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Characterization of Long Non-coding RNA Associated Proteins by RNA-immunoprecipitation

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Abstract

With the advances in sequencing technology and transcriptome analysis, it is estimated that up to 75% of the human genome is transcribed into RNAs. This finding prompted intensive investigations on the biological functions of non-coding RNAs and led to very exciting discoveries of microRNAs as important players in disease pathogenesis and therapeutic applications. Research on long non-coding RNAs (lncRNAs) is in its infancy, yet a broad spectrum of biological regulations has been attributed to lncRNAs. RNA-immunoprecipitation (RNA-IP) is a technique of detecting the association of individual proteins with specific RNA molecules *in vivo*. It can be used to investigate lncRNA-protein interaction and identify lncRNAs that bind to a protein of interest. Here we describe the protocol of this assay with detailed materials and methods.

Keywords

long non-coding RNA; RNA-binding protein; RNA-immunoprecipitation

1. INTRODUCTION

lncRNAs are operationally defined as RNA transcripts larger than 200 nt that do not appear to have coding potential (1–5). Given that up to 75% of the human genome is transcribed to RNA, while only a small portion of the transcripts encodes proteins (6), the number of lncRNA genes can be large. After the initial cloning of functional lncRNAs such as H19 (7, 8) and XIST (9) from cDNA libraries, two independent studies using high-density tiling array reported that the number of lncRNA genes is at least comparable to that of protein-

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coding genes (10, 11). Recent advances in tiling array (10–13), chromatin signature (14, 15), computational analysis of cDNA libraries (16, 17), and next-generation sequencing (RNA-seq) (18–21) have revealed that thousands of lncRNA genes are abundantly expressed with exquisite cell-type and tissue specificity in human. In fact, the GENCODE consortium within the framework of the ENCODE project recently reported 14,880 manually annotated and evidence-based lncRNA transcripts originating from 9,277 gene loci in human (6, 21), including 9,518 intergenic lncRNAs (also called lincRNAs) and 5362 genic lncRNAs (14, 15, 20). These studies indicate that 1) lncRNAs are independent transcriptional units; 2) lncRNAs are spliced with fewer exons than protein-coding transcripts and utilize the canonical splice sites; 3) lncRNAs are under weaker selective constraints during evolution and many are primate specific; 4) lncRNA transcripts are subjected to typical histone modifications as protein-coding mRNAs, and 5) the expression of lncRNAs is relatively low and strikingly cell-type or tissue-specific.

The discovery of lncRNA has provided an important new perspective on the centrality of RNA in gene expression regulation. lncRNAs can regulate the transcriptional activity of a chromosomal region or a particular gene by recruiting epigenetic modification complexes in either *cis*- or *trans*-regulatory manner. For example, Xist, a 17 kb X-chromosome specific non-coding transcript, initiates X chromosome inactivation by targeting and tethering Polycomb-repressive complexes (PRC) to X chromosome in *cis* (22–24). HOTAIR regulates the HoxD cluster genes in *trans* by serving as a scaffold which enables RNA-mediated assembly of PRC2 and LSD1 and coordinates the binding of PRC2 and LSD1 to chromatin (12, 25). Based on the knowledge obtained from studies on a limited number of lncRNAs, at least two working models have been proposed. First, lncRNAs can function as scaffolds. lncRNAs contain discrete protein-interacting domains that can bring specific protein components into the proximity of each other, resulting in the formation of unique functional complexes (25–27). These RNA mediated complexes can also extend to RNA-DNA and RNA-RNA interactions. Second, lncRNAs can act as guides to recruit proteins (24, 28, 29), such as chromatin modification complexes, to chromosome (24, 29). This may occur through RNA-DNA interactions (29) or through RNA interaction with a DNA-binding protein (24). In addition, lncRNAs have been proposed to serve as decoys that bind to DNA-binding proteins (30), transcriptional factors (31), splicing factors (32–34) or miRNAs (35). Some studies have also identified lncRNAs transcribed from the enhancer regions (36–38) or a neighbor loci (18, 39) of certain genes. Given that their expressions correlated with the activities of the corresponding enhancers, it was proposed that these RNAs (termed enhancer RNA/eRNA (36–38) or ncRNA-activating/ncRNA-a (18, 39)) may regulate gene transcription.

RNA-immunoprecipitation (RNA-IP) is a technique of detecting the association of individual proteins with specific RNA molecules *in vivo*. It can be used to investigate lncRNA-protein interaction and identify lncRNAs that bind to a protein of interest. Here we describe the protocol of this assay with detailed materials and methods.

2. MATERIALS

Prepare all solutions using ultra-pure RNase-free water and analytical grade reagents. Contamination of the *solutions* with RNase can result in RNA degradation. Use filtration or/and autoclave sterilization to ensure that all reagents and supplies used in this section are RNase-free. Use RNase ZAP to clean all equipment and work surface.

1. Sucrose
2. 1 M Tris-HCl (pH 7.4)
3. 1 M MgCl₂
4. Triton X-100
5. 1 M KCl
6. 0.5 M EDTA
7. NP-40
8. 1 M Dithiothreitol (DTT)
9. 10x Phosphate-Buffered Saline (PBS, Invitrogen, AM9625): to make 1x PBS, mix one part of 10X PBS with nine parts RNase-free water. Store at 4°C.
10. Protein A/G beads (Sigma, P9424).
11. RNase inhibitor (Invitrogen, 10777-019).
12. Protease inhibitor cocktail (Sigma, P8340).
13. TRIzol RNA extraction reagent (Invitrogen).
14. 1 mL Dounce homogenizer (Fish Scientific, FB56687).
15. Nuclear Isolation Buffer: 1.28 M sucrose, 40 mM Tris-HCl (pH 7.4), 20 mM MgCl₂, 4% Triton X-100. Put 40 mL RNase-free water in a beaker with a stir bar and dissolve 21.9 g sucrose in the beaker. Add 2 mL 1 M Tris-HCl (pH 7.5), 1 mL 1 M MgCl₂ and 2 mL Triton X-100 and mix well. Make up to a final volume of 50 mL with RNase-free water, store at 4°C.
16. RNA Immunoprecipitation (RIP) Buffer: 150 mM KCl, 25 mM Tris (pH 7.4), 5 mM EDTA, 0.5% NP-40. Mix 7.5 mL 1 M KCl, 1.25 mL 1 M Tris-HCl (pH 7.4), 500 uL 0.5 M EDTA, and 250 uL NP-40 and make up to a final volume of 48 mL with RNase-free water. Store at 4°C. Right before use, add DTT (0.5 mM final concentration), RNase inhibitor (100 U/mL final concentration) and protease inhibitor cocktail (1x final concentration).

3. METHODS

The procedures must be performed in an RNase-free environment. Use filtered-tips and RNase-free tubes and clean all equipment and work surface with RNase ZAP before starting the experiment. lncRNA-IP aims to identify lncRNA species that bind to a protein of interest. The protocol includes two parts: 1) preparing protein lysate from target cells and 2)

immunoprecipitating the protein of interest and extract protein-bound RNAs. It is up to the readers to decide the subsequent analysis on the isolated RNAs. Before harvesting cells, pre-cool 1x PBS, RNase-free water, nuclear isolation buffer and RIP buffer on ice; estimate the amount of RIP buffers needed and add RNase inhibitor and protease inhibitor cocktail to the buffer accordingly (see Note 1).

3.1.1 Whole Cell lysate preparation (see Note 2)

If nuclear RNA-protein interaction is the focus of the research, skip this step and go directly to 3.1.2 for nuclear lysate preparation.

1. Harvest cells using regular trypsinization technique and count the cell number.
2. Wash cells in ice-cold 1x PBS once and resuspend the cell pellet (1.0×10^7 cells) in 1 mL ice-cold RIP buffer containing RNase and protease inhibitors.
3. Shear the cells on ice using a dounce homogenizer with 15 to 20 strokes.
4. Centrifuge at 15,000 g for 15 min at 4°C and transfer the supernatant into a clean tube. This supernatant is the whole cell lysate.

3.1.2 Cell harvest and nuclei lysate preparation (see Note 2)

1. Harvest cells using regular trypsinization technique and count the cell number.
2. Wash cells in ice-cold 1x PBS three times and resuspend 1.0×10^7 cells in 2 mL ice-cold PBS (see Note 3).
3. Put cell suspension in 1x PBS on ice, add 2 mL ice-cold nuclear isolation buffer and 6 mL ice-cold RNase-free water into the tube and mix well, incubate the cells on ice for 20 min with intermittent mixing (four to five times).
4. Harvest nuclei by spinning the tube at 2500 g for 15 min at 4°C. The pellet contains the purified nuclei.
5. Resuspend nuclei pellet in 1 mL freshly prepared ice-cold RIP buffer containing DTT, RNase and protease inhibitors.
6. Shear the nucleus on ice with 15 to 20 strokes using a dounce homogenizer.
7. Pellet nuclear membrane and debris by centrifugation at 16,000 g for 10 min at 4°C.
8. Carefully transfer the clear supernatant (nuclear lysate) into a new tube. The supernatant is nuclear lysate.

¹It is utterly important that the experiment described above is conducted with extra precaution to avoid RNA degradation. All materials have to be RNase-free and the buffers need to be precooled on ice.

²To ensure result reproducibility, the cells need to be maintained consistently.

³The abundances of different target protein and lncRNAs may vary from cell line to cell line, therefore the amount of lysate input needs to be empirically determined for each assay. We found 1.0×10^7 cells is a good starting point. In cases more cells are needed, scale up the amount of buffer used to ensure high nuclear lysing efficiency.

3.1.3 RNA immune-precipitation and purification

1. Wash 40 uL protein A/G beads with 500 uL ice-cold RIP buffer three times. After the wash, spin down the beads at 600 g for 30 s at 4°C, take off the RIP buffer and add 40 uL RIP buffer to resuspend the beads.
2. Add the prewashed beads and 5–10 ug IgG and into the whole cell lysate from 3.1.1 or nuclear lysate from 3.1.2.
3. Incubate the lysate with IgG and beads at 4°C with gentle rotation for 1 h. Pellet the IgG with beads by centrifugation at 16,000 g for 5 min.
4. Carefully transfer the supernatant (pre-cleared nuclear lysate) into a new tube. At this point, the lysate can be divided into multiple portions of equal volume for different antibodies and corresponding controls. Take 50 uL lysate and set aside on ice as input control.
5. Add antibody of interest into nuclear lysate (see Note 4), incubate the lysate and antibody overnight at 4°C with gentle rotation.
6. The next day, add 40 uL prewashed protein A/G beads and incubate at 4°C for 1 h with gentle rotation.
7. Pellet the beads by spinning at 600 g for 30 s at 4°C, remove supernatant.
8. Wash the beads with 500 uL ice-cold RIP buffer three times, invert five to ten times during each wash and pellet the beads by spinning at 600 g for 30 s at 4°C.
9. Wash the beads with 500 uL ice-cold PBS and pellet the beads by spinning at 600 g for 30 s at 4°C, and use a fine needle or tip to remove as much PBS as possible without disturbing the beads.
10. Resuspend beads in 1 mL TRIzol RNA extraction reagent and isolate co-precipitated RNA according to manufacturer's instructions.
11. Dissolve RNA in nuclease-free water and store the RNA at –80°C for further application (see Note 5).

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⁴The amount of antibody used for each experiment need to be empirically determined. Our suggestion is to start at around 1 – 2 ug antibody per million cells.

⁵The amount of nuclease-free water used to dissolve the RNAs are determined by several factors, including the type of downstream analysis, the amount of lncRNA bound to the target protein and the cell type. We recommend the researchers start at 20 uL and adjust according to their specific situations.

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