability of any protein of interest. The versatility of this method relies on the adaptability of the immunoprecipitation conditions and the choice of the RNA, which exponentially broadens the range of potential applications.

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HIGHLIGHTS

In vitro protocol for detection of direct and indirect protein-RNA interactions

User-friendly method for *de novo* identification of RNA-binding proteins

Versatile method to assess binding preference depending on RNA type and origin

Highlights common immunoprecipitation pitfalls and potential solutions

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An Optimized Immunoprecipitation Protocol for Assessing Protein-RNA Interactions *In Vitro*

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SUMMARY

RNA-binding proteins are key regulators of cell identity and function, which underscores the need for unbiased and versatile protocols to identify and characterize novel protein-RNA interactions. Here, we describe a simple and cost-effective *in vitro* RNA immunoprecipitation (iv-RIP) method to assess the direct or indirect RNA-binding ability of any protein of interest. The versatility of this method relies on the adaptability of the immunoprecipitation conditions and the choice of the RNA, which exponentially broadens the range of potential applications.

For complete details on the use and execution of this protocol, please refer to Guallar et al. (2020).

BEFORE YOU BEGIN

Prepare the media (see "Materials and Equipment" section) and cell cultures before starting this protocol. Refer to Key Resources Table for a complete list of materials and equipment. This protocol is optimized for pluripotent stem cells and embryonic fibroblasts, both of murine origin. The versatility of this technique makes it potentially suitable for any desired cell line or primary culture with the required modifications.

RNase enzymes are ubiquitous. Work in a RNase-free zone in the lab by cleaning the area with RNase ZAP™ RNase Decontamination Solution and use RNase-free tubes, pipette tips and solutions. RNase inhibitors should be added to all buffers except those used for the protein extraction steps, which includes an RNase digestion step (See below "Preparation of Whole Cell Protein Extracts" section). For RNA preparation, incubation with immunocomplexes and visualization by gel, it is critical to prepare solutions with RNase-free water or DEPC-treated Milli-Q water (see "Materials and Equipment" section). RNase and proteinase activities are reduced with temperature, thus, unless otherwise stated, work with samples on ice or at 4°C at all times, and set the needed devices/centrifuges at 4°C.

MEF Cell Culture

Timing: 1 week

Murine embryonic fibroblasts (MEFs) are cultured directly on plastic plates and must be maintained in culture at 70%–80% confluency to avoid growth inhibition by cell contact. Avoid excessive cell dilution when performing serial passages, which might lead to cell proliferation arrest.

1. Thaw cells in the appropriate surface plate at a density of 1×10^4 cells/cm^2 and allow them to recover and start proliferating in MEF medium.
2. When they reach confluency (around 2×10^4 cells/cm^2), subculture the cells to expand them:
   a. Carefully remove the culture medium by aspiration and rinse the cells with PBS twice to remove residual α-1-antitrypsin from the fetal bovine serum (FBS) present in the culture medium which would inhibit trypsin function.
   b. Detach cells from plate by adding enough 0.05% trypsin to cover the plate surface and incubate the plate for 5 min at 37°C (for more details on trypsin volume, please refer to tables in "Materials and Equipment" section).

   Alternatives: Different enzymatic solutions to detach cells may be used instead of trypsin.

   c. Resuspend cells into a single-cell suspension by gently pipetting up and down.
   d. Transfer the resuspended cells to a sterile 15-mL tube with the appropriate amount of FBS-containing medium to neutralize the trypsin. A minimum of 3 trypsin volumes should be added for complete trypsin inactivation.
   e. Centrifuge cells for 5 min at 300 × *g* at 20°C–25°C. Cells should form a pellet after centrifugation.
   f. Carefully remove supernatant avoiding cell pellet disruption and leave a small volume of medium behind to resuspend the cells by lightly tapping the tube.
   g. Add the desired volume of the specific culture medium to the cells and transfer them to a new culture plate. Plate cells at approximately 0.8×10^4 cells/cm^2.
   h. Complete the plate volume with the specific medium.
   i. Incubate plates in a humidified 37°C incubator with 5% CO_2.
3. When desired cell number is reached, process the culture as indicated in the corresponding section below.

   Note: Primary cultures have a limited number of cell divisions. Fibroblasts display longer doubling times and a flattened morphology at higher passage number. Whenever possible use cells of earlier passages (< 6 passages).

Murine Pluripotent Cell Culture

Timing: 1 week

Pluripotent cells are cultured in 0.1% gelatin-coated dishes (see "Materials and Equipment" section).

⚠ CRITICAL: Due to their fast division rate (doubling time of around 10–12 h), pluripotent cell cultures should have their medium refreshed every day to avoid chromosomal and mitochondrial instability induced by depleted medium. Perform serial passages (1:4–1:6) every other day avoiding reaching more than 70%–80% confluence.

4. Prepare gelatin-coated plates.
   a. Prepare 0.1% gelatin by diluting 100 mL gelatin 1% into 900 mL of Milli-Q water.
   b. Filter the 0.1% gelatin using a 0.22 μm filter.
   c. Pipette gelatin 0.1% in the plate, completely covering the surface of the wells. Incubate the plate for at least 20–30 min in a 37°C cell incubator.
   d. After incubation, carefully aspirate the gelatin from the plate prior to adding the cells.

5. Thaw the cells and plate them into gelatin-coated plates with serum-leukemia inhibitory factor (LIF) medium.
6. For cell passaging, follow a similar procedure as with MEFs (see step 2 of "Before You Begin" section).

Note: Pluripotent cells form tight colonies which may take longer incubation times with trypsin (i.e., 7 min) to disaggregate. Cells will be ready to disaggregate by pipetting when they appear as floating "grape clusters" under the microscope. Then, resuspend and disaggregate cells into a single-cell suspension by gently pipetting up and down.

7. When desired cell mass/number is reached, samples are processed as indicated in each section.

Note: We recommend avoiding working with pluripotent cells after a high number of passages. Mitochondrial and chromosomal alterations could be produced once certain number of cell divisions are reached.

RNA Isolation and DNase I Treatment

Timing: 2 h

Prepare in advance the RNA that will be incubated with the immunopurified proteins or protein complexes. RNA from the same or different cell types used to immune purify proteins can be used. This protocol can be adapted to test protein affinity for total RNA, or certain RNA types that can be isolated using specific isolation methods (e.g., cytoplasmic, nuclear, messenger RNA, noncoding RNA, etc.). Notably, we have shown that the iv-RIP protocol can be applied efficiently to test the ability of RNA-binding proteins (RBPs) to interact with modified or unmodified RNAs (i.e., A-to-I edited vs unedited RNA) (Guallar et al., 2020).

8. Aspirate growth media and rinse the cells with PBS twice.
9. Enzymatically detach cells using trypsin 0.05% as above indicated (see step 2 of "Before You Begin" section).
10. Count cells before TRIzol™ addition to adjust the volume to cell number and add 0.75 mL TRIzol™ Reagent per 5–10 × 10^6 cells.

Caution: TRIzol™ and chloroform are harmful both for the researcher and environment. Always work in a chemical fume hood wearing lab coat, gloves, and eye protection.

Alternatives: Cell number could also be estimated directly by cell confluence. Add 0.3–0.4 mL of TRIzol™ Reagent per 10^5—10^7 cells. When directly lysing on the plate, do not wash cells before addition of TRIzol™ Reagent to avoid mRNA degradation. Use a cell scrapper to collect the cell lysate from the well and transfer the viscous sample with a cut tip to a 1.5-mL Eppendorf tube.

Pause Point: Samples in TRIzol™ can be stored at 4°C 12–16 h or at −20°C up to a year.

11. RNA purification:
    a. Incubate samples in TRIzol™ 5 min at 20°C–25°C to allow dissociation of the nucleoprotein complexes.
    b. Transfer the TRIzol™ samples to RNA Phasemaker™ tubes.

Note: Centrifuge the RNA Phasemaker™ tubes for 30 s at 12,000–16,000 × *g* prior to use to place the inner gel at the bottom of the tube (Figure 1). The use of RNA Phasemaker™ tubes

simplifies the recovery of the aqueous phase and increases the RNA yield up to 30%, while not interfering with downstream applications.

Alternatives: If Phasemaker™ tubes are not available, proceed directly to c.

c. Add 0.2 mL of chloroform per mL of TRIzol™ reagent.

Note: Make sure that the cap is tightly closed to avoid any leakage.

d. Shake vigorously by hand for 15 s.
e. Incubate 3 min at 20°C–25°C.
f. Centrifuge the samples at 12,000 × *g* for 15 min at 4°C.

Note: The mixture separates into a lower-red phenol-chloroform phase, an interphase and a colorless upper aqueous phase containing the RNA. Note that Phasemaker™ gel forms a barrier that separates upper and lower phases (Figure 1).

g. Transfer the aqueous phase containing the RNA, to a new RNase-free 1.5-mL tube.

⚠ CRITICAL: Avoid puncturing the Phasemaker gel with the pipette tip when aspirating the top aqueous phase, as it could lead to sample contamination.

Alternatives: If Phasemaker™ tubes are not available, be extremely careful while recovering the top aqueous phase after centrifugation to avoid transferring any of the interphase or organic layer, which contains contaminants for downstream applications such as genomic DNA and proteins.

12. Precipitate the RNA.
    a. Add 0.5–1 µL of RNase-free glycogen (1 mg/mL stock). Mix well by hand.

Note: Glycogen is a polysaccharide that traps the nucleic acids in presence of salts, forming complexes to be precipitated by centrifugation. During centrifugation, a visible pellet is

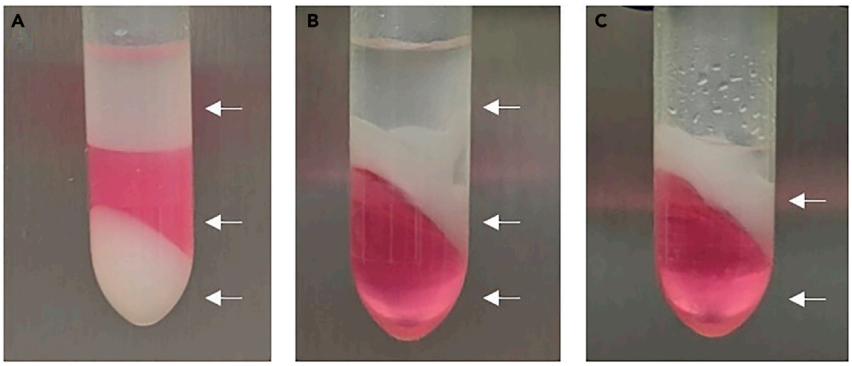

Figure 1. RNA Isolation Using RNA Phasemaker Tubes
(A) RNA Phasemaker tube containing the gel (bottom arrow) after addition of the cells in TRIZOL™ (red phase) and the chloroform (top phase) and prior to centrifugation.
(B) RNA Phasemaker tube after the centrifugation step. The gel (middle arrow) separates the lower phenol-chloroform phase and the upper aqueous phase which contains the RNA.
(C) Representation of an empty Phasemaker tube after carefully transferring the aqueous phase to another clean tube (not represented). The arrows point to the different phases present in the tube.

formed, greatly facilitating the handling of nucleic acids. Glycogen does not interfere with subsequent applications.

b. Add 0.5 mL of isopropanol to the aqueous phase per mL of TRIzol™ used for the lysis.

Note: Isopropanol and ethanol volumes used in steps 12b and 13a, respectively, are calculated relative to the volume of TRIzol™ used in step 10 of section "RNA Isolation and DNase I treatment".

c. Mix well by inversion 10 times.

⚠ CRITICAL: Mix well the isopropanol to efficiently precipitate the RNA.

d. Incubate 10 min at 20°C–25°C.
e. Centrifuge at 12,000 × *g* for 10 min at 4°C.
f. Carefully discard the supernatant with a micropipette avoiding RNA pellet detachment.

Note: RNA precipitates as a white gel-like pellet at the bottom of the tube.

13. Wash the RNA pellet.
    a. Add 1 mL of pre-chilled 75% ethanol per mL of TRIzol™.

Pause Point: The RNA can be stored in 75% ethanol for at least 1 year at –20°C, or at least 1 week at 4°C.

    b. Vortex briefly and centrifuge at 7,500 × *g* for 5 min at 4°C.
    c. Carefully discard supernatant.
    d. Briefly spin the tube again and discard the remaining supernatant with a micropipette and a small tip.
    e. Air dry the pellet on the bench for 5 min or until the pellet begins to become transparent.

Note: When the white RNA-glycogen pellet dries it becomes transparent and more difficult to visualize.

⚠ CRITICAL: Do not let the RNA pellet completely dry, as it could negatively impact on RNA solubilization. Partially dissolved RNA samples have an A230/280 ratio of <1.6.

14. Solubilize the RNA.
    a. Resuspend the RNA pellet in 20–50 μL of RNase-free water by pipetting up and down.

Note: RNA solutions too concentrated will appear as viscous and difficult to pipette, and thus lead to inaccurate concentration measurements.

    b. Incubate the samples in a heat block set at 55°C–60°C for 15 min.
    c. Quantify RNA concentration (e.g., by Nanodrop).

Note: While measuring the absorbance of your RNA sample, a ratio of A260/280 ≈ 2 is considered pure RNA.

Note: When using pluripotent cells (i.e., ESCs) or somatic cells (i.e., fibroblasts), RNA yield should be 5–8 μg per 10^6 cells lysed.

15. Remove potential traces of DNA from total RNA preparation by DNase I treatment.

a. Treat up to 100 μg total RNA with 1.5 U of DNase I (RNase-free) and add the appropriate volume of the 10× DNase I buffer with magnesium.

Note: DNase I is an endonuclease that digests single- and double-stranded DNAs. Do not use more than 1 U of DNase I, RNase-free per 1 μg of RNA.

b. Incubate at 37°C for 30 min.
c. Keep RNA samples on ice.

Note: This RNA is ready to be used in step 21 of the protocol. Although not required for the iv-RIP protocol, at this stage DNase I could be inactivated by heating at 65°C 10 min in presence of EGTA or EDTA, which will not affect downstream applications.

Pause Point: RNA can be stored after step 14 or 15 at −80°C until use.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Monoclonal ANTI-FLAG® M2 antibody produced in mouse	Sigma-Aldrich	Cat#F1804; RRID: AB_262044
Mouse TrueBlot® ULTRA: Anti-Mouse Ig HRP	Rockland Immunochemicals	Cat#18-8817-33; RRID: AB_2610851
Chemicals, Peptides, and Recombinant Proteins		
Gelatin from bovine skin	Sigma-Aldrich	Cat#G9391
Bradford Reagent	Sigma-Aldrich	Cat#B6916
Ethanol	Sigma-Aldrich	Cat#459844
Chloroform	Fisher Chemical	Cat#C/4920/15
Isopropanol	Fisher Bioreagents	Cat#BP2618-212
Diethyl Pyrocarbonate (DEPC)	Acros Organics	Cat#170250250
Bovine Serum Albumin	Fisher BioReagents™	Cat#BP1605-100
IGEPAL® CA-630	Sigma-Aldrich	Cat#I3021
RNase OUT™ Recombinant Ribonuclease Inhibitor	Invitrogen™	Cat#10777019
RNasin® RNase Inhibitor	Promega	Cat#N2111
Protease Inhibitor Cocktail	Sigma-Aldrich	Cat#P8340
Benzonase® Nuclease	Millipore	Cat#E1014
RNase A	Thermo Scientific™	Cat#EN0531
DNase I	Thermo Scientific™	Cat#EN0521
TRIzol™ Reagent	Invitrogen™	Cat#15596026
Glycogen	Roche	Cat#10901393001
Ethidium Bromide	Fisher Bioreagents	Cat#BP1302-10
Tris-Borate-EDTA Buffer	Fisher Bioreagents	Cat#BP1333-4
RNase ZAP™ RNase Decontamination Solution	Invitrogen	Cat#AM9780
Critical Commercial Assays		
qScript® cDNA SuperMix	Quantabio	Cat#95048-025
PowerUp™ SYBR™ Green Master Mix	Applied Biosystems™	Cat#A25742
Invitrogen™ Qubit™ RNA HS Assay Kit	Invitrogen™	Cat#Q32852

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Luminata Crescendo Western HRP	Millipore	Cat#WBLUR0100
Experimental Models: Cell Lines		
Mouse embryonic fibroblasts	This study	N/A
Mouse embryonic stem cells (CCE)	This study	N/A
Oligonucleotides		
Cds2 Primer Forward: ATGACCGAACTACGGCAGAG	Sigma-Aldrich	N/A
Cds2 Primer Reverse: GAGGTTGGTAGGGGAGCTG	Sigma-Aldrich	N/A
Gla Primer Forward: TCTGTGAGCTTGCGCTTTGT	Sigma-Aldrich	N/A
Gla Primer Reverse: GCAGTCAAGGTTGCACATGAAA	Sigma-Aldrich	N/A
Sppl2a Primer Forward: CATGTCATGCGTGATACTGCT	Sigma-Aldrich	N/A
Sppl2a Primer Reverse: ACCCTGATAACTACTGGCAACT	Sigma-Aldrich	N/A
Software and Algorithms		
StepOne Software v2.3	Thermo Fisher	N/A
Other		
Branson 450 Digital Sonifier	Marshall Scientific	Cat#B450
StepOnePlus™ Real-Time PCR System	Applied Biosystems™	Cat#4376600
SimpliAmp™ Thermal Cycler	Applied Biosystems™	Cat#A24811
Disposable Sterile Filter Systems	Corning™	Cat#431097
0.22µm EMD Millipore Steriflip™	Millipore	Cat#SCGP00525
MicroAMP™ Fast Optical 96-well Reaction Plates	Applied Biosystems™	Cat#4346907
Optical Adhesive Film	Applied Biosystems™	Cat#4360954
Dynabeads™ Protein G for Immunoprecipitation	Invitrogen™	Cat#10009D
Tube rotator	VWR	Cat#444-0500
DynaMag™-2 Magnet	Invitrogen™	Cat#12321D
Eppendorf® LoBind microcentrifuge tubes	Sigma	Cat#Z666505
Refrigerated Universal MPW-260R Centrifuge	MPW	Cat#10260R
Phasemaker™ Tubes for RNA	Phasemaker™	Cat#A33248
NanoDrop™ 2000 Microvolume Spectrophotometer	Thermo Scientific™	Cat#ND-2000
Qubit™ 4 Fluorometer	Invitrogen™	Cat#Q33226
Invitrogen™ Qubit™ Assay Tubes	Invitrogen™	Cat#Q32856
Immobilon-P PVDF Membrane	Millipore	Cat#IPVH00010
Corning™ Centrifuge Tubes with CentriStar™ Cap	Corning™	Cat#430791
Molecular Imager® Gel Doc™ XR+ System with Image Lab™ Software	BIO-RAD	Cat#1708195

MATERIALS AND EQUIPMENT

Cell Culture Media and Reagents

Here, we specify the culture medium and reagents employed for each cell type used during the present protocol:

> ***Note:*** Cell culture is performed in a sterile tissue culture hood under aseptic conditions and cells are maintained in a humidified 37°C incubator with 5% CO_2.

- MEF Cell Medium: High-glucose DMEM, supplemented with 10% FBS, 2 mM L-glutamine and 1% penicillin and streptomycin (P/S).

- Murine Pluripotent Stem Cell Medium (Serum-LIF medium): High-glucose DMEM, supplemented with 15% FBS, 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1% nucleoside mix, 1% P/S and 1,000 U/mL of recombinant LIF.
- Gelatin 1% (w/v): weight 10 g of product and add sterile water up to 1,000 mL. Place the solution on a hot plate magnetic stirrer at 37°C until complete solubilization is produced (the final product must present a color from light yellow to beige). After complete solubilization, autoclave to sterilize.

Note: The optimal work concentration relies on the specific cell type, research objectives and applications. Murine embryonic stem cells (ESCs) are cultured on 0.1% gelatin pre-coated plates (see "Murine Pluripotent Cell Culture" section). This 0.1% solution is prepared by diluting 1/10 the 1% gelatin solution in sterile Milli-Q water and filtered through a 0.22 μm filter.

- Reagent volumes: To homogenize cell culture conditions, we recommend using the following reagent volumes:

Culture Plates and Dishes	Approx. Growth Area (cm^2)	Culture Medium Volume (mL)	Gelatin 0.1% Volume (mL)	Trypsin Volume (mL)
12-well (single well only)	3.8	1–1.5	0.5–1	0.25–0.5
6-well (single well only)	9.5	2–3	1–2	0.5–0.75
100 mm (dish)	55	10–12	5–7	2–3
150 mm (dish)	152	30–35	12.5–15	5–7

0.1% DEPC-Treated Water

Reagent	Final Concentration	Volume (mL) for 1,000 mL
Diethyl pyrocarbonate (DEPC)	0.1%	1
Milli-Q water	n/a	999

Add the DEPC to the Milli-Q water and mix thoroughly. Incubate the solution for 12 h at 20°C–25°C protected from light. Autoclave at 100°C for 45 min to remove any traces of DEPC.

Reagent (Stock Concentration)	Final Concentration	Volume (mL) for 50 mL
NaCl (5 M)	150 mM	1.5 mL
Tris-HCl pH 7.4 (1 M)	10 mM	0.5 mL
EDTA (0.5 M)	1 mM	0.1 mL
EGTA (0.5 M)	1 mM	0.1 mL
Triton X-100 (25%)	1%	2 mL
IGEPAL (10%)	0.5%	2.5 mL
Milli-Q water	n/a	43.3 mL

Lysis Buffer 150 mM NaCl

Sterilize by filtering the Lysis Buffer with a 0.22 μm filter. Store at 4°C. This buffer is stable for no more than 1 month.

Note: Calculate the buffer volume that is necessary to perform the experiment, depending on the number of samples. Right before use add 0.2 mM sodium orthovanadate (0.4 μL/mL of buffer), 0.2 mM PMSF (2 μL/mL), 1× Protease Inhibitor Cocktail (PIC) to the buffer (1 μL/mL) (see Other Solutions below for stock concentrations).

Lysis Buffer 100 mM NaCl

Reagent (Stock Concentration)	Final Concentration	Volume (mL) for 50 mL
NaCl (5 M)	100 mM	1 mL
Tris-HCl pH 7.4 (1 M)	10 mM	0.5 mL
EDTA (0.5 M)	1 mM	0.1 mL
EGTA (0.5 M)	1 mM	0.1 mL
Triton X-100 (25%)	1%	2 mL
IGEPAL (10%)	0.5%	2.5 mL
Milli-Q water	n/a	43.8 mL

Sterilize by filtering with a 0.22 μm filter. Store at 4°C. This buffer is stable for no more than 1 month. Add fresh protease inhibitors before use (see above).

Lysis Buffer 0 mM NaCl

Reagent (Stock Concentration)	Final Concentration	Volume (mL) for 50 mL
Tris-HCl pH 7.4 (1 M)	10 mM	0.5 mL
EDTA (0.5 M)	1 mM	0.1 mL
EGTA (0.5 M)	1 mM	0.1 mL
Triton X-100 (25%)	1%	2 mL
IGEPAL (10%)	0.5%	2.5 mL
Milli-Q water	n/a	44.8 mL

Sterilize by filtering with a 0.22 μm filter. Store at 4°C. This buffer is stable for no more than 1 month. Add protease inhibitors fresh before use (see above).

RIP Buffer

Reagent (Stock Concentration)	Final Concentration	Volume (mL) for 50 mL
KCl (0.5 M)	150 mM	15 mL
Tris-HCl pH7.5 (1 M)	25 mM	1.25 mL
EDTA (0.5 M)	5 mM	0.5 mL
IGEPAL (10%)	0.5%	2.5 mL
Milli-Q water	n/a	30.75 mL

Sterilize by filtering with a 0.22 μm filter. Right before use, add 0.2 mM PMSF (2 μL), 1× PIC (1 μL), 40 U RNase OUT™ (1 μL) and 40 U RNasin® (1 μL) RNase Inhibitors per mL of buffer.

Other Solutions

Reagent	Stock Concentration
Sodium orthovanadate	0.5 M
PMSF	0.1 M
PIC	1,000×
RNase OUT™	40 U/μL
RNasin®	40 U/μL

Note: Protease Inhibitor Cocktail (PIC) mixture contains AEBSF at 104 mM, Aprotinin at 80 μM, Bestatin at 4 mM, E-64 at 1.4 mM, Leupeptin at 2 mM and Pepstatin A at 1.5 mM.

STEP-BY-STEP METHOD DETAILS

Preparation of Whole Cell Protein Extracts

Timing: 30 min

The protocol is optimized for ESC whole cell protein extracts but can be adapted to any cell type or cellular fraction.

Note: The choice of the right Lysis Buffer is critical for the quality of the sample that will be used for immunoprecipitation. Detergent (non-ionic: 0.1%–2%) and salt (0–500 mM) concentrations should be determined empirically for each protein of interest, to ensure maximum release of the proteins from the cells while maintaining native protein structures and interactions for the downstream immunoprecipitation and RNA interaction assays. Most immunoprecipitation assays use 100–150 mM salt concentrations which fall within the range of physiological concentration (approximately 137 mM).

1. Harvest cells for further processing.
   a. Proceed as previously stated to enzymatically detach the cells from the culture dish (see "Murine Pluripotent Cell Culture" section).
   b. Pellet cells by centrifugation in a swing-bucket rotor at 300 × *g* for 5 min at 20°C–25°C.
   c. Carefully discard supernatant.
   d. Wash cell pellet once with PBS.

Pause Point: Alternatively, cell pellet may be flash frozen and stored at −80°C for at least 1 year. For flash-freezing the cells, introduce the 15-mL conical tube containing the cell pellet directly in liquid nitrogen for 10 s. Store at −80°C until use.

Timing: 2 h

This step describes the processing of cell pellets to obtain whole cell protein extracts. Here, we presented an example using 15 cm cell culture plates of pluripotent ESCs at a 70%–80% confluence (≈80–100 × 10^6 cells). Adequate reagent volumes to the specific cell culture plates were used.

⚠ CRITICAL: Pre-chill tubes before use and keep the samples on ice at all times to prevent protein degradation.

Note: If frozen cell pellets are used, allow them to thaw slowly on ice before proceeding to lysis.

2. Resuspend the cells in 4 mL of ice-cold Lysis Buffer 150 mM NaCl containing fresh protease inhibitors.

Note: Both cell density and volume will affect the efficiency of cell lysis by sonication and should be optimized for each cell type. An appropriate cell density should be used to allow, on the one hand, a proper cell lysis with a short number of sonication pulses to reduce sample heating and, on the other hand, the obtention of protein extracts of high concentration (1–3 μg/μL) to avoid excessive dilution during the immunoprecipitation.

3. Mix by constant rocking for 30 min at 4°C.
4. Briefly spin down the samples at 4°C to collect the sample in the tube.

5. Sonicate the samples in a Branson Digital Sonifier using 4 cycles of 5 s ON and 30 s OFF at 50% amplitude to disrupt cells and obtain whole cell lysates.

⚠ CRITICAL: Sound level and frequency of the noise emitted during the ultrasonic processing may result in an uncomfortable noise being emitted. Operators may need to use auditive protective equipment.

⚠ CRITICAL: Mix well the cell suspension to make it homogeneous before the first sonication cycle. Sonication process heats up the sample leading to protein degradation, especially proteins larger than 80–100 kDa. If the sonicator is not coupled to a refrigeration system, perform this step with the 15-mL polystyrene tube placed in an ice-water bath (Figure 2). Use short ON pulses to reduce the heating up of the samples during sonication. Foaming will dramatically decrease the sonication efficiency.

Note: Sonication parameters are highly dependent on the device used and they need to be optimized. Check the cell suspension under the microscope for intact cells after each burst to decide the end point of the sonication (Figure 3).

6. Clear the cell lysates by centrifugation of the 15-mL tube at 4,000 × *g* for 30 min at 4°C to remove insoluble debris.
7. Collect supernatant (whole cell protein extract) and transfer to a new 15-mL tube.

Pause Point: Cells lysates may be flash frozen in liquid nitrogen and stored at −80°C until use. In the day of use, protein extracts should be thawed on ice to avoid fast freeze-thaw cycles and keep protein complexes integrity.

Immunoprecipitation – Day 1

Timing: 1 day

This step describes the immunocomplexes formation between protein and antibody.

8. Measure protein concentration with Bradford reagent using an appropriate standard (e.g., bovine serum albumin).

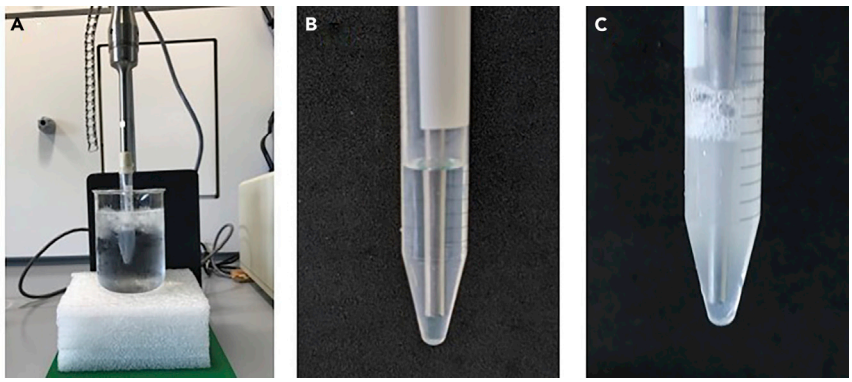

Figure 2. Sonication Setup
(A) An ice-water bath is used to avoid protein degradation due to sample heating upon sonication.
(B) Representation of correct sonicator probe setup, set deep in the sample avoiding touching the tube walls.
(C) Foaming can appear as the result of not keeping the sonication probe properly submerged into the sample.

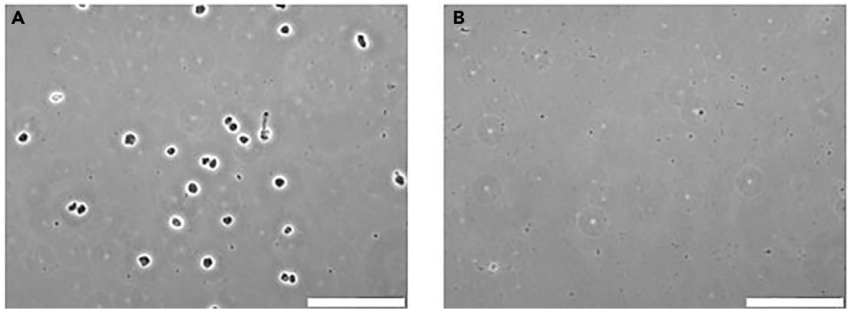

Figure 3. Monitorization under the Microscope of Cell Lysis by Sonication

(A) Cell solution before sonication.

(B) Cells are lysed by sonication and detergent presence. Scale bars represent 100 μm.

Alternatives: Protein concentration can be assessed by BCA Protein Assay, or other methods compatible with detergent concentration of the Lysis Buffer (i.e., 1% Triton X-100 and 0.5% IGEPAL).

9. Use 1 mg of whole cell protein extract for each immunoprecipitation (IP) in a Protein-LoBind Eppendorf tube.

   Note: Keep a 1% aliquot as input sample to test the immunoprecipitation by Western Blot (see Figure 5).

10. Bring the reaction to 100 mM NaCl by adding Lysis Buffer without NaCl and adjust to a final volume of 1.5 mL with Lysis Buffer 100 mM NaCl. Table 1 can be used to simplify the calculations of the buffers used in the IP.

11. Add 1 μg of antibody per mg of protein to the corresponding pre-labeled tube. Include a mock IP, using the non-immune IgG fraction from the same species in which the antibody was made.

    Note: The amount of antibody required is dependent on the antibody affinity and protein abundance, and should be empirically determined for each antibody/protein pair.

12. Add 100 U of Benzonase and $MgCl_2$ to a final concentration of 1.5 mM to each tube to remove DNA and RNA molecules.

    ⚠ CRITICAL: Benzonase is a genetically engineered endonuclease that cleaves both DNA and RNA while having no proteolytic activity. Benzonase requires 1–2 mM $MgCl_2$ to work, and its activity is reduced by high concentration of EDTA. Lack of $MgCl_2$ in your sample will result in incomplete nucleic acid removal. Use tip filters for benzonase, RNases and DNases to avoid cross contamination and degradation of the samples.

    Alternatives: This endonuclease could be replaced by a combination of DNase I and RNase A nucleases treatment.

Table 1. Volume of Each Buffer Required for Setting up the Immunoprecipitation at a Final NaCl Concentration of 100 mM

Lysis Buffer 150 mM (mL)	Lysis Buffer 100 mM (mL)	Lysis Buffer 0 mM (mL)	Total Volume (mL)
x	$y = (1 - x) \times 1.5$	$z = 1.5 - x - y$	1.5

13. Allow immunocomplexes to form by slow (i.e., 15–30 rpm), constant rotation for 12–16 h at 4°C.

Note: Tube caps can be sealed with parafilm to avoid any potential leakage.

Alternatives: Incubation time is dependent on the kinetics of each antibody to reach the equilibrium of binding. For high affinity antibodies, 2 h should be enough for immunocomplexes to form.

Immunoprecipitation – Day2

Timing: 7 h

This step describes the collection of protein-antibody immunocomplexes using magnetic Dynabeads™ Protein G and the incubation with RNA.

14. Equilibrate the magnetic beads.
    a. Add the desired volume of magnetic bead slurry to a clean 1.5-mL tube (10 μL of magnetic beads per 1 μg of antibody should be enough).

Note: Beads have a high binding capacity, increasing the bead volume could lead to a higher background binding with limited increase in specific protein purification.

Note: Resuspend magnetic beads by rocking the vial. Avoid vortexing the beads.

Alternatives: Agarose beads may also be used. Different handling procedures may apply.

    b. Add 1 mL of 100 mM NaCl Lysis Buffer to the beads.
    c. Rotate for 5 min at 4°C.
    d. Place the tube on the DynaMag™-2 magnet to separate the beads from the buffer and invert 3–4 times to facilitate their adherence to the magnet and the collection of the beads located at the tube cap.
    e. Carefully remove the supernatant (Figure 4). Avoid disrupting the magnetics beads with the micropipette tip.

    f. Repeat the wash steps (b–e) twice more.
    g. Resuspend the beads with 1 volume of 100 mM NaCl Lysis Buffer per volume of beads.
15. Add the beads to the RNA LoBind Eppendorf containing immunocomplexes from step 13.

Note: To avoid disrupting protein binding when pipetting Dynabeads™ due to mechanical forces exerted by flowing the beads solution thought the tip, cut a 1,000 μL pipette tip around 1–2 mm upstream the tip end.

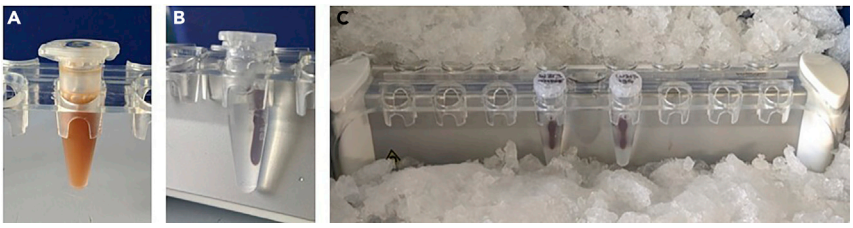

Figure 4. Magnetic Beads Handling

(A) Magnetic beads in suspension after rotating 5 min at 4°C.

(B) Magnetic beads adhere to the side of the tube facing the magnet after 3–5 min of letting the tube sit. This enables an easy removal of the supernatant.

(C) Samples in the magnet should be processed on ice to prevent protein and RNA degradation.

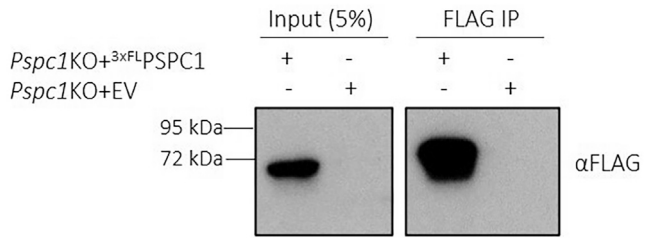

Figure 5. Validation of the Immunoprecipitation of ^{3xFL}PSPC1 with Anti-FLAG Antibody by Western Blotting

Pspc1 KO cells rescued with an empty vector (EV) control were used as negative control. The percentage of input is shown.

16. Mix by slow rotation (i.e., 15–30 rpm) on a rotamer for 2–4 h at 4°C.
17. Place the tube on the DynaMag™-2 magnet. Allow the beads to bind the magnet and the solution to become totally clear (Figure 4B).
18. Remove the supernatant.

⚠ CRITICAL: Store samples on ice during this processing step to avoid sample degradation (Figure 4C).

Note: the supernatant can be kept at −80°C to analyze by Western Blot. This fraction should be depleted of the immunoprecipitated protein.

19. Wash the beads.
    a. Add 1 mL of 100 mM NaCl Lysis Buffer with protease inhibitors.
    b. Rotate 5 min at 4°C.
    c. Place the tube on the DynaMag™-2 magnet to separate beads from the solution.
    d. When the solution becomes clear, carefully remove the supernatant using a micropipette.
    e. Repeat wash steps (a–d) five times.
20. Resuspend the beads in 500 μL of RIP Buffer with freshly added proteases and RNase inhibitors.
21. Add total RNA purified in step 15 of the "Before You Begin" section to bead-protein complexes and incubate by slow rotation for 45 min at 20°C–25°C.

Note: Whereas to facilitate the visualization of the interacting RNAs in an agarose gel higher amounts of total RNA (i.e., up to 100 μg) should be used in this step, small amount of total RNA (i.e., up to 1 μg) might be sufficient for detection by RT-qPCR (see "RNA detection by RT-qPCR" section).

Note: The amount of RNA required for assessing the interaction is highly dependent on the RBP-binding affinity and specificity and it might need to be optimized.

22. Place the tube on the DynaMag™-2 magnet. Allow the beads to bind the magnet and the solution to become totally clear.
23. Remove the supernatant.
24. Wash beads with protein-RNA complexes.
    a. Add 1 mL of RIP Buffer with protease inhibitors.
    b. Rotate 5 min at 4°C.
    c. Place the tube on the DynaMag™-2 magnet to separate beads from the solution.
    d. Carefully remove the supernatant using a micropipette.
    e. Repeat wash steps (a–d) five times.
25. Resuspend the complexes in 50 μL of RNase-free water and transfer to a new 1.5-mL tube.

Note: Save 5 μL of the immunobound complexes to validate the immunoprecipitation by Western Blot (Figure 5). Add 10 μL of Milli-Q water and 5 μL 4× Laemmli buffer and boil the samples for 5 min at 95°C. Store at −20°C until use.

26. Add 450 µL of TRIzol™ to the remaining 45 µL of beads containing the protein-RNA immunocomplexes. Mix well by vortexing 15 s to allow the elution of the RNA from the protein-antibody-bead complexes.

Note: The sample volume should not exceed 10% of the volume of TRIzol™ Reagent used for lysis.

Note: Process in a similar way 0.5%–1% of input RNA from step 15 of the "Before You Begin" section.

Pause Point: Samples can be stored at 4°C 12–16 h or at –20°C for up to a year.

Extraction of the IPed RNAs

Timing: 1.5 h

After eluting the immunocomplexes, perform RNA extraction from TRIzol™ reagent to isolate the co-immunoprecipitated (co-IPed) RNAs.

27. Co-IPed RNAs extraction.
    a. Incubate the samples at 20°C–25°C for 5 min.
    b. Add 0.2 mL of chloroform per mL of TRIzol™ reagent used in step 25.
    c. Securely cap the tube and shake vigorously or vortex briefly.
    d. Incubate 3 min at 20°C–25°C.
    e. Centrifuge samples at 12,000 × *g* for 15 min at 4°C.
    f. Angle the tube at 45° and pipette the upper aqueous solution to a new RNase-free Eppendorf.

Note: Avoid transferring any of the interphase or organic layer, which contain contaminants for downstream applications such as genomic DNA and proteins respectively.

    g. Add 0.5–1 µL of RNase-free glycogen (1 mg/mL stock).
    h. Add 0.5 mL of isopropanol per mL of TRIzol™ reagent used in step 26.
    i. Mix by inversion 10 times.
    j. Incubate the samples 10 min at 20°C–25°C.
    k. Centrifuge samples at 12,000 × *g* for 10 min at 4°C.
    l. Discard supernatant with a pipette.
    m. Add 1 mL of pre-chilled ethanol 75% per mL of TRIzol™ reagent used in step 26.
    n. Vortex briefly and centrifuge at 7,500 × *g* for 5 min at 4°C.
    o. Carefully discard all supernatant with a micropipette.
    p. Allow the pellet to air dry 5 min at 20°C–25°C.
    q. Resuspend in 20 µL of RNase-free water.
    r. Quantify IPed RNAs using Qubit RNA HS Assay Kit following manufacturer's protocol.

Alternatives: RNA concentration and quality can be assessed by Nanodrop, although its sensitivity is much lower and some carry-on contaminants may interfere with an accurate measurement.

RNA Visualization in an Agarose Gel

Timing: 2.5 h

This step describes the visualization of the IPed RNA using agarose gel electrophoresis.

28. Prepare the agarose gel.
    a. Prepare TAE 1× in DEPC-treated Milli-Q and filter it with a 0.22 μm filter to avoid the appearance of spots in the gel that may interfere with the visualization of the bands.
    b. Prepare a 1% agarose gel with the filtered TAE 1×.
    c. Clean gel bed and combs with tissue wipes that do not leave any fibers behind (e.g., VWR ® Light-Duty Tissue Wipers).
29. Treat half of the volume to be visualized in a gel with RNase A.
    a. Incubate the RNA (≤1 μg) with 25 U of RNase A (DNase and protease-free) for 20 min at 37°C.
    b. Keep on ice.

Note: At low salt concentrations (0 - 100 mM NaCl), RNase A cleaves single-stranded and double-stranded RNA as well the RNA strand in RNA-DNA hybrids. Use filter tips to avoid cross-contaminations, as RNases are very stable and not easy to inactivate.

30. Load the IPed samples (±RNase A) using an Orange G-based loading dye and run the gel for 15 min with 80 V.

⚠ CRITICAL: Do not use a Bromophenol blue-containing tracking dye as it migrates with the 370 bp bands in TAE and may interfere with the visualization of your bands.

Note: Load a small volume of an appropriate ladder two-three wells apart from the samples, so its signal does not interfere with the fainter RNA signal during the visualization. This also applies to the input RNA sample used in the IP.

31. Post-staining of the gel.
    a. Prepare a post-staining solution for the gel by adding 45 μL of ethidium bromide per 50 mL of filtered TAE
    b. Incubate the gel in the post-staining solution for 1 h at 20°C–25°C.

Caution: Ethidium bromide is an intercalant agent used to visualize nucleic acids under UV light exposition. It is toxic by inhalation and causes mutagenic and carcinogenic effects. This compound should be handled with gloves and face mask, especially during powder reconstitution. Dispense the post-staining solution in an appropriated recipient.

32. Visualize the gel in a UV transilluminator.

Note: Staining of RNA with ethidium bromide is less sensitive than dsDNA, requiring 10 times more nucleic acid for equivalent detection. The minimum RNA amount that can be detected is ≈30 ng per lane.

Alternatives: SYBR® Gold Nucleic Acid Gel Stain or other nuclei acids stains can be used to improve detection sensitivity.

RNA Detection by RT-qPCR

Timing: 4.5 h

Prepare cDNA from the co-IPed RNA sample and quantify the specific transcript abundance by real-time quantitative PCR (RT-qPCR).

33. Perform first strand cDNA synthesis from the IPed RNA obtained in step 27.

a. Set up the reaction by mixing 4 μL of 5× qScript cDNA SuperMix with the RNA (10 pg to 1 μg) and bring to 20 μL with RNase-free Milli-Q water.

Note: Input RNA should also be included for cDNA preparation. 1%–5% of total RNA purified in step 27 should be used.

b. Briefly vortex and spin down the samples.
c. Incubate: 5 min at 25°C, 30 min at 42°C and 5 min at 85°C.

Alternatives: qSCRIPT™ is a Single Step retrotranscription system which includes Oligo (dT) and random primers. Other high sensitivity retrotranscriptases can be used.

Pause Point: cDNA can be stored at −20°C until use.

Set up the RT-qPCR reaction. Plan the RT-qPCR plate for determining the abundance of a target of interest among the IPed RNA. Use triplicates for each sample (i.e., input, mock IP and IP) and gene (e.g., at least one negative control not bound by your protein of interest should be included).

34. Dilute the cDNA 1/10–1/100 for RT-qPCR.
35. Prepare a mix for each primer pair containing SYBR™ Green and the primers as follows.

	1×	10×
SYBR™ Green 2X Master Mix	7.5 μL	75 μL
Primer mix (10 μM each)	1 μL	10 μL
Milli-Q water	2.5 μL	25 μL
Total	11 μL	110 μL

36. Mix thoroughly and centrifuge briefly to spin down the contents and eliminate any air bubbles.
37. Transfer 11 μL of the master mix to the corresponding well of the 96-well plate using an electronic pipette.

Alternatives: If no electronic pipette is available, samples can be loaded with regular pipettes, although this can result in lower reproducibility between technical replicates.

38. Add 4 μL of the diluted cDNA to the appropriate wells.
39. Seal the plate with an optical adhesive cover suitable for RT-qPCR detection.

⚠ CRITICAL: Securely seal the plate to avoid sample evaporation during cycling.

40. Briefly centrifuge the plate to spin down contents and eliminate air bubbles.
41. Introduce the plate in the Thermocycler and set the appropriate program (an example is shown in Table 2).

Note: RNA target enrichment among the RBP-interactome can be calculated from RT-qPCR data following the pipeline explained in Quantification and Statistical Analysis section.

EXPECTED OUTCOMES

A successful iv-RIP of an RNA-binding protein should show (1) a good immunoprecipitation of the protein subjected to IP and (2) presence of RNA visualized by gel, which should disappear after RNase treatment. If the researcher is testing the RNA-binding ability of a novel RBP, a known RNA-binding protein for which a good IP-grade antibody is available should be subjected to iv-RIP in parallel, as a positive control. It is critical to perform quality checks on the specificity of the

Table 2. Example of Thermo Cycler Protocol for Quantitative PCR

Step	Temperature	Time	Brief Step Description
1	98°C	10 min	Initial denaturation
2	95°C	15 s	Denaturation (Cycling stage)
3	55°C	15 s	Primer annealing (Cycling stage)
4	72°C	40 s	Extension (Cycling stage) + Measure fluorescence
5	Go to step 2, 39× more times		
6	95°C	5 s	Melting curve + Measure fluorescence
7	65°C	1 min	
8	Melting curve: 65°C to 97°C increment +5°C		
9	97°C	15 s	
10	4°C	Hold	

antibody and the identity of the visualized nucleic acids (i.e., DNA vs RNA). As a negative control, the RBP knockout (KO) cell line should be incubated with the same antibody used for the immunoprecipitation. As an alternative, the lysate can be incubated with the non-immune IgG fraction from the same species in which the antibody was made, as a measure for the specificity of a binding event relative to background.

An example is shown for PSPC1 immunoprecipitation in a *Pspc1* CRISPR-cas9 knockout (*Pspc1*KO) background rescued with a construct overexpressing a 3xFLAG-tagged PSPC1 or an empty vector control (Figure 5).

Direct binding of PSPC1 to RNA was visualized by gel (Figure 6). Depending on the input RNA used and the RBP-binding preference, a different band pattern can be observed. Indirect interactions can also be tested by iv-RIP and confirmed by knocking out the candidate RBP partner suspected to mediate the interaction, as we previously described (Guallar et al., 2018).

If some target RNAs are predicted to be bound by the RBP, the binding level can be validated by RT-qPCR. As an example, MDA5 binding to several ADAR1-modified versus unmodified targets is shown in Figure 7. Immunopurified MDA5 protein from whole cell extracts was incubated with total RNA from *Adar1* wild-type (WT) or KO cells. It is crucial to include an input sample to normalize background abundance to increase the confidence of the immunoprecipitated RNA. As previously predicted, we were able to validate a higher enrichment of unedited RNAs (from *Adar1* KO cells) (e.g., *Sppl2a*, *Cds2*, *Gla*) among MDA5-interacting RNAs compared to edited ones (from *Adar1* WT cells).

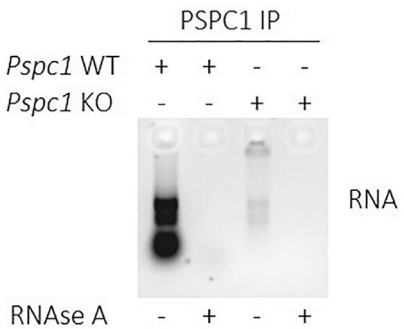

Figure 6. Agarose Gel Analysis of RNA Bound by PSPC1 Protein Complexes in Wild-Type (WT) and *Pspc1* Knockout (KO) ESCs
IPed RNA was incubated with RNase A to validate the identity of the visualized nucleic acids.

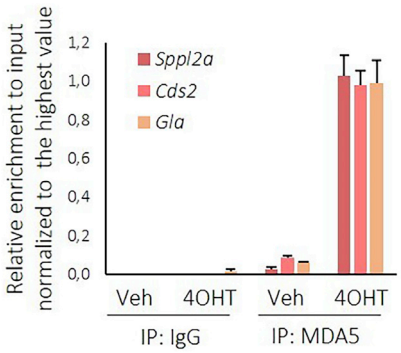

Figure 7. Determination by RT-qPCR of the Binding of the RBP of Interest to Specific RNA Targets

MDA5 or IgG immunoprecipitation was performed and immunocomplexes were incubated with total RNA from wild-type (*Adar1*$^{FI/FI}$+Veh) or *Adar1* knockout (*Adar1*$^{FI/FI}$+4OHT) fibroblasts at day 7 of reprogramming with OSKM. Relative enrichment of MDA5 binding is shown compared to control RNA (*Adar1*$^{FI/FI}$+Veh) and normalized to highest value. Data are shown as mean ± SEM.

Other Applications

For high-throughput analysis (i.e., RNA-seq) proceed to RNA fragmentation as needed. Fragmentation of RNA can be performed using NEBNext Magnesium RNA Fragmentation Module kit, after optimization. The incubation time of RNA with the Fragmentation buffer is optimized to ensure proper fragment length to ensure that the resulting RNA fragments are suitable for library preparation.

QUANTIFICATION AND STATISTICAL ANALYSIS

The binding of a protein to a target RNA of interest is determined by RT-qPCR and calculated with the Percent Input Method. In brief, signals obtained from the iv-RIP are divided by signals obtained from an input RNA sample. This input sample represents the amount of RNA used in the iv-RIP. Table 3 represents an example of Ct values obtained after iv-RIP of an RBP of interest and RT-qPCR of the IPed RNAs.

Given that the RNA input used for RT-qPCR represents a fraction of the total RNA incubated with the RBP during immunoprecipitation, a corrected Ct value for the input must be calculated, to calculate the estimated Ct value that would have been obtained by RT-qPCR if total input RNA had been analyzed:

$$Ct_{corrected}=Ct-\log_2(1/dilution)$$

In the example shown in Table 3, a 5% of the RNA input (1/20 dilution) was analyzed by RT-qPCR:

$$Ct_{corrected}=Ct-\log_2(20)$$

Note: For accurate quantitation, adjust the amount of input RNA (0.1%–5%) so that the Ct values of both the input and the IP samples are within the values of the standard curve.

Note: Always include an RNA that is not bound by the RBP of interest as a background control for each RT-qPCR.

Table 3. Triplicate Measurements of the Presence of a Specific RNA in the Input, Negative IP Control and IP Sample Are Determined

Raw data									
	Input (5%)			Negative Control for IP (RBP IP in KO Cells or IgG IP in WT Cells)			RBP IP		
RNA 1 (not target)	Ct1a	Ct1b	Ct1c	Ct3a	Ct3b	Ct3c	Ct5a	Ct5b	Ct5c
RNA 2 (target of protein X)	Ct2a	Ct2b	Ct2c	Ct4a	Ct4b	Ct4c	Ct6a	Ct6b	Ct6c

Ct means threshold cycles of RT-qPCR.

Then calculate the average $Ct_{corrected}$ for each gene ($\overline{Ctx}$) in the input and the ΔCt for each RNA in the immunoprecipitated samples as indicated in Table 4.

Then, calculate the average of ΔCt values for each target RNA and sample (i.e., negative control and RBP IP). Finally, calculate the percent input immunoprecipitated which represents the percentage of the target RNA from the total RNA present in the input which is recovered after *in vitro* incubation with the protein:

$$\text{Percentage of target RNA immunoprecipitated} = (2^{-\Delta Ct} \times 100)$$

LIMITATIONS

In contrast to other methods that include a cross-linking step that stabilizes the protein-RNA interactions occurring *in vivo*, iv-RIP is performed under native conditions, which may limit the detection to only stable protein-RNA interactions. Moreover, given that low stringent washing is performed in order to maintain potential indirect RNA-binding interactions it is critical to include negative controls that allow to detect false positive results. Optimally, KO tissues or cells lacking the protein that is subjected to immunoprecipitation should be used as negative controls. Alternatively, immunoprecipitation using an IgG control can be used to detect background RNA binding to the beads. Furthermore, multiple biological replicates should be analyzed given the intrinsic variability in RNA-RBP immunoprecipitation assays. On the other hand, purified RNAs are usually correlated with their abundance as demonstrated by the fact that ribosomal RNAs are the principal species contaminating protein purifications (Darnell, 2010). Together, these factors could lead to the masking of specific but low abundant interactions by non-specific highly abundant transcripts (Darnell, 2010).

TROUBLESHOOTING

Problem

Lower RNA yield than expected (related to RNA Isolation; Before You Begin Section)

Potential Solutions

To work with RNA instruments, tips, and tubes DNase and RNase free, should be used, as recommended in this protocol. Working material (gloves, benches, pipettes…) may be also cleaned with RNase inactivating solutions before its use to ensure work area and materials are RNase free.

- Make sure to establish RNase-free work conditions and there is no cross contamination with RNases.
- Avoid puncturing Phasemaker™ gel when pipetting the aqueous phase. Do not attempt to draw off the entire aqueous layer after phase separation.
- Be careful when washing the RNA pellet and decanting the supernatant because the pellet can detach from the wall of the tube very easily.
- Make sure RNA pellets are dried after wash step and before adding RNase-free water to avoid ethanol contamination but without letting them dry too long to avoid solubilization issues.
- Make sure pellet is completely solubilized pipetting the sample repeatedly and heat the sample to 50°C–60°C for 15 min.

Table 4. Explanation of How to Determine the ΔCt Values for Each Target RNA and Sample

ΔCt							
	Input (100%)	Negative control for IP (RBP IP in KO cells or IgG IP in WT cells)			RBP IP		
RNA 1 (not target)	$\overline{Ct1}$	Ct3a-$\overline{Ct1}$	Ct3b-$\overline{Ct1}$	Ct3c-$\overline{Ct1}$	Ct5a-$\overline{Ct1}$	Ct5b-$\overline{Ct1}$	Ct5c-$\overline{Ct1}$
RNA 2 (target of protein X)	$\overline{Ct2}$	Ct4a-$\overline{Ct2}$	Ct4b-$\overline{Ct2}$	Ct4c-$\overline{Ct2}$	Ct6a-$\overline{Ct2}$	Ct6b-$\overline{Ct2}$	Ct6c-$\overline{Ct2}$

Problem
Cell lysate is cloudy after sonication (related to Preparation of Whole Cell Protein Extracts; step 5)

Potential Solution
An efficient sonication is an essential step for a good cell lysis. During this process, when bubbles grow to sizes greater than their resonance size, sonication becomes ineffective at producing cavitation and can cause defects like scattering or bubble shielding. To avoid these problems, more suitable pulse times and intervals can be considered. It is also important maintaining the sample at 4°C at all times to avoid the increase of the temperature to prevent protein degradation.

- Ensure that the proper volume of Lysis Buffer is used.
- Increase sonication time.

Problem
Excessive foaming at sonication (related to Preparation of Whole Cell Protein Extracts; step 5)

Potential Solution

- Increase duration of OFF intervals at sonication.
- Make sure the probe is properly introduced into the buffer, avoiding touching the tube walls.
- Centrifuge the tube to remove the existent foam, as it will promote further foaming.

Problem
No detection of target proteins (related to Immunoprecipitation; step 25)

Potential Solution

- Check the expression profile of your protein of interest in your cells. If there is low level of target protein expression, increase the amount of lysate used or prepare the protein lysate from other cell types with higher expression of the protein of interest.
- Make sure that you are using the recommended amount of antibody. An optimization of the antibody amount may be required.
- Use 1.5-mL Protein-LoBind® tubes to avoid loss of protein immunocomplexes which can bind to the walls of the tube during immunoprecipitation.
- Magnetic beads settle to the bottom of the tube over time. Be sure to mix properly the magnetic beads before aliquoting the sample to check the immunoprecipitation by Western Blot.
- During immunocomplexes washes, wait for the complete binding of magnetic beads to the wall of the tube before removing supernatant, to avoid immunocomplexes aspiration.

Problem
Unspecific protein detection (related to Immunoprecipitation; step 25)

Potential Solution

- More stringent wash conditions could be used if the RBP-antibody affinity is strong.
- Sufficient washing steps are critical to ensure efficient removal of non-specific RNA-protein complexes.

Problem
Lack of IP-grade antibody for immunoprecipitation (related to Immunoprecipitation; step 25)

Potential Solution

Proteins for which there is no validated IP-grade antibody can be purified by constructing a vector for overexpression of a N- or C-terminal peptide tagged (e.g., FLAG, HA…)-protein or through introducing the tag by CRISPR/cas9 genome modification of the endogenous locus. A control IP with the anti-tag antibody can be used in the WT cell line in parallel, to provide a specificity metric for the IP. Tag size and location (N- or C-terminus) can have an impact in protein folding and/or function, and should be taken into consideration.

Problem

Genomic DNA contamination (related to RNA Visualization in an Agarose Gel; step 32)

Potential Solution

Treat the RNA sample with DNase before immunocomplexes formation and RNA incubation to eliminate this possibility, as indicated in step 15 in Before You Begin section.

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Diana Guallar (diana.guallar@usc.es).

Materials Availability

No materials were generated in this study.

Data and Code Availability

No data or code were generated in this study.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.W, M.F., and D.G.; Methodology, A.F.-I., V.G.-O., J.A.P., M.F., and D.G.; Writing, A.F.-I., V.G.-O., J.A.P., M.F., and D.G.; Supervision and Funding Acquisition, J.W., M.F., and D.G.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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