RNA-biotin based pulldown assays for the detection of siRNA targeted genomic regions and siRNA directed histone modifications (PROT32)

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Last reviewed: 19 June 2007 by Jeannie Lee, Department of Genetics (and Pathology), Harvard Medical School, Boston, MA, USA.

Introduction

The recent discovery of RNA interference and in particular the observation that siRNAs can modulate gene expression at the level of transcription, i.e. small-interfering RNA (siRNA) directed transcriptional gene silencing (TGS) in Human cells (Matzke and Birchler 2005; Morris 2005) has illustrated the fact that RNA may be far more intricately involved in epigenetics than was previously assumed. To determine more clearly how siRNAs are interacting with the homologous genomic regions in the nucleus in human cell cultures we designed several RNA-biotin based pulldown assays which can be used alone or in combination with other known assays such as ChIP and Flag-tagged pulldown assays. Three protocols are explained in detail here. The first protocol is essentially a dual-pulldown assay employing Flag-tagged DNMT3A or antibody of choice for an endogenous protein and 5' biotin linked antisense RNA (Figure 1a), while the second protocol is a triple pulldown assay which essentially expands upon the dual pulldown to incorporate a third pulldown which is an iteration of the ChIP and is a pulldown for H3K27^{me3+} (Figure 1b). The third assay described here is the biotin-RNA pulldown of a low-copy RNA that spans the siRNA targeted promoter region (Figure 2). Data generated from this assay is currently in submission.

Procedure

Part I: Detection of Flag-tagged proteins binding 5' biotin labeled siRNAs in vitro

1. A minimum of ~4.0×10^6
   cells are required/sample and clearly the more cells to begin with the better the chances of a successful pulldown. A total of 4.0×10^6
   293 HEK cells (~70% confluent) are plated and 24 hours later transfected with 15µg of a RNA Polymerase II expressing flag-tagged construct plasmid. We generally use Lipofectamine 2000™ (Invitrogen) at a 1:3 ratio (e.g. 1µl plasmid to 3µl lipofectamine). The plasmid and lipofectamine are mixed together, vortexed, pulse spun, and then 50µl of DMEM (without Fetal bovine serum, FBS) added. The entire mixture is again vortexed followed by a pulse spin, and then added drop wise (randomly around the plate) of the previously plated cell cultures (Figure 1a);
2. Forty-eight hours later the cell cultures are washed in cold 1xPBS, then scrapped from the plate and collected in 1ml of cold 1x PBS. The cultures are then pelleted at ~2,000 RPM for 4 minutes and the pellet re-suspended in 500µl of cold lysis buffer
   on ice for 10 minutes and then centrifuged 5,000 RPM for 5 minutes at 4°C. The pellet is considered the nuclear fraction while the solution is the cytoplasmic fraction. Save the cytoplasmic fraction on ice for step 3. To isolate the final nuclear fraction the pellet is re-suspended in 500µl of lysis buffer and incubated on ice for 10 minutes, centrifuged at 5,000 RPM for 5 minutes at 4°C and the solution saved as the nuclear fraction with the pellet discarded (comment 1 and comment 2);
3. The resultant lysates (cytoplasmic and nuclear fractions) are then mixed, 125µl of each of the cytoplasmic and nuclear fraction, and incubated for 3 hours at 4°C with 500nM 5'biotin end-labeled siRNA under gentle agitation, i.e. a rocking plate (Figure 1a). We utilized the EF1 alpha specific siRNA, EF52, shown previously to direct TGS in human cells (Morris et al. 2004). The EF52 siRNA was constructed with a 5' biotin tag on either the sense or antisense strand (City of Hope Protein and synthesis core facility) and various iterations were generated by first heating the RNAs (sense or antisense +/- biotin) to 70°C for 5 minutes and then mixed together and room temperature cooled, i.e. biotin tagged sense+antisense, sense+biotin tagged antisense, biotin tagged sense+biotin tagged antisense. The biotin tagged sense or antisense can also

be used alone, as well as the sense and an antisense lacking the 5' biotin tag (as a controls). We have found that the antisense alone co-immunoprecpitates with the flag-tagged DNMT3A (Weinberg 2005). Biotin linked RNAs can easily be generated by numerous companies. We now generally use IDT technologies 5' biotin linked RNAs;

4. Following the 3 hour incubation Dynabeads™ M-280 Streptaviden magnetic beads ($7x10^7$ beads, ~100µl/sample) are added to the respective samples (note 1);
5. The resultant siRNA/Flag complexes are then eluted with magnetic bead binding, the solution can be discarded (or saved as input) and the beads (putatively bound with the siRNA/Flag complexes) washed 3 to 5 times with 2x wash buffer (note 2);
6. Following the final wash the bound siRNA/protein complexes are eluted from the avidin-biotin bound beads by incubation in 100µl of elution buffer at 65°C for 5 minutes;
7. The eluted complexes can then be electrophoresed in denaturing PAGE and subjected to western blot analysis with an anti-Flag antibody for detection of the particular flag-tagged protein of choice (Figure 1a).

Part II Triple immunoprecipitation: H3K27 ChIP/-flag-DNMT3A/biotin-RNA

1. A total of 4.0×10^6
   293T cells are plated out and 24 hours later transfected plated (~70% confluent at the time of transfection) with the flag-tagged protein expressing plasmid of choice (15µg, Lipofectamine 2000™ Invitrogen). The following day the flag-tagged protein expressing plasmid transfected cultures are transfected again with 100nM EF52 biotin labeled siRNA (antisense or sense alone) using Lipofectamine 2000™ or stock 3.4µM MPG (3µl/ml of media described in (Morris et al. 2004)) (note 3).

Pulldown (1), Antibody specific

2. Forty-eight hours following the biotin linked sense or antisense siRNA transfection the cultures are collected and a chromatin immunoprecipitation (ChIP) assay is performed by first adding formaledehyde directly to tissue culture media to a final concentration of 1% for 10 minutes at room temperature under gentle rocking (Figure 1b);
3. Stop the crosslinking reaction by adding glycine to a final concentration of 0.125M and continue to rock or spin at room temp for 5 minutes;
4. Next, pour off media and rinse plates with cold 1X PBS+1/1000 PMSF, aspirate and then add 1.5ml of PBS+1/1000 PMSF and scrape the plates to remove the cells. Collect the cultures and centrifuge 4 minutes at 2000 RPM;
5. Remove the PBS+1/1000 PMSF by aspiration and re-suspend cell pellet in 1ml ChIP lysis buffer plus the protease inhibitors 1/1000 PMSF and 50 units of RNAse inhibitor. The final volume of cell lysis buffer should be sufficient so that there are no clumps of cells. Incubate on ice for 10 minutes;
6. Centrifuge at 5,000 rpm for 5 minutes at 4°C to pellet the nuclei;
7. Re-suspend nuclei in 600µl of ChIP lysis buffer+1/1000 PMSF+50 units of RNAse inhibitor and incubate on ice for 10 minutes;
8. Sonicate chromatin while keeping samples on ice (1 interval for 20 seconds at power setting of "3" on the Branson 50 cell machine). Immediately centrifuge at 14,000 RPM for 10 minutes at 4°C;
9. Carefully remove the supernatant and transfer to a new tube. Preclear chromatin by adding Protein A/Salmon Sperm (10µl). Incubate on a rotating platform at 4°C for 15 minutes, no longer. Centrifuge at 14,000 RPM for 5 minutes (comment 3);
10. Transfer supernatant to a clean tube and divide equally among your samples. Be sure to include a "no antibody" control sample. Adjust the final volume of each sample with lysis buffer if required. Sample volumes should be between 400-800µl/sample to allow ample mixing during the overnight incubation. Add 1µg of antibody to each sample and incubate on the rotating platform at 4°C overnight;
11. Add 20µl Protein A/Salmon sperm sephrose beads to each sample and incubate at room temperature for 15 minutes on a rotating platform (comment 3);
12. Centrifuge the samples containing the Protein A/Salmon spern sepharose beads (10,000 RPM for 1 minute at 4°C) and save the supernatants as unbound fraction (input for step 21 below);
13. Wash the pelleted Protein A/Salmon sperm sepharose beads bound to the putative protein of interest twice with:
    a. 1ml ChIP lysis buffer;
    b. 1ml of ChIP high salt buffer;
    c. 1ml of ChIP wash buffer;

    (note 4)

Pulldown (2), Flag-tag specific

14. After the last wash elute the complex by adding 100µl of ChIP elution buffer. Incubate the samples at 65°C for 10 minutes and then centrifuge at 14,000 RPM for 3 minutes. Save 20µl of the elutes as (Flag-Input). Next, take the remaining ~80µl of elute to clean tubes containing 40µl of EZVIEW™ Red anti-Flag M2 affinity gel beads (Sigma™) which have been pre-treated with a wash in 250µl of TBS-mod buffer, vortexed and centrifuged for 30 seconds at 6,000 RPM (comment 4);
15. Gently mix (rotating plate) the cell lysate/ChIP pulldown with the antibody-M2 beads for 2-3 hours or overnight at 4°C;
16. Capture the Flag-antibody-M2 immunoprecipitated complexes by pulse centrifugation (15 seconds at 3,000 RPM). Wash

the bead/complexes 3 times in 500µl ice-cold TBS-mod buffer (comment 4);
17. To elute the putative ChIP-Flag complexes (by competition with 3x Flag-peptide) add 100µl of TBS-mod buffer+3µl (5µg/µl 3X Flag Peptide) to each pelleted bead/complex, incubate 30-60 minutes at 4°C with gentle shaking;
18. Centrifuge the resin for 30 seconds at 10,000 RPM, transfer the supernatants (which should now contain the ChIP/Flag eluted complexes) to fresh tubes containing100µl (6-7×10^8 beads/ml) of Dynabeads™ M-280 Streptaviden (Figure 1b).

Pulldown (3), RNA-biotin specific

19. Incubate the elute/Dynabead™ slurry at 4°C for 15 minutes on an orbital shaker followed by capture with a magnetic bead separator. The captured beads are washed (carefully as to not agitate the beads) 3 times with 2x wash buffer;
20. After the last wash (2x wash buffer) the complexes are eluted in 100µl of 2x elute buffer at 65°C for 5 minutes (Figure 1b);
21. The elutes are then reverse cross-linked by adding 1µl Rnase A (10mg/ml) and 20µl of 5M NaCl. Incubate the samples in a 65° water bath or heat block for 4-5 hours to overnight (note 5);
22. After the reverse cross-linking the elutes are treated with 10µl of 0.5M EDTA, 20µl of 1M Tris-HCL, pH 6.5 and 2µl of 10mg/ml Proteinase K and incubated in 45°C water bath for 1 hour;
23. Recover DNA/RNA by Phenol/Chloroform extraction and ethanol precipitation (note 6);
24. Re-suspend the pelleted DNA in ~30µl of water and perform PCR or real-time PCR analysis for the siRNA targeted gene compared to the respective control(s).

Part III: Detection of pRNAs by 5'biotin EF52 pull-down assay

1. A minimum of ~4.0×10^6 cells are required/sample. Twenty-four hours following the plating (~70% confluent) the cultures are transfected with the 5' biotin linked sense (control) or antisense (treatment) RNAs (70-100nM final concentration (Ting et al. 2005; Kim, Villeneuve et al. 2006)) targeted to the particular promoter of choice with Lipofectamine 2000™ (Invitrogen) at a 1:3 ratio (e.g. 1µl biotin-tagged RNA (for final 70-100nM concentration) to 3µl lipofectamine). However, the RNAs are first heated to 70°C for 10 minutes to allow the relaxation of any untoward secondary structures. The RNAs and lipofectamine are then mixed together, vortexed, quickly spun, and then 50µl of DMEM (without fetal bovine serum, FBS) added. The entire mixture is again vortexed followed by a quick spin, and then added drop wise (randomly around the plate) to the previously plated cell cultures;
2. Twenty-four hours following transfection the treated cultures can be crosslinked by adding 1% formaledehyde directly to tissue culture media to a final concentration of 1% for 10 minutes at room temperature under gentle rocking and stopped by the addition of glycine to a final concentration of 0.125M and continue to rock or spin at room temp for 5 minutes. The cells are then washed (1xPBS), scrapped from the plate if adherent, and then genomic DNA extracted (Qiagen™ DNeasy kits). The extracted genomic DNA (200µl elute) is then used to detect promoter specific transcripts (pRNAs) with the biotin linked pull-down assay (Figure 2) (comment 5);
3. From the 200µl elute 30µl should be saved as input and the remaining 170µl from the extracted genomic DNA (Qiagen™ DNeasy) is mixed with 170µl of modified lysis buffer. During this same time 100µl of Dynabeads™ M-280 Streptaviden should be washed with 300µl of modified lysis buffer (comment 6);
4. Next, mix the pre-washed Dynabeads™ M-280 Streptaviden beads with the 170µl of extracted genomic DNA elute/ modified lysis buffer solution (15 minutes at R/T under constant motion);
5. Following the 15 minute incubation the beads are captured using a magnetic bead separator (Invitrogen™) and washed 3 times with 2x wash buffer;
6. After the third wash the bound beads were eluted using elution buffer II, incubated at 65°C for 10 minutes and separated using the magnetic bead separator;
7. The final ~100µl elute is then assessed for promoter specific DNA and/or RNA by PCR and RT-PCR respectively (note 7).

Materials & Reagents

ChIP lysis buffer	30ml final volume: 1.5 ml 1M Hepes 1.05ml 4M NaCl 3ml 10% Triton X (Tx-100) 300µl 10% NAD 24.15ml H_20
ChIP high salt buffer	30ml final volume: 1.5ml 1M Hepes 3.75ml 4M NaCl 3ml 10% Triton X (Tx-100) 300µl 10% NAD 21.45ml H_20

ChIP wash buffer	30ml final volume: 150µl 2M Tris 1.7ml 4.4M LiCl 60µl 0.5M EDTA 1.5ml 10% NAD 1.5ml 10% NP-40 25.09ml H_20
ChIP elution buffer	20ml final volume: 500µl 2M Tris 2ml 10% SDS 400µl 0.5M EDTA 17.1ml H_2O
cold lysis buffer	1mM PMSF 20units RNasin 10mM Tris-HCl pH 7.4 10mM NaCl 3mM $MgCl_2$ 0.1mM DTT 0.5% NP40
wash buffer	10mM Tris pH 8.0 1mM EDTA 0.5M NaCl
elution buffer	Tris-Cl pH 6.0 1mM EDTA 2.0M NaCl 0.5M $MgCl_2$
TBS-mod buffer	50mM Tris HCl 400mM NaCl pH 7.4
2x elute buffer	10mM Tris-HCL pH 6.0 1mM EDTA 2.0M NaCl
modified lysis buffer	0.5% NP-40 300mM NaCl 20mM Hepes pH 7.0 2mM $MgCl_2$
elution buffer II	10mM Tris-HCl pH 6.0 1mM EDTA 2.0M NaCl

Author Notes

1. The avidin/magnetic beads are first washed in lysis buffer (~500µl) followed by magnetic separation, aspiration, and then re-suspended in 100µl of lysis buffer. The 100µl bead slurry is then mixed with the siRNA/Flag-Tagged containing lysates and incubated at 4°C for an additional hour.
2. Good washing is required to reduce the background.
3. While MPG can deliver the siRNA directly to the nucleus it can also be cytotoxic at high volumes.
4. For each wash incubate the samples on a rotating platform at room temperature for 3 minutes and then centrifuge at 14,000 RPM for 1 minute. Try to remove as much buffer as possible without aspirating the pelleted complex. Efficient washing is critical to reduced background.
5. Include the input/starting sample saved for each sample from step 12 should also be reverse the cross-linked during this step.
6. A glycogen carrier (Ambion) is recommended for visualization of the subsequent pellet.
7. If assaying for promoter specific RNAs it is important to Dnase treat the elutes (Turbo DNA-free™, Ambion) and then perform an RT reaction (+/- RT) followed by PCR with primers specific for the siRNA targeted promoter (Figure 2).

Reviewer Comments

Reviewed by: Jeannie Lee, Department of Genetics (and Pathology), Harvard Medical School, Boston, MA, USA.

1. The author describes a protocol to capture small RNA-associated proteins or DNA/RNA complexes. The procedures are based on immuno-affinity purification and chromatin immunoprecipitation (ChIP). The protocol could be an attractive means to verify small RNA associated complexes. Given its novelty and the timeliness of such a protocol, there would be much value in making the protocol available to the public. The protocol as described appears extremely difficult and may not be easy for the average investigator to reproduce, considering the sequential IP steps and the nature of small RNAs. Some of the concerns are noted below.
2. The author uses the same buffer to extract nuclear fraction as that for cell lysis containing low salt (10mM NaCl). This would be a higher salt concentration buffer. Response: We use the same lysis buffer for both the initial lysis and then take the resultant pellet and re-lyse with the lysis buffer followed by sonication. This is not a very precise nucleus vs. cytoplasmic fractionation. Certainly upping the salt concentration could prove advantagous when assessing the 2 fractions.
3. "Flag-Input" the author describes should be "unbound" The author should recover supernatant as "Input" before adding the anti-Flag-M2 beads. Response: This has been changed accordingly and the saved input is now in step 14 of Part-II.
4. For preparation of genomic DNA by Qiagen DNeasy kit, guanidine hydrochloride would be used at the lysis step. Can DNA (or RNA)-siRNA hybrid maintain the formation under a denaturing condition without cross-link? Response: We have had success with and without the cross-link. Greater success is with > number of cells. If one is trying to detect RNA/protein interactions using this method crosslinking should be used. If one is only trying to detect a promoter associated RNA then crosslinking is not necessary with > numbers of cells, i.e. ~4×10^6. However, if one wants to detect the presence of a promoter associated RNA the easiest manner is via RT PCR with oligos designed to specifically amplify the promoter region. This of course is in retrospect as we first performed pulldown assays to detect these variant RNA species. The details will be published soon (Han et.al. PNAS, 2007, in press). We have changed this in the text accordingly.
5. What is "blocked ProteinA/Salmon Sperm"? If this means beads like Protein A agarose or sepharose beads blocked with salmon sperm, the centrifuge to recover the beads would be too fast. It appears that the remainder of this section uses beads, but they are not mentioned. Response: We have removed the "blocked" statement as this seems misleading. The spin is correct we are trying to add the protein A/Salmon sperm to reduce the nonspecific background. We then spin a 14k to remove the bound fractions and retain the unbound as our elute for the antibody pulldown. Though clearly we could reduce the spin. In the bead recover in section (11) we have added the sepharose beads at step (11) and then wash accordingly. We have changed the text accordingly to make this section read more clearly.
6. The author tries to isolate undigested genomic DNA binding with biotin-siRNA. How long is the average length of genomic DNA prepared by the Qiagen kit? Response: We have used this method with sonication and detected the promoter associated RNA but also without sonication. We do not know the average size of the resulting DNA following the Qiagen based extraction.

Figures

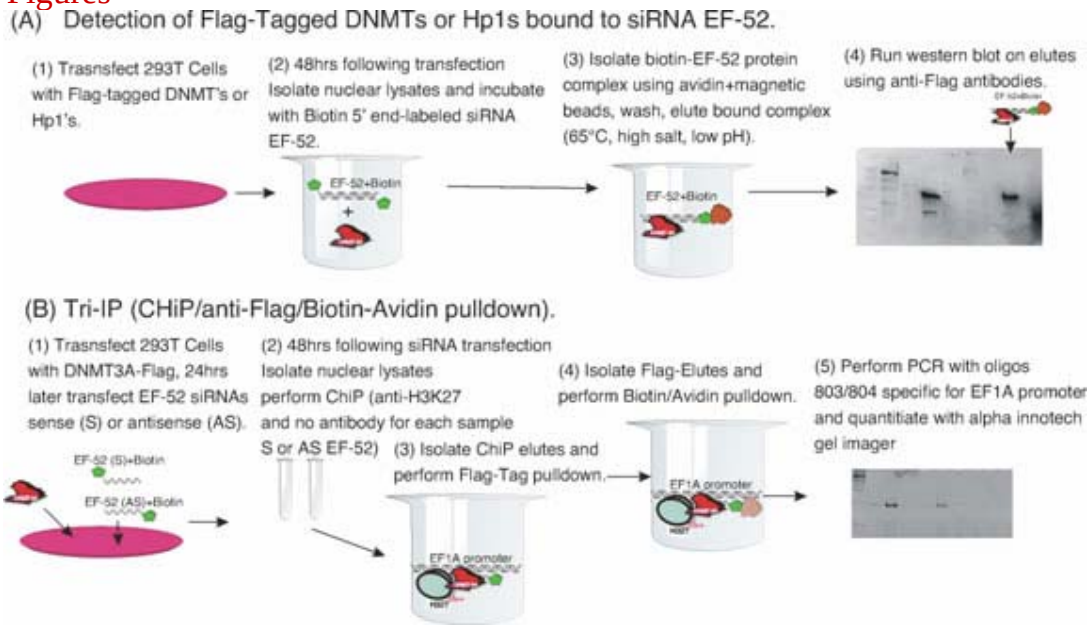

Figure 1: A) Methodology for detection of flag-tagged DNMTs or HP1s associated with 5' biotin labeled siRNA EF52. Schematic methodology is shown for detection from cell lysates of biotin labeled siRNAs in complex with flag-tagged DNMTs or HP1s (alpha, beta, or gamma). B) Triple immunoprecipitation assay for detection of $H3K27^{me3+}$, flag-tagged DNMT3A, siRNA EF52 and the targeted EF1A promoter. Methodology is shown for performing first a ChIP for $H3K27^{me3+}$ and then a Flag-immunoprecipitation followed by a biotin/avidin pulldown for biotin labeled siRNAs, or specifically the sense or antisense RNA. The resultant elutes are then utilized in PCR for the targeted EF1A promoter.

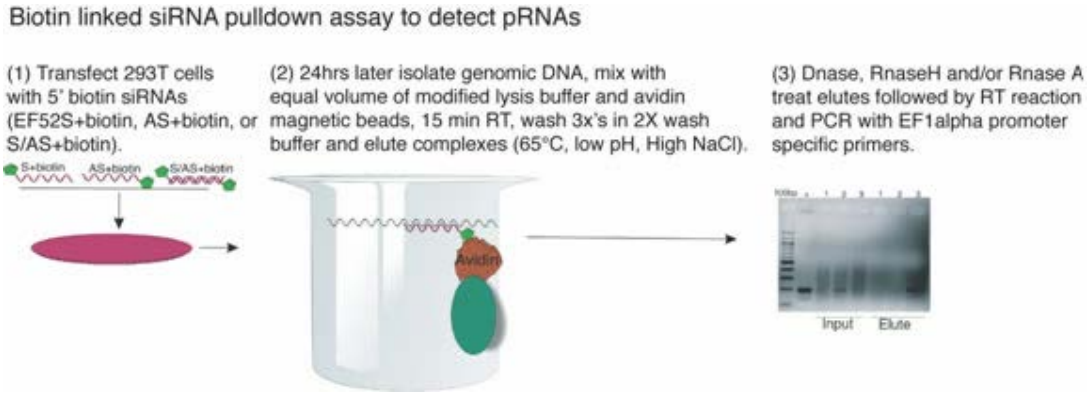

Figure 2: 5' biotin linked siRNA pull down assay to detect low-copy promoter specific RNAs. 293T or 293-R5-GFP cells (4×10^5/well, 6 well plate) or 4×10^6/10cm plate were transfected with 5' biotin linked EF52 sense, antisense or sense/antisense RNAs (100nM). Twenty-four hours later the cultures were isolated, washed 1 time in 1x PBS, genomic DNA isolated (Qiagen™ Dneasy), and exposed to avidin magnetic beads (Dynal™). The final elutes, following the binding of the biotin linked RNAs with the avidin magnetic beads and washing, were then DNAse, RNAse H or RNAse A treated, RT (BioRad™ iScript) reacted and PCR amplified.

References

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