Chapter 4

Detecting RNA–Protein Interaction Using End-Labeled Biotinylated RNA Oligonucleotides and Immunoblotting

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Abstract

RNA–protein interaction can be detected by RNA pull-down and immunoblotting methods. Here, we describe a method to detect RNA–protein interaction using RNA pull down and to identify the proteins that are pulled-down by the RNA using immunoblotting. In this protocol, RNAs with specific sequences are biotinylated and immobilized onto Streptavidin beads, which are then used to pull down interacting proteins from cellular extracts. The presence of a specific protein is subsequently verified by SDS- polyacrylamide gel electrophoresis and immunoblotting with antibodies. Interactions between the SMN RNA and the PSF protein and between the caspase-2 RNA and the SRSF3 protein (SRp20) in nuclear extract prepared from HeLa cells are illustrated as examples.

Key words Spliceosome, RNA binding protein, RNA–protein interaction, Biotinylation, Streptavidin, RNA pull-down, Immunoblotting

1 Introduction

The method for detecting RNA–protein interaction by RNA pull-down and immunoblotting is one of many methods in studying RNA–protein interaction [1–4]. The main principle of the method described here is to use streptavidin agarose beads to pull-down the biotin-labeled RNA and its associated proteins under the specified conditions [5, 6]. The streptavidin agarose can be used to effectively and specifically pull down biotinylated RNA–protein complexes because of the strong affinity between biotin and streptavidin [7–10]. Here we describe how to pull-down spliceosomal RNA-binding proteins using biotin labeled RNA and streptavidin agarose beads, and how to detect the RNA interacting proteins by immunoblotting.

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2 Materials

All the reagents applied in this experimental protocol are molecular biology grade (unless indicated otherwise), and dissolved in ultrapure water (unless indicated) which is obtained through three steps of purification: firstly, impurity particle depletion procedure with 10 μm and 5 μm filters, secondly, impurity compounds depletion procedure through carbon filter, thirdly and finally, deionizing procedure through reverse osmosis system. The ultrapure water has an electrical resistivity of 18.2 million ohm-cm at 25 °C. All reagent solutions are prepared and stored in stock at room temperature (unless indicated otherwise). The diethylpyrocarbonate (DEPC) treated water is not applied in the experiments unless indicated otherwise.

All the procedures in RNA pull down protocol are carried out at 4 °C unless otherwise specified, and all the solutions applied to this experiment are kept 4 °C overnight before the experiment. All the procedures in protein immunoblotting are carried out at room temperature otherwise specified.

2.1 RNA Pull-Down

1. Biotin-labeled RNAs of 10 nucleotides in length, the biotin residue is at the 5′ end (obtained commercially; ours are from the Bioneer Corporation (Fig. 1).
2. Streptavidin agarose washing buffer: 20 mM Tris–HCl, pH 7.5, 100 mM KCl, 2 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF.
3. RNA–streptavidin interaction buffer: 20 mM Tris–HCl, pH 7.5, 300 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF.
4. Strepavidin agarose beads.
5. Nuclear extracts prepared from HeLa cells as the source of the RNA-binding proteins.

SMN-WT:	biotin-AAGAAGGAAG
SMN mutant:	biotin-AAGAAUUAAG
Casp2 WT:	biotin-UUCUUCAUCC
Casp2 mutant:	biotin-UGAAGCGUCC

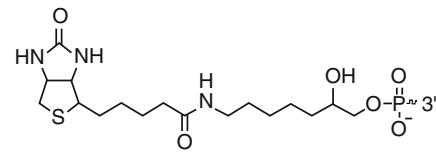

Fig. 1 Synthetic, biotin-labeled RNA. The sequences of the four 5′ end-biotin-labeled RNA decamers are shown. SMN is Survival of Motor Neuron, Casp2 is Caspase-2, and WT is wild-type. These sequences are from the messenger RNA of the respective gene. The structure of the biotin moiety is depicted

2.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting

1. 30 % acrylamide with 29:1 ratio of acrylamide and bis-acrylamide.
2. 1.5 M Tris–HCl, pH 8.8.
3. 10 % ammonium persulfate (APS).
4. *N,N,N′,N′*-tetramethylethane-1,2-diamine (TEMED).
5. 5× protein loading buffer: 60 mM Tris–HCl (pH 6.8), 25 % glycerol, 2 % SDS, 14.4 mM β-mercaptoethanol, 0.1 % bromophenol blue.
6. SDS PAGE gel running buffer: 25 mM tris base, 192 mM glycine, 0.1 % SDS (*see* **Note 1**).
7. SDS PAGE gel transfer buffer: 25 mM tris base, 192 mM glycine.
8. Nitrocellulose membrane blocking buffer: 5 % skim milk, 10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1 % Tween 20.
9. Immunoblotting buffer: 5 % skim milk, 10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1 % Tween 20.
10. Antibodies against the RNA-binding proteins (the antibody against PSF protein is from Santa Cruz and the antibody against SRSF3 protein is from Invitrogen).
11. ECL Western Blotting substrate (Thermo Scientific Pierce).
12. X-ray film (FUJI Incorporated).

3 Methods

3.1 Preparation of Biotin Labeled RNA

RNA can be obtained through two different ways, chemical synthesis or in vitro transcription. The shorter RNAs are practically feasible to be synthesized chemically while longer ones are suitable to be transcribed from a DNA template by in vitro transcription. There are also two different ways to label RNA with biotin, end labeling and body incorporation. In this protocol, end labeled biotin RNAs are used.

3.2 Preparation of Streptavidin Agarose Beads

1. Commercially obtained 50 % streptavidin agarose beads are mixed well by shaking in a cap-sealed tube.
2. 5 μl of streptavidin agarose beads is taken out into 1.5 ml Eppendorf (EP) tubes using end-cut yellow tip (*see* **Note 2**).
3. 500 μl ice-chilled streptavidin washing buffer is added into streptavidin agarose bead containing 1.5 ml EP tubes, and pipetting the slurry 4–5 times with end-cut blue tip.
4. The EP tubes are centrifuged at 5000 (470 × *g*) revolutions per minute (RPM) for 1 min at 4 °C in a bench top centrifuge.
5. The supernatant is discarded using gentle pipetting with blue tip (*see* **Note 3**).

3.3 Binding Biotin Labeled RNA onto Streptavidin Agarose Beads

1. The EP tubes containing prepared streptavidin agarose are refilled with 500 μl of streptavidin washing buffer and gently pipetted 4–5 times with end cut blue tip.
2. 1 μl (100 pmol) of biotin labeled RNA is applied to above EP tubes except one of these EP tubes as negative control (*see* **Note 4**).
3. These EP tubes are incubated at 4 °C for 1–4 h under 100 RPM rotation in a rotator (*see* **Note 5**).
4. The EP tubes are then centrifuged at 5000 RPM for 1 min at 4 °C to precipitate the biotin labeled RNA bound streptavidin agarose bead.
5. The supernatant (streptavidin washing buffer) from EP tubes above are discarded with pipetting gently with blue tip (*see* **Note 3**).
6. 500 μl of the RNA–streptavidin interaction buffer is applied to the EP tubes with pellets of the biotin labeled RNA bound streptavidin agarose beads, and suspend the pellets with pipetting for 4–5 times with end cut blue tips on (*see* **Note 6**).
7. The EP tubes with biotin labeled RNA bound streptavidin agarose beads are centrifuged at 5000 RPM for 1 min at 4 °C in bench top centrifuge.
8. The supernatant of EP tubes above are discarded with gentle pipetting with blue tip.
9. The processes from **steps 6–8** listed above are repeated two more times.

3.4 Binding of Proteins to the Immobilized Biotin Labeled RNA

1. 500 μl of the RNA–streptavidin interaction buffer is applied to the EP tubes containing biotin labeled RNA bound streptavidin agarose bead, and suspend the agarose slurry with gentle pipetting with end cut blue tip for 4–5 times.
2. 100 μl of nuclear extract with a protein concentration of 3–5 μg/μl from HeLa cells are applied to each of above EP tubes.
3. The EP tubes with nuclear extract and biotin labeled RNA bound streptavidin slurry are kept at 4 °C overnight (about 12 h) under 100 RPM rotation in a rotator.
4. The EP tubes with nuclear extract and biotin labeled RNA bound streptavidin agarose bead are centrifuged at 5000 RPM for 1 min at 4 °C in bench top centrifuge.
5. The supernatant of EP tubes above are discarded with gentle pipetting with blue tip.
6. 500 μl of the RNA–streptavidin agarose interaction buffer is applied to the EP tubes with pellets of the nuclear extract and biotin labeled RNA bound streptavidin agarose bead, and suspend the pellets with pipetting for 4–5 times with end cut blue tips on (*see* **Note 6**).

7. The EP tubes with nuclear extract and biotin labeled RNA bound streptavidin agarose bead are centrifuged at 5000 RPM for 1 min at 4 °C in bench top centrifuge.
8. The supernatant of EP tubes above are discarded with gentle pipetting with blue tip.
9. The processes from **steps 6–8** listed above are repeated two more times.

3.5 Elution of the Bound Proteins from the Streptavidin Agarose Beads

1. 16 μl of 1× protein loading dye are applied into each EP tube obtained from above procedure, in which there is the complex of RNA binding proteins and biotin labeled RNA bound streptavidin slurry.
2. The pellet in each EP tube containing 1× protein loading dye, RNA bind proteins and biotin labeled RNA bound streptavidin slurry is suspended with gentle pipetting for 4–5 times with yellow tip (*see* **Note 7**).
3. The EP tubes are heated in heat block at 80 °C for 5 min (*see* **Note 8**).
4. The EP tubes are centrifuged at 11,000 RPM for 10 min at room temperature.
5. The supernatant (about 10 μl) of the EP tubes above is gently transfer to new fresh EP tubes with matched marker on the top of caps of EP tubes (*see* **Note 9**).

3.6 Analysis of the RNA Interacting Proteins by Immunoblotting

1. The mini gel plates are cleaned with 70 % ethanol and assembled with spacers according the manufacturer's instructions.
2. 12 ml of 10 % SDS-PAGE gel solution are prepared by adding 4 ml of 30 % acrylamide with 29:1 ratio of acrylamide and bisacrylamide, 3 ml of 1.5 M Tris–HCl, pH 8.8, and 4.95 ml of double distilled H_2O. Just before pouring SDS-PAGE gel preparation solution into assembled mini gel plates, adding 50 μl of 10 % APS, 2.5 μl of TEMED, and mixing with a magnetic stirrer or shaking by hand (*see* **Notes 10** and **11**).
3. Immediately, the 10 % SDS PAGE gel preparation solution prepared above is poured into the space of pre-assembled mini gel plates followed by the comb is inserted, and keep at room temperature for about an hour to get gel formation completely. In our study, the 1.0 mm spacer and combs are preferred while this is completely case dependent (*see* **Note 12**).
4. The prepared mini gel plate is assembled to the gel running apparatus according the manufacturer's instructions followed by fulfilling running buffer to both anode side and cathode side space to have the electricity go through anode, running buffer, wells of gel, gel body, running buffer, and cathode.

5. Eluted 10 μl of the biotin labeled RNA bound proteins samples from Subheading 3.5 are applied to the SDS-PAGE gel and electrophoresed at 120 voltage (V) about 2 h until the bromophenol blue dye is at the bottom of the gel.
6. The mini gel is removed from mini gel plate and set into mini gel nitrocellulose transferring apparatus according the manufacturer's instruction.
7. The protein bands in mini gel are transferred into nitrocellulose membrane at constant electric current of 300 mA for about 1 h (*see* **Note 13**).
8. The nitrocellulose membrane is removed from transferring apparatus and appropriately trimmed before put into a container for blocking (*see* **Note 14**).
9. The nitrocellulose membrane is blocked in 10–20 ml blocking solution (*see* **Note 15**), for 1 h with gentle shaking at room temperature.
10. The blocking solution is exchanged with 10 ml fresh blocking plus 1 μl first antibody (*see* **Note 16**), and incubated at room temperature for another 1 h (*see* **Note 17**).
11. The first antibody incubating solution is discarded and the nitrocellulose membrane is washed with 20 ml TBS-T buffer for 5 min with gentle shaking at room temperature.
12. The nitrocellulose membrane is washed for 5 times by repeating above procedure.
13. The nitrocellulose membrane is then incubated with 10 ml of TBS-T plus 0.5 μl of HRP conjugated secondary antibody that recognize the C fragment of first antibody for 1 h at room temperature.
14. The secondary antibody incubating solution is discarded and the nitrocellulose membrane is washed with 20 ml TBS-T buffer for 5 min with gentle shaking at room temperature.
15. The nitrocellulose membrane is washed for 5 times by repeating above procedure.
16. The nitrocellulose membrane is then spread with 0.5 ml of mixture of ECL solution A and solution B according to the manufacturer's instruction for 1 min in the dark room.
17. The nitrocellulose membrane is taken out and put onto a transparent overhead projector sheet, and suck any leftover liquid from the edge of nitrocellulose membrane with a paper tower.
18. The nitrocellulose membrane is covered with one layer of wrap, and set into X-ray film cassette.
19. One sheet of X-ray film is exposed to the nitrocellulose membrane for 1–15 min until the optimized results are obtained. Figure 2 shows a couple of representative results from this protocol which have already been published in the journal of

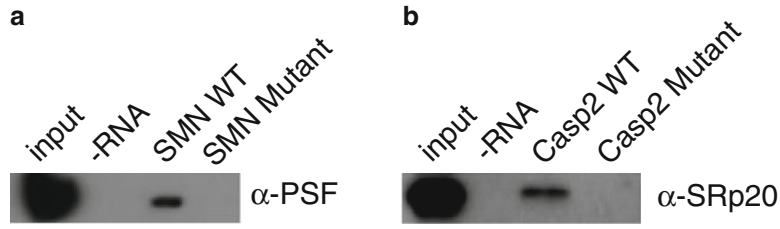

Fig. 2 Immunoblots of two RNA-binding proteins pulled down with biotinylated RNA. Nuclear extracts were incubated with biotinylated RNAs immobilized on Streptavidin agarose beads and the bound proteins were eluted and probed with specific antibodies: (**a**) an anti-PSF antibody used at a 1:5000 dilution [11] and (**b**) an anti-SRp20 antibody used at a 1:1000 dilution [12]. The PSF protein is only pulled down by the SMN wild-type RNA fragment, while the SRp20 protein is only pulled down by the Caspase-2 RNA fragment with the wild type sequence. The proteins were not pulled down with RNAs with a mutant sequence of without RNA on the beads. Both figures have already been published in the journal of Biochimica et Biophysica Acta [11, 12], and the permission documents for this reprint were obtained from the publishing company Elsevier

Biochimica et Biophysica Acta [11, 12]. The permission documents for the reprint here were obtained from the publishing company Elsevier ahead.

4 Notes

1. There are a couple of references that use different recipes, for example, some protocols suggest 250 mM glycine. We compared the results using different recipes and found no significant difference among different concentrations of glycine. We choose the 192 mM concentration of glycine because the time needed to dissolve is more reasonable and the higher concentration of glycine often needs heating during preparation [13, 14].
2. In order to collect the 50 % streptavidin agarose beads evenly, the end of yellow tip must be cut to enlarge the opening for passing streptavidin agarose beads, the number of EP tubes depends on the experimental design, in any case, one of the EP tubes must designated as negative control and no biotin labeled RNA will be added in this EP tube.
3. The supernatant should not be drained out fully, because the pellet is easy to suspend by any slight disturbs.
4. The molar ratio of biotin labeled RNA and streptavidin should be more than 2:1, more exactly, the streptavidin binding sites must be fully occupied by biotin labeled RNA, otherwise non-specific biotin containing proteins will contaminate the pull-down sample and irrelevant bands may show up in the final assay.

5. The duration of incubating time for making biotin labeled RNA bound streptavidin agarose beads depends on the length of biotin labeled RNA, if the biotin labeled RNA is less than 10 nucleotides, then the duration of incubating time is about 1 h; if the length of the RNA is more than 40 nucleotides, then the duration of incubating time is about 4 h.
6. The blue tips must be changed whenever they are used for EP tubes with different components, for example, the tubes with pellet of streptavidin, or the tubes with the pellet of biotin labeled RNA bound streptavidin.
7. The pellets shall not be suspended by vortexing, otherwise, there would be a lot of bubbles and the beads would stick to the side wall of EP tubes. Even with additional centrifugation, the bubbles would not disappear completely, which makes it difficult to separate the supernatant and the pellet cleanly.
8. The reason to set the heating temperature at 80 °C is that 80 °C is sufficient for denaturing most proteins in the presence of 1× protein loading buffer. Many references suggest boil protein samples at 100 °C in 1× protein loading dye before loading to SDS-polyacrylamide gel for electrophoresis. The protein denaturing efficacy may be improved at 100 °C, however, this will increase the risk of losing protein samples due to spilling or explosion [13–15].
9. The precipitates should never be disturbed when taking out the supernatant by pipetting. Otherwise the immunoblotted bands will be not sharp, especially when the quality of the antibody you work with is not high.
10. The percentage of SDS-PAGE gel depends on the protein bands you want to separate. The relationships between protein molecular weight and SDS-PAGE gel percentage have been described [13]. There are two different protocols to prepare SDS-PAGE gel, one of them has the separating gel at the bottom part and the stacking gel on the upper part, another one has no stacking gel at all, the whole gel are composed only of separating gel. We compared results from these two different protocols and found no significant differences. Therefore, it is not necessary to use SDS-PAGE gels with an upper stacking gel.
11. There is another difference in SDS-PAGE gel preparation recipes, one is with 0.1 % SDS in SDS-PAGE gel preparation solution and another one is without 0.1 % SDS. We have also compared the results from these two different recipes and found no significant differences. The reason is probably that SDS runs faster compared with proteins so that the PAGE gel becomes saturated with 0.1 % SDS before the proteins migrate into SDS-PAGE gel from the loading well. The handling of SDS-PAGE gel preparation without 0.1 % SDS is much sim-

pler because it is less likely to form bubbles during SDS-PAGE gel preparation.

12. The comb width and well numbers are decided according to the volume of loading sample. 10-well, 1 mm thickness comb can be used for loading about 25–30 μl of sample, while 15-well ones about 15–20 μl of sample. 10-well, 1.5 mm thickness comb can be applied to loading about 35–40 μl of sample, while 15-well ones about 25–30 μl of sample [13].
13. The efficacy of protein transfer in this step must be checked with the transfer efficacy of pre-stained protein size marker. Generally, the protein transfer efficacy depends on mini gel percentage, molecular weight of protein, and the electric current applied. After transfer protein bands from mini gel to nitrocellulose, the mini gel must be carefully checked to see if there are pre-stained protein size markers left in mini gel or not; the percentage of mini gel or the transferring electric current may need to be adjusted accordingly.
14. The membrane must be kept in the container in a position in which protein transferred side is always on the top side.
15. There are at least three different recipes for blocking solution, one 3–5 % skim milk in TBS-T buffer, another one is 1 % BSA in TBS-T, and the last one is the combination of the first two, 3–5 % skim milk plus 1 % BSA in TBS-T. The efficacy of these different blocking solutions for immunoblotting shall be checked for each application.
16. The fold of dilution to use for the first antibody can range from 100× to 20,000×; it must be tested as soon as the antibody is obtained commercially, even there is a recommended dilution.
17. The incubation condition of nitrocellulose membrane with first antibody is case dependent. Some antibodies bind specifically to the target protein (antigen) with very little non-specific binding, but some others could have a lot of non-specific bands. To decrease non-specific bands, one can try various blocking solutions—3 % skim milk, 1 % BSA, or the combination of the two. One can also apply the blocking solution in all first antibody incubating solution.

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