Video Article

Isolation of Cognate RNA-protein Complexes from Cells Using Oligonucleotide-directed Elution

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Abstract

Ribonucleoprotein particles direct the biogenesis and post-transcriptional regulation of all mRNAs through distinct combinations of RNA binding proteins. They are composed of position-dependent, cis-acting RNA elements and unique combinations of RNA binding proteins. Defining the composition of a specific RNP is essential to achieving a fundamental understanding of gene regulation. The isolation of a select RNP is akin to finding a needle in a haystack. Here, we demonstrate an approach to isolate RNPs associated at the 5' untranslated region of a select mRNA in asynchronous, transfected cells. This cognate RNP has been demonstrated to be necessary for the translation of select viruses and cellular stress-response genes.

The demonstrated RNA-protein co-precipitation protocol is suitable for the downstream analysis of protein components through proteomic analyses, immunoblots, or suitable biochemical identification assays. This experimental protocol demonstrates that DHX9/RNA helicase A is enriched at the 5' terminus of cognate retroviral RNA and provides preliminary information for the identification of its association with cell stress-associated huR and junD cognate mRNAs.

Video Link

The video component of this article can be found at <https://www.jove.com/video/54391/>

Introduction

Post-transcriptional gene expression is precisely regulated, beginning with DNA transcription in the nucleus. Controlled by RNA binding proteins (RBPs), mRNA biogenesis and metabolism occur in highly dynamic ribonucleoprotein particles (RNPs), which associate and dissociate with a substrate precursor mRNA during the progression of RNA metabolism¹⁻³. Dynamic changes in RNP components affect the post-transcriptional fate of an mRNA and provide quality assurance during the processing of primary transcripts, their nuclear trafficking and localization, their activity as mRNA templates for translation, and the eventual turnover of mature mRNAs.

Numerous proteins are designated as RBPs by virtue of their conserved amino acid domains, including the RNA recognition motif (RRM), the double-stranded RNA binding domain (RBD), and stretches of basic residues (e.g., arginine, lysine, and glycine)⁴. RBPs are routinely isolated by immunoprecipitation strategies and are screened to identify their cognate RNAs. Some RBPs co-regulate pre-mRNAs that are functionally-related, designated as RNA regulons⁵⁻⁸. These RBPs, their cognate mRNAs, and sometimes non-coding RNA, form catalytic RNPs that vary in composition; their uniqueness is due to various combinations of associated factors, as well as to the temporal sequence, location, and duration of their interactions⁹.

RNA immunoprecipitation (RIP) is a powerful technique to isolate RNPs from cells and to identify associated transcripts using sequence analysis¹⁰⁻¹³. Moving from candidate to genome-wide screening is feasible through RIP combined with a microarray analysis¹⁴ or high-throughput sequencing (RNAseq)¹⁵. Likewise, co-precipitating proteins may be identified by mass spectrometry, if they are sufficiently abundant and separable from the co-precipitating antibody^{16,17}. Here, we address the methodology for isolating RNP components of a specific cognate RNA from cultured human cells, although the approach is alterable for soluble lysates of plant cells, fungi, viruses, and bacteria. Downstream analyses of the material include candidate identification and validation by immunoblot, mass spectrometry, biochemical enzymatic assay, RT-qPCR, microarray, and RNAseq, as summarized in **Figure 1**.

Given the fundamental role of RNPs in controlling gene expression at the post-transcriptional level, alterations in the expression of component RBPs or their accessibility to cognate RNAs can be detrimental for the cell and are associated with several types of disorders, including

neurological disease¹⁸. DHX9/RNA helicase A (RHA) is necessary for the translation of selected mRNAs of cellular and retroviral origins⁶. These cognate RNAs exhibit structurally-related cis-acting elements within their 5' UTR, which is designated as the post-transcriptional control element (PCE)¹⁹. RHA-PCE activity is necessary for the efficient cap-dependent translation of many retroviruses, including HIV-1, and of growth regulatory genes, including *jund*^{6,20,21}. Encoded by an essential gene (*dhx9*), RHA is essential to cell proliferation and its down-regulation eliminates cell viability²². The molecular analysis of RHA-PCE RNPs is an essential step to understanding why RHA-PCE activity is necessary to control cell proliferation.

The precise characterization of the RHA-PCE RNP components at steady state or upon physiological perturbation of the cell requires the selective enrichment and capture of the RHA-PCE RNPs in sufficient abundance for downstream analysis. Here, retroviral PCEgag RNA was tagged with 6 copies of the cis-acting RNA binding site for the MS2 coat protein (CP) within the open reading frame. The MS2 coat protein was exogenously co-expressed with PCEgag RNA by plasmid transfection to facilitate the RNP assembly in growing cells. RNPs containing the MS2 coat protein with cognate MS2-tagged PCEgag RNA were immunoprecipitated from the cell extract and captured on magnetic beads (**Figure 2a**). To selectively capture the RNP components bound to the PCE, the immobilized RNP was incubated with an oligonucleotide complementary to sequences distal to the PCE, forming an RNA-DNA hybrid that is the substrate for RNase H activity. Since PCE is positioned in the 5' terminal of the 5' untranslated region, the oligonucleotide was complementary to the RNA sequences adjacent to the retroviral translation start site (gag start codon). RNase H cleavage near the gag start codon released the 5' UTR complex from the immobilized RNP, which was collected as the eluent. Thereafter, the sample was evaluated by RT-PCR to confirm the capture of PCEgag and by SDS PAGE and immunoblot to confirm the capture of the target MS2 coat protein. A validation of the PCE-associated RNA binding protein, DHX9/RNA helicase A, was then performed.

Protocol

Buffer Compositions
Wash Buffer:
50 mM Tris-HCl, pH 7.4
150 mM NaCl
3 mM MgCl ₂
Low Salt Buffer:
20 mM Tris-HCl, pH 7.5
10 mM NaCl
3 mM MgCl ₂
2 mM DTT
1x protease inhibitor cocktail EDTA-free
RNase Out 5 µl/ml (RNase inhibitor)
Cytoplasmic Lysis Buffer:
0.2 M Sucrose
1.2% Triton X-100
NETN-150 Wash Buffer:
20 mM Tris-HCl, pH 7.4
150 mM NaCl
0.5% NP-40
3 mM MgCl ₂
10% Glycerol
Binding Buffer:
10 mM HEPES pH 7.6
40 mM KCl
3 mM MgCl ₂
2 mM DTT
5% glycerol

Table 2: Buffer compositions.

1. Preparation of the Cells and the Affinity Matrix

1. Culture a cell line of interest to sub-confluence (80%) in a 10 cm dish. Use 10 cm plates for independent immunoprecipitations (IP) of a FLAG-tagged NLS-MS2 coat protein. Perform the IP using antisera to the FLAG-epitope tag. When expressing the FLAG-tagged MS2 coat protein plasmid, transfect cells 24-48 hr in advance of harvest²⁰.
NOTE: A particular RNP may be enriched from nuclear or cytoplasmic lysates or a biochemically-fractionated preparation, such as fractions of a sucrose gradient. It is recommended to harvest the lysate from non-transfected cells in parallel to institute an additional negative control.
2. Transfer 60 μ l per IP of protein G magnetic bead slurry to a 1.7 ml microcentrifuge tube.
3. Place the tube on a magnet rack for microcentrifuge tubes to separate the beads from the storage solution.
4. Remove the storage solution by carefully drawing it up with a micropipette.
5. Remove the tube from the magnet rack.
6. Wash and equilibrate the beads with 600 μ l (10 times the used volume of beads) of 1x wash buffer (20 mM Tris-HCl, pH 7.4; 3 mM MgCl₂; and 150 mM NaCl) and end-over-end rotation for 3 min at room temperature.
7. Place the tube on the magnet rack and remove the wash buffer.
8. Add 10 volumes (600 μ l) of 1x wash buffer and immunoprecipitating FLAG antibody to the equilibrated protein G magnetic beads (according to the amount recommended by the manufacturer for an immunoprecipitation) and rotate end-over-end at room temperature for at least 30 min to conjugate the immunoprecipitating antibody. Use the corresponding isotype IgG as a suitable antibody negative control.
9. Place the tube on the magnet rack to collect the beads and to remove the supernatant.
10. Remove the tube from the magnet rack and wash the antibody-conjugated beads with 10 volumes (600 μ l) of 1x wash buffer and rotation for 3 min at room temperature. Repeat this step twice.
11. Place the tube on the magnet rack and remove the wash buffer.

2. Harvesting the RNPs

NOTE: Prepare the RNPs during the incubation time after step 1.8.

1. Remove culture medium from the cells by aspiration and wash the cells twice with 1-5 ml of ice-cold 1x phosphate-buffered saline (PBS). Use a cell scraper to dislodge wet, adherent cells prior to collection by centrifugation at 226 x g for 4 min at 4 °C.
2. Add 375 μ l of ice-cold, low-salt buffer (20 mM Tris-HCl, pH 7.5; 3 mM MgCl₂; 10 mM NaCl; 2 mM DTT; 1x protease-inhibitor cocktail, EDTA-free; and 5 μ l/ml RNase Inhibitor) to the cell pellet and allow swelling by placing it on ice for 5 min.
NOTE: Tailor the volume of low-salt buffer according the size of the cell pellet. For 1.2 x 10⁶ cells from a 10 cm plate, 375 μ l of buffer is sufficient.
3. To collect the cytoplasmic cell lysate, add 125 μ l of ice-cold lysis buffer (0.2 M sucrose/1.2% Triton X-100), and then perform 10 strokes with a Dounce homogenizer that was pre-chilled in an ice bucket.
NOTE: To collect the total cell extract, standard RIPA lysis buffer is recommended for solubilizing the nucleoplasm/chromatin.
4. Spin in a microfuge at top speed (16,000 x g) for 1 min; this will clear the supernatant of debris and nuclei.
5. Transfer the supernatant to a new 1.7 ml microcentrifuge tube that has been on ice. Determine the total protein concentration by a standard, laboratory-preferred method, such as the Bradford Assay²³. Reserve an aliquot of at least 10% for a Western blot analysis, to be used as an input control. Use the harvested cell lysate immediately for immunoprecipitation or store it at -80 °C for future analysis.
NOTE: In our experience, these samples can be stored and used several months later for immunoprecipitation analysis without a compromise of integrity.

3. Immunoprecipitation

1. Add the desired volume of the harvested cell lysate, based upon the protein concentration determined in step 2.5, to the target antibody-conjugated beads. Using 1x wash buffer, bring the total volume up to 600 μ l and rotate end-over-end for 90 min at room temperature.
NOTE: This time period is sufficient to generate a robust isolation of high-affinity RNP complexes while minimizing non-specific binding. Dilute alternative RNP sources, such as fractions of a sucrose gradient, at least 1:1 with 1x wash buffer, and then incubate them with previously-prepared bead-antibody complexes, as mentioned above.
2. After 90 min of incubation, place the IP tube on the magnet rack to collect the beads. Reserve the supernatant as flow-through.
NOTE: This first flow-through is an important control sample to measure IP efficiency. An immunoblot assay will provide an indication of IP efficiency, as well as the specificity of the investigated interactions. It is recommended to keep this supernatant for downstream analysis.
3. Wash the RNP-bound, antibody-conjugated beads with 10 volumes (600 μ l) of ice-cold NETN-150 wash buffer (20 mM Tris-HCl, pH 7.4; 150 mM NaCl; 3 mM MgCl₂; 0.5% NP40; and 10% glycerol) under rotation for 3 min at room temperature. Repeat the step 3 times.
NOTE: This buffer differs from the lysis buffer and effectively reduces weak or non-specific associations.
4. Following the final wash, resuspend the immobilized RNP complexes in 60 μ l of ice-cold 1x binding buffer (10 mM HEPES, pH 7.6; 40 mM KCl; 3 mM MgCl₂; 5% glycerol; and 2 mM DTT) and reserve 10% of the immobilized complex beads for Western blot and 20% for RNA isolation.

4. Elution

1. Adjust the remaining volume to 100 μ l with ice-cold 1x binding buffer and heat the tube at 70 °C for 3 min.
2. To isolate cognate RNA-protein complexes, add a ~30-nt DNA oligonucleotide that is complementary to the 3' sequence boundary of the RNA of interest and incubate for 30 min at room temperature with gentle rocking (100 nM of oligonucleotide is sufficient).
NOTE: The oligonucleotide is antisense to the nucleotide residues adjacent to the translation start-site of the PCE construct used as an example in this protocol. Appropriate sequence complementarity is provided by the ~40% G-C content. Design the antisense oligonucleotide for efficient hybridization to the minimum complementary region of the target RNA.

3. Add 5-10 units of RNase H to the tube and incubate at room temperature for 1 hr to cleave the RNA of the RNA:DNA hybrid. Transfer the supernatant to a sterile 1.7 ml microcentrifuge tube; this sample contains the captured RNP complexes of interest.
NOTE: Depending upon the accessibility of the cognate RNA, two or more rounds of RNase H treatment may be useful to increase the sample abundance for the downstream analyses.
4. Use 20% of the eluate in a Western blot for known associated proteins and 20% of the eluate for RNA isolation followed by RT qPCR. The remaining 60% can be used for mass spectrometry or to identify protein components.
5. In parallel, subject an aliquot of the reserved cell lysate to RNA isolation and RT-PCR analysis. This assessment provides an indication of the enrichment of RNA within an RNP complex.
NOTE: Use the isolated protein preparation in a control western blot to ascertain that the RNase H cleavage was effective in releasing the RNP from the immunoprecipitated complex.

5. Protein Electrophoresis and Western Blot Analysis

1. Subject approximately 10-20% of the total sample to SDS-PAGE and immunoblot analysis according to standard laboratory protocol.
NOTE: This step serves to validate IP efficiency and specificity prior to downstream RNA analysis. It can also be used to assess the protein composition of the isolated RNP complex.

6. Collection of the Immunoprecipitated RNA

NOTE: RNA isolation can be done by Trizol method or following the described protocol.

1. Resuspend half of the total sample in 750 μ l of acid guanidinium thiocyanate reagent and incubate at room temperature for 5 min prior to extracting the RNA from the captured PCE-RNP complexes.
2. Add 200 μ l of chloroform to the tube, shake vigorously for 10 sec, and incubate at room temperature for 3 min.
3. After centrifugation at 16,000 $\times$ g for 15 min at 4 $^{\circ}$ C, collect the aqueous phase into a fresh tube and add an equal volume of isopropanol. Mix well and incubate at room temperature for at least 10 min. Add 1 μ l of glycol blue to the sample and store it at -20 $^{\circ}$ C in the freezer for efficient precipitation or processing at a future date.
4. Centrifuge the tube at 16,000 $\times$ g for 10 min at 4 $^{\circ}$ C. Carefully collect and discard the supernatant so as to not disturb the RNA pellet; the use of a p-200 micropipette tip is recommended to facilitate this process.
5. Add 500 μ l of 75% ethanol to each tube, vortex, and centrifuge the tubes at 16,000 $\times$ g for 5 min at 4 $^{\circ}$ C. Carefully collect and discard the supernatant as in step 6.4. Air-dry the pellet for 2-3 min and resuspend in 100 μ l of RNase-free water.
NOTE: Do not prolong the time the pellet air-dries in order to avoid a problem with resuspension.
6. Apply 100 μ l of RNA sample to an RNA clean-up column. Process it using the manufacturer's protocol. Elute the RNA in 30 μ l of RNase-free water and store at -80 $^{\circ}$ C for up to 3 months.
NOTE: This isolated RNA is suitable for downstream analysis by RT-realtime PCR (qPCR), microarray, and RNA sequencing. Depending upon the abundance of the RNP and the efficiency with which the RNP of interest is isolated, the same lysate may be subjected to two or more rounds of IP to generate sufficient RNA applicable for the downstream analyses.

7. RNA Reverse Transcription and Amplification of cDNA by PCR

1. Subject the isolated RNA to reverse transcription by a random primer with a high-quality reverse transcriptase (RT), according to the manufacturer's instructions.
2. To amplify the RNA of interest, design a gene-specific antisense primer within the RNase H cleavage sequence. Use similar amounts of the antisense oligonucleotide, a random primer, or an oligo-dT primer (See **Materials Table**).
NOTE: The oligo-dT primer and poly-adenylated mRNA provide positive control reactions for the RT-PCR reactions.
3. Reserve 5% of the RT reaction (1 μ l) for a preparative qPCR done in tandem with negative-control lysate IP and positive-control DNA samples to define the cutoff and produce a standard curve. If the CT value of the preparative qPCR is beyond the range of the standard curve, dilute the RT reaction in consecutive 1:5 dilutions and repeat step 7.2.
NOTE: For an RNA sequencing and mass spectrometry technique, please see the Supplementary Method file. [Please click here to view this Supplementary Method file. \(Right-click to download.\)](#)

Representative Results

Prior RIP results identified retroviral gag RNAs and selected cellular RNAs that co-precipitate with DHX9/RHA, including HIV-1⁶, junD⁶ and huR (Fritz and Boris-Lawrie, unpublished). The retroviral 5' UTR has been demonstrated to co-precipitate with DHX9/RHA in the nucleus and to co-isolate in the cytoplasm on polyribosomes. It is uniquely defined as the *cis*-acting post-transcriptional control element (PCE)⁶. To isolate PCEgag RNA-RHA ribonucleoprotein complexes (RNPs) formed in proliferating cells, we performed FLAG-MS2 RNP immunoprecipitation in transfected HEK293 cell lysates. The results show efficient precipitation of the FLAG-MS2 protein. Notably, DHX9/RHA is identified within this RNP complex and not within the IgG isotype control, nor within the negative-control HEK293 cell lysate (**Figure 2A**). The matrix containing the RNPs was incubated with a complementary 30-nt oligonucleotide, and then an RNase H cleavage of the RNA-DNA hybrid was performed. Upon RNase H digestion, the eluates were analyzed by Western blotting. The demonstrated RNase H cleavage of PCEgag RNA at the RNA-DNA hybrid position released the RNP complex specifically bound to the 5' UTR. The DHX9/RHA-associated RNPs were determined to be enriched in the eluents. Importantly, the FLAG-MS2-bound RNPs remained with the antibody-conjugated beads (**Figure 2B**). The co-immunoprecipitation of the MS2 stem-loop containing retroviral PCEgag RNA in cells and in eluents was validated by RT-PCR and qRT-PCR (**Figure 2C**). The results confirmed a select association between DHX9/RHA and the retroviral 5' UTR, which has been highlighted as a unique RNP important for targeted translation control⁶.

Figure 1: RNP isolation and the enrichment of specific RNPs associated with the 5' UTR of PCEgag by RNase H cleavage. Workflow showing the steps to isolate a selected ribonucleoprotein formed *de novo* in cells, its collection by oligonucleotide-guided RNaseH cleavage, and the downstream analysis of RNA and protein components. Summary of affinity chromatography using a high-affinity interaction between multimers for the MS2 RNA stem-loop and the MS2 coat protein-FLAG fusion protein to capture PCE RNA on FLAG beads. [Please click here to view a larger version of this figure.](#)

Figure 2: A PCEgag RNA containing 6 MS2 RNA binding sites was captured by an immobilized FLAG-tagged MS2 coat protein, and specific RNPs associated with the 5' UTR were released by oligonucleotide-directed RNase H cleavage. HEK293 cells were co-transfected with pAR200 (a plasmid containing the PCEgag and 6x MS2 loops in the HIV intron) and a plasmid expressing the FLAG epitope-tagged MS2 protein with a nuclear localization sequence (NLS). Cells were collected and the cytoplasm was isolated at 48 hr post-transfection. The cytoplasmic lysates were subjected to immunoprecipitation with either FLAG antibody or an IgG control. **(A)** The IP efficiency was determined by immunoblot using an anti-FLAG antibody. DHX9/RHA, a PCE binding protein, served as a positive control; PCE-containing RNA immunoprecipitated with the MS2 protein. Lanes 1-3: Input proteins used for IP. Lanes 4-5: FLAG IP of FLAG MS2 overexpressed lysate and HEK293 cytoplasmic cell lysate. Lane 6: IgG IP of FLAG MS2 overexpressed lysate. Lanes 7-9: Flow-through of the IPs. **(B)** 5' UTR-bound RNPs were enriched by RNase H cleavage. Lanes 1-3: Eluates of the 5' UTR-bound protein by RNase H. Lanes 4-6: proteins bound to antibody-conjugated beads. **(C)** Reverse transcriptase PCR and real-time quantitative PCR of specific RNA bound to different fractions of RNPs. [Please click here to view a larger version of this figure.](#)

Discussion

The RNP isolation and cognate RNA identification strategy described here is a selective means of investigating a specific RNA-protein interaction and of discovering candidate proteins co-regulating a specific RNP in cells.

The advantage of using oligonucleotide-directed RNase H cleavage to isolate RNPs is the ability to capture and specifically analyze the RNP cis-acting RNA element over heterogeneous RNPs bound downstream to the cis-acting RNA element of interest. Because the abundance of a cognate RNA in a given RNP is a minor fraction of the collected RNPs isolated without RNase H cleavage, the major disadvantage to this workflow is the scarcity of the cognate RNP. In our experience, this limitation necessitated 5- to 10-fold more starting material than conventional RNA IP for detection in sensitive immunoblot and proteomics analyses. Another advantage provided by immobilizing the RNP is the convenience

of repeating the RNase H treatment and collecting additional eluate. In our experience, 2-4 rounds of RNase H treatment were advisable to collect additional eluate.

In this protocol, key technical concerns are maintaining the optimal temperature and ensuring the sterile handling of the reagents. All reagents should be RNase- and protease-free. The integrity of the RNA sample is a potential pitfall to consider if an RNA-protein interaction is not detectable.

This technique requires the efficient lysis of the cells to access the RNPs in a suitable quantity for detection. While an important caveat is incomplete cell lysis, vigorous treatments may increase non-specific interactions with the RNA. Therefore, the experimental conditions should be measured in order to enrich specific protein-RNA interactions. However, the use of high-stringency washes may eliminate important yet transient interactions. Therefore, the washing conditions are another variable to consider in the specific requirements of a particular experiment.

RIP efficiency is a powerful technique to isolate cognate RNA-protein partners, but some transient or weak interactions that are of physiological importance are lost during the binding or washing steps. Cross-linking of the mRNP complex is an option to consider in an analysis. Moreover, physiological growth conditions should be kept in mind while analyzing regulatory RNPs, as the expression level of the cognate RNA or RBP may be subject to physiological fluctuations under certain conditions. Furthermore, the type of downstream analysis will also play a role in selecting the lysis and washing conditions during the immunoprecipitation.

In addition, the successful immunoprecipitation requires that the cell lysate be significantly dilute (at least 1:1). 1x wash buffer (20 mM Tris-HCl, pH 7.3; 3 mM MgCl₂; and 150 mM NaCl) is the selected diluent, as its composition does not affect RNP integrity, solution solubility, or antibody-antigen interactions.

For downstream mass spectrometry, samples should be free from salts, including Na⁺, Cl⁻, and Tris, as well as some detergents. If necessary, components of buffers-the ionic compounds-may be reduced by dialysis or ionic exchange filtration, and in some cases, volatile buffers are useful in the final steps. In cases when antiserum is used to isolate an epitope-tagged protein expressed by transfection, Western blot validation of the recombinant protein using initial cell lysate should be executed before further analysis. In case an epitope tag is used (*i.e.*, FLAG, MYC, GFP, *etc.*), the exposure of the tag is crucial to the success of the antibody binding step. Freeze-thaw of samples should be avoided.

The RIP technique has been widely employed to elucidate diverse mechanisms of post-transcriptional gene control^{10,25}. This includes the identification of novel protein-mRNA, protein-microRNA, and protein-protein interactions^{10,25}. Here, we provide a comprehensive protocol for the determination of RNP composition within a cell culture. Our method allows for the targeted and genome-wide assessment of critical protein-RNA interactions, as well as for the capture of rare and/or transient RNP complexes.

The application of this technique has contributed to our characterization of RNAs bound by DHX9/RNA helicase A and helped us to define the critical post-transcriptional regulation of retroviruses and the cellular proto-oncogenes junD^{6,26} and huR (Fritz and Boris-Lawrie, unpublished data). We expect the application of this technique in future studies to further our understanding of the critical mechanisms for gene control, including those mediated by long non-coding RNP complexes.

Disclosures

The authors have nothing to disclose.

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