Chapter 3

Biotin–Streptavidin Affinity Purification of RNA–Protein Complexes Assembled In Vitro

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Abstract

RNA–protein complexes are essential for the function of different RNAs, yet purification of specific RNA–protein complexes can be complicated and is a major obstacle in understanding the mechanism of regulatory RNAs. Here we present a protocol to purify RNA–protein complexes assembled in vitro based on biotin–streptavidin affinity. In vitro transcribed RNA is labeled with ^{32}P and biotin, ribonucleoprotein particles or RNPs are assembled by incubation of RNA in nuclear extract and fractionated using gel filtration, and RNP fractions are pooled for biotin–streptavidin affinity purification. The amount of RNA–protein complexes purified following this protocol is sufficient for mass spectrometry.

Key words Biotin, Streptavidin, Affinity purification, RNA–protein complexes

1 Introduction

Recent years have witnessed rapid progresses in RNA research, and a major finding is the emerging of large quantity of long noncoding RNAs, but their functions are still largely unknown. Since RNA–protein interactions are the fundamental bases for RNA functions, purifying RNA–protein complexes and revealing the main components in the complexes would provide deep insights into the mechanisms on how these RNAs achieve their regulatory roles [1, 2].

However, purification of RNA–protein complexes formed in vivo or assembled in vitro remains to be a daunting technical challenge in many labs. Major obstacles encountered are degradation of RNAs, optimization of RNP assembly conditions, effective separation of the RNP from nonspecific binding, and the amount of RNPs available for downstream analysis. Current strategies in RNA–protein complex purification include biotin–streptavidin system [3], MS2 coat protein and modified MS2 hairpin [4], PP7 coat protein and PP7 hairpin, aptamer streptoTag and streptomycin [5], or S1m aptamer and streptavidin [6].

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Here we describe the most traditional yet effective system using biotin and streptavidin. Biotin ($C_{10}H_{16}N_2O_3S$, M.W. 244.31) is a water soluble vitamin, also known as vitamin B_7, vitamin H, coenzyme R or biopeiderm, whereas streptavidin is a protein of 52.8 kD purified from the bacterium *Streptomyces avidinii*. Tetramers of streptavidin have extremely high affinity to biotin with the dissociation constant of 10^{-14} mol/L, which is one of the strongest non-covalent interactions found in nature so far. The high binding affinity between biotin and streptavidin is due to extensive network of hydrogen bonds, hydrophobic interactions, conformation complementarity and stabilization.

The biotin–streptavidin system is widely used in protein purification [7] and nano-biotechnology as it is resistant to many harsh conditions such as denaturants, detergents, organic solvents, proteolytic enzymes, extreme pH and temperature. In addition, uniform labeling is used instead of end labeling for biotin incorporation in this protocol, which would enhance the RNA recovery efficiency. Finally, the small size of biotin may minimize the interference of the biological activity of labeled substrate. For instance, pre-mRNA labeled with optimized amount of biotin could be efficiently spliced in vitro.

This protocol requires a minimum of 4 days, which is seemingly time and labor consuming compared to some fast protocols. However, the amount of RNP purified following the protocol is considerably higher and is quite sufficient for mass spectrometry and other downstream analysis.

2 Materials (*See* Note 1)

2.1 In Vitro Transcription

1. 10xNTPs: 4 mM ATP and CTP; 2 mM UTP and GTP.
2. Cap analog: 20 mM (New England Biolabs).
3. Biotin-16-UTP: dilute to 0.3 mM (Roche).
4. UTP-[α-^{32}P] (Perkin Elmer).
5. RNasin Inhibitor: 40 u/μl (Promega).
6. T7 RNA polymerase: 50 u/μl (New England Biolabs).
7. 5x Transcription buffer: Mix 2 ml of 1 M Tris-HCl 7.6, 300 μl of 1 M $MgCl_2$, 1 ml of 100 mM spermidine, and autoclaved milli-Q water for a total volume of 10 ml; aliquot after filter with a syringe filter (*see* **Note 2**).

2.2 RNA Purification and Gel Separation

1. RQ1 RNase-free DNase (Promega).
2. Glycogen (Roche).
3. Phenol–chloroform–IAA (25:24:1, pH 6.6)(Ambion).
4. 5 M ammonium acetate: dissolve 19.25 g in water and bring the volume to 50 ml, filter.

5. 100 % ethanol.
6. 70 % ethanol.
7. Formamide dye: mix 0.4 ml 0.5 M EDTA, 0.8 ml 2.5 % xylene cyanol, 0.8 ml 2.5 % bromophenol blue, 16 ml formamide, 2 ml sterile H_2O; aliquot and store at −20 °C.
8. 6.5 % denaturing PAGE gel solution: combine 81.25 ml 40 % acryl–bis (29:1), 215 g urea, 50 ml 10x TBE, and add H_2O to 500 ml. Filter and store at 4 °C.
9. RNA ladder: for example, ϕX174 DNA/HinfI dephosphorylated markers (Promega).
10. ATP-[γ-^{32}P] (Perkin Elmer).
11. TEMED(Sigma).
12. 10 % ammonium persulfate (APS): dissolve 1 g in 10 ml H_2O, filter, aliquot and store at −20 °C.
13. 3 MM Whatman filter paper.
14. Plastic membrane (Saran Wraps).

2.3 In Vitro RNP Assembly

1. 12.5 mM ATP: add 250 μl 0.5 M ATP to 9.75 ml water.
2. 0.5 M Creatine Phosphate diTris salt (Sigma).
3. 80 mM $MgCl_2$: add 1.6 ml 1 M $MgCl_2$ to 18.4 ml water.
4. RNP assembly buffer: Mix 1 ml 1 M HEPES pH 7.6, 1.65 ml 3 M KCl, and add water to 50 ml, filter.
5. Nuclear extract (*see* **Note 3**).

2.4 Fractionation of the RNP by Gel Filtration

1. Sephacryl S-500 HR, store at 4 °C(GE Healthcare).
2. 10xColumn buffer: 200 mM HEPES pH 7.6, 600 mM KCl, 25 mM EDTA, 1 % Triton X-100, store at 4 °C.
3. 1xColumn buffer: dilute 100 ml 10x column buffer in 890 ml water, then add 10 ml freshly made 10 % sodium azide, store in the cold room.
4. 1.5 ml capless tube.
5. 3 MM filter paper.
6. Saran membrane.

2.5 Biotin–Streptavidin Affinity Purification

1. Low salt buffer: Mix 10 ml HEPES pH 7.6, 25 ml 3 M KCl, 2.5 ml 0.5 M EDTA, 500 μl Triton X-100, 1.25 ml 2 M DTT, and add water to 100 ml. Filter and store at 4 °C.
2. 3 M KCL: dissolve 112 g in H_2O and add H_2O to 500 ml, filter and store at 4 °C.
3. Streptavidin agarose resin (Thermal Scientific).
4. Protease inhibitor, EDTA free: dissolve 1 mini-tablet in 500 μl low salt buffer (Roche).

5. 4x stacking gel buffer: dissolve 30.3 g Tris-base in water, add 20 ml 10 % SDS, adjust pH to 6.8, bring up to 500 ml with water, filter and store at 4 °C.
6. PGB (Protein gel buffer) : Mix 12.5 ml 4x stacking gel buffer, 10 ml glycerol, 20 ml 10 % SDS, 0.5 ml 1 % bromophenol blue, and add water to 45 ml; aliquot 990 μl in each tube and store at −20 °C. Add 10 μl 2 M DTT before use.
7. Proteinase K: resuspend 100 mg (Roche) in 10 ml autoclaved milli-Q water, aliquot and store at −20 °C.
8. 2x PK buffer: Mix 10 ml 1 M Tris pH 8.0, 2.5 ml 0.5 M EDTA, 3 ml 5 M NaCl, 10 ml 10 % SDS, and add H_2O to 50 ml; filter and store at room temperature. Warm up in 37 °C water bath before use if precipitates are observed.

2.6 Sample Preparation for Mass Spectrometry

1. NuPAGE Novex 4–12 % Bis-Tris protein gels (Life Technologies).
2. The NuPAGE MOPS SDS buffer (Life Technologies).
3. NuPAGE antioxidant (Life Technologies).
4. SilverQuest staining kit (Life Technologies).
5. Trichloroacetic acid (TCA) (Sigma).
6. Acetone (HPLC grade).

3 Methods (*See* Note 4)

3.1 In Vitro Transcription

1. Assemble the following reaction at room temp:

DNA template (*see* **Note 5**)	1.0 ug
10x NTPs	2.5 μl
1 %Triton	2.5 μl
20 mM Cap analog	2.5 μl
200 mM DTT	1.5 μl
0.3 mM Biotin-16-UTP	0.8 μl
RNasin Inhibitor	0.5 μl
5xTranscription buffer	5.0 μl
UTP-[α-^{32}P]	1.0 μl
T7 RNA polymerase	1.5 μl

Add H_2O to a total volume of 25 μl.

2. Incubate at 37 °C in a water bath for 1 ~ 2 h.

3. Add 1 μl RQ1 RNase-free DNase, incubate for 10 min at 37 °C.
4. Add H_2O to a final of 200 μl, remove 2 μl for measuring radioactivity.

3.2 RNA Purification and Gel Separation

1. Add equal volume of phenol/chloroform, vortex and then spin at 13,500 *g* (12,000 rpm) for 5 min, transfer 160 μl supernatant to another Eppendorf tube.
2. Add 2 μl 20 mg/ml glycogen, 200 μl 5 M Ammonium Acetate and 950 μl 100 % ethanol, mix and centrifuge at 13,500 *g* (12,000 rpm) for 20 min.
3. Wash the pellet once with 70 % ethanol, resuspend in 20 μl H_2O, remove 1 μl for counting.
4. Count the samples aliquoted before and after purification using a liquid scintillation counter for 2 min, calculate the incorporation rate and the concentration of RNA (*see* **Note 6**).
5. Aliquot 1 μl of the RNA sample and dilute to 1 ng/μl, then mix 1–3 μl of the RNA dilution with formamide dye, incubate in 70–80 °C water for 10 min, vortex, spin down, and load the sample(s) on 6.5 % denaturing PAGE gel.
6. After gel separation, peel off the gel using filter paper and cover with saran membrane, dry the gel with a vacuum drier. Expose the gel to X-ray film or phosphoimager screen (Fig. 1) (*see* **Note 7**).

3.3 In Vitro RNP Assembly

1. In each of the four 2.0 ml Eppendorf tubes (*see* **Note 8**), add:

12.5 mM ATP	20 μl
0.5 M Creatine phosphate	20 μl
80 mM $MgCl_2$	20 μl
Dilution buffer	150 μl
Nuclear extract	150 μl
RNA	500 ng
H_2O	140 μl
Total	500 μl

2. Mix well and incubate at 30 °C for 30–60 min, put on ice and then load on the gel filtration column.

3.4 Fractionation of the RNP by Gel Filtration

1. Wash the gel filtration column thoroughly, soak Sephacryl S-500 HR resin in the column buffer (*see* **Note 9**).

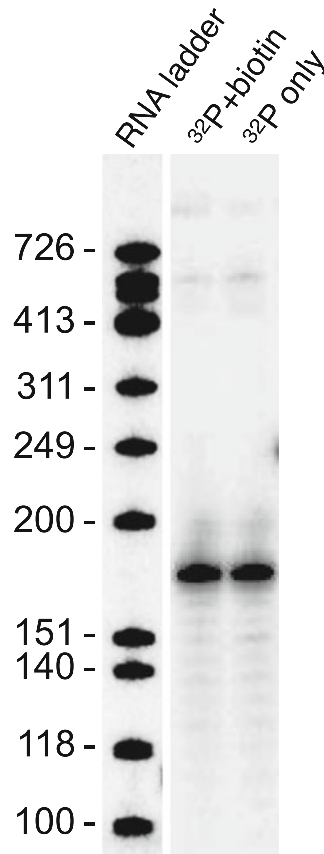

Fig. 1 In vitro T7 transcripts separated on 6.5 % denaturing PAGE. ^{32}P + biotin: transcripts were double labeled with ^{32}P and biotin; ^{32}P: transcripts were labeled with ^{32}P only

2. Attach stop-cock and tubing to the column outlet and put the column on a ring stand, inject milli-Q water into the column from the tubing using syringe to remove air bubbles.
3. In the cold room, attach the column to peristaltic pump, pour the resin into the column against a glass rod steadily.
4. Turn on the pump to start packing and keep adding the resin before it is fully packed (*see* **Note 10**).
5. When the column is packed to about 2.5 in. from the top, swirl the rod above the resin to create a completely flat surface for loading.
6. Mix 250 μl nuclear extract with 250 μl dilution buffer, block the column by running the mixture.
7. Load the assembled RNP complexes onto the column very carefully and collect fractions using capless tubes (*see* **Note 11**).

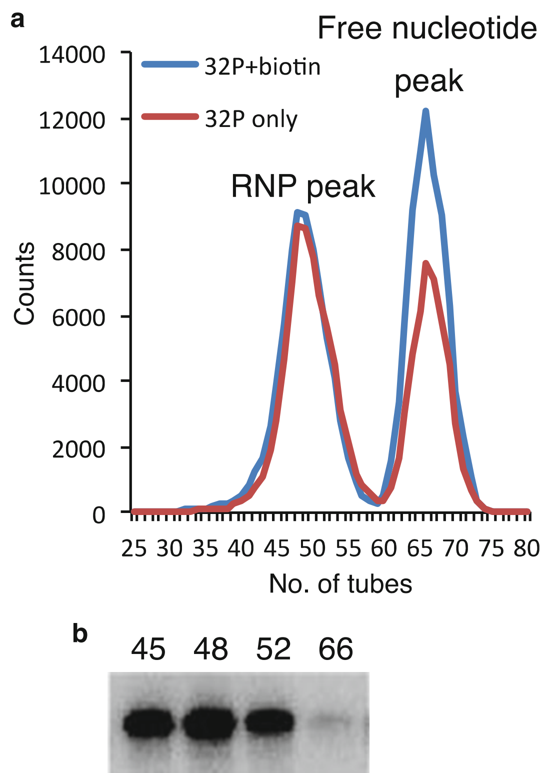

Fig. 2 Chromatography of the gel filtration fractions. (**a**) Chromatography showing the RNP peak and free nucleotide peak; (**b**) RNA purified from an aliquot of the designated fraction

8. Remove 50 μl from fractions 25 to 80 for counting and draw the chromatography (Fig. 2a) (*see* **Note 12**).
9. Remove an aliquot from randomly selected fractions of both peaks, add 5 μl proteinase K, 100 μl 2xPK buffer and H_2O to the final volume 200 μl, vortex and then incubate at 37 °C for 10 min.
10. Add 2 μl glycogen, 200 μl RNA phenol, vortex and then spin for 5 min at 13,500g (12,000 rpm) at room temperature.
11. Transfer 160 μl supernatant to another tube, add 600 μl 100 % ethanol, spin at room temperature for 20 min after vortex.
12. Wash once with 70 % ethanol, air dry briefly and add 10 μl of formamide dye, incubate in 70–80 °C water for 10 min, vortex and spin down, samples are ready for loading on RNA gel.
13. Run the gel and expose, RNA should be observed in samples taken from the RNP peak but not in samples from the free nucleotide peak (Fig. 2b).

3.5 Biotin–Streptavidin Affinity Purification

1. Pool the fractions from the RNP peak together and adjust the salt concentration to 150 mM with 3 M KCL, transfer to 15 ml tube (*see* **Note 13**).
2. Add 100–150 μl of streptavidin agarose resin and 1/100 V of protease inhibitor, Rotate at 4 °C overnight.
   The amount of streptavidin agarose resin added in the affinity purification should be optimized. To calculate the recovery efficiency, take equal aliquots from pooled fractions before streptavidin agarose resin is added and the supernatant after biotin–streptavidin binding, purify RNA and run RNA gel, quantify the bands.
   Recovery efficiency = 1-(Band intensity after/band intensity before)
   For example, the recovery efficiency before and after optimization is 43 % (1–57 % = 43 %) vs 84 % (1–16 % = 84 %), whereas there is almost no recovery without biotin (Fig. 3).
3. Wash with 10 ml 150 mM low salt buffer for four times, rotate at 4 °C for 10 min for each wash.
4. Transfer the agarose resin to 1.5 ml Eppendorf tube, Add 150 μl PGB and rotate at room temp for 10 min, incubate in hot water (70–80 °C) for 2 min.
5. Spin down the resin at 2000*g* (4500 rpm), carefully transfer the supernatant to another tube and spin again, transfer the supernatant to the same tube. The supernatant is ready for gradient gel after boiling or mass spectrometry after precipitation (*see* **Note 14**).

3.6 Sample Preparation for Mass Spectrometry

1. Aliquot about 10 % elute, boiling and run gradient gel, stain the gel with silver staining kit (Fig. 4) (*see* **Note 15**).
2. For mass spectrometry, dilute the elute to 1 ml and add 100 μl 100 % TCA, vortex for 15 s, place on ice for 15 min (*see* **Note 16**).

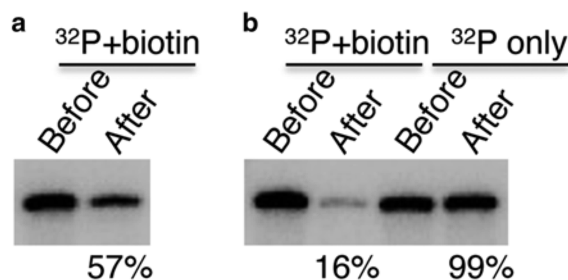

Fig. 3 Opitimization of the amount of streptavidin agarose resin enhances RNA recovery efficiency. (**a**) RNA recovery efficiency before optimization; (**b**) RNA recovery efficiency with optimized amount of agarose resin

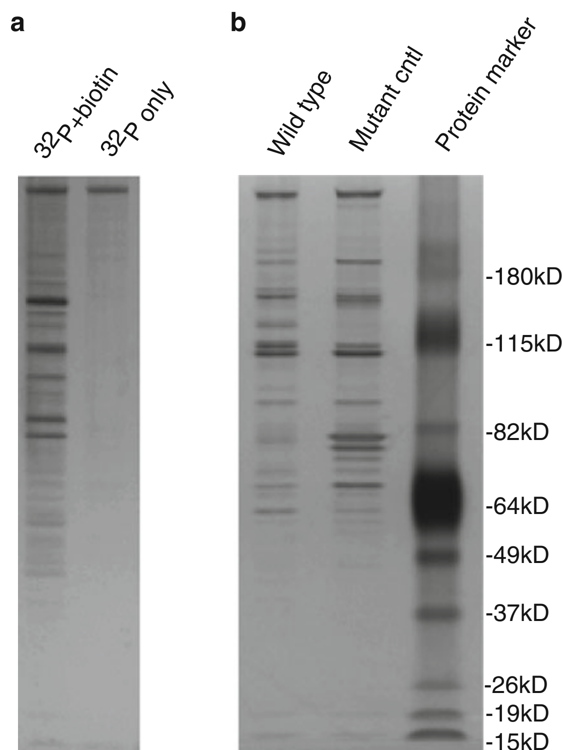

Fig. 4 Two examples of silver staining of purified RNPs after separation on gradient SDS-PAGE. (**a**) Silver staining showing RNP was purified only from biotin-labeled sample; (**b**) RNPs assembled on wild type transcripts and mutant control

3. Spin at full speed in a table-top microcentrifuge for 15 min, 4 °C, remove the supernatant.
4. Wash pellet with 100 μl ice-cold acetone twice, spin for 30 min each time.
5. Remove acetone and air dry the pellet, samples are ready for mass spectrometry (*see* **Note 17**).

3.7 Day-to-day Task

Day 1:

Pack the column

In vitro transcription

RNA purification

Run the RNA gel

Day 2:

In vitro RNP assembly

Gel filtration fractionation

Day 3–4:

Fraction counting/chromatography

RNA purification from selected fractions

Affinity purification

Day 4:

Run the gradient gel

Silver staining

TCA precipitation.

4 Notes

1. The supplier information is provided for convenience rather than a requirement.
2. All the reagents and chemicals should be of the highest grade. We routinely use autoclaved milli-Q water instead of DEPC-treated water. All the reagents should be stored at −20 °C.
3. Store nuclear extract at −80 °C and aliquot all the other reagents and store at −20 °C. Nuclear extract can be from bulk or small scale preparation. For small scale nuclear extract preparation, see the reference by Folco EG, et al. 2012 [8]. For RNPs present in the cytoplasm, replace nuclear extract with S100 [9].
4. Before the experiments, keep in mind that solution I from the miniprep kit is the major source for RNase contamination since large amount of RNase A is added to the solution. Other sources for RNase contamination include dust and hands. Therefore, DO NOT perform any RNA-related experiments on the same bench where miniprep is carried out and always keep the RNA bench CLEAN!
5. DNA template could be linearized plasmid or PCR products containing T7 promoter sequence. Transcription buffer should be added last to avoid precipitates. Follow instructions on working with isotope and have proper protections. For negative control, omit Biotin-16-UTP.
6. Calculation the incorporation rate and the concentration of RNA:

   Incorporation rate (%) = Counts after × 2 × 10/(counts before)

   For example, if the counts before purification is 12,500 and counts after purification is 60,000, then the incorporation rate is 96 %.

   Incorporation rate (%) = 60,000 × 2 × 10/12,500 = 96%

   Concentration (ng/μl) = Incorporation rate × 1000 × 6.25/20

(for two reactions, replace 6.25 by 12.5; for four reactions, replace 6.25 by 25)

For example, if the incorporation rate is 96 %, then the concentration of the RNA is 300 ng/μl.

Concentration (ng/μl) = 96 % × 1000 × 6.25/20 = 300 ng/μl

7. It is critical to run the T7 transcripts on RNA gel since this will provide the information about the length and the quality of the transcripts and whether there is any unspecific transcript. The RNA ladder should be end labeled using ATP-[γ-^{32}P].
8. You may use a 50 ml Falcon tube if you prefer to do single tube incubation. The RNP assembly conditions may need to be optimized.
9. A 0.75 m × 1.5 cm column is recommended, the bed volume is ~90 ml. The column should be clean and RNase free; soak and wash with 0.5 % sodium azide if necessary. You may need to use other Sephacryl resin depending on the size of the RNP assembled for best resolution.
10. Do not let the packed resin dry out during packing! It will ruin the column.
11. Protect yourselves properly during loading and shield off the fractionation area since it is radioactive! The fractionation speed should be adjusted so that fractions of 1 ml are collected in each tube.
12. There is no need to count fractions 1 to 24 since they do not contain any RNA. The chromatography should be a smooth curve, if jigsaw peaks are observed, the column may be ruined or the order of the fractions for counting is mixed up.
13. The concentration of KCl in fractions is 60 mM, which should be taken into account when adjusting the salt concentration. The salt concentration can be adjusted up to 250 mM if more stringent binding and wash condition is preferred.
14. Keep the samples warm in winter to avoid precipitates. Be very careful not to transfer any agarose resin after the second spin. It may be helpful not to transfer all the supernatant at this point.
15. It is critical and essential to perform the control that only labeled with ^{32}P but without bibotin (^{32}P only) since it will help to determine whether the affinity purification is successful or not. As showed in Fig. 4a, this lane should be blank or only faint bands could be observed.
16. Add TCA directly without dilution of the elute may lead to significant amount of cloudy precipitates which will ruin the experiment.
17. You should NOT be able to see the pellet or only tiny pellet can be seen. Large pellet means bad sample preparation and you may not get results from mass spectrometry.

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