Studying RNP Composition with RIP

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Abstract

RNA is never left alone throughout its life cycle. Together with proteins, RNAs form membraneless organelles, called ribonucleoprotein particles (RNPs) where these two types of macromolecules strongly influence each other's functions and destinies. RNA immunoprecipitation is still one of the favorite techniques which allows to simultaneously study both the RNA and protein composition of the RNP complex.

Keywords

RNA-binding protein
Immunoprecipitation
Ribonucleoprotein

1. Introduction

Post-transcriptional mechanisms regulating gene expression depend on a combined action of RNA-binding proteins (RBP) and RNAs. Soon after the start of transcription RNA molecules associate with a distinct class of RBPs, giving rise to macromolecular, functional units called ribonucleoprotein particles (RNP), which control either gene expression or the functions of the components present in the complex. In this contest, RBPs crucially regulate key steps of RNA metabolism, including splicing, polyadenylation, capping, export, localization, editing, translation, and turnover. In addition, RNAs present in the

RNP can also regulate interactions, localization, and functions of the RBPs [1, 2, 3, 4].

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RBPs interact with different affinities with RNA. They are characterized by the presence of one or more RNA-binding domains (RBDs), containing 60–100 residues that usually associate with RNA in a structure and sequence-dependent manner. To date, more than 40 RBDs have been identified, the most common class being the RNA recognition motif found in >50% of the RBPs. Other RBDs include the K-homology (KH) domain, the Larp module, zinc fingers, double-stranded RNA-binding motifs, DEAD-box domains, and the Piwi/Argonaute/Zwille (PAZ) domain [5, 6, 7, 8].

Because the RNP composition determines the fate of any given mRNA in virtually all phases of its life cycle, it is perhaps not surprising that changes in RNP composition or function are increasingly recognized as causes of human disease. Alterations in the expression and localization of RBPs can influence the expression levels of oncogenes and tumor-suppressor genes and can lead to diverse cancer-related cellular phenotypes such as proliferation, angiogenesis, and invasion [9, 10, 11]. Further, misregulated assembly of RNPs in the central nervous system often leads to neurodegenerative diseases such as amyotrophic lateral sclerosis and spinal muscular atrophy [12, 13, 14].

A detailed insight into the composition of these highly dynamic RNPs, their assembly, and remodeling is pivotal for understanding how they control gene expression. Substantial advances have been made towards this goal through recent technologies in the analysis of RNAs and RNPs.

Here we describe one of the most basic method, the RNA immunoprecipitation (RIP), which relies on the immunoprecipitation of RNP complexes typically achieved through the use of an antibody targeting one of the RBP in the RNP complex, with subsequent isolation, analysis, and identification of RNAs bound to the RBP. Such identification can be obtained by qRT-PCR in the case of known targets or by RNA-sequencing in the case of unknown targets or when a more comprehensive, potentially unbiased analysis is needed. The main advantages of the RIP protocol are that it gives the possibility of isolating RNP complexes, allowing identification of components other than mRNAs, such as

other regulatory or RNA-processing proteins, as well as small noncoding RNAs. In addition, RIP is an ideal method for studying remodeling of RNPs during the dynamic processes of post-transcriptional gene expression [15].

The protocol we present here is based on the RIP protocol published by Keene and colleagues but with few modifications. All steps of the procedure from harvesting cells to RNA extraction are given. In addition, we provide several hints as a guide for optimizing specific steps of the experiment [16].

As an example to illustrate the protocol, we describe how we performed a RIP of the DHX30 RNA helicase and its associated RNAs starting from HCT116 cells.

2. Materials

2.1. Tissue Cell Culture Components

1. HCT116 is a human colorectal carcinoma cell line and was maintained in RPMI (Gibco) media supplemented with 10% FBS, antibiotics (100 units/mL penicillin plus 100 mg/mL streptomycin), and 2 mM L-glutamine (*see* **Note 1**).

2.2. Sample Collection Components

1. Phosphate-buffered saline (PBS) for cell culture (pH 7.4): 10× PBS contains potassium phosphate monobasic (KH_2PO4) 1.440 g/L, sodium chloride (NaCl) 90 g/L, sodium phosphate dibasic ($Na_2HPO\ 4_4\ -7H_2O$) 7.950 g/L.
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2. Polysome lysis buffer (PLB): 100 mM Potassium chloride (KCl), 5 mM magnesium chloride ($MgCl_2$), 10 mM HEPES (pH 7.0), 0.5% Nonidet P-40 (NP40), 1 mM dithiothreitol (DTT), 100 U/mL RNase Out, 1× complete protease inhibitor cocktail (Merk). To prepare 5 mL PLB add 50 µL of 1 M HEPES (pH 7.0), 500 µL of 1 M KCl, 25 µL of 1 M $MgCl_2$, and 100 µL of 25% NP40 to 4207.5 mL of nuclease-free H_2O. Then add 5 µL of 1 M DTT, 12.5 µL RNase Out, and 100 µL of 50× protease inhibitor cocktail (*see* **Note 2**).

2.3. RNP Immunoprecipitation Components

1. Protein A/G dynabeads: This choice depends on the species and IgG type of the antibody to be conjugated.

2. Antibodies: antibody recognizing an RBP of interest, and an isotype-matched control antibody (here: rabbit anti-DHX30 (Bethyl A302-218A), and Normal Rabbit anti-IgG (Merck-Millipore 12-370)).

3. NT2 buffer: 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM $MgCl_2$,

0.05% NP40.

To make 50 mL of NT2 buffer, add 2.5 mL of 1 M Tris (pH 7.4), 1.5 mL of 5 M NaCl, 50 µL of 1 M $MgCl_2$, and 100 µL of 25% NP40 to 45.85 mL nuclease-free H_2O.

4. RNasin.
5. Dithiothreitol (DTT).
6. TRIZOL.
7. Chloroform, isopropanol, and glycogen.

3. Methods

3.1. Tissue Culture and RNP Lysate Collection

1. Maintain HCT116 cells in the RPMI cell growth medium at 37 °C and 5% CO_2. For a complete experiment, two 15 cm dishes at 70–80% of confluence will be needed.
2. Decant the growth medium, place the dish on ice, wash the adherent cells with ice-cold PBS, collect the cells off the dish using a scraper, and transfer them into a pre-chilled 50 mL conical tube. Spin down the cells

   at 200 × *g* at 4 °C for 5 min. Wash the cell pellet with cold PBS, spin down again, and aspirate the supernatant as much as possible, without disturbing the cell pellet.
3. Resuspend the cell pellet in 1 mL of PLB buffer by pipetting up and down. Allow the lysate to chill on ice for 5 min before immediately freezing and storing the pellet at −80 °C until you proceed with the experiment (*see* **Note 3**).

3.2. Antibody Coating of Protein A/G Beads

For the immunoprecipitation of DHX30 protein, we use magnetic protein A dynabeads and coat them manually with rabbit anti-DHX30 antibody or normal rabbit IgG, according to the manufacturer's descriptions.

1. Vortex the dynabeads for 30 s and transfer 25 µL of beads per sample into two nuclease-free low-retention 1.5 mL tubes. One will be used for

the RBP of interest and the other one for the control RIP.

2. Wash the beads two times with 500 μL of NT2. Collect the beads using a magnetic stand and resuspend beads by pipetting up and down with low-affinity filter tips.

3. Dissolve 3–5 μg of specific antibody or isotype-matched IgG in 100 μL of NT2 buffer supplemented with protease inhibitors, mix by pipetting up and down, and add it to the beads (*see* **Note 4**).

4. Incubate the beads at 4 °C on a rotating wheel at 20 rpm for at least 2 h. (Alternatively, incubate at 4 °C on a rotating wheel, overnight, the day before the immunoprecipitation).

5. Immediately before usage, wash the antibody-coated beads two times with an ice-cold NT2 buffer supplemented with RNase and protease inhibitors to remove unbound antibody and potential contaminants.

6. After the final wash, resuspend the designated amount of antibody-coated beads per sample in 720 μL of ice-cold NT2 buffer. Add to each sample/tube 40 U/mL of RNase inhibitors, 1× protease inhibitors, and

   DTT to a final concentration of 1 mM, and keep them on ice until the lysate samples are ready to proceed with the IP.

3.3. Immunoprecipitation Reaction and RNA Extraction

1. Thaw the cell lysate (**step 3** of Subheading 3.1) on ice.

2. Centrifuge the lysate at 4 °C at 20,000 × *g* for 30 min to clear the sample.

3. Transfer the cleared lysate into a new pre-chilled microfuge tube and store it on ice. Be careful not to disturb the pellet that is formed. At least 800 μL of lysate will be collected.

4. Take two 40 μL aliquots of the cleared lysate of each sample and store them on ice. The first one represents total cellular RNA as input for the RIP and will be needed for a subsequent qRT-PCR or RNA-seq library preparation. The total RNA input is used to calculate and quantify the enrichment of specifically RBP-bound transcripts. The second aliquot is used for control western blotting to verify the input RBP amount. Add 80 μL of NT2 buffer to both aliquots. This matches the total RNA and

80 μL of NT2 buffer to both aliquots. This matches the total RNA and protein concentration to the subsequent immunoprecipitation steps.

5. For each sample wash 10–15 μL of protein A/G magnetic beads as in **step 2** of Subheading 3.2. Add 360 μL of lysate to the washed beads and proceed with the pre-clearing step incubating 1 h on a rotating wheel, at 4 °C. This step reduces background signal (*see* **Note 5**).

6. Use the magnetic stand to collect the beads and transfer the pre-cleared lysate to the beads coated with either the antibody recognizing the RBP and the control IgG from **step 6** of Subheading 3.2 (*see* **Note 6**).

7. Slowly rotate the IP sample at 4 °C tumbling end over end for 4 h or overnight (*see* **Note 7**).

8. Use the magnetic stand to collect the beads and transfer the supernatant into a new tube. Take a 40 μL aliquot of the supernatant and store it on ice for a subsequent control western blotting. This aliquot serves as a control for estimating the IP efficiency.

9. Wash the beads five times with 1 mL of ice-cold NT2 buffer by pipetting up and down with low-affinity filter tips. At the last washing step, dissolve the beads in 400 μL of the washing buffer and take 40 μL of the beads slurry and store it on ice for IP WB control. This sample, together with the input and supernatant, serves to control for effective RBP immunoprecipitation and potential loss of RBP during the washing steps (*see* **Note 8**).

10. Release the RNA from the RNP complexes by adding 1 mL of TRIZOL reagent directly to the tube (*see* **Note 9**).

11. Isolate RNA following the manufacturer's descriptions from either TRIZOL or by using the column-based Direct-Zol RNA MiniPrep kit.

12. Resuspend or elute the RNA in up to 15 μL RNase-free water for subsequent RNA quantification. RNA can be stored at 80 °C for months (*see* **Note 10**).

3.4. RNP Immunoprecipitation Controls

As mentioned before, in the case the RNA targets of the RBP are known, RIP technique can be suitable to analyze the composition and remodeling of its RNP

in particular experimental conditions. Instead, if the targets of the RBP are still unknown, after RIP it is possible to proceed with RNA-seq library preparation. In both cases it is important to perform control experiments to assess enrichment of both the RBP and RNA components of the RNP being immunoprecipitated.

1. Perform SDS–PAGE followed by Western blotting running the collected lysate samples, i.e., input, unbound, and IPs and incubating the membrane with the antibody of your RBP of interest and control antibody (*see* Fig. 1a and **Note 11**).

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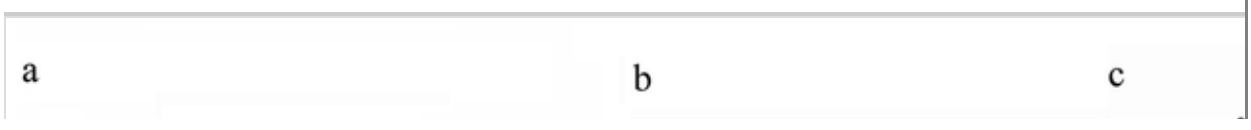
2. If you have information about candidate transcripts bound to your RBP,

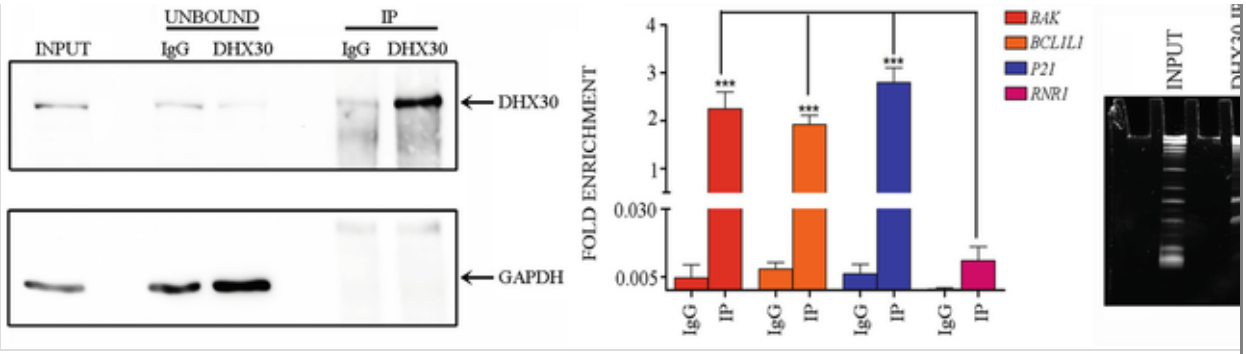
   perform qRT-PCR on the RNA samples (total RNA input, IP, and isotype-matched IP control) (*see* Fig. 1b and **Note 12**).

3. Instead, if no information about the potential targets is available and therefore it is not possible to proceed with control qRT-PCR, it is suggested to perform a denaturing RNA Urea-PAGE running input and IPs. In this way the best conditions for the RNA immunoprecipitation can be found and checked before proceeding with library preparation and RNA-seq (*see* Fig. 1c, **Note 13** and [17]).

Fig. 1

An example of RIP results. (**a**) The efficiency and specificity of immunoprecipitation of DHX30 antibody was verified by western blot, comparing unbound fractions and immunoprecipitates obtained with the specific antibody or the control IgG. (**b**) RNA immunoprecipitation (RIP) assays using a DHX30 antibody in HCT116 cells. Data are plotted as the percentage of input fold enrichment relative to the signal obtained for each mRNA examined in the control immunoprecipitation (IgG). Bars plot the average and the standard deviation of three biological replicates. *P*-value was calculated comparing the amount of each sample with the amount of *RNR1*, a negative control; ***$p < 0.001$, Student's t test. (**c**) Specific RNAs are enriched in the IP DHX30 eluates. DHX30-specific antibody or control IgG was used to immunoprecipitate DHX30 containing RNPs. RNAs were isolated and run onto 9.7% Urea-Denaturing PAGE. The gel was then stained with Sybr Gold (ThermoFisher) (Modified from Rizzotto et al. Cell Reports 2020, PMID 32234473)





4. Notes

1. Studying the RNP composition in a physiological context is highly recommended. However, IP-grade antibodies are not always commercially available for a specific RBP. In this case it is necessary to transfect and ectopically express an epitope-tagged RBP, and immunoprecipitate it using the antibody against the tag.

2. RNA samples must be handled cautiously to protect them from degradation caused by nucleases and heat. Always wear gloves. Keep samples at 4 °C as much as possible. Clean the workbench with nuclease-inhibitor solutions like RNase AWAY or equivalent. All solutions and buffers used for handling RNA samples should be prepared with pure and nuclease-free water and processed with nuclease-free filter tips in nuclease-free low-retention reaction tubes.

3. If a cycle of freezing and thawing is necessary to complete the cell lysis and to avoid post-lysis reassortment, on the contrary, one more cycle can determine the disruption of the complexes and degradation of the components.

4. It is recommended to set the amount of the antibody before starting with the RIP experiment. An excessive amount can increase a nonspecific signal, on the contrary using a too small amount can lead to a loss of signal.

5. RNAs have a certain grade of nonspecific affinity with protein A/G magnetic beads. It is strongly recommended to perform a pre-clearing step. However, this procedure can dramatically reduce the capture of low abundance RNA species.

6. Keene and other colleagues suggest to dilute PBL lysates ten times in NT2 buffer following these conditions: 850 μL of NT2, 100 μL of PBL

lysates, 20 mM EDTA, 1 mM DTT, 40 U/mL of RNase inhibitors, 1× protease inhibitors [16]. However, at least for DHX30 immunoprecipitation, we observed better results by diluting PBL lysates only three times in NT2 buffer.

7. Generally, 4 h are sufficient for the binding and it is recommended to avoid RNA degradation. However, when the RBP or the RNAs being investigated are expressed at low levels, overnight incubation is suggested.

8. Washing step is critical to reduce background signal. It is necessary to test the conditions for the specific RNP being investigated. To reduce background, it may be necessary to use more stringent wash conditions, such as adding from 0.5 M to 3 M urea, 0.1% or less SDS, or deoxycholate to the NT2 wash buffer. All tubes should be kept on ice as much as possible while working quickly during the washing process to reduce degradation.

9. Use of glycogen and overnight precipitation at −20 °C can improve the efficiency of the RNA recovery and extraction.

10. The volume of resuspension/elution can be adjusted to the expected yield. Of course, this will affect RNA concentrations for subsequent procedures. For accurate RNA quantification of the RIP samples use sensitive methods (e.g., Qubit RNA IQ Assay for the Qubit 4.0 Fluorometer, ThermoFisher).

11. This western blot control is necessary to verify if the RBP of interest was sufficiently immunoprecipitated from the sample. Expect to see a depleted signal in your supernatant and enrichment in your IP/bead fraction. However, complete RBP depletion in the supernatant fraction is not required. For the nonspecific isotype control IP, there should not be a signal in the IP/bead fraction for your RBP of interest. While IP conditions are being optimized, it is recommended to include an aliquot of the IP wash step to assess RBP loss due to too harsh buffer conditions.

12. Quantify the RNA samples and reverse transcribe approximately 1–10 ng of each RNA sample into cDNA. Verify the enrichment of candidate-bound transcript using qRT-PCR and include several controls of potentially non-bound transcripts. It is critical to observe enrichment

of the candidate-bound transcript in the RNP IP sample over input and isotype-matched control IP sample.

13. Even if sensitive RNA quantification methods are now available, before proceeding with expensive library preparation and sequencing, it is suggested to perform a denaturing RNA Urea-PAGE. Using an 8–9% gel and staining with Sybr Gold expect to see a faint or no signal in the control IgG and an appreciable signal in the IP.

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