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Video Article

Detection of RNA-binding Proteins by *In Vitro* RNA Pull-down in Adipocyte Culture

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Abstract

RNA-binding proteins (RBPs) are emerging as a regulatory layer in the development and function of adipose. RBPs play a key role in the gene expression regulation at posttranscriptional levels by affecting the stability and translational efficiency of target mRNAs. RNA pull-down technique has been widely used to study RNA-protein interaction, which is necessary to elucidate the mechanism underlying RBPs' as well as long non-coding RNAs' (lncRNAs) function. However, the high lipid abundance in adipocytes poses a technical challenge in conducting this experiment. Here a detailed RNA pull-down protocol is optimized for primary adipocyte culture. An RNA fragment from androgen receptor's (AR) 3' untranslated region (3'UTR) containing an adenylate-uridylylate-rich element was used as an example to demonstrate how to retrieve its RBP partner, HuR protein, from adipocyte lysate. The method described here can be applied to detect the interactions between RBPs and noncoding RNAs, as well as between RBPs and coding RNAs.

Video Link

The video component of this article can be found at <http://www.jove.com/video/54207/>

Introduction

RBPs are proteins that bind to the double or single stranded RNA in cells and participate in forming RNA-protein complexes. RBPs may bind a variety of RNA species, including mRNA and long noncoding RNAs (lncRNAs), and exert their influence at post-transcriptional levels. Both RBPs and lncRNAs are emerging as novel regulators in adipose development and function^{1,2,3}. To understand the mechanism of RBP- and lncRNA-mediated regulation of cellular pathways, it is often necessary to detect the interaction between a specific RNA molecule and one or several RBPs, and, sometimes, to identify the full spectrum of protein partners of a RNA transcript. However, the experiment can be challenging due to the high content of lipids in adipocytes. The RNA pull-down protocol described here can be employed to retrieve the protein partners of a specific RNA from the adipocyte lysates of primary cultures^{4,5}.

Rationale for this protocol is summarized as follows. An RNA bait is *in vitro* transcribed from an DNA template, biotin-labeled and conjugated to streptavidin-coated magnetic beads⁴. The RNA bait is incubated with cellular lysates to allow for the formation of RNA-protein complexes, which are subsequently pulled down on a magnetic stand. More specifically, the RNA pull-down protocol described below use an RNA fragment from AR 3'UTR as the bait to retrieve HuR protein, a universally expressed RBP bound to 3'UTR of mRNAs^{6,7}, from the adipocyte lysate of primary culture. To test the binding specificity of this assay, a non-relevant RNAs as well as blank streptavidin beads is included as control. This protocol is compatible with western blotting or mass spectrometry (MS) to confirm the capture of a specific RBP or to identify the full repertoire of captured RBPs, respectively⁸.

Several techniques are available to study RNA-protein interactions. To reveal RNAs bound by a given RBP, RIP (RNA immunoprecipitation) and CLIP (UV crosslinking and immunoprecipitation) can be applied. In contrast, to identify protein partners of a given RNA, RNA pull-down, ChIRP (chromatin isolation by RNA purification), CHART (capture hybridization analysis of RNA targets) and RAP (RNA antisense purification) can be applied. In comparison with the later ones, RNA pull-down technique takes less effort to set up. It can be employed to capture proteins *in vitro* and *in vivo*. The *in vivo* approach of RNA pull-down, which is technically more challenging than its *in vitro* counterpart, preserves RNA-protein interactions by crosslinking in cells, captures aptamer-tagged RNAs of interest from cells, and subsequently detects bound RBPs. RNA pull-down can be used to enrich low abundant RBPs, and to isolate and identify the RNA-protein complexes that have diverse functional roles in controlling cellular regulation^{9,10}.

Protocol

NOTE: The RNA of interest in the context of this study is a fragment of AR 3'UTR.

1. Preparation of Biotin-labeled RNA

- To obtain the RNA, amplify T7-AR-oligo by PCR, followed by *in vitro* transcription using a commercial kit and following manufacturer's instructions¹¹.
NOTE: Primers for PCR are listed in **Table 4**: T7-AR-F and T7-AR-R.
- For non-specific binding control, amplify a partial sequence of the firefly luciferase (FL) DNA by PCR from psiCHECK-2 plasmid, followed by *in vitro* transcription.
NOTE: Primers for PCR are listed in **Table 4**: hluc(+)-T7-probe-F and hluc(+)-probe-R. **Table 4**. Sequences of template and primers for PCR amplification.
- For a 50 μ l PCR reaction, mix 50 ng DNA (oligo or plasmid), 4 μ l dNTPs (2.5 mM of each individual dNTP), 5 μ l 10x reaction buffer, 1 μ l 2.5 U/ μ l DNA polymerase, 2 μ l 10 μ M forward primer, 2 μ l 10 μ M reverse primer, and nuclease-free water.
 - Run PCR amplification in a thermal cycler using the following program. STEP1: one cycle of 95 °C for 2 min; STEP2: 35 cycles of 95 °C for 30 sec, 55 °C for 30 sec and 72 °C for 30 sec (AR) or 60 sec (FL); STEP3: one cycle of 72 °C for 10 min.
- Synthesize biotinylated RNAs using an *in vitro* transcription kit following manufacturer's instructions¹¹. In this experiment, use PCR products as templates for RNA synthesis. For a 20 μ l *in vitro* transcription reaction, mix 200 ng PCR amplified DNA, 2 μ l of each individual NTP, 1 μ l 10mM biotin-CTP, 2 μ l 10x reaction buffer, and 2 μ l T7 RNA polymerase. Incubate the mixture at 37 °C for 4 hr.
- Following *in vitro* transcription, treat reaction mixture with 1 μ l 2 U/ μ l DNase at 37 °C for 15 min to degrade template DNA. Finally, dilute the biotinylated RNA to a total volume of 60 μ l for subsequent purification.
- Purify biotinylated RNAs to remove salts and unincorporated nucleotides by using purification columns following manufacturer's instructions¹². Measure RNA concentration and store at -80 °C for subsequent pull-down assays.
- To ensure proper secondary structure for RNA of interest in RNA-protein interaction, heat 50 μ l biotinylated RNA (50 pM) at 90 °C for 2 min, chill on ice for 2 min, then supply with 50 μ l 2x RNA structure buffer (**Table 3**), and allow for the RNA folding at RT for 30 min^{13,14}.

2. Preparation of RNA-conjugated Beads

NOTE: Use a magnetic stand for separation of the magnetic beads and supernatant.

- Resuspend the streptavidin-coated magnetic beads in the original vial by brief vortexing following manufacturer's instructions. Transfer 150 μ l resuspended beads to a 1.5 ml tube. Place the tube on magnet for 1 min. Discard supernatant. Keep bead pellet.
NOTE: In RNA pull-down assays, when aliquoting resuspended beads from original vial, use 50 μ l beads for an assay followed by western blotting, and 200 μ l beads for an assay followed by MS.
- Wash beads once by re-suspending bead pellet with 1 ml of the manufacturer's recommended 1x W&B (binding and washing) buffer (**Table 3**), followed by rotating the tube for 5 min at room temperature in a rotator. Discard supernatant. Keep bead pellet.
- To inactivate RNase on beads, wash beads twice, each time by re-suspending bead pellet with 400 μ l buffer A. Buffer A is an alkaline solution containing 0.1 M NaOH and 0.05 M NaCl (**Table 3**). Rotate the tube for 2 min at room temperature in a rotator. Discard supernatant. Keep bead pellet.
- To remove NaOH from beads, wash beads twice, each time by re-suspending bead pellet with 400 μ l buffer B (**Table 3**), followed by rotating the tube for 2 min at room temperature in a rotator. Discard supernatant. Keep bead pellet.
- Resuspend bead pellet with 300 μ l 2x W&B buffer, split beads equally and transfer into three 1.5 ml tubes (100 μ l beads per tube). Supply the bead-containing tubes with 100 μ l RNase free water, 100 μ l biotinylated FL RNA and 100 μ l biotinylated AR 3'UTR RNA, respectively.
- Incubate each bead mixture for 1 hr at room temperature with rotation. Place tubes on magnet for 1 min. Discard all supernatant. Keep bead pellets.
- Wash beads twice, each time by re-suspending each bead pellet with 400 μ l 1x W&B buffer, followed by rotating tubes for 5 min at room temperature in a rotator.
- Place tubes on magnet for 1 min. Discard all supernatant. Resuspend each bead pellet with 50 μ l nuclear isolation buffer (**Table 3**).

3. Preadipocyte Isolation and Adipogenesis Induction

- As described in previous publications⁵, dissect preadipocytes from the interscapular brown fat pad (C57BL6 wild type mice, 3 weeks old).
- Grow and *in vitro* differentiate the preadipocytes⁵. Cells are ready for downstream application 4-5 days after initiation of differentiation.

4. Preparation of Cellular Lysate from Primary Adipocytes

NOTE: Ensure all procedures of cell lysate preparation are made on ice, and all buffers are chilled on ice. Pre-chill dounce glass homogenizers on ice.

- Grow and differentiate preadipocytes in 15 cm dishes⁵ (see section 3.2). Each dish provides adequate cells for one RNA pull-down assay. On day 4 or day 5 of differentiation, discard cell culture medium, and wash cells once with 1x PBS.
- To harvest cells, add 10-15 ml 1x PBS to cells, scrape to collect cells in a 50 ml tube, and pellet cells by centrifugation at 200 x g for 5 min at RT. Discard supernatant by using 1 ml pipette.

3. Resuspend cell pellet in 2 ml hypotonic buffer (**Table 3**), and incubate cells on ice for 15 min. Transfer cells to a 15 ml dounce glass homogenizer, and shear cells mechanically on ice with 20 strokes.
4. Pellet cell nuclei by centrifugation at 3,300 x g for 15 min at 4 °C. Keep nuclei pellet on ice for further use (see section 4.6). Transfer supernatant to 1.5 ml tubes, add 3M KCl to supernatant to obtain a final concentration of 150 mM, and spin at 20,000 x g for 15 min at 4 °C.
5. Following centrifugation, carefully remove lipid layer on the top by using 1 ml pipette, and collect supernatant as the cytoplasmic cellular lysate.
6. Resuspend nuclei pellet (see section 4.4) in 1 ml nuclear isolation buffer. Transfer nuclei to a 15 ml dounce glass homogenizer using 1 ml pipette, and shear nuclei mechanically on ice with 100 strokes. Pellet nuclear debris by centrifugation at 20,000 x g for 15 min at 4 °C, and collect supernatant as the nuclear cellular lysate.
7. Either combine the nuclear and cytoplasmic cellular lysates, or use each fraction separately, for downstream pull-down application. In this demo experiment, these two fractions of cell lysate were combined at a volume ratio of 1:1.
8. Add RNase inhibitor to this unfractionated cell lysate to obtain a final concentration of 0.5 U/μl. Set aside 100 μl of cell lysate as input control for western blotting¹⁵.

5. Binding and Elution of RBP

1. Split cell lysate into three equal aliquots (1 ml cell lysate in a 1.5 ml tube), and incubate with blank, FL RNA-conjugated and AR 3'UTR RNA-conjugated beads (see section 2.8), respectively, for 3 hr at 4 °C with rotation.
2. Place tubes on magnet for 1 min. Collect supernatant using a 1 ml pipette, followed by RNA isolation from supernatant to confirm RNA integrity. Isolate RNA by using an acid guanidinium thiocyanate-phenol solution following manufacturer's instructions (**Table 2**). Assess the RNA intactness by analyzing 28S and 18S subunits of ribosomal RNA. Keep bead pellets on ice.
3. Wash the magnetic beads six times, each time by resuspending each bead pellet with 1 ml nuclear isolation buffer containing 40 U RNase inhibitor and 0.25% IGEPAL, followed by rotating tubes for 2 min at room temperature in a rotator. Use a magnetic stand for separation of the magnetic beads and supernatant. Discard all supernatant. Keep bead pellets on ice.
4. Use following steps to elute RNA-protein complexes from beads for western blotting. First, resuspend each bead pellet in 75 μl nuclear isolation buffer; then, add 25 μl 4x western blot loading buffer (containing SDS) to obtain a total volume of 100 μl. Finally, boil beads in this 100 μl solution at 95 °C for 5 min.
NOTE: Use following steps to elute RNA-protein complexes from beads for MS. STEP1: resuspend each bead pellet in 100 μl elution buffer (**Table 3**); STEP2: incubate bead samples for 2-3 hr at room temperature with rotation; STEP3: centrifuge and collect protein-containing supernatant; STEP4: concentrate protein solutions to 20-30 μl before submission for MS.
5. Centrifuge each 100 μl of bead sample at 12,000 x g for 30 sec at 4 °C, and collect supernatant. Aliquot 15 μl supernatant for western blot analysis (see section 6.1 and 6.2). Probe the resulting membrane with the diluted HuR mouse monoclonal antibody (1:1,000 dilution), overnight.

6. Western Blotting for Verification of RBP

1. Separate RBPs by SDS-PAGE using standard techniques.
2. Conduct western blotting using standard techniques by probing for the RBPs associated with the RNA of interest.
NOTE: In this demo experiment, HuR mouse monoclonal antibody was used for the immunodetection of HuR protein.
3. Incubate the resulting membrane with the diluted HuR antibody (1:1,000 dilution) in 5% w/v nonfat dry milk, 1x TBS, 0.1% Tween-20 at 4 °C with gentle shaking, overnight. Wash membrane three times with 1x TBST. Incubate membrane with the secondary antibody (goat anti-mouse IgG-HRP) at room temperature for 1 hr. Wash membrane. Develop and record signal.

Representative Results

In this demo experiment, an AR RNA fragment was used as the bait to capture its binding protein HuR. Both a FL RNA bait that is non-relevant to HuR protein, and an aliquot of unconjugated blank streptavidin beads served as negative controls. RNA-protein interactions can occur either in nucleus or cytoplasm, and this pull-down protocol can be applied to either total or fractionated (nuclear or cytoplasmic) cell lysates. Analysis of proteins by western blotting shows that the sample of 15 μl unfractionated cell lysate (see section 4.8), as no-bead input control, gave a positive result, indicating that HuR protein is detected in the adipocyte lysate of mouse primary culture by using the monoclonal antibody. The AR RNA elution fraction gave a positive result, indicating that HuR protein is detected in the adipocyte lysate by using the AR RNA-conjugated beads for the RNA pull-down assay. Both the FL RNA elution fraction and the blank bead sample gave negative results, indicating that nonspecific interactions between FL RNA fragment and HuR protein, as well as between the blank beads and HuR protein are not detectable by this RNA pull-down approach. Results of these two negative controls also indicate that a specific interaction between the AR RNA fragment and HuR protein is successfully detected by the RNA pull-down protocol described here.

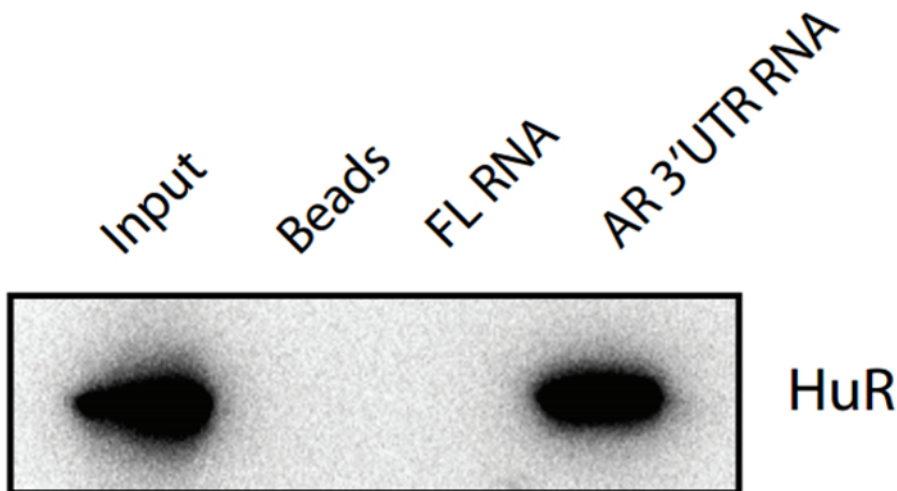


Figure 1: Western Blot Verification of HuR Captured by the RNA Pull-down Assays. Input: reconstituted cell lysate (cytoplasmic + nuclear lysate); Beads: the sample from blank streptavidin-coated magnetic beads; FL RNA: streptavidin beads bound with the FL mRNA; AR 3'UTR RNA: streptavidin beads bound with the AR 3'UTR mRNA; Primary antibody: HuR mouse monoclonal antibody; Secondary antibody: goat anti-mouse IgG-HRP; Abbreviation, FL: firefly luciferase, AR: androgen receptor; [Please click here to view a larger version of this figure.](#)

Centrifuge
Thermal cycler
Spectrophotometer
Rotator
Protein gel electrophoresis and blotting apparatus
Magnet

Table 1: Major Equipment Used in this Study. Major equipment used in this study.

<i>In vitro</i> transcription kit
Biotinylated RNA
Spin column for recovery of DNA and RNA fragments
Streptavidin magnetic beads
HuR mouse monoclonal antibody
Goat anti-mouse IgG-HRP
Nuclease-free molecular biology grade water
Phosphate buffer saline
RNase inhibitor
Protease inhibitor
Pfu DNA polymerase
Acid guanidinium thiocyanate-phenol solution

Table 2: Major Reagents Used in this Study. Major reagents used in this study.

2x RNA structure buffer
20 mM Tris-HCl (pH 7.4)
0.2 M KCl
20 mM MgCl ₂
2 mM DTT
0.8 U/μl RNase inhibitor
Buffer A
0.1 M NaOH
0.05 M NaCl
Buffer B
0.1 M NaCl
1x W&B (Binding and Washing) buffer
5 mM Tris-HCl (pH7.4)
1 M NaCl
1x Hypotonic buffer
10 mM Tris-HCl (pH 7.4)
10 mM KCl
2 mM MgCl ₂
1 mM DTT
1 mM PMSF
1x protease inhibitor
1x Nuclear isolation buffer
25 mM Tris-HCl (pH 7.4)
150 mM KCl
2 mM MgCl ₂
1 mM DTT
0.5% IGEPAL (or 0.25% IGEPAL for elution of RBPs)
1 mM PMSF
1x protease inhibitor
0.4 U/μl RNase inhibitor (or 40 U/ml RNase inhibitor for elution of RBPs)
1x Elution buffer
2 mM biotin in 1x PBS

Table 3: Major Solutions Used in this Study. 2x RNA structure buffer (see section 1.7); Buffer A (see section 2.3); Buffer B (see section 2.4); 1x W&B (Binding and Washing) buffer (see sections 2.2, 2.5, 2.7); 1x Hypotonic buffer (see section 4.3); 1x Nuclear isolation buffer (see sections 2.8, 4.6, 5.3, 5.4); 1x Elution buffer (see NOTE following section 5.4).

Template and primers	Sequences
T7-AR-oligo	5'- TCT AAT ACG ACT CAC TAT AGG GCT GGG CTT TTT TTT TCT CTT TCT CTC CTT TCT TTT TCT TCT TCC CTC CCT AGC TTA TGA CCG TGG CAG TCT -3'
T7-AR-F	5'- CTA ATA CGA CTC ACT ATA G -3'
T7-AR-R	5'- AGA CTG CCA CGG TCA TAA GC -3'
hluc(+)-T7-probe-F	5'-TAATACGACTCACTATAGGGAGCCCCTGCTAACGACATTTACAACGAG-3'
hluc(+)-probe-R	5'-CATAATCATAGGGCCGCGCACACAC-3'

Table 4: Sequences of Template and Primers for PCR Amplification. T7-AR-oligo: DNA template for PCR amplification (see section 1.1); T7-AR-F: forward primer for PCR amplification (see section 1.1); T7-AR-R: reverse primer for PCR amplification (see section 1.1); hluc(+)-T7-probe-F: forward primer for PCR amplification (see section 1.2); hluc(+)-probe-R: reverse primer for PCR amplification (see section 1.2).

Discussion

lncRNAs and RBPs have vital roles in health and disease, however the molecular mechanisms of these molecules are poorly understood. Identification of proteins that interact with lncRNA molecules is a key step towards elucidating the regulatory mechanisms. Enrichment of RBPs by the RNA pull-down assay system is based on in-solution capture of interacting complex so that proteins from a cell lysate can be selectively extracted using biotinylated RNA baits bound to streptavidin magnetic beads. Several other methods such as ChIRP, CHART and RAP may achieve the same goal, however these methods takes more effort to set up than the RNA pull-down technique^{16,17,18}.

The main strength of RNA pull-down is that it is relatively a simple protocol and easy to perform. Both CHIRP and RAP involve a crosslinking step that preserves naturally occurring RNA-protein complexes and the design of a full set of antisense oligonucleotides (90 nucleotides long in RAP, and 20 nucleotides long in ChIRP) tiling the entire target RNA^{16,17,18}. RNA folding is a critical step in the RNA pull-down assays. The main limitation of this protocol is that the target RNAs are synthesized *in vitro* and may not be correctly folded into native structure to allow proper binding with its RBP(s). Misfolded non-native RNAs may either fail to form RNA-protein interactions and invalidate functional assays, or form interactions that only occur *in vitro* under nonphysiological conditions. Although the pull-down protocol described here adopted a well-recognized procedure for RNA folding^{13,14} (see section 1.7), it doesn't guarantee proper folding for other RNA transcripts.

RNA pull-down is often coupled with RIP as a complementary approach to detect the RNA(s) bound by the RBP of interest¹⁹. Another critical step in this protocol is the removal of lipid fraction from total cell lysate (see section 4.5) because the high lipid abundance in mature adipocytes presents a major technical challenge. The protocol described here is particularly developed for use with adipocytes. However, the basic components of this protocol and the stringency of all buffers used can be altered and adapted for application to other cell culture models. For tissues that have high levels of endogenous RNase, different RNA pull-down protocols may be applied.

RNA pull-down, which uses RNAs of interest to identify the associated RBPs, is an effective and efficient technique to probe the pivotal functions and mechanisms of lncRNAs, which have a wide range of roles in human cells. Future development of RNA-based capture, including RNA pull-down, will be needed to address challenges with defining the components, assembly and function of RNA-protein complexes, as well as generating sufficient RBPs from low abundant RNA-protein complexes for MS.

Disclosures

No conflicts of interest declared.

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