

Video Article

# Desthiobiotin-Streptavidin-Affinity Mediated Purification of RNA-Interacting Proteins in Mesothelioma Cells

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## Abstract

The *in vitro* RNA-pulldown is still largely used in the first steps of protocols aimed at identifying RNA-binding proteins that recognize specific RNA structures and motifs. In this RNA-pulldown protocol, commercially synthesized RNA probes are labeled with a modified form of biotin, desthiobiotin, at the 3' terminus of the RNA strand, which reversibly binds to streptavidin and thus allows elution of proteins under more physiological conditions. The RNA-desthiobiotin is immobilized through interaction with streptavidin on magnetic beads, which are used to pull down proteins that specifically interact with the RNA of interest. Non-denatured and active proteins from the cytosolic fraction of mesothelioma cells are used as the source of proteins. The method described here can be applied to detect the interaction between known RNA binding proteins and a 25-nucleotide (nt) long RNA probe containing a sequence of interest. This is useful to complete the functional characterization of stabilizing or destabilizing elements present in RNA molecules achieved using a reporter vector assay.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/57516/>

## Introduction

Gene expression and the final level of the gene product can be tightly regulated by affecting the mRNA stability and mRNA translation rate<sup>1</sup>. These post-transcriptional regulatory mechanisms are exerted through the interactions of non-coding RNA and/or RNA-binding proteins (RBPs) with targeted mRNA. It is usually the 3' untranslated region of mRNA (3' UTR - belonging to the non-coding portion of the genome<sup>2</sup>) that contains specific *cis*-regulatory elements (CRE), which are recognized by *trans*-acting factors such as miRNA or RBPs<sup>3</sup>. The best-studied *cis*-element within the 3' UTR, is the adenine-rich element (ARE) motif, which is recognized by specific AU-binding proteins (AUBP), and, in turn, induces either mRNA degradation/deadenylation (ARE-mediated decay) or mRNA stabilization<sup>4</sup>.

The size of the 3' UTR of calretinin mRNA (CALB2) is 573 bp long and contains a putative AUUUA pentamer, as predicted by AREsite2, a bioinformatic tool<sup>5</sup>. Consistent with the presence of a putative ARE motif, the pmirGLO vector-reporter assay demonstrated a stabilization role of this element within CALB2 mRNA<sup>6</sup>. Finally, the *in vitro* RNA-pulldown was used to identify the AUBP that stabilizes calretinin mRNA through the ARE motif.

Since all non-coding RNAs interact with proteins<sup>7</sup>, the *in vitro* RNA-pulldown is a good way and first-of-choice assay for identifying RNA-interactors to aid in deciphering molecular mechanisms. In this RNA-pulldown method, commercially synthesized RNA probes, which were labeled with a modified form of biotin (desthiobiotin) at the 3' terminus of the RNA strand, were used. The RNA-desthiobiotin is immobilized through interaction with streptavidin on magnetic beads, which are used to pull down proteins that specifically interact with the bound-RNA of interest. Non-denatured and active proteins from the cytosolic fraction of mesothelioma cells are used as the source of proteins. Such RNA-bound proteins are eluted from the magnetic beads, run through a 12% SDS-PAGE gel, transferred to a membrane, and probed with different antibodies.

In the standard streptavidin-biotin affinity purification procedure, harsh denaturation conditions are required to disrupt the strong irreversible biotin-streptavidin bond to elute the bound proteins<sup>8</sup>, which could lead to the dissociation of protein complexes. Unlike biotin, desthiobiotin reversibly binds to streptavidin and is competitively displaced with a buffered solution of biotin, allowing for the gentle elution of proteins, and avoiding the isolation of naturally biotinylated molecules<sup>9</sup>, suggesting that the technique is ideal for isolating native protein complexes under native conditions.

*In vitro* binding conditions and stringency, which are determined by salt concentration, reducing agents and detergent percentage, should be close to those present in the cellular context in order to identify true *in vivo* interactions. The binding conditions implemented herein have been previously demonstrated as appropriate for the identification of the HuR as an AU-binding protein<sup>10</sup>. This approach could save time, since optimization of proper binding conditions can be time-consuming and challenging. In addition, this method could be used as a starting protocol for any RNA-pulldown experiment and can be gradually optimized by changing the concentration of salts and detergents, changing the glycerol

percentage, and adding other salts. Moreover, we demonstrated that even a short 25-nucleotide RNA-probe harboring a pentamer ARE-motif could be used to demonstrate interaction with a specific AUBP.

## Protocol

### 1. Preparation of Cytosolic and Nuclear Protein Fraction

1. Plate  $4 \times 10^6$  ACC-MESO-4 cells in a T150 cell culture flask grown in RPMI - 1640 medium supplemented with 10% FBS, 1x penicillin/streptomycin (100x), and 2 mM L-Glutamine (200 mM). When cells reach a confluence of 80-90% ( $\sim 5-5.5 \times 10^6$  cells), proceed with protein extraction of nuclear and cytosolic fraction.  
NOTE: ACC-MESO-4 cell line was obtained from the RIKEN BioResource Centre<sup>11</sup>.
2. Aspirate medium, wash the cells by adding 15 mL of 1x PBS, tilt the plate gently a few times and aspirate the 1x PBS. Add 3 mL of 0.25% Trypsin-EDTA and incubate the culture flask at 37 °C for 3 min.  
NOTE: Look at the cells under the microscope for detachment; if still attached tap the flask against the palm of a hand 3 times.
3. Add 7 mL of RPMI-1640 medium supplemented with 10% FBS, 1x penicillin/streptomycin (100x), and 2 mM L-Glutamine (200 mM), collect the cells in a 15 mL tube, and spin down at 200 x g for 5 min.
4. Aspirate medium and resuspend cell pellet with 1.5 mL of 1x PBS, then transfer the cells into a 1.5 mL microcentrifuge tube. Spin down at 500 x g and 4 °C for 5 min. Discard the supernatant.  
NOTE: From this step onwards, perform all centrifugation steps at 4 °C and keep all reagents on ice.
5. Resuspend the cell pellet with ice-cold 1.5 mL of 1x PBS and spin down the cells at 500 x g and 4 °C for 3 min. Discard the supernatant.  
NOTE: The required amounts of CER I, CER II, and NER reagents are 10, 0.55, and 5 volumes of estimated cell pellet packed volume, respectively; the following protocol assumes a volume of 20  $\mu$ L. We recommend preparing only the required amount of reagent CER I.
6. Add 200  $\mu$ L of ice-cold cytoplasmic extraction reagent (CER I) supplemented with 2  $\mu$ L of 1x proteinase inhibitor, e.g., dilute 1 tablet of Protease Inhibitor (EDTA-free) in 500  $\mu$ L of water to obtain 100x stock. This amount of reagent CER I is enough to lyse 20  $\mu$ L of a cell pellet packed volume.
  1. To estimate the cell pellet packed volume, compare the tube containing the cell pellet with a 1.5 mL tube filled with 10, 20, 50 or 100  $\mu$ L of 1x PBS.
7. Resuspend the cell pellet by vigorously vortexing the tube for 15 s and incubate the tube on ice for 10 min. Add 11  $\mu$ L of ice-cold cytoplasmic extraction reagent II (CER II) to the tube, vigorously vortex for 5 s, and incubate 1 min on ice.
8. Vortex vigorously for 5 s and centrifuge the tube at 16,000 x g and 4 °C for 5 min. Transfer the supernatant to the clean pre-chilled tube on ice. The supernatant is the cytoplasmic fraction that is further used in the RNA-pulldown experiment.
9. Resuspend the remaining pellet in 100  $\mu$ L of ice-cold nuclear extraction reagent (NER) supplemented with 1  $\mu$ L of proteinase inhibitor (100x) and vigorously vortex for 15 s. We recommend preparing only the required amount of NER reagent supplemented with protease inhibitors.
10. Incubate the sample on ice for 40 min with occasional vortexing for 15 s (every 10 min).
11. Centrifuge the tube at 16,000 x g for 10 min. Transfer the supernatant (this is the nuclear protein fraction) to a clean pre-chilled tube.
12. Immediately proceed by measuring protein concentration using the bicinchoninic (BCA) method (see **Table of Materials**). To control for protein purity of each fraction, perform immunoblotting (**Figure 1**) against  $\alpha$ -tubulin (positive detection only in the cytosolic fraction) and Poly ADP-ribose polymerase - PARP (positive detection only in the nuclear fraction). For protein visualization, refer to section 4.
13. In order to keep protein activity and their native state, prepare 25  $\mu$ L of aliquots of cytosolic and nuclear extracts. Snap-freeze these aliquots by placing them in liquid nitrogen for 5 s and store directly at -80 °C.

### 2. Labeling of RNA with Desthiobiotin

1. Have RNA probes commercially synthesized and HPLC purified. Resuspend with nuclease-free water at 10  $\mu$ M.  
NOTE: Probe sequences are listed in **Table 1**.
2. Use 50 pmol of RNA per RNA-pulldown reaction. Thaw and keep all reagents on ice except for PEG 30%, used to label 3' terminus of RNA oligo.
3. Transfer 5  $\mu$ L of 10  $\mu$ M RNA probes, labeled as CALB2 3' UTR (ARE), CALB2 3' UTR (mtARE), and Unrelated-RNA (IRE), into 0.5 mL thin-wall microcentrifuge tubes and incubate in a PCR machine at 85 °C for 5 min. Place the tubes immediately on ice.  
NOTE: This step is important, as it promotes the relaxation and accessibility of the RNA probe for labeling.
4. For a single 30  $\mu$ L reaction, add to the RNA-containing tube the following 10  $\mu$ L mix: 3  $\mu$ L of nuclease free water, 3  $\mu$ L of 10x RNA Ligase Reaction Buffer, 1  $\mu$ L of RNase Inhibitor (40 U/ $\mu$ L), 1  $\mu$ L of Desthiobiotinylated Cytidine Bisphosphate (1 mM), and 2  $\mu$ L of T4 RNA ligase (20 U/ $\mu$ L).  
NOTE: To prepare master mix A for 3 samples in excess (3.2), mix 9.6  $\mu$ L of nuclease free water, 9.6  $\mu$ L of 10x RNA Ligase Reaction Buffer, 3.2  $\mu$ L of RNase Inhibitor (40 U/ $\mu$ L), 3.2  $\mu$ L of Desthiobiotinylated Cytidine Bisphosphate (1 mM), and 6.4  $\mu$ L of T4 RNA ligase (20 U/ $\mu$ L).
5. Add carefully 15  $\mu$ L of PEG 30% to the reaction. Use another tip to mix the reaction. Incubate the reaction overnight at 16 °C.
6. The next day, prepare the following: 5 M NaCl (fresh/nuclease-free), chloroform: isoamyl alcohol in 24:1 ratio (e.g., per reaction - 96  $\mu$ L chloroform and 4  $\mu$ L of isoamyl alcohol), ice-cold 100% ethanol, and ice-cold 70% ethanol.
7. Add 70  $\mu$ L of nuclease-free water to the RNA-labeling reaction tubes. Add 100  $\mu$ L of chloroform: isoamyl alcohol and vortex briefly. Spin-down at 13,000 x g for 3 min. Carefully remove ONLY the upper phase and transfer to a new nuclease-free 1.5 mL tube; avoid touching the lower phase.
8. Add 10  $\mu$ L of 5 M NaCl, 1  $\mu$ L of glycogen and 300  $\mu$ L of ice-cold 100% ethanol. Place the tube at -20 °C for 2 h.  
NOTE: At this step, the experiment can be continued on the following day.
9. Centrifuge at 13,000 x g and 4 °C for 15 min. Carefully discard the supernatant without disturbing the pellet. Add 300  $\mu$ L of ice-cold 70% ethanol and centrifuge again at 13,000 x g and 4 °C for 5 min.
10. Discard the supernatant completely and air-dry the pellet (15 min). Resuspend the pellet in 20  $\mu$ L of nuclease-free water.  
NOTE: Proceed with RNA-pulldown on the same day.

11. Incubate the RNA at 90 °C for 2 min and place on ice. Place the labelled-RNA on ice during the following step of pre-washing of the streptavidin magnetic beads.

NOTE: A longer incubation time may damage the RNA probe.

### 3. RNA-Protein Pulldown

1. **Pre-washing streptavidin-magnetic beads and incubation with desthiobiotin-RNA**
  1. Use 50  $\mu$ L of streptavidin magnetic beads per 50 pmol RNA. However, this should be optimized depending on the experiment.
  2. Vortex the tube with streptavidin-magnetic beads for 15 s, and quickly remove 200  $\mu$ L (master mix; enough for 3+1 reactions) into a clean 1.5 mL safe-lock tube using cut pipette tips. Place the tube on a magnetic stand so that the beads collect at the side of the tube and wait 1 min. Remove the resuspension liquid.
  3. To wash the beads, remove the tube from the magnetic stand, add 400  $\mu$ L of 0.1 M NaOH, 0.05 M NaCl solution, and gently pipet up and down several times. Place the tube back on the magnetic stand. Wait 1 min and collect the supernatant. Repeat this step again.
  4. Wash the beads with 200  $\mu$ L of 100 mM NaCl. Remove the supernatant.
  5. Add 200  $\mu$ L of 20 mM Tris (pH 7.5), resuspend beads by pipetting, place the tube on the magnetic stand, wait for 1 min and remove the supernatant. Repeat the step.
  6. Remove the tube from the magnetic stand, add 200  $\mu$ L of 1x RNA Capture buffer, and resuspend streptavidin magnetic beads by briefly vortexing. Remove 50  $\mu$ L of streptavidin magnetic beads and add to each labeled-RNA tube using a cut pipette tip. Incubate the tubes for 30 min at room temperature on a roller.
2. **Binding of protein to RNA**
  1. Place the tubes into a magnetic stand, wait 1 min, and remove the supernatant.
  2. Add 50  $\mu$ L of 20 mM Tris (pH 7.5) to the beads and resuspend by pipetting. Place the tubes into a magnetic stand, wait 1 min, and remove supernatant. Repeat this step.
  3. Add 100  $\mu$ L of 1x Protein-RNA binding buffer to the beads and resuspend by pipetting.
  4. In the meantime, prepare the following mix: 10  $\mu$ L of 10x Protein-RNA binding buffer, 30  $\mu$ L of 50% glycerol, 50  $\mu$ g of cytoplasmic proteins, and nuclease-free water up to 100  $\mu$ L.  
NOTE: Prepare master mix B for 3 samples in excess (3.3) as follows: 33  $\mu$ L of 10x Protein-RNA binding buffer, 99  $\mu$ L of 50% glycerol, 165  $\mu$ g of cytoplasmic proteins and nuclease-free water up to 330  $\mu$ L. Keep the tube of master mix B on ice.
  5. Place the tubes into magnetic stand, wait 1 min and collect the supernatant. Add 100  $\mu$ L of master mix B, resuspend by gently pipetting up and down, and avoid creating bubbles. Incubate reaction tubes for 60 min at 4 °C on a roller.
3. **Washing and elution**
  1. Place the tubes into a magnetic stand, collect the supernatant (which is flow through - FT), and transfer into a new tube.  
NOTE: Keep this FT on ice for later analysis.
  2. Pipette 100  $\mu$ L of 1x Wash buffer on the beads, resuspend gently, put back into the magnetic stand, wait 1 min, and discard the supernatant. Repeat this step 2 times.
  3. Add 40  $\mu$ L of Elution Buffer to the magnetic beads, mix by pipetting up and down, and incubate at 37 °C on a tube shaker (950 rpm) for 30 min.
  4. Place the tubes into a magnetic stand, wait 1 min, and collect eluted sample (which is eluate - E). Place on ice and use for downstream analysis.  
NOTE: At this step, each tested RNA probe consists of a flow-through (FT) and eluate (E) sample.

### 4. Polyacrylamide Electrophoresis and Western Blot Analysis

NOTE: See Table 2 for recipes for the buffers used in this section. Perform a Western blot according to the Laemmli method<sup>12</sup>.

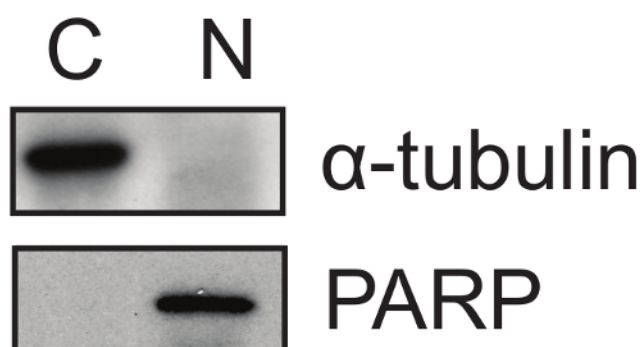
1. Handcast 12% SDS-PAGE gels (10-wells, 1.5 mm spacer, 66  $\mu$ L). Prepare a running gel mix: 4 mL of 30% acrylamide-bisacrylamide stock solution, 2.4 mL of Lower Tris buffer, 3.2 mL of dH<sub>2</sub>O, 9.6  $\mu$ L of TEMED, 96  $\mu$ L of 10% ammonium persulfate solution (APS). Prepare stacking gel mix: 3.25 mL of dH<sub>2</sub>O, 0.5 mL of 30% acrylamide - bisacrylamide stock solution, 1.3 mL of Upper Tris buffer, 10  $\mu$ L of TEMED, 20  $\mu$ L of 10% APS.
2. Add 16.6  $\mu$ L of 6x Laemmli buffer (e.g., 14 mL of Tris/HCl/SDS pH 6.8 (Upper Tris buffer), 2 g of SDS, 1.86 g of DTT, 6 mL of glycerol, and 0.012% bromophenol blue; stored at -20 °C) into the FT sample, and 6.6  $\mu$ L of 6x Laemmli buffer into E sample. Incubate samples for 5 min at 95 °C.
3. Load the samples (20  $\mu$ L of FT and whole Elute ~40  $\mu$ L) and run them for 30 min at 60 V, continuing with 20 V overnight.
4. Using a semi-dry electroblotting system, transfer proteins onto a PVDF membrane for 80 min at 15 V.  
NOTE: The PVDF membrane must be activated before performing protein transfer. Incubate the membrane in 100% methanol for 1 min, followed by a 2 min incubation in ultrapure water.
5. After the protein transfer, incubate the SDS-PAGE gel with Coomassie solution for 3 h, followed by three steps of washing with ultrapure water, each 30 min.
6. At the end of protein transfer, let the PVDF membrane dry for 1 h. Reactivate the membrane as indicated in step 4.5. Block the membrane 1 h with 5% BSA in 1x TTBS.
7. Incubate the membrane with the first antibody: HuR or mesothelin, both diluted 1:1000 in 5% BSA in 1x TTBS for 4h at room temperature or overnight at 4 °C.
8. Wash the membrane three times for 7 min in 1x TTBS, and incubate with rabbit anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody diluted 1:10,000 in 5% BSA, 1x TTBS.
9. Visualize proteins using enhanced chemiluminescence and a digital imager.

## Representative Results

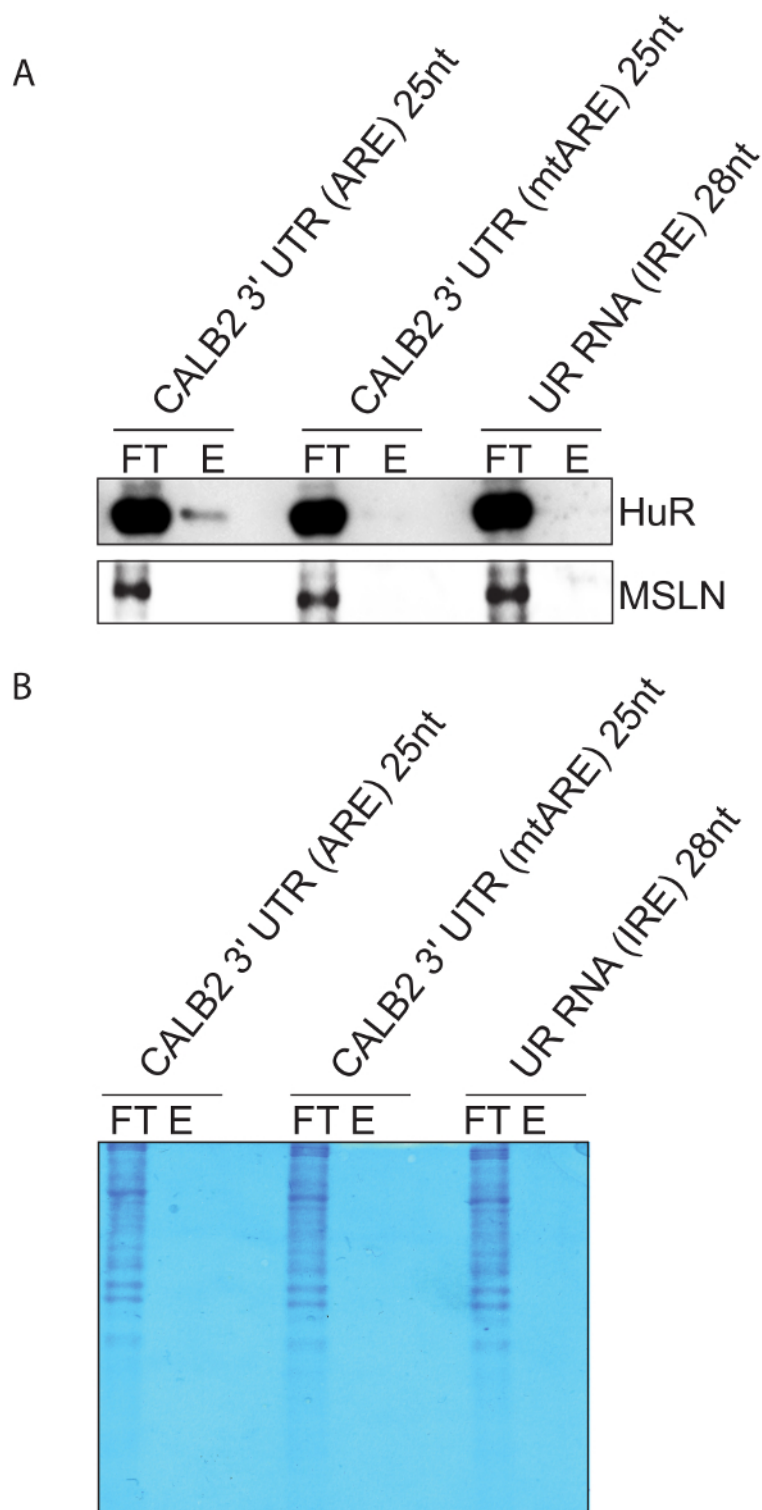
In this experiment, a 25-nt long fragment of calretinin 3' UTR harboring ARE motif (CALB2 3' UTR (ARE) 25-nt) was used to test whether it binds specifically to the Human-antigen R (HuR) protein, a known mRNA stabilizer. To test the specificity of the ARE element, a 25-nt RNA probe CALB2 3' UTR (mtARE) containing an ARE-motif mutation, which was previously shown to abolish the stabilization effect of the ARE motif, was used<sup>6</sup>. The third RNA probe represents the negative control, which is a 28-nt unrelated RNA that contains the well-defined iron-responsive element (Unrelated RNA (IRE)), known to bind cytosolic iron-responsive protein<sup>13,14</sup>. Since HuR is predominately localized within the nucleus but functions as a mRNA-stabilizer in the cytosol<sup>15</sup>, nuclear/cytosolic extraction was performed to obtain active proteins from the cytosol.

To demonstrate the purity of the nuclear/cytosolic fractions, proteins were analyzed by Western blot, which showed that  $\alpha$ -tubulin was only detected in the cytosolic fraction, whereas PARP protein was detected only in the nuclear fraction, as anticipated (**Figure 1**). The eluate from the CALB2 3' UTR ARE-probe demonstrated binding of HuR whereas HuR was absent in the eluate from the mutant probe CALB 3' UTR (mtARE). HuR was also absent in the eluate from the unrelated RNA probe which binds the iron-responsive protein (**Figure 2A**). To further demonstrate the specificity between the CALB 3' UTR (ARE) and HuR, the membrane was additionally probed with anti-mesothelin (MSLN) antibody, as this protein does not interact with RNA. Eluates from all three samples showed no presence of mesothelin. Taken together, this indicates that the stabilizing ARE motif within CALB2 3'UTR can specifically bind HuR protein.

Coomassie staining of the gel (**Figure 2B**) shows that equal amounts of proteins were used to incubate with the three different RNA probes. Because of the low amount of cytosolic extract used and transfer to the membrane, this staining did not allow for detection of proteins in the eluate (E) lanes, which were otherwise detected by Western blot analysis.



**Figure 1: Western blot analysis of 5  $\mu$ g of cytosolic and nuclear protein fraction.** PARP protein is present in the nuclear fraction (N) but absent from the cytosolic (C) fraction.  $\alpha$ -tubulin is present in the cytosolic fraction (C) but absent in the nuclear fraction (N). [Please click here to view a larger version of this figure.](#)



**Figure 2: Western blot shows that HuR is captured by 25-nt CALB2 3' UTR (ARE).** **A)** FT: flow through after incubation with RNA probe; E: proteins eluted upon incubation and binding to RNA probes. Probes: CALB2 3' UTR (mtARE) - 25-nt fragment of calretinin 3' UTR that contains 3 mutated nucleotides within the ARE motif; UR RNA (IRE) - 28-nt RNA probe with a well-defined iron-responsive element that binds the iron-responsive proteins. The membrane was further probed for mesothelin (MSLN), a protein that does not interact with RNA. **B)** Coomassie staining of the gel after protein transfer, demonstrating that equal amounts of proteins were incubated with RNA probes. [Please click here to view a larger version of this figure.](#)

RNA probe	Sequence	Concentration
CALB2 3' UTR (ARE) 25nt	UCGCUGUAUGAUUUAGGCUUCUAUG	10 $\mu$ M
CALB2 3' UTR (mtARE) 25nt	UCGCUGUAUGGUCUGGGCUUCUAUG	10 $\mu$ M
Unrelated RNA - IRE 28nt	UCCUGCUUCAACAGUGCUUGGACGGAAC	10 $\mu$ M

**Table 1: Sequences of RNA probes**

<b>Lower Tris buffer for running gel</b>	
1.5 M	Tris Base
0.40%	SDS solution
adjust pH to 8.8 using HCL (6 mol/L)	
fill up with	dH2O
<b>Upper Tris for stacking gel SDS-PAGE</b>	
500 mM	Tris base
0.4%	SDS solution
Adjust pH to 6.8 using HCL (6 mol/L)	
fill up with	dH2O
<b>10X Running buffer</b>	
250 mM	Tris base
1.92 M	Glycine
Adjust pH to 8.3 using HCL (6 mol/L)	
fill up with	dH2O
Filter or autoclave and store at 4°C	
<b>1X Running buffer</b>	
100 mL	10X Running buffer
0.05%	SDS solution
Fill up to 1 L with	dH2O
<b>10X Transfer Buffer</b>	
480 mM	Tris base
390 mM	Glycine
0.38%	SDS solution
fill up with	dH2O
Note: Filter 0.22 um and Store at 4 °C	
<b>1X Transfer buffer (for semi-dry system)</b>	
20 mL	10X Transfer Buffer
40 mL	methanol
fill up to 200 mL	dH2O
<b>10X TBS (Tris-buffered saline)</b>	
250 mM	Tris base
1.36 M	NaCl
27 mM	KCl
Adjust pH to 7.4	
fill up with	dH2O
Filter	
<b>1X TTBS (Tris-buffer saline with 0.1 Tween-20)</b>	
1X	10X TBS
0.10%	Tween-20
fill up with	dH2O
<b>RNA Capture Buffer (1X)</b>	
20 mM	Tris (pH 7.5)
1 M	NaCl
1 mM	EDTA



<b>Protein-RNA Binding Buffer (10x)</b>	
200 mM	Tris (pH 7.5)
500 mM	NaCl
20 mM	MgCl <sub>2</sub>
1%	Tween-20
<b>Wash Buffer (1x)</b>	
20 mM	Tris (pH 7.5)
10 mM	NaCl
0.1%	Tween-20
<b>Elution Buffer</b>	
4 mM	biotin
20 mM	Tris (pH 7.5)
50 mM	NaCl
<b>Coomassie solution</b>	
0.02%	Coomassie G-250
5%	Aluminiumsulfate-(14-18)-hydrate
10%	EtOH
2%	ortho-phosphoric acid

**Table 2: Recipes**

## Discussion

3' UTRs belong to the non-coding genome<sup>3</sup>, and all non-coding RNAs can interact with proteins in order to exert their function<sup>7</sup>. When the mammalian genome was found to be pervasively transcribed and produced a significant portion of long noncoding RNAs<sup>16</sup>, emerging evidences demonstrated that these long-noncoding RNAs function in regulating gene expression as they interact with chromatin-remodeling complexes<sup>17</sup>. This knowledge was gained by utilizing the RNA-pulldown assay. Thus, to start deciphering interactors of a specific RNA, the first step could be *in vitro* biochemical assays such as RNA-pulldown, as it is simple to perform.

Of note, not only sequence motifs but also the secondary structure of RNA plays a critical role in RNA functionality as they form domains essential for interacting with proteins. The secondary structure can vary in physiological and *in vitro* conditions, and RNA-pulldown may lead to the identification of falsepositives. For example, RNA-pulldown identified EZH2, a component of the polycomb repressive complex 2 (PRC2), as an interactor of X inactive specific transcript (*Xist*)<sup>18</sup>, whereas a recent study that used RNA antisense purification coupled with mass spectrometry (RAP-MS) identified other interactors such as SHARP (SMRT and HDAC associated repressor protein), SAF-1 (scaffold attachment factor A) and LBR (lamin B receptor)<sup>19</sup>. Therefore, if no other functional tests support the interaction between an RNA and a given protein, findings should be further supplemented with a complementary protein-centric approach, such as immunoprecipitating the given protein, followed by extraction and characterization of the bound RNA.

The herein protocol presented has been used to detect HuR binding to the ARE motif in CALB2 3' UTR, which had previously been functionally characterized by pmirGLO-luciferase assay<sup>6</sup>. Due to the small amount of proteins, this protocol is not suitable for downstream analysis such as mass-spectrometry where larger protein amounts are necessary. Instead, the protocol can be used to revalidate the RNA-interactors identified by mass spectrometry, but using a significantly lower amount of proteins.

Since the method includes working with RNA, which is susceptible to degradation, we recommend cleaning the working surfaces with a RNase-decontaminating solution in addition to standard laboratory good practice. If possible, perform preparation of RNA labeling and purification under laminar flow. The purity of the RNA oligo may also affect the binding conditions; thus, the commercially synthesized probes were HPLC purified. If modified, longer and extremely pure RNA oligos are required, use PAGE purification method. Prepare aliquots of native proteins by snap freezing, therefore avoiding damaging of proteins due to the slow freezing approach and repeated freezing and thawing.

## Disclosures

The authors declare that they have no competing financial interests.

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