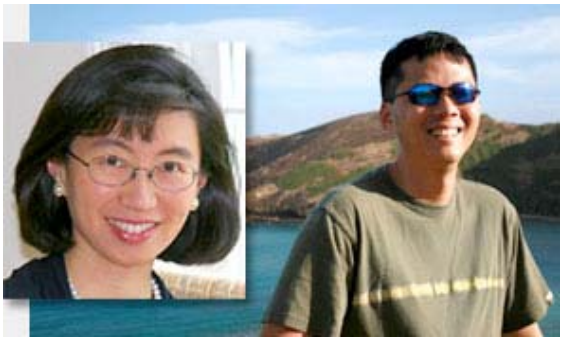
RNA-chromatin immunoprecipitations (RNA-ChIP) in mammalian cells (PROT28)



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Introduction

RNA-protein interactions play important roles within the cell. Using a variation of the widely-used chromatin immunoprecipitation (ChIP) assay, the potential association of cellular RNAs and candidate proteins can be evaluated in a process named “RNA-ChIP”. This technique has been successfully used in mammalian cells, for example to examine the relationship of noncoding RNAs with histone proteins (References 1, 2) or to examine interactions between viral RNAs and proteins in the host mammalian cell (Reference 3). In RNA-ChIP, RNA-protein interactions are fixed by reversible chemical cross-linking with formaldehyde followed by immunoprecipitation with antibodies against the candidate protein(s). RNAs that are associated with the protein are detected by reverse transcriptase-PCR (RT-PCR). The following procedure was used to examine protein-RNA interactions in mouse embryonic stem cells, but can be modified for other cell types.

Acknowledgement: This procedure is based closely on a protocol kindly provided by Sandra Gilbert (see Reference 2).

Procedure

Isolation of cells and cross-linking

1. Embryonic stem cells were grown in a gelatinized 75-cm^2 flask on a feeder cell layer to ~70-80% confluence.
2. Cells were washed twice with PBS and trypsinized. 1x10^7 cells were added to a 15ml conical tube, pelleted, and resuspended in 10ml PBS.
3. Formaldehyde was added to a final concentration of 1%, and crosslinking was performed for 10 minutes at room temperature (see note 1).
4. Glycine was added to a final concentration of 125mM to quench crosslinking, and the cells pelleted again.
5. The pellet was washed twice with ice-cold PBS containing 1x protease inhibitor cocktail.

Lysis and sonication

The cell pellet was resuspended in 200µl of Buffer A
and placed on ice for 10 minutes. The crude nuclei fraction was pelleted by microcentrifugation for 5000 rpm for 5 minutes at 4ºC. The pellet was washed once in Buffer A without NP-40, then resuspended in 500µl of Buffer B and incubated on ice for 10 minutes.

Lysates were sonicated three times on finely-crushed dry ice using a Branson Sonifier at constant power, output=7, and continuous sonication for 30 seconds (see note 2). Samples were rested at 4ºC for at least 30 seconds between sonication sessions to allow solutions to cool.

After sonication, insoluble elements were cleared by microcentrifugation at maximum speed (~14k) for 10 minutes at 4ºC.

Immunoprecipitation

The sonicate was diluted 10-fold into IP Buffer
to a final volume of 1ml per immunoprecipitation reaction. A 1% aliquot was preserved as an input sample and frozen at -80ºC

until the reverse crosslinking step. Antibodies were added to each tube (including an equal amount of a normal IgG control) and immune complexes were allowed to form by slow mixing on a rotating platform at 4ºC overnight. Although antibody concentrations will vary, a guideline can be to begin within the range of 0.5–5µg/ml (see note 3).

To collect immune complexes, 50µl of Protein A/G Agarose-PLUS (Santa Cruz) was added to each tube and slow mixing rotation continued for 2 hours. Immune complexes were "pulled down" by gentle centrifugation at 1000 rpm for 2 minutes at 4ºC.

Washes

Each immune complex was washed five times (1 ml wash, 5 minutes each). After each wash, complexes were pelleted by gentle centrifugation (1000 rpm, 1 minute) and the wash buffer aspirated using a clean pipet tip:

1. Low-salt wash
2. High-salt wash
3. LiCl wash
4. TE pH 8
5. TE pH 8

Elution and reversal of crosslinking

Immune complexes were eluted by addition of 250µl Elution Buffer, which is prepared freshly each time. Samples were vortexed briefly, incubated for 15 minutes with rotation mixing, then supernatant collected after centrifugation (8000 rpm, 2 minutes). Elution was repeated and eluates combined for a total of 500µl.

NaCl was added to a final concentration of 200mM (including the input samples) then placed at 65ºC for at least 2 hours to reverse crosslinking (see note 4 and note 5). Next, 20µl of 1M Tris-Cl pH 6.5, 10µl of 0.5M EDTA, and 20µg of Proteinase K was added to each sample and incubated at 42ºC for 45 minutes.

Samples were subjected to phenol:chloroform:isoamyl alcohol extraction and ethanol precipitation with Glycoblue (Ambion) as a carrier. Pellets were washed once in 75% ethanol, air-dried briefly, and resuspended in 20µl of DEPC-treated water.

Detection of RNA

DNA from the samples was removed by the use of DNAse I (Turbo DNA-free, Ambion). RNAs can be detected by any standard reverse-transcriptase-PCR protocol. I used Superscript III RT (Invitrogen) and performed PCR with Amplitaq Gold (Perkin-Elmer). A control reaction omitting the reverse transcriptase should be performed to rule out DNA contamination.

Materials & Reagents

Buffer A	(with and without NP40) 5mM PIPES (pH 8.0) 85mM KCl *0.5% NP40* 1x Roche protease inhibitors cocktail SUPERase•in (50 U/ml)
Buffer B	1% SDS 10mM EDTA 50mM Tris-HCl pH (8.1) 1x Roche protease inhibitors cocktail SUPERase•in (50 U/ml)
IP Buffer	0.01% SDS 1.1% Triton X-100 1.2mM EDTA 16.7mM Tris (pH 8.1) 167mM NaCl 1x Roche protease inhibitors cocktail SUPERase•in (50 U/ml)
Low-salt wash	0.1% SDS 1% Triton X-100 2mM EDTA

	20mM Tris-HCl (pH 8.1) 150mM NaCl
High-salt wash	0.1% SDS 1% Triton X-100 2mM EDTA 20mM Tris-HCl (pH 8.1) 500mM NaCl
LiCl wash	0.25M LiCl 1% NP40 1% deoxycholate 1mM EDTA 10mM Tris-HCl (pH 8.1)
Elution Buffer	1% SDS 0.1M $NaHCO_3$ SUPERase•in (50 U/ml)

Solutions

All solutions should be used exclusively for RNA-ChIP experiments, since small amounts of contamination will be easily detected with the amplification steps in this procedure. Solutions should be prepared carefully with RNAse-free (e.g., DEPC-treated) water and RNAse-free reagents, separated into different aliquots, and stored at 4ºC. Just prior to use, a concentrated stock solution of Roche protease inhibitor cocktail (25X) should be added to the buffer/solution to 1X concentration. I also added RNAse inhibitors to many as denoted below. This could be adjusted, especially for Buffer A, depending on whether the cell type of interest is thought to contain abundant endogenous RNAses.

Specialty reagents

- Turbo DNA-free (Ambion, Catalog #1907)
- SUPERase•in (Ambion, Catalog #2694)
- GlycoBlue (Ambion, Catalog #9515)
- Proteinase K, RNA-grade (Invitrogen, Catalog #25530-049)
- Complete tablets (protease inhibitor cocktail), EDTA-free (Roche, Catalog #04693132001)
- Protein A/G-PLUS Agarose (Santa Cruz Biotechnology, Catalog #sc-2003)
- Superscript III reverse transcriptase (Invitrogen, Catalog #L1016-01)
- Amplitaq Gold (Perkin-Elmer, Catalog #N8080246)

Common laboratory solutions (make with RNAse-free ingredients or keep a separate RNA-only aliquot/bottle):

- Phosphate-buffered saline, pH 7.4 (PBS)
- Formaldehyde solution (37%)
- 1M glycine
- TE pH 8.0 (10 mM Tris-Cl pH 8, 1 mM EDTA)
- 1M Tris pH 6.5
- 20% SDS
- 0.5M EDTA
- Phenol:chloroform:isoamyl alcohol (25:24:1)

Authors Notes

n.b. This procedure is susceptible to both contamination and RNA degradation. It is very helpful to have dedicated RNAse-free reagents, centrifuges, and pipetors for this procedure and to use aerosol-barrier pipet tips to prevent cross-contamination. In addition, due to the instability of RNA we recommend performing the procedure continuously—that is, not to freeze away the procedure at any point and continue several days later as can be done at several points in “conventional” ChIP.

1. The optimal number of cells used in each immunoprecipitation will vary. Two of the important variables include the characteristics of the protein target and the antibody used. Use of higher-affinity/efficiency antibodies or targeting of histone proteins, for instance, may favor detection of the RNA-protein interaction and require less crosslinking time. On the other hand, detection of transcriptional factors may require longer crosslinking time (i.e., 30 minutes).
2. It may not be the experimenter’s intent to map the interaction between the protein to specific regions of the RNA, but to simply evaluate if the RNA associates with the protein at all. If this is the case, it is not necessary to shear the DNA/RNA to very small fragments, and may be detrimental to the RNA if repeated rounds of sonication cause the solution to be

overheated. In either case, do not leave the solution on crushed dry ice when it is not being sonicated, as the SDS is prone to precipitation.

3. Many protocols include a “pre-clearing” step in which the samples are exposed to a “pre-immune” or normal IgG serum to reduce non-specific background before the immunoprecipitation step. In my hands, this step has not been necessary but can be added if a high background is seen.
4. It may be useful to vary the amount of time for reversing the crosslinks. In my hands, 2 hours seemed to be a workable balance between having the chemical crosslinks reversed versus the concern of exposing the RNA to elevated temperatures for prolonged periods. I did not allow the crosslinking reversal reaction to proceed overnight.
5. An alternative to the remaining steps listed after this point is to isolate the RNA from the eluate by Trizol or Trizol LS reagent (Invitrogen). The reader is referred to another review which describes this alternative procedure [see Reference 3].

Reviewer Comments

Reviewed by: Kevin V. Morris, Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA, USA.

1. Comments by this reviewer have already been integrated into the protocol where appropriate.

Figures

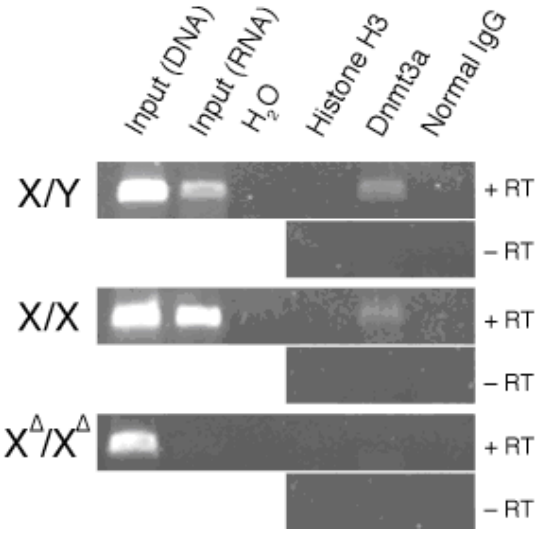

Figure 1.
An example of an RNA-ChIP experiment to examine interactions between the DNA methyltransferase, Dnmt3a, and the noncoding RNA, *Tsix* (see Reference 1). In wild-type male (X/Y) and female (X/X) cells, *Tsix* RNA can be amplified from a lysate immunoprecipitated by the Dnmt3a antibody. As expected, in cells with a null mutation for *Tsix* (X^{Δ}/X^{Δ}), the RNA is not detected.

References

1. Sun BK, Deaton AM, Lee JT. A transient heterochromatic state in Xist preempts X-inactivation choice without RNA stabilization. Mol Cell 2006 (21): 617-628.
2. Gilbert SL, Pehrson JR, Sharp PA. XIST RNA associates with specific regions of the inactive X chromatin. J Biol Chem 2000 Nov 24: 275(47): 36491-4.
3. Riranjanakumari S, Lasda E, Brazas R, Garcia-Blanco MA. Reversible cross-linking combined with immunoprecipitation to study RNA-protein interactions in vivo. Methods 2002 (26): 182-190.