Chapter 7

RIP: RNA Immunoprecipitation

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Abstract

The relevance of RNA-protein interactions in modulating mRNA and noncoding RNA function is increasingly appreciated and several methods have been recently developed to map them. The RNA immunoprecipitation (RIP) is a powerful method to study the physical association between individual proteins and RNA molecules in vivo. The basic principles of RIP are very similar to those of chromatin immunoprecipitation (ChIP), a largely used tool in the epigenetic field, but with some important caveats. The approach is based on the use of a specific antibody raised against the protein of interest to pull down the RNA-binding protein (RBP) and target-RNA complexes. Any RNA that is associated with this protein complex will also be isolated and can be further analyzed by polymerase chain reaction-based methods, hybridization, or sequencing.

Several variants of this technique exist and can be divided into two main classes: native and cross-linked RNA immunoprecipitation. The native RIP allows to reveal the identity of RNAs directly bound by the protein and their abundance in the immunoprecipitated sample, while cross-linked RIP leads to precisely map the direct and indirect binding site of the RBP of interest to the RNA molecule.

In this chapter both the protocols applied to mammalian cells are described taking into account the caveats and considerations required for designing, performing, and interpreting the results of these experiments.

Key words RNA immunoprecipitation, Native RIP, Cross-linked RIP, Protein-RNA interaction

1 Introduction

The interest in the interaction between proteins and RNAs as key aspect of gene regulation has increased over the last decade [1]. The growing expansion in sequencing technologies has facilitated the investigation of the transcriptome at unprecedented depth [2]. Therefore, the importance of messenger RNA (mRNA) processing, including alternative splicing, nuclear export, subcellular localization, and editing in producing diverse isoforms and in controlling the stability and translation of mRNAs, has largely reported [3–5]. Moreover, the identification of diverse classes of noncoding RNAs (ncRNAs), including many thousands of long noncoding RNAs (lncRNAs), has increasingly emerged [6, 7].

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All aspects of controlling gene expression, either by small regulatory RNAs, like microRNAs, and lncRNAs involve RNA-protein interactions. Indeed, RNA molecules can interact with proteins through their secondary or tertiary structure to create ribonucleoprotein complexes (RNPs). The central function of RNPs in mRNA processing and ncRNA function are well-established concepts. Thus, the main challenge for understanding RNA-mediated biological processes is identifying the RNAs associated with RNA-binding proteins (RBPs) in a cellular context.

Historically, individual mRNA targets have been identified using in vitro techniques such as cross-linking with ultraviolet light, nitrocellulose filter binding, and RNA electromobility shift assays (REMSAs; [8]). Although these methods have provided ample biochemical information, they are inadequate to identify unknown RNA targets when starting with an RBP. Furthermore, bioinformatic algorithms have been developed to search for novel mRNA targets of particular RBPs, but the efficacy of such approaches is not complete because they identify RNA-binding sites of few nucleotides which therefore appear more frequently among mRNAs than expected.

More recently, the predominant methods for exploring RNA-protein interactions are based on protein immunoprecipitation [9]. These methods require knowledge of the protein; therefore they are not useful for identifying the proteins that interact with a given RNA transcript. RNA immunoprecipitation (RIP) is an antibody-based technique used to identify RNA-protein interactions in vivo [10, 11]. Specific ribonucleoprotein complexes can be immunoprecipitated from a cellular lysate with an antibody raised against the protein of interest. Every RNA interacting with this protein complex can also be isolated and further examined by PCR-based methods, hybridization, or massive sequencing [11–13]. Bioinformatic tools have been developed to map reads to their transcripts of origin and to identify protein-binding sites, in case of cross-linked-based methods.

Binding maps of several RNA-binding proteins across the transcriptome have been created by using these techniques, thus providing key insights into how mRNA processing is regulated in the cell [14]. Also, early insights into the proteins interacting with lncRNAs, such as the protein of the Polycomb-repressive complex 2 (PRC2), have been gained with these approaches [15–17].

There are several variants of these methods, which can be divided into two main groups: native [12, 13, 17, 18] and cross-linked RNA immunoprecipitation [19–22]. Native methods detect RNA-protein complexes in physiological conditions. Although these approaches are valuable because preserve the native complexes existing in the cell, they nevertheless have several shortcomings. The first and well described is that ribonucleoproteins can re-associate after cell lysis, and therefore not accurately reproduce the interactions that occur in vivo [23].

Moreover, the presence of abundant transcripts with nonspecific interactions may lead to the underestimation of specific RNA-protein interactions. Indeed, ribosomal RNAs are often the largest contaminating RNA species in protein purifications [24]. Because of these concerns, the nature of the interactions detected by these methods has been quite debated. For instance, many lncRNAs, as well as mRNAs, were identified as interacting with PRC2 by using native protocols [13]. However, a recent study has claimed that virtually all transcripts may interact with PRC2 in the cell [25]. Thus, the biological significance of identified lncRNA-PRC2 interactions is currently subject of discussion, with scientists arguing that they are merely nonspecific interactions [26]. Yet, it is undoubted that some of these lncRNAs-PRC2 interactions have been confirmed and exhibit well-defined functional roles [15, 16, 27]. Given that the extent to which nonspecific RNA-protein associations are detected by the native approaches is not clearly quantifiable, the interactions identified with these methods often require further experimental validation, such as through the integration of multiple distinct experimental approaches [16, 28].

To prevent the re-association of proteins and RNA after cell lysis and to "freeze" their interactions in the cell, cross-linking agents can be used to fix all the interactions. UV cross-linking may be used to identify direct interactions between RNA and proteins with the limitation that UV-cross-linking is not reversible [19]. Cross-linking agents that are reversible may be more beneficial for subsequent characterization of the associated molecules [21]. One of the reversible cross-linking agents is formaldehyde, which is able to rapidly preserve cellular complexes in their native state, and to rapidly penetrate the cell membrane. These are the qualities that have led to its broad application in methods such as RNA immunoprecipitation [22]. An additional general weakness shared by both the approaches, the native and cross-linked RNA immunoprecipitation, concerns the quality of the antibody raised against the protein of interest. Indeed, the efficacy of the result is highly dependent on the antibody used and the abundance of the target ribonucleoprotein.

As shown in Fig. 1, either cross-linked or non-cross-linked RNA-protein complexes from living cells are involved in an RIP assay. These complexes are isolated by immunoprecipitation using a specific antibody towards the protein of interest. After reversing the cross-links, the interacting RNA can be analyzed by reverse transcription, followed by a polymerase chain reaction.

Immunoprecipitation of RNPs may be followed by genomic analysis using microarrays, known as RIP-Chip, or more recently using next-generation sequencing methods known as RIP-Seq. These are powerful high-throughput techniques for in vivo identifying RNA targets associated to specific proteins in cellular context. A considerable amount of bioinformatic analysis is necessary

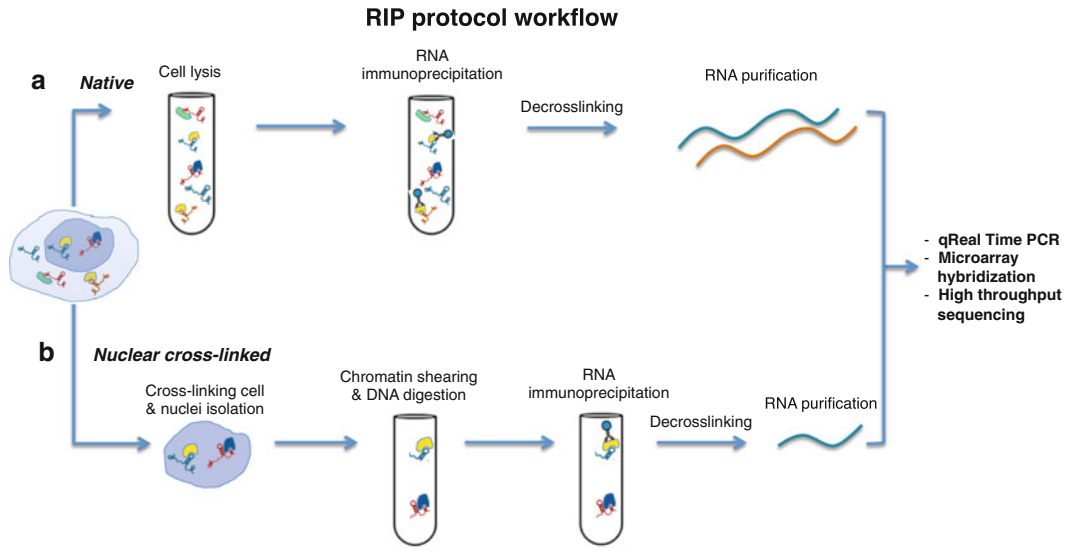

Fig. 1 Schematic representation of native and cross-linked RIP protocols. (**a**) For the native RIP, the harvested cells are directly lysed. The immunoprecipitation is performed using an antibody raised against the protein of interest. The RNA species are purified and analyzed by qPCR, microarray hybridization, and/or next-generation sequencing. (**b**) In the cross-linked protocol, live cells are treated by formaldehyde or another cross-linking agent (e.g., UV light) to fix the RNA/protein interactions. After the nuclear extraction, the chromatin is sheared and the DNA is degraded by DNase treatment. The immunoprecipitation is performed using specific antibody against the protein of interest. Following the immunoprecipitation and the reverse cross-linking to release the immunoprecipitated RNA, the RNA is extracted subsequently analyzed by qPCR, microarray hybridization, and/or next-generation sequencing

for processing raw array data to create a list of target gene mRNAs (or transcriptional fragments, in the case of RNA sequencing) as well as for statistical interpretation and analysis of the data. Primary data analysis often imposes further computational and experimental validation of putative identified associated RNA and potential RBP-binding sites [29, 30].

Here, we describe the detailed native and cross-linked RNA immunoprecipitation protocols allowing to select multiple RNA molecules, expressed in a specific cellular context, which are directly and/or indirectly interacting with the protein of interest.

2 Materials

Prepare all solutions using ultrapure water and analytical grade reagents.

1. Phosphate-buffered saline (PBS) pH 7.4 (stable at room temperature).
2. Polysome lysis buffer (10× PLB): 1000 mM KCl, 50 mM $MgCl_2$, 100 mM HEPES-NaOH pH 7, 5% Nonidet P-40

(NP-40). Prepare 10× stock buffer and store it at room temperature. Before using it, prepare PLB dilution to 1× and add 1 mM dithiothreitol (DTT), 200 units/ml RNase OUT, and EDTA-free Protease Inhibitor Cocktail.

3. NT-2 buffer (5×): 250 mM Tris-HCl pH 7.4, 750 mM NaCl, 5 mM $MgCl_2$, 0.25% NP-40. Store the stock buffer at 4 °C. Before use prepare NT-2 buffer 1× dilution.
4. NET-2 buffer: 1× NT-2 buffer supplemented with 20 mM EDTA pH 8.0, 1 mM DTT, 200 units/ml RNase OUT.
5. Formaldehyde solution: 50 mM HEPES-KOH, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 11% formaldehyde.
6. Cell lysis buffer: 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 0.5% NP-40. Before use add 1 mM DTT, 200 units/ml RNase OUT, and EDTA-free Protease Inhibitor Cocktail.
7. Nuclei resuspension buffer: 50 mM HEPES-NaOH pH 7, 10 mM $MgCl_2$. Before use add 1 mM DTT, 200 units/ml RNase OUT, and EDTA-free Protease Inhibitor Cocktail.
8. Immunoprecipitation buffer: 150 mM NaCl, 10 mM Tris–HCl pH 7.4, 1 mM EDTA, 1 mM EGTA pH 8, 1% Triton X-100, 0.5% NP-40. Before use add 1 mM DTT, 200 units/ml RNase OUT, and EDTA-free Protease Inhibitor Cocktail.
9. Proteinase K digestion buffer: 1× NT-2 buffer supplemented with 1% sodium dodecyl sulfate (SDS), 1.2 mg/ml Proteinase K.

3 Methods

3.1 Lysate Preparation (Native RIP)

1. Grow cells in an appropriate culture medium, stimulate or treat them, if necessary, and collect them when they are at ~80–90% of confluence (*see* **Note 1**).
2. Count cells using a hemacytometer. Consider to use ~2–5 mg of total protein extract for each immunoprecipitation which corresponds to ~$5–20 \times 10^6$ mammalian cells.
3. Collect cells by centrifugation at $1000 \times g$ for 5 min at 4 °C and discard the supernatant.
4. Wash cells twice with 1× ice-cold PBS. Collect cells by centrifugation at $1000 \times g$ for 5′ at 4 °C and discard the supernatant.
5. Resuspend cells in equal pellet volume of polysome lysis buffer. Pipette up and down to break clumps of cells (*see* **Note 2**).
6. Incubate on ice for 5 min.
7. Store at −80 °C to promote the cell lysis. The lysate may be stored for several months to −80 °C.

3.2 Preparation of Magnetic Beads and Immobilization of Antibodies (Native RIP)

1. Completely resuspend protein-A- or protein-G-coated magnetic beads before taking magnetic beads by pipetting or end-over-end rotation (*see* **Note 3**).
2. Add 75 µl protein-A or -G magnetic beads into a 1.5 ml tube and wash twice with 0.5 ml of NT-2 buffer.
3. Resuspend the beads in 100 µl of NT-2 and add 5 µg of the antibody of your interest and the negative control to the tubes.
4. Incubate with rotation for 1 h at room temperature to allow the binding of antibody to coated beads.
5. Centrifuge the tubes at 5000 × *g* for 15 s, place them on the magnetic rack, and remove the supernatant using a vacuum aspirator (*see* **Note 4**).
6. Remove the tubes from the rack, add 1 ml of NT-2, mix them by pipetting, spin at 5000 × *g* for 15 s, place the tube in the magnetic support, and remove the supernatant using a vacuum aspirator. Repeat this step another five times for a total amount of six washes.
7. After the sixth wash, resuspend the beads with 900 µl of NET-2 buffer and keep them in the ice (*see* **Note 5**).

3.3 Immunoprecipitation of Protein-RNA Complexes (Native RIP)

1. Centrifuge the cell lysate at 20,000 × *g* for 10 min at 4 °C, remove 100 µl of supernatant, and add it to each antibody-bead reaction. The final volume of the immunoprecipitation reaction will be 1 ml.
2. Take 10 µl (10%) of the cell lysate supernatant and place it in a new tube labeled "Input" (*see* **Note 6**). Store it at −80 °C until starting RNA immunoprecipitation.
3. Incubate all tubes on rotating wheel for 3 h up to overnight at 4 °C.
4. After the overnight incubation, spin down the tubes, place them on the magnetic support in the ice, incubate for 1 min, and discard the supernatant (*see* **Note 7**).
5. Remove the tubes from the magnet, add 1 ml of ice-cold NT-2 to each tubes, and vortex the samples vigorously.
6. Spin down the tubes, place them on the ice-cold magnetic separator, incubate for 1 min, and discard the supernatant.
7. Repeat **steps 5** and **6** another five times with 1 ml of ice-cold NT-2.

3.4 Lysate Preparation (Cross-Linked RIP)

1. Grow cells in an appropriate culture medium, stimulate or treat them, if necessary, and collect them when they are at ~80–90% of confluence (*see* **Note 1**).
2. Count cells using a hemacytometer. Consider to use ~2–5 mg of total protein extract for each immunoprecipitation, which corresponds to ~5–20 × 10^6 mammalian cells.

3. Add to cell suspension the necessary volume of formaldehyde solution to have 1 % final concentration. Incubate for 10 min at room temperature (*see* **Note 8**).
4. Stop the cross-linking reaction adding one-tenth the volume of 2.66 M glycine, and incubate for 5 min at room temperature and then 10 min on ice (*see* **Note 9**).
5. Wash cells twice with 1× ice-cold PBS. Collect cells by centrifugation at 1000 × *g* for 5′ at 4 °C and discard the supernatant.
6. Resuspend cells in 4 ml cell lysis buffer and incubate for 10–15 min in ice.
7. Homogenize by Dounce with 10 strokes with pestle A and 40 strokes with pestle B to allow the release nuclei.
8. Recover nuclei by centrifugation at 1000 × *g* for 10 min at 4 °C.
9. Resuspend nuclei with 3 ml of nuclei resuspension buffer and sonicate the nuclei to obtain DNA fragments in a range between 1000 and 200 bp.
10. After the sonication, add 250 unites/ml of DNase to the chromatin and incubate for 30 min at 37 °C.
11. Stop the DNase reaction adding EDTA to a final concentration of 20 mM.

    Adjust the sample with 1 % Triton X-100, 0.1 % sodium deoxycholate, 0.01 % SDS, and 140 mM NaCl.

3.5 Preparation of Magnetic Beads and Immobilization of Antibodies (Cross-Linked RIP)

1. Completely resuspend protein-A- or protein-G-coated magnetic beads before taking magnetic beads by pipetting or end-over-end rotation (*see* **Note 3**).
2. Add 75 µl protein-A or -G magnetic beads into a 1.5 ml tube and wash twice with 0.5 ml of nuclei resuspension buffer supplemented with 1 % Triton X-100, 0.1 % sodium deoxycholate, 0.01 % SDS, and 140 mM NaCl (*see* **Note 4**).
3. Resuspend the beads in 100 µl of complete nuclei resuspension buffer and add 5 µg of the antibody of your interest and the negative control to the tubes.
4. Incubate with rotation for 1 h at room temperature to allow the binding of antibody to coated beads.
5. Centrifuge the tubes at 5000 × *g* for 15 s, place them on the magnetic rack, and remove the supernatant using a vacuum aspirator.
6. Remove the tubes from the rack, add 1 ml of complete nuclei resuspension buffer, mix them by pipetting, spin at 5000 × *g* for 15 s, place the tube in the magnetic support, and remove the supernatant using a vacuum aspirator. Repeat this step another five times for a total amount of six washes.

7. After the sixth wash, resuspend the beads with 75 µl of complete nuclei resuspension buffer and keep them in the ice.

3.6 Immunoprecipitation of Protein-RNA Complexes (Cross-Linked RIP)

1. Centrifuge the cell lysate at 20,000 × *g* for 10 min at 4 °C, remove 975 µl of supernatant, and add it to each antibody-bead reaction. The final volume of the immunoprecipitation reaction will be 1 ml.
2. Take 9.75 µl (1%) of the cell lysate supernatant and place it in a new tube labeled "Input" (*see* **Note 6**). Store it at −80 °C until starting RNA immunoprecipitation.
3. Incubate all tubes on rotating wheel for 3 h up to overnight at 4 °C.
4. After the overnight incubation, spin down the tubes, place them on the magnetic support in the ice, incubate for 1 min, and discard the supernatant.
5. Remove the tubes from the magnet, add 1 ml of ice-cold Immunoprecipitation buffer to each tubes, and vortex the samples vigorously.
6. Spin down the tubes, place them on the ice-cold magnetic separator, incubate for 1 min, and discard the supernatant.
7. Repeat **steps 5** and **6** another five times with 1 ml of ice-cold immunoprecipitation buffer.

3.7 RNA Purification (Native and Cross-Linked RIP)

1. Resuspend each immunoprecipitate in 150 µl of Proteinase K buffer. To each "Input" add 107 µl of NT-2, 15 µl SDS 10%, and 18 µl of Proteinase K, to reach a total volume of 150 µl. Incubate all tubes at 55 °C for 30 min with shaking to digest the proteins.
2. After 30 min of incubation, spin down all tubes, place them in the magnetic rack, transfer the supernatants in a new tubes, and add to each of them 250 µl of NT-2.
3. Add 400 µl of phenol:chloroform:isoamyl alcohol (125:24:1) to each tube and vortex vigorously for 15 s. Centrifuge the tubes at 20,000 × *g* for 10 min at room temperature to separate the phases.
4. Carefully remove 350 µl of the aqueous phase without disturbing the protein interface. Place it in a new tube and add 400 µl of chloroform. Vortex the tubes for 15 s and centrifuge them at 20,000 × *g* for 10 min at room temperature to separate the phases.
5. Gently take 300 µl of the aqueous phase and place it in a new tube. To each tube add 50 µl of ammonium acetate 5 M, 15 µl of LiCl 7.5 M, 5 µl glycogen (5 mg/ml), and 850 µl of absolute ethanol.

6. Keep at −80 °C for 1 h to overnight to allow the RNA precipitation, then centrifuge at 20,000 × *g* for 30 min at 4 °C, and discard the supernatant gently.
7. Wash the pellet with 500 µl of cold 80 % ethanol.
8. Centrifuge at 20,000 × *g* for 15 min at 4 °C. Discard the supernatant carefully and air-dry the pellets.
9. Resuspend the pellets in 10–20 µl of RNase-free water and place the tube on ice.
10. Treat all the volume of each sample with DNase to remove residual contaminant DNA in the further analysis.

3.8 Gene-Specific Studies

1. The reverse transcription of the immunoprecipitated and input RNAs may be carried out with any commercially available reverse transcription enzyme and kit that use random examers as primers. Consider preparing two RT+ and one RT− for each sample, starting from the same volume of DNase-treated RNA.
2. After the cDNA synthesis of the positive and negative IPs and input, perform the real-time PCR preparing the dilution 1:8 with H_2O of all cDNAs [e.g., to have 16 µl of cDNA dilution add 2 µl cDNA stock + 14 µl pure H_2O].
3. For the protocol of the single reaction follow the datasheet of chosen supermix. To robust results it is important to perform triplicates for each samples.

3.9 qPCR Analysis

1. Use the software of the real-time instrument to monitoring the amplification reaction. At the end of the run, in the log-scale view, the slopes of the amplification curves for all the assays should be parallel to each other to be comparable.
2. In the log-scale view of the amplification curve, manually position the threshold near the mid-point of the linear range. The threshold value should be the same for all the triplicate reactions in the same gene study.
3. Check the dissociation curve to confirm that each reaction produces a single specific product. In this case the chart should appear a single peak at a melting temperature (Tm) greater than 75 °C.
4. Export all Ct with appropriate labels in an Excel spreadsheet.
5. Calculate the average Ct between replicates.
6. Normalize each IP fractions' Ct average to the input fraction Ct average for the same qPCR Assay (ΔCt) to account for sample preparation differences:

$$\mathbf{\Delta Ct[normalized\ RIP]} = (\text{Average Ct[RIP]} - (\text{Average Ct[Input]} - \log_2(\text{Input Dilution Factor})))$$

where Input Dilution Factor = (fraction of the input RNA saved)$^{-1}$

7. Calculate the % input for each RIP fraction (linear conversion of the normalized RIP ΔCt):

   $$\textbf{\% Input} = 2^{(-\Delta Ct[\text{normalized RIP}])}$$

8. It is possible to adjust the normalized RIP fraction Ct value for the normalized background (negative control) fraction Ct value (first ΔΔCt):

   $$\textbf{\Delta\Delta Ct[negative control]} = \Delta Ct[\text{normalized ChIP}] - \Delta Ct[\text{negative control}]$$

9. Calculate RIP fold enrichment above the sample-specific background (linear conversion of the first ΔΔCt):

   $$\textbf{Fold Enrichment} = 2^{(-\Delta\Delta Ct\,[ChIP/NIS])}$$

3.10 Microarray Processing

Several platforms are available to perform the microarray hybridization assay after the purifying the immunoprecipitated RNAs. They share the same limit that is based on the supervised concept of technique. In fact, the microarray method allows investigating the presence of annotated genes in the immunoprecipitated material. Bioinformatic analysis of the data coming from these experiments is commonly performed with the "Human Gene 1.0 ST Array" from Affimetrix and the Agilent's "GeneSpring GX10" software. A typical analysis is carried out on triplicates of each sample (INPUT RNA and positive and negative IPs). The replicates in each set are subject to the basic quality control including the correlation study and the principal component analysis (PCA). PLIER16 algorithm, an iterative pipeline that is followed by the filtering for the 20th–100th percentile, is often used to obtain the gene abundance estimation in each set. Using the same algorithm it is possible to extract a list of genes that show a minimum twofold increase of measured abundance in the treatment versus input. A one-way analysis of variance (ANOVA) may be performed on this RNA subset.

3.11 Sequencing

1. The analysis of whole transcriptome interacting with a specific protein (RIP-Seq) may be performed using the next-generation sequencing. This technology is potentially unbiased if the choice of the library preparation is correct. For instance, the poli(A) separation will reduce much the RNA population if most of bound RNAs are noncoding RNAs (ncRNAs) or the introduction of the size selection step will discard the small ncRNAs.
2. Another bias that must be considered is that considering the less abundant RNAs. For this reason a depth of sequencing minimum of ~30 million of reads is suggested [29].
3. After the sequencing, the produced reads can be aligned to the reference genome with either Tophat or Botwie tools.
4. For the native RIP-seq, the estimation of the abundance of each RNA molecules in the samples can be analyzed using

programs to assemble transcriptomes from RNA-Seq data and quantify their expression [e.g., Cufflinks]. The estimated abundance of each type of RNA in the RIP sample can be normalized against the proper input and then compared with the values in the negative control, after the normalization.

5. In case of cross-linked RIP-seq, the analyses after mapping the reads are different from the native procedure because only RNA fragments selected for the interaction with the protein of interest should be enriched in the immunoprecipitated sample. The position of the protein-binding site on the RNA transcript is mapped by using the peak caller algorithms as used for the ChIP [e.g., MACS1.4]. After detecting those positions, it is also possible to evaluate the presence in the peak of a frequent motif with appropriate motif discovery tools [e.g., MEME suite]. These type of studies allow understanding if the binding of protein is mediated by a sequence recognition.

4 Notes

1. The generic precaution on working with RNA is to use instruments, tips, and tubes DNase and RNase free. Gloves, benches, and pipettes may be accurately cleaned before the use.
2. During cell lysate preparations occasionally vortex the cell pellet-PLB mixture to promote thawing. Once the cells are fully thawed, vortex vigorously to allow cell lysis. Poor vortexing may result in a low amount of protein-RNA available for the immunoprecipitation.
3. The type of beads utilized for the immunoprecipitation depends on the immunoglobulin isotype and species. It is useful to check the bead manufacturer's binding chart to determine the best choice of beads. In most cases, mouse monoclonal antibodies have stronger affinity for protein G and rabbit polyclonal antibodies have stronger affinity for both protein G and protein A. To solve this problem it is possible to prepare a 1:1 stock mixture of protein A- and protein G-coated magnetic beads, washed with the proper buffer, and stored at 4 °C with 0.02 % sodium azide.
4. To remove the supernatant, place the tubes in the magnetic support and wait for the complete settling of beads on the tube site that interact with the magnet. While aspirating the supernatant, be sure to change tips between the samples.
5. The presence of EDTA in the NET-2 buffer avoids the immunoprecipitation of ribosomal RNA disrupting the interaction between these molecules and the ribosomal proteins.
6. The input will be used to generate the standard curve in the further real-time PCR analysis. It is essential to compare the

sample to a negative control since detecting the enrichment from a specific RNA alone may not indicate an existent interaction. One control is to normalize the level of an RNA observed after purification to its abundance in total lysate (the input sample in RIP assay). Moreover, interactions can also occur due to unspecific associations with the purification resin or other reagents of the procedure. To measure these artifactual associations, other proteins can be used as negative controls. However, the negative control should be carefully selected, as a non-RNA-binding protein is likely to have lower nonspecific RNA binding.

7. The immunoprecipitation efficiency of the chosen antibody should be verified by Western blot in SDS-PAGE. Therefore, it is recommended to store additional 10 μl of the input material and compare it with the abundance of the protein of interest in 100 μl of the first unbound and 100 μl of the bead suspension after the sixth wash.
8. To efficiently cross-link adherent cells, after harvesting and counting them, it is necessary to wash them with ice-cold PBS and perform the cross-linking in cold PBS. As in the ChIP assay, the time of the cell exposure to the formaldehyde is important to obtain optimal results. A too short time of incubation with formaldehyde leads to the underestimation of RNA-protein interaction due to the incomplete cell fixation. A too long time of cross-linking increases the number of protein fixed together, thereby reducing the shearing efficiency and increasing nonspecific signals.
9. To perform an efficient sonication is a crucial step to obtain a good quality of immunoprecipitation. The sonication is strictly dependent on the cell type, and the density and amount of treated cells. It is also important to preserve protein degradation incubating the sample at 4 °C and using a medium power intensity with the alternation of the pulse step with an OFF step to avoid the increase of the temperature. The sonication efficiency could be analyzed on an aliquot of sheared chromatin which has to be de-cross-linked at 56 °C for 4 h. After the reverse cross-linking the DNA is purified with a common protocol using phenol:chlorofom:isolamyl alcohol (24:24:1) or with specific kits to DNA extraction. The sheared and purified DNA is loaded on agarose gel 1.5 % in TAE and for 30 min at 100 V. After the running, the gel is incubated for 5 min in EtBr solution (40 mg in 100 ml H_2O) and then in pure H_2O for 10 min. The fragment length is checked by using UV lamp. A good sonication should show an important enrichment of fragment between 1000 and 200 bps. If the DNA is not sheared enough repeat the sonication steps.

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