Title

Purification of mRNA Processing Complexes Using an RNA Affinity Approach

Authors

Wang, Xiuye

Shi, Yongsheng

Publication Date

2017

DOI

10.1007/978-1-4939-7204-3_5

Chapter 5

Purification of mRNA Processing Complexes Using an RNA Affinity Approach

Xiuye Wang and Yongsheng Shi

Abstract

Multiple mRNA processing steps, including splicing and 3′ processing, take place in macromolecular complexes that contain many proteins and sometimes RNA molecules. A key challenge in the mRNA processing field has been to define the structure-function relationship of these sophisticated molecular machines. A prerequisite for addressing this challenge is to develop tools for purifying mRNA processing complexes in their native and intact forms that are suitable for functional and structural studies. Among many methods that have been developed, RNA affinity-based methods are most widely applied. In these methods, RNA molecules that are substrates to mRNA processing machineries are fused with an affinity tag, incubated with cellular extracts/lysates to allow for the assembly of mRNA processing complexes, and finally the assembled complexes are purified using RNA affinity tag. In this chapter, we will overview RNA affinity-based purification methods and describe in detail one such method, MS2-tagging, and its application in the purification of mRNA 3′ processing complexes. Although these methods were originally developed for purifying mRNA processing complexes, they should be applicable to purification of other RNA-protein complexes as well.

Key words mRNA processing complex, Affinity purification, MS2

1 Introduction

mRNA processing takes place in several large macromolecular complexes. The mammalian spliceosomes consist of five small nuclear RNAs (snRNAs) and over one hundred proteins [1]. Mammalian 3′ processing complex contains over 20 core components and ~65 associated proteins [2]. In addition, these large molecular machines are highly dynamic. For example, the assembly of mammalian spliceosomes goes through multiple stages, from E, A, B, to C complexes, in which splicing reactions take place [1, 3]. The complexity and dynamic nature of mRNA processing complexes present great challenges to the study of their structure-function relationships.

A number of methods have been developed for the purification of mRNA processing complexes and they can be classified into two

Yongsheng Shi (ed.), *mRNA Processing: Methods and Protocols*, Methods in Molecular Biology, vol. 1648,
DOI 10.1007/978-1-4939-7204-3_5,

general types. The first type is protein affinity-based methods. For example, immunoprecipitation of a number of epitope-tagged yeast splicing factors has recently been used to purify spliceosomes at different stages [4–6]. The advantages of this method include its simplicity and the ability to elute the tagged proteins under native conditions. In fact, spliceosomes purified using this method have been successfully studied by cryo-electron microscopy (cryo-EM) [7], providing near atomic resolution structures for the first time. The main disadvantage of this method, however, is the lack of specificity: all tagged proteins, including free proteins and those assembled into mRNA processing complexes, are purified in a complicated mixture. To achieve the purity required for some biochemical or structural studies, additional purifications steps are usually needed.

The second type of method for purifying mRNA processing complexes is based on RNA affinity. In this method, an RNA substrate for a specific mRNA processing machinery is fused with an affinity tag and then incubated with cell lysates/extract to allow for the assembly of the proper complex. The mRNP complexes are then purified using immobilized proteins or small molecules that specifically bind to the RNA affinity tag. Many RNA affinity tags have been introduced for this purpose, including biotin [8], artificially selected aptamers [9, 10], and several hairpin structures from bacteriophages [11]. Biotinylated RNA substrates have long been used to various mRNA processing complexes [12, 13]. This method takes advantage of the avid and specific interaction between biotin and streptavidin and allows purification under stringent conditions with a high level of recovery. However, biotin-based affinity purification also has its drawbacks: biotin labeling of RNA is not 100% efficient and, as a result, a significant portion of the RNA substrates is unlabeled and thus needs to be removed before purification. More importantly, it is very difficult to elute the purified mRNP complexes in its intact form for subsequent functional and/or structural studies. On the other hand, aptamer or natural hairpin-based RNA affinity tags offer both high specificity and the possibility to elute the mRNP complexes under native conditions. One of the most widely used RNA affinity purification methods is based on a stem-loop structure from the MS2 bacteriophage, which is specifically bound by the MS2 coat protein [2, 14]. In this method, several copies of the MS2 hairpin (<20 nucleotides) are fused to an RNA substrate and the RNA is incubated with a fusion protein between the MS2 coat protein and the maltose binding protein (MBP). The resultant RNP is then incubated with cell lysates/extract under proper conditions to assemble the mRNA processing complex. Subsequently, the assembled mRNP complexes are retrieved by using amylose beads, which specifically bind to the MBP moiety of the MBP-MS2 fusion protein. Finally, the bead-bound complexes can be eluted under native conditions and

used for functional and/or structural studies. When used in combination with other purification steps, such as glycerol gradient centrifugation or gel filtration, this MS2-based RNA affinity method has enabled the purification of various spliceosomes (E, A, B, C and post-splicing mRNPs) [14–16] and mRNA 3′ processing complexes [2]. Therefore, RNA affinity purification has greatly facilitated the identification of novel mRNA processing factors and the study of the structure-function relationships of mRNA processing complexes. Next, we will provide a detailed protocol for applying this method for the purification of mRNA 3′ processing complex. It is important to point out that the protein- and RNA-affinity-based purification methods are not mutually exclusive and can be used in combination to obtain highly purified mRNP complexes.

2 Materials

2.1 RNA Substrate Preparation by In Vitro Transcription

1. DNA templates for 3MS2-tagged RNA substrates
   A plasmid that contains the coding sequence for your RNA substrate driven by a T7 or SP6 promoter. Three copies of the MS2 hairpin, the sequence of which is shown below, can be inserted either upstream or downstream of the RNA substrate sequence:
   5′-CGTACACCATCAGGGTACGAGCAAGCCCATTG CGTACACCATCAGGGTACGACTAGTACATTCGT ACACCATCAGGGTACG-3′ (MS2 sequences are underlined)
   SV40 late poly(A) site (SVL) is a commonly used substrate for in vitro 3′ processing assays [2]. A mutant version of SVL with one nucleotide mutation in the poly(A) signal (AAUAAA to AACAAA) is deficient in recruiting mRNA 3′ processing factors and thus is used as a negative control [2]. The 3× MS2 affinity tag was inserted upstream of SVL or SVLm (Fig. 1). For additional considerations for designing an RNA substrate, *see* **Note 1**.
2. T7 or SP6 RNA polymerase.
3. Nucleotide triphosphate mix (NTPs) for making non-radiolabeled RNAs: 5 mM/each.

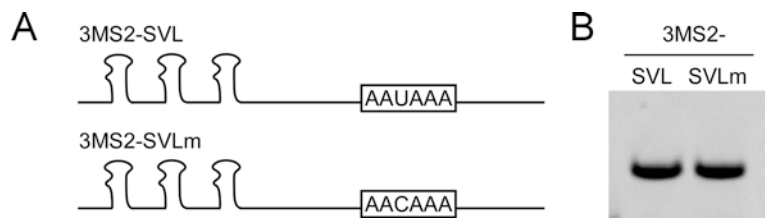

Fig. 1 (**a**) Schematic of poly(A) site and AAUAAA mutant tagged by 3× MS2 stem loops. (**b**) RNA detection of 3MS2-poly A site by 8% Urea-PAGE

4. NTPs for [α-^{32}P] UTP labeling: 1 mM UTP and 5 mM/each for ATP, CTP, and GTP.
5. [α-^{32}P] UTP, 10 μCi/μl.
6. 5× transcription buffer: 200 mM Tris (pH 7.9), 30 mM $MgCl_2$, 10 mM spermidine, and 50 mM NaCl.
7. 40 U/μl RNaseOUT or RNase inhibitor.
8. RNase-free DNase I.
9. Phenol/chloroform with isoamyl alcohol (PCI) (pH 4.3).
10. 100% ethanol and 75% ethanol.
11. GlycoBlue or glycogen (for RNA precipitation).
12. 10 M ammonium acetate.
13. 2× RNA loading buffer: 80% (v/v) formamide, 1× TBE, 0.1% (w/v) bromophenol blue, and 0.1% (w/v) xylene cyanol FF.
14. RNA elution buffer: 0.5 M ammonium acetate, 10 mM $MgCl_2$, 1 mM EDTA, and 0.2% SDS.
15. A spectrophotometer.
16. A liquid scintillation counter.
17. A phosphorimager.
18. Mineralight lamp (multiband UV-254/365 nm).

2.2 RNA Affinity Purification

1. pMBP-MS2 plasmid (Addgene: 65,104).
2. BL21 (DE3) *E. coli* strain.
3. Luria-Bertani (LB) liquid medium: 10 g NaCl, 10 g Tryptone, 5 g yeast extract and 950 ml H_2O, and autoclave it.
4. Lysis buffer for MS2-MBP purification: 20 mM Tris–HCl (pH 7.5), 200 mM NaCl, and 0.5 mM PMSF.
5. 1 M creatine phosphate (CP).
6. 10 μg/μl tRNA.
7. 40 U/μl RNaseOUT or RNase inhibitor.
8. HeLa nuclear extract (NE) [17].
9. 50 μg/μl heparin.
10. 10× Tris-Glycine: 500 mM Tris-base and 500 mM Glycine.
11. 4% PAGE: 1 ml of 40% Polyacrylamide gel solution (polyacrylamide: bisacrylamide = 80:1), 1 ml of 10× Tris-Glycin, 100 μl of 10% ammonium persulfate (APS), 10 μl TEMED and 7.9 ml H_2O.
12. TBE buffer: 90 mM Tris–HCl (pH 8.0), 90 mM borate, and 2 mM EDTA.
13. 5× Native gel loading buffer: 25% (v/v) Glycerol in TBE buffer with 0.25% (w/v) bromophenol blue.

14. 10% glycerol gradient solutions: 20 mM HEPES (pH 7.9), 60 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, and fresh 0.5 mM DTT.
15. 30% glycerol gradient solutions: 20 mM HEPES (pH 7.9), 60 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 30% glycerol, and fresh 0.5 mM DTT.
16. RNA pulldown buffer: 20 mM Hepes-KOH, pH 7.9, 100 mM KCl, 1 mM $MgCl_2$, 1% Triton X-100, and fresh 0.5 mM DTT.
17. Maltose.
18. Amylose resin.
19. Prechilled acetone.

3 Methods

3.1 Prepare RNA Substrates by In Vitro Transcription

1. Linearize the template DNA plasmid by digesting 1 μg of the plasmid with a restriction enzyme that cuts immediately downstream of the RNA substrate sequence.
2. Purify the digested DNA and dissolve in 10 μl H_2O.
3. Set up in vitro transcription reaction as follows:

   10 μl of DNA template (1 μg).

   2.5 μl of 100 mM DTT.

   2.5 μl of NTP mix (5 mM of each NTP).

   0.5 μl of 5 U/μl RNaseOUT.

   5 μl of 5× Transcription buffer.

   1 μl of T7/SP6 polymerase.

   3.5 μl of H2O.

   25 μl (total volume).

   The above reaction is for making unlabeled RNAs. For making radiolabeled RNA: set up in vitro transcription as described above with the following modifications: add 1–3 μl [α-^{32}P] UTP and use an NTP mix that contains 1 mM of UTP and 5 mM/each of ATP, GTP, and CTP (*see* **Notes 2** and **3**).
4. Incubate at 37 °C for 2 h.
5. Add 1 μl of RNase-free DNase I and incubate at 37 °C for another 15 min.
6. Add 175 μl of H_2O and 200 μl PCI and vortex for 10 s.
7. Spin down at 16,000 × *g* for 15 min at 4 °C.
8. Retrieve 180 μl supernatant, and add 50 μl 10 M ammonium acetate, 750 μl of 100% ethanol, 1 μl of glycogen, and mix well.
9. Put on dry ice for at least 30 min.
10. Spin at 16,000 × *g* for 20 min at 4 °C. A pellet should be clearly visible at the bottom of the tube.

11. Remove the supernatant and wash the pellet with cold 75% ethanol.
12. Spin at 12,000 × *g* for 10 min at 4 °C.
13. Air dry the pellet for 10 min at room temperature.
14. Resuspend RNA pellet in 50 μl H_2O.
15. Measure RNA concentration using a NanoDrop spectrophotometer. For the quantification of radiolabeled RNA, do not use a spectrophotometer to measure concentration! Instead, take out 1% of the in vitro transcription reaction (R_{before}) and 1% of the final RNA (R_{after}). Measure the radiation levels of R_{before} and R_{after} with a liquid scintillation counter and calculate the molar amount of the radiolabeled RNA product using the following formula:

    RNA amount = (2.5 μl × 1 mM × R_{after}/R_{before})/(the number of Us in the RNA)
16. Resolve 200 ng RNA on 8% Urea-polyacrylamide gel in 1× RNA loading buffer.
17. Stain the gel with SYBR green for 30 min in the dark to check the quality of RNA.
18. (***Optional step***) If a significant amount of degraded or truncated RNAs are detected, gel purify the RNA to obtain the full-length fragment. To this end, resolve the RNA products from the in vitro transcription reaction on an 8% urea acrylamide gel, visualize the RNA band(s) with a phosphorimager (for radiolabeled RNA) or long wave light of ultraviolet light (for unlabeled RNA), and cut out the band containing the full-length RNA. Put the gel piece in a 1.5 ml tube with 400 μl RNA elution buffer. Rotate the tube at 37 °C for 1 h. and PCI extract twice. Precipitate the RNA as described above.

3.2 Preparation of MBP-MS2 Fusion Protein

1. Transform pMBP-MS2 into BL21 (DE3) strain.
2. Pick a single colony and inoculate 2 ml LB plus 100 μg/ml ampicillin.
3. Incubate the starter culture in a shaker (200 rpm) at 37 °C overnight.
4. Transfer 500 μl of the starter culture into 500 ml LB plus 100 μg/ml ampicillin.
5. Incubate in a shaker (200 rpm) at 37 °C until $O.D_{600}$ reaches ~0.5.
6. Add IPTG to a final concentration of 1 mM.
7. Further incubate in a shaker at 37 °C for 3 h.
8. Harvest cells by centrifugation at 5000 × *g* for 10 min.
9. Carefully decant the media and resuspend the cell pellet in 40 ml prechilled PBS buffer, and transfer to a 50 ml falcon tube.

10. Spin at 5000 × *g* for 10 min.
11. Carefully decant the supernatant and resuspend the cell pellet in 10 ml lysis buffer.
12. Sonicate the cells in 5 ml aliquots (output 3, 10 s for four times) on ice.
13. Spin at 9400 × *g* for 20 min.
14. (***Optional step***) As MBP-MS2 protein tends to bind to nucleic acids, which may interfere with the purification and subsequent analyses, it is recommended to remove the nucleic acids by adding $CaCl_2$ to 10 mM and 5 μl of micrococcal nuclease to the cleared lysate.
15. Wash 500 μl amylose beads with lysis buffer and then mix the beads with the cell lysate.
16. Rotate for 1 h at 4 °C.
17. Load the mix onto a column and let the cell lysate pass through the column by gravity flow.
18. Wash the beads with 5 ml lysis buffer for three times.
19. Wash the column with 10 ml of PBS.
20. Elute the MBP-MS2 protein by adding 500 μl elution buffer (PBS + 15 mM maltose) to the beads. Incubate for 2 min, spin at 1000 × *g* for 5 min, and collect the supernatant. Repeat this step until no protein can be detected in the eluate by using Bradford assay. Usually, all proteins are eluted in the first 3 elutions.
21. Dialysis against 2 l of Buffer D twice for at least 2 h each time at 4 °C.
22. Measure protein concentration using Bradford assay and store purified protein in small aliquots at −80 °C.

3.3 Test the Interaction Between MBP-MS2 and RNA Substrate In Vitro

It is necessary to check the binding ability and optimize the ratio of MS2-MBP protein to RNA. Excess RNA or protein maybe reduces pulldown efficiency to some extent.

1. Mix 2 pmol RNA substrate with 10 ~ 100 pmol MS2-MBP (use several different amounts) in 10 μl RNA pulldown buffer, respectively.
2. Incubate on ice for 20 min.
3. Add 2.5 μl 5× native gel loading buffer and mix well.
4. Resolve 10 μl on a 1.5% agarose gel and identify the lowest amount of protein that can shift all of the RNAs. (For radioactivity labeled RNA, 4% PAGE (80:1) is a better choice and check signal by phosphorimaging. Run the gel in 1× tris-glycine buffer in a cold room at 100 v for 210 min, and then expose to phosphorimager.)

   For 3MS2-SVL and 3MS2-SVLm, the molar ratio of 1:20 between RNA and MS2-MBP is optimal.

3.4 RNA Affinity Purification of mRNA 3′ Processing Complex

1. Set up the cleavage/polyadenylation reaction as follows: 5 pmol of 3MS2-SVL or 3MS2-SVLm RNA, 100 pmol of MS2-MBP, and add RNA pulldown buffer to bring the total volume up to 50 μl.
2. Incubate for 30 min on ice.
3. Add 5 μl of 0.1 M ATP, 10 μl of 1 M CP, 5 μl of 10 μg/μl tRNA, 200 μl NE, 230 μl H_2O and mix well (*see* **Note 4**).
4. Incubate for 20 min at 30 °C.
5. Put sample on ice and add 4 μl of 50 μg/μl heparin to mix well (*see* **Note 5**).
6. Add 50 μl prewashed amylose beads to the reaction and rotate for 1 h at 4 °C (*see* **Note 6**).
7. Spin the mixture at 2000 × *g* for 2 min and remove the supernatant.
8. Add 1 ml RNA pulldown buffer and rotate for 15 min.
9. Spin the mixture at 2000 × *g* for 5 min and remove the supernatant.
10. Repeat the wash three times.
11. Elute complex by adding 100 μl elution buffer (RNA pulldown buffer plus 12 mM maltose) to the beads and rotate for 20 min at 4 °C.
12. Spin down for 2 min at 2000 × *g* and transfer the supernatant to a new 1.5 ml EP tube.
13. Repeat **step 11** and combine eluates.
14. Add 800 μl cold acetone and keep it at −20 °C for 20 min (up to overnight).
15. Spin down proteins at 16,000 × *g* for 20 min at 4 °C.
16. Air dry pellet for 10 min at room temperature.
17. The protein pellet can be analyzed by mass spectrometry to identify mRNA processing factors, or by SDS-PAGE and silver staining (Fig. 2) or Western blotting analyses.

3.5 Glycerol Gradient Sedimentation (Optional Step)

When a substrate RNA is incubated with cell extracts, multiple distinct complexes may form, including heterogeneous complexes that consist of mostly hnRNPs, and mRNA processing complexes of different stages (ref for both spliceosome and 3′ processing complexes). Although the one-step affinity purification method is usually sufficient for identification of mRNA processing factors (provided that proper negative control samples are used), additional purification steps may be necessary to obtain a homogenous population of mRNA processing complexes for structural and/or functional studies. Gradient sedimentation resolves particles according to their sizes and is suitable for further

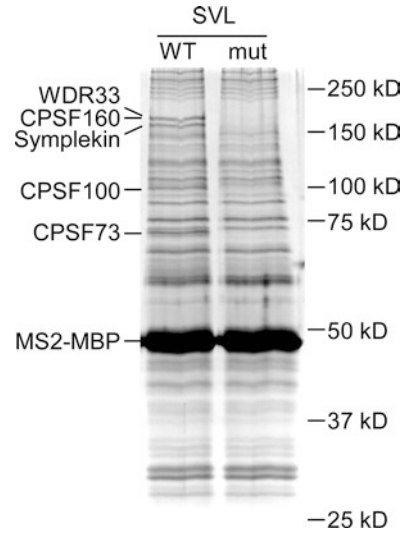

Fig. 2 Poly(A) factors pull downed by 3MS2-SVL poly(A) site RNA. Silver staining was performed after RNA pulldown. Only wild-type poly(A) site can pull down poly(A) factors, which are indicated by their respective names

purification of mRNA processing complexes. Detailed protocols are described below:

1. Make a 10–30% glycerol gradient (12 ml) using a gradient maker in SW41 centrifuge tubes, and leave it at 4 °C for 1 h or more.
2. Carefully load the in vitro mRNA processing reaction on the top of the gradient.
3. Load the gradient onto an ultracentrifuge and spin at 80,000 × *g* for 15 h.
4. Manually collect 0.5 ml fractions from the top.
5. Measure radioactivity of each fraction.
6. Pick specific peak fractions that contain your desired mRNA processing complexes (combine fractions if multiple fractions are found in a peak) and mix with 50 μl amylose beads that have been prewashed with wash buffer.
7. Rotate for 30 min (up to 2 h) at 4 °C.
8. Follow **step 7** of Subheading 3.4.

4 Notes

1. Several factors need to be considered for designing RNA substrates. First, the RNA substrate sequence has to be tested beforehand to ensure that it allows the assembly of the desired processing complex in vitro. Second, the 3MS2-tagged RNA substrates should be carefully compared with the untagged RNA to ensure that the tag does not interfere with the processing reaction.

Third, as many cellular proteins bind promiscuously to RNAs, it is essential to include negative control RNA substrates for affinity purification. The ideal negative controls are mutant versions of the RNA substrates, which contain a small number of substitutions that abolish the assembly of the processing complex.

2. For glycerol gradient sedimentation, it is necessary to use [α-^{32}P] UTP-labeled RNA to determine in which fractions the 3′ processing complex are by measuring the intensity of radioactivity. When the formal assay is conducted non-radioactivity labeled RNA are used, and it is easy to pick certain fractions the target complex is in.
3. Lots of RNA are required for this assay and T7 RNA polymerase can meet this demand. For poly(A) RNA substrate used in this chapter, about 7 μg RNA can be obtained from a 25 μl transcription reaction. Scale up if more RNA is required for the further assay.
4. Pulldown efficiency depends on interaction ability between RNA and relevant complexes. The amount of proteins from 500 μl RNA pulldown reaction in this chapter can be used for ten western blot analyses. Reaction volume can be scaled up based on further assay.
5. Addition of heparin is critical for reducing background. However, excessive heparin also decreases pulldown efficiency. The final concentration depends on the motif strength in RNA. For example, more heparin can be used for stronger poly(A) site. It is necessary to optimize heparin concentration for each poly(A) site.
6. Poly-prep chromatography columns are recommended if the reaction is more than 2 ml. It is very convenient to use column after incubation at 37 °C for incubation with amylose resin, washing, and elution.

Acknowledgment

This study was supported by grants from the National Institutes of Health (GM090056 and CA177651) and the American Cancer Society (RSG-12-186).

References

1. Will CL, Luhrmann R (2011) Spliceosome structure and function. Cold Spring Harb Perspect Biol 3(7):a003707
2. Shi Y, Di Giammartino DC, Taylor D, Sarkeshik A, Rice WJ, Yates JR 3rd, Frank J, Manley JL (2009) Molecular architecture of the human pre-mRNA 3′ processing complex. Mol Cell 33(3):365–376
3. Matera AG, Wang Z (2014) A day in the life of the spliceosome. Nat Rev Mol Cell Biol 15(2):108–121
4. Grote M, Wolf E, Will CL, Lemm I, Agafonov DE, Schomburg A, Fischle W, Urlaub H, Luhrmann R (2010) Molecular architecture of the human Prp19/CDC5L complex. Mol Cell Biol 30(9):2105–2119

5. Khanna M, Van Bakel H, Tang X, Calarco JA, Babak T, Guo G, Emili A, Greenblatt JF, Hughes TR, Krogan NJ, Blencowe BJ (2009) A systematic characterization of Cwc21, the yeast ortholog of the human spliceosomal protein SRm300. RNA 15(12):2174–2185
6. Tardiff DF, Rosbash M (2006) Arrested yeast splicing complexes indicate stepwise snRNP recruitment during in vivo spliceosome assembly. RNA 12(6):968–979
7. Yan C, Hang J, Wan R, Huang M, Wong CC, Shi Y (2015) Structure of a yeast spliceosome at 3.6-angstrom resolution. Science 349(6253): 1182–1191
8. Leppek K, Stoecklin G (2014) An optimized streptavidin-binding RNA aptamer for purification of ribonucleoprotein complexes identifies novel ARE-binding proteins. Nucleic Acids Res 42(2):e13
9. Iioka H, Loiselle D, Haystead TA, Macara IG (2011) Efficient detection of RNA-protein interactions using tethered RNAs. Nucleic Acids Res 39(8):e53
10. Hartmuth K, Urlaub H, Vornlocher HP, Will CL, Gentzel M, Wilm M, Luhrmann R (2002) Protein composition of human prespliceosomes isolated by a tobramycin affinity-selection method. Proc Natl Acad Sci U S A 99(26):16719–16724
11. Keryer-Bibens C, Barreau C, Osborne HB (2008) Tethering of proteins to RNAs by bacteriophage proteins. Biol Cell 100(2):125–138
12. Rodgers JT, Patel P, Hennes JL, Bolognia SL, Mascotti DP (2000) Use of biotin-labeled nucleic acids for protein purification and agarose-based chemiluminescent electromobility shift assays. Anal Biochem 277(2):254–259
13. Hou S, Shi L, Lei H (2016) Biotin-streptavidin affinity purification of RNA-protein complexes assembled in vitro. Methods Mol Biol 1421: 23–34
14. Zhou Z, Sim J, Griffith J, Reed R (2002) Purification and electron microscopic visualization of functional human spliceosomes. Proc Natl Acad Sci U S A 99(19):12203–12207
15. Bessonov S, Anokhina M, Will CL, Urlaub H, Luhrmann R (2008) Isolation of an active step I spliceosome and composition of its RNP core. Nature 452(7189):846–850
16. Yoshimoto R, Kataoka N, Okawa K, Ohno M (2009) Isolation and characterization of post-splicing lariat-intron complexes. Nucleic Acids Res 37(3):891–902
17. Dignam JD, Lebovitz RM, Roeder RG (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 11(5):1475–1489