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Methods for the Study of Long Noncoding RNA in Cancer Cell Signaling

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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 noncoding RNAs and led to very exciting discoveries of microRNAs as important players in disease pathogenesis and therapeutic applications. Research on long noncoding RNAs (lncRNAs) is in its infancy, yet a broad spectrum of biological regulations has been attributed to lncRNAs. Here, we provide a collection of detailed experimental protocols for lncRNA studies, including lncRNA immunoprecipitation, lncRNA pull-down, lncRNA northern blot analysis, lncRNA in situ hybridization, and lncRNA knockdown. We hope that the information included in this chapter can speed up research on lncRNAs biology and eventually lead to the development of clinical applications with lncRNA as novel prognostic markers and therapeutic targets.

Keywords

Long noncoding RNA; RNA immunoprecipitation; RNA pull-down; In situ hybridization; Northern blots; Short hairpin RNA

1 Introduction

Cancer is a genetic disease involving multistep changes in the genome [1]. While up to 75% of the human genome is transcribed to RNA, only less than 2% of the genome encodes protein-coding transcripts, leaving most of the genome to noncoding RNA transcripts [2, 3].

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The recent discovery of the noncoding RNA genes has dramatically altered our understanding on cancer genetics. In the last decade, the functional significance of small non-coding RNA, microRNA, in tumorigenesis and progression has been extensively documented; yet research on long noncoding RNA (lncRNA) is still in its infancy [4-8]. lncRNAs are operationally defined as RNA genes larger than 200 nucleotides that do not appear to have protein coding potential [4-8]. Recent studies demonstrated that lncRNAs act as key regulators of development, differentiation, apoptosis, and cell proliferation, all of which have been implicated in tumor initiation and progression. In addition, the expression of lncRNAs has been found to be remarkably deregulated by epigenetic and genomic alterations in tumors. In various experimental systems, lncRNAs have reported to have tumor suppressor or oncogene activity. Therefore, it is reasoned that lncRNAs may play important roles in the development of cancer, hence represent the leading edge of cancer research. Investigations on lncRNA functions in cancer will lead to a greater understanding of molecular mechanisms of this disease, and eventually lead to the development of lncRNA-based novel applications in cancer diagnosis and therapeutic management.

1.1 The Human Genome Contains Many Thousands of Unexplored lncRNAs

lncRNAs are operationally defined as RNA transcripts larger than 200 bp that do not appear to have coding potential [4-8]. Given that up to 75% of the human genome is transcribed to RNA, while only a small portion of the transcripts encodes proteins [3], the number of lncRNA genes can be large. After the initial cloning of functional lncRNAs such as H19 [9, 10] and XIST [11] 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-coding genes [12, 13]. Recent advances in tiling array [12-15], chromatin signature [16, 17], computational analysis of cDNA libraries [18, 19], and next-generation sequencing (RNA-seq) [20-23] have revealed that thousands of lncRNA genes are abundantly expressed with exquisite cell-type and tissue specificity in human. In fact, the GENCODE consortium (version 31) within the framework of the ENCODE project recently reported 48,227 lncRNA transcripts originating from 17,904 lncRNA genes in human. 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.

1.2 lncRNAs Regulate Gene Expression and Protein Functions Via Various Mechanisms

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 noncoding transcript, initiates X chromosome inactivation by targeting and tethering Polycomb-repressive complexes (PRC) to X chromosome in cis [24-26]. 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

[14, 27]. Based on the knowledge obtained from studies on a limited number of lncRNAs, 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 [27-29]. These RNA mediated complexes can also extend to RNA-DNA and RNA-RNA interactions. lncRNAs can also act as guides to recruit proteins [26, 30, 31], such as chromatin modification complexes, to chromosome [26, 31]. This may occur through RNA-DNA interactions [31] or through RNA interaction with a DNA-binding protein [26]. In addition, lncRNAs have been proposed to serve as decoys that bind to DNA-binding proteins [32], transcriptional factors [33], splicing factors [34-36], or miRNAs [37]. Some studies have also identified lncRNAs transcribed from the enhancer regions [38-40] or a neighbor locus [20, 41] 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 [38-40] or ncRNA-activating/ncRNA-a [20, 41]) may regulate gene transcription.

1.3 lncRNA Expression Is Deregulated in Human Cancer

The advances in high-throughput RNA quantification technologies unveiled a profound deregulation of the lncRNome in human cancer. First, lncRNA expression profiles are dramatically different between tumors and their adjacent normal tissues. A comprehensive study analyzing lncRNA expressions in 5860 tumor samples from 13 cancer types and 424 normal specimens from nine matching tissue types from the Cancer Genome Atlas project revealed that dysregulation of expression of lncRNA is common in cancer [42]. Second, given that lncRNA expression patterns are more tissue-specific that those of protein coding genes [22, 23], it has been proposed that lncRNA expression signatures may be able to accurately determine the developmental lineage and tissue origin of human cancers. Third, the association between the expressions of several lncRNAs, such as MALAT-1 [43], HOTAIR [15], PCAT-1 [21], and LET [44], and cancer metastasis have been identified by high-throughput profiling studies and validated by further independent investigations, suggesting that lncRNAs may also serve as robust biomarkers in predicting cancer prognosis and survival.

1.4 lncRNAs Serve as Tumor Suppressor Genes or Oncogenes

Though studies on lncRNAs are still in its early stage, it is clear that lncRNAs are involved in regulating proliferation [33, 36, 45], differentiation [16, 31, 46-48], migration [15, 20] and apoptosis [30, 49]. Therefore, it is reasoned that deregulation of lncRNA expression may contribute to the development and progression of cancer. In fact, some lncRNAs have been shown to function as oncogenes or tumor suppressors. For example, HOTAIR [14] can induce metastasis [15] by operating as a tether that links EZH2/PRC2 and LSD1, therefore coordinating their epigenetic regulatory functions [27]. ANRIL, an antisense lncRNA of the CDKN2A/CDKN2B gene, represses INK4A/INK4B expression [50] by binding to CBX7/PRC1 [28] and SUZ12/PRC2 [29]. On the other hand, deleting Xist resulted in the development of highly aggressive myeloproliferative neoplasm and myelodysplastic syndrome with 100% penetrance in female mice [51].

1.5 lncRNAs Represent Promising Biomarker and Therapeutic Candidates for Cancer Diagnosis and Treatment

The ability to fully characterize cancer genome contributed significantly to the development of biomarkers and therapeutic applications for cancer diagnoses and treatments. PCA3, a prostate cancer-associated lncRNA [52], is the first prominent example of lncRNA as a novel biomarker. The noninvasive method to detect PCA3 transcript in urine has been developed and used clinically to detect prostate malignancy [53]. The transition from lncRNA-based diagnostics to lncRNA-based therapies is also under intensive investigations. The rapid advances in oligonucleotide/nanoparticle therapy create realistic optimism for developing lncRNA-based therapeutic tools for cancer treatment. Although the majority of cancer-related studies still focus on the protein-coding genes, given that almost 75% of the genome is transcribed to RNAs and initial studies on a handful of lncRNAs clearly demonstrated their functional significance in cancer development and high potential in clinical applications, we argue that investigations on lncRNAs is the leading edge of cancer research.

1.6 Methods in lncRNAs Research

In the following sections, we provide detailed protocols on characterizing lncRNA expression and functions. They are RNA-immunoprecipitation (RNA-IP), RNA pull-down, Northern blot analysis on lncRNA expression, In situ hybridization (ISH) of lncRNA and lncRNA knockdown, respectively. As one major mechanism for lncRNA to exert its function is to serve as a scaffold via RNA-protein interaction, it is important to investigate which lncRNAs are binding to a protein of interest. RNA-IP is developed to identify lncRNA species that bind to a protein of interest. On the other hand, if the research focus is to identify the proteins that are bound to a given lncRNA, lncRNA pull-down will help to identify the protein molecules that interact with a specific lncRNA (Fig. 1). Moreover, as a novel class of RNA transcripts, it is important to characterize the expression of lncRNAs in various systems. While the northern blot can be used to determine lncRNA abundance and identify differ splicing variants of a given lncRNA (Fig. 2); lncRNA in situ hybridization can provide information regarding the expression level of a given lncRNA, more importantly, it can reveal the cellular or tissue localization of the lncRNA of interest. Knocking down the expression of a target gene has been a gold standard assay to elucidate its endogenous function. To this aspect, we also included two lncRNA knockdown protocols in this chapter. We hope this chapter can help the readers to develop assays for their lncRNA research which will lead to a better understanding on the roles of lncRNAs in carcinogenesis and other pathological conditions.

2 Materials

Prepare all solutions using ultrapure 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.

2.1 lncRNA-Immunoprecipitation

1. Sucrose.
2. 1 M Tris-HCl (pH 7.4).
3. 1 M $MgCl_2$.
4. Triton X-100.
5. 1 M KCl.
6. 0.5 M EDTA.
7. NP-40.
8. 1 M Dithiothreitol (DTT).
9. 10× phosphate-buffered saline (PBS): to make 1× PBS, mix one part of 10× PBS with nine parts RNase-free water. Store at 4 °C.
10. Protein A/G beads.
11. RNase inhibitor.
12. Protease inhibitor cocktail.
13. TRIzol RNA extraction reagent.
14. 1 mL Dounce homogenizer.
15. Nuclear Isolation Buffer: 1.28 M sucrose, 40 mM Tris–HCl (pH 7.4), 20 mM $MgCl_2$, 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 $MgCl_2$, 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 μL 0.5 M EDTA, and 250 μL 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 (1× final concentration).

2.2 lncRNA Pull-Down

1. Sucrose.
2. 1 M Tris-HCl (pH 7.4).
3. 1 M $MgCl_2$.
4. Triton X-100.
5. 1 M KCl.
6. NP-40.
7. 1 M NaCl.

8. 1 M Dithiothreitol (DTT).
9. 10× phosphate-buffered saline (PBS): to make 1× PBS, mix one part of 10× PBS with nine parts RNase-free water. Store at 4 °C.
10. DNA template (*see* Note 1).
11. Restriction enzyme.
12. Vanadyl-ribonucleoside complex (VRC).
13. 10× Biotin RNA labeling mix (Roche)
14. T7 RNA polymerase (10 U/μL) and 5× transcription buffer (Agilent).
15. RNase inhibitor.
16. Protease inhibitor cocktail.
17. DNase I 2000 U/mL.
18. Straptavidin agarose beads.
19. 0.5 M EDTA (pH 8.0).
20. Yeast tRNA.
21. Nuclear Isolation Buffer: 1.28 M sucrose, 40 mM Tris–HCl (pH 7.4), 20 mM $MgCl_2$, 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 $MgCl_2$, 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.
22. 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 μL 0.5 M EDTA, and 250 μL 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 (1× final concentration).
23. NT2 Buffer: 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM $MgCl_2$, 0.05% NP-40. Store at 4 °C. For 50 mL NT2 buffer, mix 2.5 mL 1 M Tris–HCl, 5 mL 150 nM NaCl, 1 mL 1 mM $MgCl_2$, 2.5 mL 1% NP-40, add 39 mL RNase-free water to a final volume of 50 mL, filter stock solution, store at 4 °C. Right before use, add RNase inhibitor (100 U/mL final concentration), Vanadyl-ribonucleoside complex (VRC, 400 nM final concentration), DTT (1 mM final concentration), EDTA (20 mM final concentration), and protease inhibitor cocktail (1× final concentration).
24. RNA structure buffer: 10 mM Tris (pH 7.0), 0.1 M KCl, 10 mM $MgCl_2$.

1. The desired DNA template should be a plasmid containing a promoter for in vitro transcription (i.e., T7 or T3) and a target sequence whose 5′ end is placed as close as possible to the 3′ end of the promoter. We usually use pBluescript SK(+) and transcribe the target sequence using T7 polymerase. Minimize any unnecessary addition of non-lncRNA sequence into the plasmid to avoid impropriate RNA folding.

25. 1 mL Dounce homogenizer (Fish Scientific).
26. Agarose gel electrophoresis supplies for DNA fragment purification.
27. Quick Spin Columns for radiolabeled RNA purification Sephadex G-50.
28. Gel Extraction Kit (Qiagen).
29. BCA protein assay kit.
30. 2× Laemmli loading buffer: 4% SDS, 120 mM Tris–HCl (pH 6.8), 0.02% bromophenol blue and 0.2 M DTT. Mix 4 mL 10% SDS, 1.2 mL 1 M Tris–HCl (pH 6.8), 200 μL 1% bromophenol blue, and 2 mL 1 M DTT, and add milliQ water to make the final volume to 10 mL. Make 500 μL aliquots to minimize the freeze-and-thaw cycles.

2.3 lncRNA Northern Blot Analysis

2.3.1 DIG labeled RNA Probe Synthesis

1. DNA template (*see* Note 1).
2. Restriction enzyme.
3. 10× DIG RNA labeling mix (Roche).
4. T7 RNA polymerase (10 U/μL) and 5× transcription buffer (Agilent).
5. Dnase I 2000 U/mL.
6. Agarose gel electrophoresis supplies for DNA fragment purification.
7. Quick Spin Columns for radiolabeled RNA purification Sephadex G-50.
8. Gel extraction kit.

2.3.2 Separating RNA by Electrophoresis

1. Nucleic acid agarose.
2. 55 °C water bath.
3. 10× denaturing gel buffer (Invitrogen).
4. Heat block.
5. Gel electrophoresis apparatus.
6. 3-(*N*-morpholino)propanesulfonic acid (MOPS).
7. Sodium acetate.
8. 0.5 M EDTA.
9. 10× MOPS buffer: 200 mM MOPS, 50 mM sodium acetate, 20 mM EDTA, adjust pH to 7.0. To make 1× MOPS gel running buffer, mix one part of 10× MOPS buffer with nine parts of RNase-free water.
10. RNA loading buffer (Invitrogen).

11. Ethidium bromide (only if RNA visualization is needed).
12. DIG labeled RNA marker (Roche).

2.3.3 Transferring RNA to the Membrane

1. 20× SSC (3 M NaCl in 0.3 M sodium citrate (pH 7.0)).
2. Razor blade.
3. 3 M Filter paper.
4. Positively charged nylon membrane.
5. Blunt end forceps.
6. Paper towel.
7. RNase-free flat- bottomed container as buffer reservoir.
8. Clean glass pasture pipette as roller.
9. Light weights (150–200 g) object serving as weight during transfer.
10. Supports of the reservoir (i.e., a stack of books).
11. Stratalinker® UV Crosslinker.

2.3.4. Probe–RNA Hybridization

1. 20× SSC.
2. 10% SDS.
3. DIG easy Hyb Granules (Roche).
4. 68 °C shaking water bath.
5. Heat block.
6. Hybridization oven.
7. Hybridization bags.
8. Low Stringency Buffer: 2× SSC with 0.1% SDS.
9. High Stringency Buffer: 0.1× SSC with 0.1% SDS.

2.3.5 Detection of Probe–RNA Hybrids

1. Washing and Blocking buffer set (Roche).
2. Anti-DIG-alkaline phosphatase antibody (Roche).
3. NBT/BCIP Stock Solution.
4. CDP-Star, Ready-to-Use (Roche).
5. TE buffer: 10 mM Tris–HCL, 1 mM EDTA, adjust pH to approximately 8.

2.4 lncRNA In Situ Hybridization

1. 8-well chamber slide.
2. 10× Phosphate-Buffered Saline (PBS): to make 1× PBS, mix one part of 10× PBS with nine parts RNase-free water. Store at 4 °C.
3. 4% paraformaldehyde (in RNase-free PBS).
4. 0.1 M triethanolamine.
5. Acetic anhydride.
6. 0.2 M HCl in RNase-free water.
7. 20× SSC.
8. Formamide.
9. 50× Denhardt solution (Sigma).
10. Dextran sulfate. To make 50% Dextran sulfate solution, dissolve 5 g Dextran sulfate in 10 mL RNase-free water, stir at room temperature until completely dissolved.
11. Yeast tRNA.
12. Prehybridization buffer: 2× SSC, 50% formamide, 1× Denhardt solution, and 1 mg/mL yeast tRNA. For 50 mL hybridization buffer, add 1 mL 20× SSC, 5 mL formamide, 200 μL 50× Denhardt solution, and 50 mg yeast tRNA, and make up a final volume of 50 mL with RNase-free water.
13. Hybridization buffer: 2× SSC, 50% formamide, 1× Denhardt solution, 10% dextran sulfate, 1 mg/mL yeast tRNA. For 50 mL hybridization buffer, add 1 mL 20× SSC, 5 mL formamide, 200 μL 50× Denhardt solution, 10 mL 50% dextran sulfate, and 50 mg yeast tRNA, and make up a final volume of 50 mL with RNase-free water.
14. DIG Wash and Block Buffer Set (Roche).
15. Anti-DIG-alkaline phosphatase antibody.
16. NBT/BCIP stock solution (Roche).
17. TE buffer: 10 mM Tris–HCL, 1 mM EDTA, adjust pH to approximately 8.
18. Humidity oven.

2.5 lncRNA Knockdown

2.5.1 lncRNA Knockdown Using siRNAs

1. siRNA targeting lncRNA of interest: sources of siRNAs include (1) ready-to-use linecodeRNA from http://www.thermoscientificbio.com/ and (2) custom-designed siRNAs (*See* Note 2 for a list of siRNA design websites and *see* Note 3 for a list of siRNA synthesis vendors).
2. Control siRNA.

3. siRNA transfection reagents. We use Lipofectamine RNAi max from Invitrogen, but there are a handful of similar reagents that one can choose from. *See* Note 4 for a list of siRNA transfection reagents.

4. Opti-MEM.

2.5.2 lncRNA Knockdown Using shRNAs

1. pLKO.1,
2. shRNA (designed at http://www.broadinstitute.org/rnai/public/seq/search).
3. MISSION® pLKO.1-puro Non-Mammalian shRNA Control Plasmid DNA (Sigma).
4. pRSV-Rev (Addgene 12253).
5. pMDLg/pRRE (Addgene 12251).
6. pMD2G (Addgene 12259).
7. HEK293T cells.
8. RPMI 1640 media.
9. Fetal Bovine serum.
10. Penicillin-Streptomycin solution with 10,000 units penicillin and 10 mg streptomycin/mL.
11. FuGENE6.
12. 0.45 μm disc filter.
13. Hexadimethrine bromide (polybrene): to make 4 mg/mL stock, dissolve 40 mg powder in 10 mL MilliQ water and filter through 0.22 μm disc filter, make 500 μL aliquot and store at −20 °C.

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[2] List of siRNA design websites:

a. siDESIGN center at http://www.thermoscientificbio.com/.
b. BLOCK-iT™ RNAi Designer at http://rnaidesigner.invitrogen.com/rnaiexpress/.
c. http://www.broadinstitute.org/rnai/public/seq/search.

[3] List of siRNA synthesis vendors:

a. http://www.idtdna.com/.
b. http://www.invitrogen.com.
c. http://www.genscript.com/.
d. http://www.thermoscientificbio.com/.
e. http://www.sigmaaldrich.com/.

[4] List of siRNA transfection reagents:

a. Lipofectamine RNAiMax (Invitrogen).
b. DharmaFECT Transfection Reagents (Thermo Scientific).
c. X-tremeGENE siRNA transfection reagent (Roche).

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 staring the experiment.

3.1 lncRNA–Immunoprecipitation

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, precool 1× 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 5).

3.1.1 *Whole Cell Lysate Preparation (*See Note 6)—

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 1× 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 15–20 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 6)

1. Harvest cells using regular trypsinization technique and count the cell number.
2. Wash cells in ice-cold 1× PBS three times and resuspend 1.0×10^7 cells in 2 mL ice-cold PBS (*see* Note 7).
3. Put cell suspension in 1× 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.

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5. It 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.
6. To ensure result reproducibility, the cells need to be maintained consistently.
7. The 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.

6. Shear the nucleus on ice with 15–20 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.

3.1.3 RNA Immune-Precipitation and Purification

1. Wash 40 μL protein A/G beads with 500 μL 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 μL RIP buffer to resuspend the beads.
2. Add the prewashed beads and 5–10 μg IgG and into the whole cell lysate from Subheading 3.1.1 or nuclear lysate from Subheading 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 (precleared 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 μL lysate and set aside on ice as input control.
5. Add antibody of interest into nuclear lysate (*see* Note 8), incubate the lysate and antibody overnight at 4 °C with gentle rotation.
6. The next day, add 40 μL 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 μL 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 μL 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 coprecipitated 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 9).

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8. The amount of antibody used for each experiment need to be empirically determined. Our suggestion is to start at around 1–2 μg antibody per million cells.
9. The 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 μL and adjust according to their specific situations.

3.2 lncRNA Pull-Down

lncRNA pull-down aims to identify proteins that bind to a lncRNA of interest. The protocol includes three parts: (1) synthesis and labeling the lncRNA of interest; (2) preparing protein lysate from target cells; (3) pull-down labeled lncRNA with its interacting proteins. The readers can decide the subsequent analyses on the lncRNA-bound proteins.

Before harvesting cells, precool PBS, water, RIP buffer and NT2 buffer on ice; estimate the amount of buffers needed and add VRC, EDTA, DTT, RNase inhibitor, and protease inhibitor to the buffers accordingly (*see* Note 5).

3.2.1 Biotinylated RNA Synthesis by In Vitro Transcription

1. DNA template preparation: Linearize 3–4 μg of the plasmid containing the desired template DNA (*see* Note 1) with a suitable restriction enzyme at the 3′ end of the insert (*see* Note 10).
2. DNA template purification: run the digested DNA by DNA gel electrophoresis, excise the band of correct size and extract DNA from agarose using Gel Extraction Kit.
3. In vitro synthesis of biotinylated RNA using T7 RNA polymerase: add the components listed in Table 1 into an RNase-free tube on ice, mix thoroughly, centrifuge briefly and incubate at 37 °C for 2 h. After incubation, add 2 μL Dnase I (RNase-free) into the reaction and incubate at 37 °C for 15 min to remove DNA template. Stop the reaction by adding 0.8 μL 0.5 M EDTA (pH 8.0).
4. Biotinylated RNA purification of using G-50 Sephadex Columns: Before use, gently invert the column several times to resuspend the medium. Remove the top cap and then remove the bottom tip (*see* Note 11). Drain the buffer in the column by gravity and then centrifuge at 1100 × *g* for 2 min to eliminate residua buffer. Place the column in an upright position (*see* Note 12) with a new collection tube, apply the RNA sample (up to 100 μL) to the center of the column carefully (*see* Note 13) and centrifuge for 4 min at 1100 × *g* at 4 °C. The elution contains the purified biotinylated RNA. Determine the RNA concentration and store at −80 °C.

3.2.2 *Whole Cell Lysate Preparation (* See Note 6)—If nuclear RNA-protein interaction is the focus of the research, skip this step and go directly to Subheading 3.2.3 for nuclear lysate preparation.

1. Harvest cell by regular trypsinization (~10^7 cells) and wash cells with ice-cold 1× PBS once.

10. Make sure the restriction enzyme digests efficiently and generate a 5′ overhang.
11. The top-to-bottom sequence is necessary to avoid creating vacuum and uneven flow of buffer.
12. Maintaining the column in an upright position is very important, especially after centrifugation. Tipping the column can cause back-flow of the RNA sample and reduce the yield after purification.
13. Avoid applying the sample to the side of the column or overloading the column, since it will reduce the yield and purify of the RNA.

2. Resuspend cell pellet in 1 mL ice-cold RIP buffer containing RNase and protease inhibitors.
3. Shear the cell pellet on ice using a Dounce homogenizer with 1–20 strokes.
4. Centrifuge at 15,000 × *g* for 15 min at 4 °C to clear the cell lysate. The supernatant contains whole cell lysate.

3.2.3 *Nuclear Lysate Preparation (*See Note 6)

1. Cell harvest and nuclei isolation: harvest cells using regular trypsinization technique, wash cell pellet in ice-cold 1× PBS once and resuspend 1.0×10^7 cells in 2 mL ice-cold 1× PBS. Put cell suspension 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. Harvest nuclei by spinning the tube at 2500 × *g* for 15 min at 4 °C. The pellet contains the purified nuclei.
2. Nuclei lysis: resuspend nuclei pellet in 1 mL freshly prepared ice-cold RIP buffer containing RNase and protease inhibitors. Shear the nuclei on ice with 15–20 strokes using a Dounce homogenizer. Pellet nuclear membrane and debris by centrifugation at 15,000 × *g* for 15 min at 4 °C. Carefully transfer the clear supernatant into a new tube. The supernatant contains nuclear lysate (*see* Note 7).
3. Preclear lysate: take 60 μL Straptavidin agarose beads slurry and wash the beads with precooled NT2 buffer three times. After wash, spin down the beads at 12,000 × *g* for 1 min and resuspend the bead in 60 μL precooled NT2 buffer. Add the prewashed Straptavidin agarose beads into the whole cell lysate (Subheading 3.2.2) or nuclear lysate (Subheading 3.2.3) and incubate at 4 °C for at least 1 h with gentle rotation. Centrifuge the lysate briefly, carefully transfer the supernatant into a new tube, and determine the protein concentration using BCA protein assay. Save 3–5% of the lysate as input.

3.2.4 *RNA Pull-Down (*See Note 14)

1. Dilute ~10 picomole biotinylated RNA into 40 μL of RNA structure buffer and heat the tube at 90 °C for 2 min, immediately transfer the tube on ice and incubate for another 2 min. Then let the tube sit at room temperature for 20 min to allow proper RNA secondary structure formation.
2. Add the properly folded RNA from previous step into 200 μg of precleared lysate from Subheading 3.2.4., and supplement with tRNA to a final concentration of 0.1 μg/μL (*see* Note 15). Incubate at 4 °C for 2 h with gentle rotation.

14. The amount of total protein used in each assay need to be empirically determined based the specific questions the researchers try to address. If the interaction between a specific lncRNA and a target protein is to be tested, the abundance of the target protein in the nuclear lysate need to be taken into account in determining the amount of total protein used in the assay.
15. The purpose of adding tRNA in the pull-down assay is to reduce nonspecific binding. Therefore, the amount of tRNA added to each reaction can be optimized according to specific conditions.

3. Add 60 μL prewashed Streptavidin Agarose Beads and incubate for 1 h at 4 °C.
4. At the end of the incubation, centrifuge at 12,000 × *g* 4 °C for 1 min and take off the supernatant. Wash the beads with 1 mL ice-cold NT2 buffer at 4 °C 5 times (*see* Note 16).
5. After the last wash, carefully remove any residual buffer without disturbing the beads.
6. Add 40 μL 2× Laemmli loading buffer and boil the beads in loading buffer for 5–10 min, centrifuge at 12,000 × *g* for 1 min at room temperature and transfer the supernatant, which contain the lncRNA interacting proteins, into a new tube and store at −80 °C for further analysis.

3.3 *lncRNA Northern blot Analysis (*See Note 17)

lncRNA Northern blot analysis aims to characterize lncRNA expression. The protocol includes five parts: (1) RNA probe synthesis and labeling; (2) RNA sample electrophoresis; (3) RNA transfer; (4) RNA-probe hybridization; and (5) RNA-probe hybrid detection.

3.3.1 DIG Labeled RNA Probe Synthesis by In Vitro Transcription—

The DIG labeled RNA probe synthesis is very similar to the biotinylated RNA synthesis described in Subheading 3.2.1. The differences between the two procedures are:

1. Since the probe needs to be complementary to the target sequence, the probe RNA is transcribed from the 3′ end of the target sequence. We clone the gene of interest in reverse orientation to make the in vitro transcription template for Northern probes.
2. Use DIG labeling mix in place for the biotin-label mix.

3.3.2 Separating RNA Samples by Electrophoresis

1. Gel setup:
   a. Wipe the gel rack, tray and combs with RNAZap, rinse with water, and let dry.
   b. Weight 100 g agarose in a clean glass flask and mix with 90 mL RNase-free water. Melt the agarose completely by heating with a microwave. Put the flask with melted agarose in a 55 °C water bath.
   c. In a fume hood (*see* Note 18), add 10 mL 10× denaturing gel buffer to the gel mix that is equilibrated to 55 °C. Mix the gel solution by gentle swirling to avoid generating bubbles. Slowly pour the gel mix into the

16. We found that sometimes increasing the number of washes can greatly reduce the background, therefore it is recommended to optimize the wash condition for each specific assay.
17. Northern blot analysis is a golden-standard in RNA detection and analysis. There are many protocols developed by laboratories specialized in RNA research or companies. The protocol described here is adapted from the NorthernMax procedure from Invitrogen and DIG application manual for filter hybridization from Roche. In our hands, this protocol is time efficient and gives satisfying results without using radioactivity.
18. Always cast the gel in a fume hood as the denaturing solution contains formaldehyde. Solidified gels can be wrapped up and stored at 4 °C for overnight.

gel tray, pop any bubbles or push them to the edges of the gel with a clean pipette tip. The thickness of the gel should be about 6 mm. slowly place the comb in the gel. Allow the gel to solidify before removing the comb.

d. Right before RNA electrophoresis, place the gel tray in the electrophoresis chamber with the wells near the negative lead and add 1× MOPS gel running buffer in the chamber until it is 0.5–1 cm over the top of the gel (*see* Note 19).

2. RNA electrophoresis

a. Mix no more than 30 μg sample RNA with 3 volumes of RNA loading buffer (*see* Notes 20 and 21). To destruct any secondary structure of the RNAs, incubate the RNA with loading buffer at 65 °C for 15 min using a heat block. Spin briefly to collect samples to the bottom of the tube and put the tubes on ice (*see* Note 22).

b. Carefully draw the RNAs in the tip without trapping any bubbles at the end of pipette tip, place the pipette tip inside of the top of the well, slowly push samples into the well and exit the tip without disturbing the loaded samples. If markers are needed, load one lane with DIG-labeled RNA marker.

c. Run the gel at 5 V/cm (*see* Note 23).

d. (Optional) Stain the gel with ethidium bromide and visualize the RNA under UV (*see* Note 24).

3.3.3 Transfer RNA from Agarose Gel to the Membrane

1. Material preparation:

a. Use a razor blade to trim the gel by cutting through the wells and discard the unused gel above the wells. For marking the orientation, make a notch at a corner.

b. Cut the membrane to the size slightly larger than the gel. Make a notch at a corner to align the membrane with gel in the same orientation. Handle the membrane with care—only touching the edges with gloved hands or blunt tip forceps.

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19. Do not let gel soaked in running buffer for more than 1 h before loading.
20. Load no more than 30 μg total RNA in each lane. As the binding capacity of the membrane is limited, more RNA loaded does not guarantee a stronger signal. Overloading can lead to the detection of minor degradation of targeted RNAs.
21. If the total volume of sample and dye exceed the capacity of the wells, it is necessary to concentrate the RNA by precipitation and suspend the pellet in smaller volume of water before adding the loading dye.
22. Use a heat block instead of a water bath to avoid contaminating the samples with water.
23. The voltage is decided by the distance between the two electrodes (not the size of the gel). Usually, the run takes about 2 h. If the run is longer than 3 h, exchange the buffer at the two end chambers to avoid the pH gradient.
24. RNA gels that stained with Ethidium bromide are not suitable for Northern blot analysis. Therefore, if a visual examination or photograph of total RNA samples is needed as a reference for the northern blot, we suggest the researchers to either run the same set of samples on a separate gel or stain with Ethidium bromide the gel just for visualization; or de-stain the gel before continuing northern blot analysis. If a gel will be subjected to Northern analysis after UV visualization, avoid prolonged exposure of the gel to UV light.

c. Cut eight pieces of filter paper the same size of the membrane.

d. Cut a stack of paper towels that are 3 cm in height and 1–2 cm wider than the gel.

e. Pour 20× SSC into a flat-bottomed container that has bigger dimension of the agarose gel. This serves as the buffer reservoir and can also be used to wet the paper and membrane. Put the reservoir on a support (i.e., a stack of books) so that its bottom is higher than the paper towel stack.

f. Cut three pieces of filter paper that are large enough to cover the gel and long enough reach over to the reservoir. These papers serve as the bridge to transfer buffer from the reservoir to the gel.

2. Transfer set up

a. Stack paper towel on a clean bench and put three pieces dry filter paper on top.

b. Wet two more pieces of filter paper and put on top of the dry filter paper. Gently roll out any bubbles between the filter paper layers.

c. Carefully put the membrane on top of the wet filter paper. Gently roll out any bubbles between the membrane and the filter papers.

d. Put the trimmed gel onto the center of the membrane with the bottom of the gel touching the membrane (i.e., the gel plane that faces down during electrophoresis will be in contact with the membrane), align the notches of the gel and membrane. Roll out bubbles between the membrane and the gel.

e. Place three more pieces prewet filter paper on top of the gel and roll out bubbles between filter paper layers.

f. Wet the three pieces of paper bridge and place them with one end on top of the stack and the other end in buffer reservoir. Make sure there is no bubble between any layers of paper (*see* Note 25).

g. Place a 150–200 g object with the size similar to the gel on top of the stack.

h. Transfer the gel for 15–20 min per mm of gel thickness. It usually takes about two hours (*see* Note 26).

3. RNA crosslink: disassemble the transfer stack carefully and rinse the member with 1× MOPS gel running buffer to remove residual agarose. Blot off excessive liquid and immediately subject the membrane to crosslink treatment. Cross

[25] It is essential to ensure that the only way for the transfer buffer to run from reservoir to the dry paper stack is through the gel. Therefore, extra care is needed to assemble the stack properly to avoid shortcut. The most common shortcut happens between the bridge and the paper beneath the gel. One can cover the edges of the gel with Parafilm to prevent this from happening.

[26] Transfer longer than 4 h may cause small RNA hydrolysis and reduce yield.

linking the RNA to the membrane with Stratalinker® UV Crosslinker using the autocrosslink setting (*see* Note 27). Air-dry the membrane at room temperature. At this point, the membrane can be subjected to hybridization immediately or stored in a sealed bag between two pieces filter paper at 4 °C for several month before hybridization.

3.3.4 *Hybridization of DIG-Labeled Probes to the Membrane (*See Note 28)

1. Prehybridization.
   a. Reconstitute the DIG easy Hyb Granules: add 64 mL RNase-free water into one bottle of the DIG easy Hyb Granules, stir for 5 min at 37 °C to complete dissolve the granules. DIG easy Hyb buffer will be used in prehybridization and hybridization. The reconstituted DIG easy Hyb buffer is stable at room temperature for up to 1 month.
   b. For every 100 cm^2 membrane, 10–15 mL Hyb buffer should be used for prehybridization. Measure the appropriate amount of Hyb buffer for prehybridization and place it in a clean tube and prewarm it in a 68 °C water bath (*see* Note 29).
   c. Put the membrane in a hybridization bag, add the prewarmed Hyb buffer from the previous step, seal the bag properly and incubate the membrane in Hyb buffer at 68 °C for at least 30 min with gentle agitation (*see* Note 30). Prehybridization can be up to several hours as far as the membrane remains wet.
2. Hybridization.
   a. For every 100 cm^2 membrane, 3.5 mL Hyb buffer is needed for hybridization. Measure the appropriate amount of Hyb buffer for hybridization and place it in a clean tube and prewarm it in a 68 °C water bath (*see* Note 29).
   b. Determine the amount of RNA probe needed (*see* Note 31) and place it into a microcentrifuge tube with 50 μL RNase-free water. Denature the probe by heating the tube at 85 °C for 5 min and chill on ice immediately.
   c. Mix the denatured probe with prewarmed Hyb buffer by inversion.

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27. The autocrosslink Mode of Stratalinker® UV Crosslinker delivers a preset exposure of 1200 μJ to the membrane and takes about 40 s. Other methods of crosslinking RNA to membrane are available and can be used at this step as well.
28. Once the membrane is wet during prehybridization, it is important to avoid it getting dry during the hybridization and detection process. Dried membrane will have high background. Only if the membrane will not be stripped and reprobed, it can be dried after the last high stringency wash and stored at 4 °C for future analysis.
29. For most northern blot hybridization using DIG Easy Hyb buffer, 68 °C is appropriate for both prehybridization and hybridization. In cases of more heterologous RNA probes being used, the prehybridization and hybridization temperature need to be optimized.
30. Prehybridization/hybridization can be performed in containers other than bags, as far as it can be tightly sealed. Sealing the hybridization container can prevent the release of NH_4, which changes the pH of the buffer, during incubation.
31. For RNA probe synthesized by in vitro transcription, it is recommended that the probe concentration should be 100 ng per mL Hyb buffer.

d. Remove prehybridization buffer from the membrane and immediately replace with the prewarmed hybridization buffer containing the probe.

e. Seal the bag properly and incubate the membrane in probe-containing Hyb buffer at 68 °C overnight with gentle agitation (*see* Note 30).

f. The next day, prewarm the High Stringency Buffer to 68 °C and pour Low Stringency Buffer in an RNase-free container at room temperature and make sure it is enough to cover the membrane.

g. Cut open the hybridization bag, remove the Hyb buffer, and immediately submerge the membrane in the Low Stringency Buffer.

h. Wash the membrane twice in Low Stringency Buffer at room temperature for 5 min each time with shaking.

i. Wash the membrane twice in High Stringency Buffer at 68 °C for 5 min each time with shaking (*see* Note 32).

3.3.5 Detection of DIG-Probe-Target RNA Hybrids

1. Localizing the probe-target hybrid with anti-DIG antibody.

   a. Transfer the membrane from the last wash in High stringency buffer to a plastic container with 100 mL Washing buffer. Incubate for 2 min at room temperature and discard the Washing buffer.

   b. Add 100 mL Blocking buffer onto the membrane and incubate for more than 30 min (up to 3 h) with shaking at room temperature.

   c. Dilute anti-DIG-alkaline phosphatase antibody at the ratio of 1:5000 in Blocking buffer and incubate the membrane in 20 mL diluted antibody for 30 min at room temperature with shaking.

   d. Wash membrane twice with 100 mL ofWashing buffer for 15 min each time at room temperature.

2. Visualizing probe-target hybrids using chromogenic or chemiluminescent method (*see* Note 33).

   a. Equilibrate the membrane in 20 mL Detection buffer for 3 min at room temperature. If using the chromogenic method, prepare the color substrate solution while equilibrating the membrane.

   b. For chromogenic detection:

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32. If the probe is less than 80% homologous to the target RNA, the high stringency wash should be performed at a lower temperature, which needs to be empirically determined.

33. The DIG probe-target RNA hybrids can be detected in two ways. One uses chemiluminescent method, whereas the other uses chromogenic method. The chemiluminescent method is sensitive and fast, but it requires the usage of the films and the accessibility of a darkroom. The chromogenic method requires no film or dark room and different targets can be detected simultaneously using different colored substrate. However, the chromogenic method may not be sensitive enough for low-abundant targets.

- Put the membrane with the RNA side facing up in a container and incubate in 10 mL color substrate solution in the dark without shaking.
- When the desired intensity for the band is observed, discard the color substrate solution and rinse the membrane in 50 mL of TE buffer for 5 min (*see* Note 34).
- Document the result by photographing the membrane (*see* Note 35).

c. For chemiluminescent detection:

- Put the membrane with the RNA side facing up on a plastic sheet (i.e., cut out of a hybridization bag) and add 20 drops of CDP-Star, Ready-to-Use reagent.
- Immediately cover the membrane with another sheet to evenly distribute the reagent without creating any bubbles.
- Incubate for 5 min at room temperature.
- Squeeze out excess reagent and seal the bag.
- Develop the membrane with an X-ray film in a dark room (*see* Note 35).

3.4 lncRNA In Situ Hybridization

lncRNA in situ hybridization aims to characterize and quantify lncRNA expression in cells. The protocol includes three parts: (1) RNA probe synthesis and labeling, (2) cell preparation and pretreatment, and (3) RNA-probe hybridization and detection.

3.4.1 DIG Labeled RNA Probe Synthesis by In Vitro Transcription—

The DIG labeled RNA probe synthesis is very similar to the biotinylated RNA synthesis described in Subheading 3.2.1. The differences between the two procedures are:

1. Both sense and anti-sense probes need to be synthesized (*see* Note 36). The antisense probe contains the complementary sequence to the target gene and the sense probe contains the target gene sequence. The hybridization signal from the antisense probe represents target gene expression while the sense probe is used as negative control.

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34. At this step, if there are multiple membranes, process one at a time. Depending on the abundance of target RNAs, the band may appear as quickly as a few minutes after adding the chromogenic agents. The reaction can be stopped when the band reaches a desired intensity.
35. If reprobing is needed, photograph the result while the membrane is wet and proceed to stripping and reprobing. If no reprobing is needed, dry the membrane, document the result by photograph and store the dried membrane in a clean bag at room temperature.
36. During assay development for each specific gene target, it is critical to know whether the hybridization is specific to the gene of interest. One way to ensure hybridization specificity is to include samples hybridizing with sense probe (containing the target sequence, serving as negative controls) at the same concentration as those with the antisense probe (containing the complementary sequence). If the control probe gives comparable signal as the target probe does, the hybridization may not be specific enough to the gene of interest and optimization of the probe sequence, probe concentration, hybridization condition, and wash stringency will be needed.

2. To generate antisense probes, we clone the gene of interest in reverse orientation and transcribe it using in vitro transcription as described in Subheading 3.2.1.

3. DIG labeling mix is used in place for the biotin-label mix during in vitro transcription.

3.4.2 Cell Preparation and Pretreatment

1. Culture the cells of interest on multiwell chamber glass slides. At the day of experiment, the cells should be 60–80% confluent. We use the 8-chamber glass slide to reduce the amount of hybridization buffer and probes used. The amount of reagents used in the following protocol is for 8-well chamber slides. Scale up or down according to the cell culture device used in each specific experiment.

2. On the day of the experiment, wash the cells with 400 μL 1× RNase-free-PBS per well, then fix the cells using 200 μL 4% PFA per well at room temperature for 10 min. Wash the fixated cells three times with 1× PBS at room temperature.

3. Add acetic anhydride into 0.1 M triethanolamine to make the final acetic anhydride concentration 0.25% (v/v, *see* Note 37). Pretreat cells with 0.1 M triethanolamine containing 0.25% acetic anhydride for 10 min at room temperature. Wash the cells with 1× PBS for 5 min afterward.

4. Permeablize the cells with 0.2 M HCl for 10 min and wash the cells twice with 1× PBS, 5 min each time, at room temperature.

5. The slides can be either subject to hybridization immediately or stored in 1× PBS at 4 °C for a couple of days before proceeding to the next step.

3.4.3 In Situ Hybridization and Detection of Probe–Target Hybrid

1. Add 200 μL prehybridization buffer (hybridization buffer without probe and dextran sulfate, see Subheading 2 for details) into each well, incubate at 60 °C for 2 h (*see* Note 38).

2. During prehybridization, take the amount of probe needed for the experiment (*see* Note 39) and denature the probes by heating the probes at 85 °C for 10 min and immediately cool down on ice for at least 5 min.

3. Add denatured probes into the hybridization buffer. To ensure assay specificity, always use a control probe for each target probes, *see* Note 36 for details.

4. Remove the prehybridization buffer from chambers and add 200 μL hybridization buffer containing denatured probes on to the slide and incubate at 60 °C in humidity oven with lid on overnight.

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37. The acetic anhydride need to be added freshly each time and discard any leftover 0.1 M triethanolamine with acetic anhydride.
38. To avoid evaporation of the buffer, we put on the lid for the chamber slide and use the humidity oven with temperature set at 60 °C for the incubation.
39. The concentrations of different probes need to be empirically determined. We found that 100–400 ng probes per mL hybridization buffer are a good starting point.

5. The next day, wash cells by adding 500 μL 0.1× SSC with 50% formamide in each well and incubate the slides with lid at 60 °C for 30 min twice (*see* Note 40).
6. Then wash the cells by adding 500 μL 2× SSC to each well and incubate for 5 min at room temperature twice (*see* Note 40).
7. Wash the cells with 500 μL washing buffer per well at room temperature for 5 min.
8. Add 200 μL blocking buffer per well and incubate for 1 h at room temperature.
9. While the cells are incubated with blocking buffer, dilute the anti-digoxygenin-alkaline phosphate antibody in blocking buffer (*see* Note 41).
10. Discard the blocking buffer. Add 100 μL diluted antibody per well and incubate for 1 h at room temperature.
11. Wash the slides with 500 μL wash buffer per well for 15 min at room temperature twice.
12. Incubate the slides in 500 μL detection buffer per well for 10 min at room temperature. Meanwhile, dilute 200 μL NBT/BCIP stock solution in 10 mL detection buffer.
13. Discard the detection buffer and add 500 μL diluted NBT/BCIP solution per well and incubate cells in the dark (up to 16 h).
14. When the desired color intensity is observed (*see* Note 42), stop the color reaction by discarding the NBT-BCIP solution and incubating with TE buffer for 15 min.

3.5 Method for shRNA Knockdown

Like mRNAs, the endogenous lncRNA expression can be down-regulated using two RNA silencing-mediated approaches. One is transfecting small-interfering RNAs (siRNAs) targeting the gene of interest into cells. The other is stably expressing gene-specific RNA hairpins (shRNAs) in target cells. The siRNA approach provides acute gene downregulation and allows for gradient knockdown of target gene expression by adding different amounts of siRNAs; whereas the shRNA-approach provides sustained downregulation of the target lncRNA, making it more suitable for experiments that take a long period to get the end-point readout. The following section will provide protocols for both methods.

3.5.1 lncRNA Knockdown Using siRNAs—

The following protocol provides siRNA concentration and the amount of transfection reagents based on Lipofectamine RNAiMax transfection protocol. If you are using different transfection reagents, please follow the specific instructions from the manufacturers.

40. The wash condition can be optimized by adjusting the salt concentration and temperature.
41. The recommended range for anti-DIG antibody is from 1:500 to 1:2000. The optimal concentration needs to be empirically determined.
42. The process usually takes from 5 min to 2 h. Stop the reaction once the desired signal is visible.

1. Make 20 μM siRNA stock solution with RNase-free water, aliquot into 20 μL per tube and store at −80 °C. It is highly recommended to reduce the freeze-and-thaw cycles of siRNA stocks.
2. The day before transfection, plate cells in 24-well plate so that it will be 60–80% confluent the next day.
3. On the day of transfection, calculate the amount of control and targeting siRNAs needed for each well according to Table 2 (*see* Note 43). Dilute the siRNA and transfection reagents in Opti-MEM in separate tubes, mix together and incubate at room temperature for at least 20 min.
4. Without removing any media from the wells, add the siRNA and transfection reagent mixture to the wells.
5. Incubate the cells with siRNA/transfection reagents for at least 6 h (*see* Note 44).
6. Remove the media containing siRNA/transfection reagents and feed the cells with fresh media.
7. Harvest the infected cells in TRIzol 48–72 h posttransfection.
8. Check target gene knockdown efficiency by quantitative RT-PCR of RNA from control and targeting siRNA transfected cells.

3.5.2 lncRNA Knockdown Using shRNAs

1. Generating shRNA knockdown constructs.
   a. Design shRNA sequence targeting specific lncRNA at http://www.broadinstitute.org/rnai/public/seq/search (*see* Note 45).
   b. Synthesize the DNA containing the shRNA sequence of choice and clone it into lentiviral vector pLKO.1.
   c. Purify pLKO.1 constructs (containing specific shRNA or scramble controls) and other packing constructs (pRSV-Rev and pMDLg/pRRE) using QIAGEN Plasmid *Plus* Maxi Kit (*see* Note 46).
2. Packing virus using 293T cells.
   a. Plate HEK293T cells in 6-well plate the day before transfection so it will be 80% confluent the next day.
   b. Change cells into antibiotic-free media before transfection.
   c. Mix 4 μg plasmid cocktail and 12 μL FuGENE6 transfection reagent according to the FuGENE protocol. The composition of the plasmid cocktail is shown in Table 3:

43. The amounts of siRNA and transfection reagents for each cell line need to be empirically determined.
44. Leaving the transfection reagents on cells for extended period of time may cause cell toxicity.
45. It is common to clone five or more shRNA and choose the two that gives the highest knockdown efficiency for future analysis.
46. We prefer to use the Qiagen Plasmid *Plus* Maxi Kit. However, any plasmid isolation kit that gives high quality DNA for efficient transfection can be used. A good mini-prep kit can be also used to isolate small amount plasmid during pilot experiments

d. Add the FuGENE-DNA mixture onto cells.

e. Eight hours after transfection, change into regular media with antibiotics.

f. Forty-eight hours after transfection, harvest virus by collecting the culture media and filter through 0.45 μm disc filter to get rid of cell debris.

g. The virus stock can be aliquot and store at −80 °C for future use.

3. Cell Infection.

a. Plate target cells in 6-well plate the day before infection so it will be 50–60% confluent the next day.

b. Mix three parts of virus with one part of culture media (i.e., 3 mL virus with 1 mL medium) and add polybrene to final concentration of 8 μg/mL.

c. Add virus mixture directly onto cells and incubate overnight.

d. After 24 h of incubation, change media or split cells depending on the cell confluency.

e. Harvest the infected cells in TRIzol 72–96 h postinfection.

f. Check target gene knockdown efficiency by quantitative RT-PCR of RNA from scramble and shRNA infected cells.

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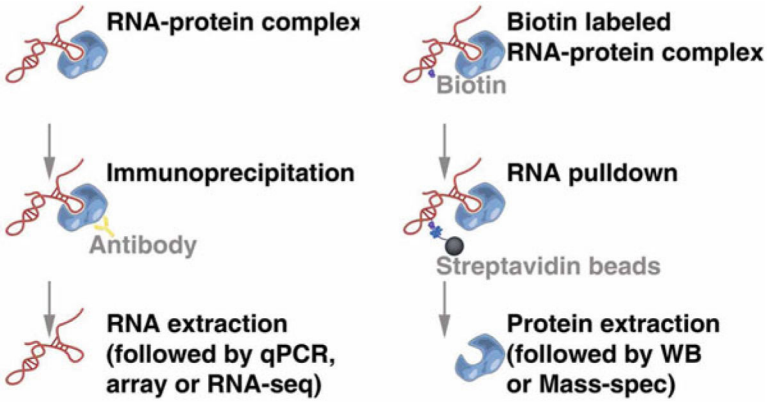

Fig. 1.

Schematic diagram of lncRNA IP and lncRNA pull-down. (**a**) lncRNA IP, to identify lncRNA molecules interacting with a protein of interest. (**b**) lncRNA pull-down, to identify proteins interacting with a specific lncRNA

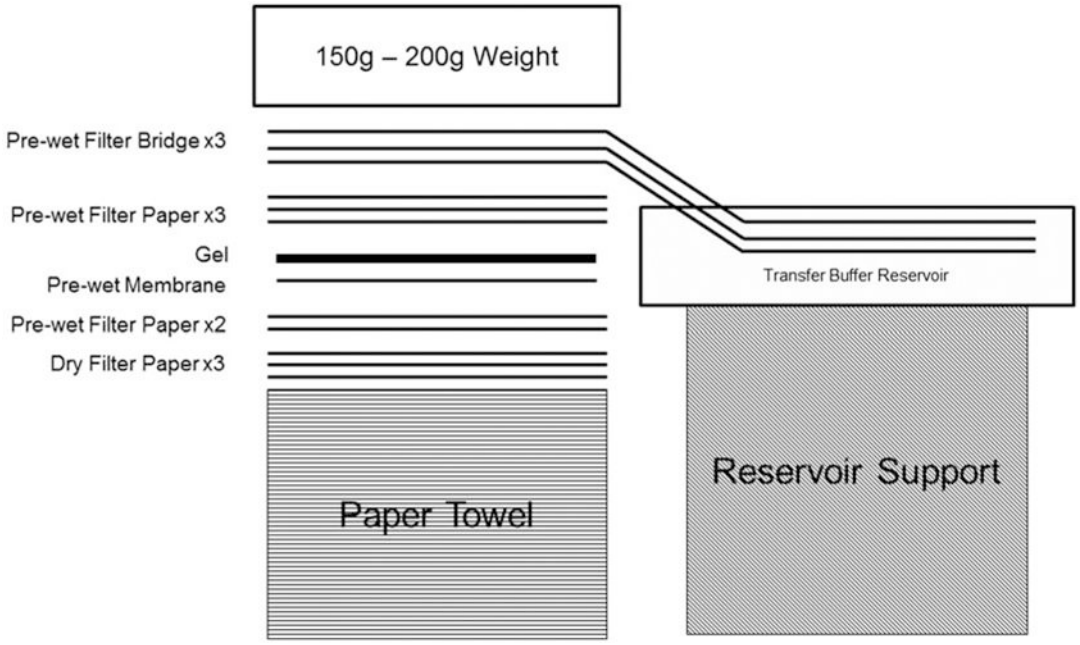

Fig. 2.

Schematic diagram of downward transfer of northern blot analysis (adapted from Northern Max kit instruction, Invitrogen)

Table 1

Components for in vitro transcription of template DNA (20 μL system)

Linearized plasmid DNA (1 μg) or PCR product (100–200 ng)	
Biotin RNA labeling mix (10×)	2 μL
5× transcription buffer	4 μL
T7 RNA polymerase (20 U/ μL)	2 μL
RNase-free sterile water	Up to 20 μL

Table 2

siRNA transfection components and composition

Reagent per well for 24-well plate	Amount of reagent
Opti-MEM for siRNA dilution	50 μL
siRNA	0.6–30 pmol
Opti-MEM for RNAiMax dilution	50 μL
RNAiMax	0.5–1.5 μL

Table 3

Plasmid composition for pLKO.1 virus packaging

Plasmid	μg
pRSV-Rev	0.65
pMDLg/pRRE	1.3
pMD2G	0.65
pLKO.1	1.4