Purification of Functional RNA-Protein Complexes Using MS2-MBP

This protocol describes the use of MS2-MBP to purify functional RNA-protein complexes, a method also known as maltose-binding protein (MBP)–affinity purification. This approach combines gel filtration and an affinity-chromatography strategy using the bacteriophage MS2 coat protein, which binds to a specific RNA-hairpin structure. The experimental design is illustrated in Figure 27.3.1. This method has been used to isolate human spliceosomes (complexes composed of pre-mRNA, small nuclear RNAs, and proteins which are responsible for the removal of introns from pre-mRNA) assembled on a well-characterized model pre-mRNA. However, the method can be adapted to isolating other RNA-protein complexes assembled in vitro. For the isolation, three MS2 coat protein–binding sites (hairpins) are inserted into the RNA of interest by constructing the appropriate clone. For isolation of the spliceosome, these hairpins are inserted at the 3′ end of exon 2 (Fig. 27.3.1A). A fusion protein containing the MS2 coat protein and maltose-binding protein (MS2-MBP) is bound to the hairpins (Fig. 27.3.1B). This RNA/MS2-MBP complex is then assembled into spliceosomal complexes by incubation in HeLa nuclear extracts under splicing conditions (Fig. 27.3.1C). Spliceosomes are partially purified by Sephacryl S-500 gel filtration chromatography (Fig. 27.3.1D) and then affinity selected by binding to amylose resin (Fig. 27.3.1E). After washing, spliceosomes are eluted under gentle conditions using maltose elution buffer (Fig. 27.3.1F).

Materials

- E. coli strain (e.g., DH5α) containing plasmid expressing MS2-MBP fusion protein
- Plasmid DNA encoding desired RNA and MS2 binding sites
- Buffer A: 10 mM sodium phosphate buffer, pH 7.0 (APPENDIX 2), filtered and degassed
- Buffer B: 10 mM sodium phosphate buffer, pH 7.0 (APPENDIX 2)/1 M NaCl, filtered and degassed
- Dialysis buffer: 10% (v/v) glycerol in PBS (see APPENDIX 2 for PBS)
- [3P]UTP (sp. act., 800 Ci/mmol; UNIT 3.4)
- Splicing dilution buffer (see recipe)
- 1.5% agarose gel (UNIT 2.5A)
- 12.5 mM ATP
- 80 mM MgCl2
- 0.5 M creatine phosphate
- 10 mg/ml HeLa cell nuclear extract (UNIT 12.1)
- Gel filtration buffer (see recipe)
- Amylose resin (New England Biolabs)
- Phosphate-buffered saline (PBS; APPENDIX 2)
- Maltose elution buffer (see recipe)
- HiTrap Heparin HP column (Amersham Biosciences) and FPLC system (UNIT 10.10) with gradient maker
- Dialysis cassettes (MWCO 10,000; Pierce)
- Centrioc-50 centrifugal concentrator (Amicon)
- 50-ml polypropylene tubes (e.g., Falcon)
- Packed 1.5/50 cm Sephacryl S-500 HR column and a gel-filtration system (UNIT 10.9)
Figure 27.3.1 Experimental design of RNA-protein complex purification using MS2-MBP. (A) Schematic of pre-mRNA with three MS2 binding sites (hairpins) at the 3'-end of exon 2. (B) Pre-mRNA bound by MS2-MBP protein. (C) Spliceosomes are formed on pre-mRNA substrates. (D) Gel-filtration profile of the spliceosome purification. V indicates position of void volume; S indicates the position of spliceosomes; H indicates the position of heterogeneous RNA-protein complexes (H complex). Radiolabeled $^{32}$P (cpm) in each fraction are shown in the y axis and fractions numbers are shown in the x axis. (E) Binding of spliceosomes to amylose beads via MS2-MBP. (F) Eluted spliceosomes.
Scintillation counter capable of Cerenkov counting
Data graphing software: e.g., Microsoft Excel or Cricket Graph from Computer
Associates International
End-over-end rotator

Additional reagents and equipment for purification of MS2-MBP protein from E.
coli (UNIT 16.6), dialysis (APPENDIX 3C), preparation of radiolabeled RNA (UNIT 14.3,
Support Protocol 1), mobility-shift RNA-binding assay (UNIT 12.2), agarose gel
electrophoresis (UNIT 2.5A), and gel-filtration chromatography (UNIT 10.9)

NOTE: All of the reagents should be RNase free (UNIT 4.1) and all procedures involving
RNA-protein complex isolation should be carried out at 4°C or on ice.

Prepare MS2-MBP fusion protein and RNA substrate
1. Express and purify MS2-MBP protein as described in UNIT 16.6.

   The MS2-MBP protein purified by this step binds E. coli nucleic acid and will not bind
substrate RNA well. Thus, further purification of MS2-MBP via an FPLC heparin column
(see step 2) is recommended.

2. Apply the MS2-MBP protein to a HiTrap Heparin column on an FPLC system per
the manufacturer’s recommendations.

3. Elute the MS2-MBP by a linear gradient of 0 M NaCl (buffer A) to 1 M NaCl (buffer
B) in 10 mM sodium phosphate buffer, pH 7.0. Reload the flowthrough onto the
column two to three times to increase the binding efficiency and final yield of
MS2-MBP.

   The nucleic acid will be eluted at about 100 mM NaCl and the MS2-MBP will be eluted at
around 300 mM NaCl.

4. Pool the peak fractions containing MS2-MBP in a dialysis cassette (MWCO 10,000)
and dialyze against dialysis buffer for 2 hr at 4°C (also see APPENDIX 3C).

5. Concentrate the protein, if necessary, using a Centricon-50 centrifugal concentrator.

   The typical final concentration of MS2-MBP is 5 mg/ml. The protein can be stored at
−80°C for future use.

6. Transcribe RNA substrates from the plasmid DNA bearing desired sequences and
MS2 binding sites using a T7, T3, or Sp6 RNA polymerase transcription system as
described in UNIT 14.3, Support Protocol 1, except label the RNA substrate with 32P

   The typical concentration of the RNA products is 0.2 mg/ml. The products can be stored at
−80°C.

Carry out an MS2-MBP/RNA binding assay
7. Perform a binding assay similar to that described in UNIT 12.2 to check the binding
efficiency of purified MS2-MBP to the substrate RNA and to optimize the molar ratio
of MS2-MBP to the RNA substrate. For spliceosome purification, mix a 100-fold
molar excess of MS2-MBP protein (from step 4 or 5) with pre-mRNA (i.e., the labeled
RNA from step 6), or, for other complexes, prepare a series of dilutions of the
MS2-MBP or the RNA, in splicing dilution buffer and incubate 30 min on ice. Assay
for binding by monitoring samples on a 1.5% agarose gel.

   The MS2-MBP/RNA complex will migrate more slowly than the unbound RNAs. Thus, it is
recommended that the binding reaction be performed in a series of dilutions of MS2-MBP
or RNA to find out the best ratio of MS2-MBP to RNA so that all of the RNA is bound by
MS2-MBP.
**Assembly of the RNA-protein complexes**

The following steps are carried out to form spliceosomes in a 2.4-ml splicing reaction. Other RNA-protein complexes should be prepared on a large scale using appropriate cell extracts and conditions.

8. Combine 10 µl of 0.2 mg/ml radiolabeled RNA substrate (step 6) and 20 µl of 5 mg/ml MS2-MBP (step 4 or 5) in a 1.5-ml microcentrifuge tube and incubate for 30 min on ice.

   The mol. wt. of the RNA used for spliceosome purification is ~132 kD; the mol. wt. for MS2-MBP is ~60 kD.

9. Add 172 µl of splicing dilution buffer to the tube, mix, and keep on ice for another 20 min.

10. Transfer the above reaction to a 50-ml polypropylene test tube and add the following reagents:

    - 720 µl splicing dilution buffer
    - 480 µl H₂O
    - 96 µl 12.5 mM ATP
    - 96 µl 80 mM MgCl₂
    - 96 µl 0.5 M creatine phosphate
    - 720 µl 10 mg/ml HeLa cell nuclear extract

   Gently mix the reactions and incubate at 30°C for the appropriate time.

   The time is dependent upon the complex to be assembled. A pilot experiment is recommended to determine the optimal incubation time. For spliceosomal C complex formation, 40 min of incubation is necessary.

**Purify the RNA-protein complexes**

11. Load the mixture onto a 1.5/50 cm Sephacryl S-500 gel filtration column that has been equilibrated in gel-filtration buffer (*UNIT 10.9*).

   This column is appropriate for the spliceosome; for other complexes it may be necessary to test a few columns to find one that gives optimal separation of the RNA-protein complex.

12. Separate the protein complex by gel filtration (*UNIT 10.9, Basic Protocol 2*) at a flow rate of 1.0 ml/min, collecting the first 80 1.0-ml fractions.

   The gel-filtration step takes about 14 hr. Thus, it is recommended that the complexes be assembled in the afternoon and the gel filtration be run overnight.

13. While the column fractions are still in the cold room, remove 25 µl from each fraction starting at no. 25 and continuing through no. 80. Determine the cpm in each fraction by Cerenkov counting. Draw a graph of the spliceosome separation using, e.g., Excel or Cricket with the fraction number as the x axis and cpm as the y axis and determine the peak of the spliceosome as shown in Figure 27.3.1D.

   The distribution of RNAs and proteins after gel filtration can be analyzed by denaturing polyacrylamide gel to check RNAs and SDS-PAGE to check proteins (*UNIT 10.2A*).

14. Pool fractions from the desired peak(s) containing the RNA-protein complexes.

15. Wash amylose resin in PBS by mixing 1 vol beads with 10 vol of PBS. Prepare a 50% (v/v) suspension of the amylose resin in PBS. Add 30 to 60 µl of the suspension per ml of pooled fractions (the actual bead volume will be ~15 to 30 µl/ml fractions). Incubate 4 hr to overnight at 4°C with rotation on an end-over-end rotator.
16. Centrifuge 3 min at 2000 \( \times g \), 4\(^\circ\)C, in a tabletop centrifuge, then carefully remove the supernatant.

17. Wash the amylose resin (now containing the spliceosome complex) by adding 10 ml of gel-filtration buffer and rotating the tubes 10 to 20 times. Spin down the resin as described in step 16 and discard the supernatant. Repeat this washing step 3 to 5 times. Finally, transfer the washed amylose resin to a new 1.5-ml microcentrifuge tube.

18. Carefully remove all liquid from the amylose resin. Add 300 \( \mu l \) maltose elution buffer and rotate 30 to 60 min at 4\(^\circ\)C.

*If it is necessary to keep the complex highly concentrated, a smaller volume of maltose elution buffer can be used, but the elution efficiency will be lower.*

19. Centrifuge the resin as in step 16. Retain the supernatant, which contains the purified spliceosomes.

20. Repeat the elution (steps 17 to 19) as desired to recover more complexes.

*Typically, 75% of the complexes will be eluted in the first elution followed by 15% in the second elution.*

**REAGENTS AND SOLUTIONS**

*Use Milli-Q-purified water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2; for suppliers, see APPENDIX 4.*

**Gel filtration buffer**
- 20 mM HEPES, pH 7.9
- 60 mM NaCl
- 0.1% (v/v) Triton X-100
- 0.01% (w/v) Na\(_3\)N
- Store up to 2 months at 4\(^\circ\)C

**Maltose elution buffer**
- 20 mM HEPES, pH 7.9
- 60 mM NaCl
- 10 mM 2-mercaptoethanol
- 12 mM maltose
- 1 mM PMSF (optional)
- Store up to 1 week at 4\(^\circ\)C

**Splicing dilution buffer**
- 20 mM HEPES, pH 7.9
- 100 mM KCl
- Store up to 1 year at −20\(^\circ\)C

**COMMENTARY**

**Background Information**

Biological machines composed of proteins and RNAs play essential biological roles. For example, the spliceosome, which is composed of pre-mRNA, five small nuclear RNAs (snRNAs), and \( \sim \)145 proteins, is responsible for the removal of introns from pre-mRNA, an essential step in eukaryotic gene expression. Over the past 20 years, a great deal of progress has been made in identifying spliceosomal components and critical RNA-RNA, RNA-protein, and protein-protein interactions. However, much less is known about the precise timing of these interactions during spliceosome assembly. In addition, little is understood about the factors that mediate the dynamics. Progress in understanding the mechanisms of pre-mRNA spicing has been hampered, in part,
because it has not been possible to isolate spliceosomes that are both highly purified and functional. Thus, it is difficult to establish the functional significance of many of the factors present in the spliceosomes.

The previously used biotin/avidin spliceosome purification method (Reed, 1990; Bennett et al., 1992) is inefficient, and requires high salt (250 mM), which dissociates some of the spliceosomal components. In addition, the bound complexes cannot be released from the affinity resin in an intact form. The MBP-MS2 method overcomes these problems, allowing efficient isolation of functional complexes in a highly pure form.

Other affinity tags were screened for their suitability for spliceosome purification. These studies revealed either high background (protein A tag) or low binding (GST or histidine tag). In contrast, significant levels of binding and low background were obtained with the maltose-binding protein (MBP). MBP affinity offers the added advantage of mild elution conditions, requiring only maltose. The MBP is fused to bacteriophage R17/MS2 coat protein, which binds to a specific hairpin RNA (Carey et al., 1983; Bardwell and Wickens, 1990; LeCuyer et al., 1995; Graveley and Maniatis, 1998). Thus, after engineering the hairpin structures into RNA, the RNA-protein complexes can be affinity-selected via MS2-MBP. A gel-filtration step that had been previously established to separate spliceosomes (Reed, 1990) was combined with MS2-MBP affinity selection. This dramatically improved the specificity of the spliceosome purification and indicated promise for isolation of other types of RNA-protein complexes. In addition, the number of MS2 binding sites and the ratio of MS2-MBP to RNA were both optimized to increase the efficiency of complex formation and purification (Zhou et al., 2002a).

Using the MS2-MBP purification method, highly purified and functional spliceosomal complexes were obtained (Das et al., 2000; Zhou et al., 2002b), the protein components were identified by mass spectrometry, and the structure of complexes was visualized by electron microscopy (Zhou et al., 2002a,b). The spliced mRNPs complex that functions in nucleocytoplasmic export has also been isolated, and it retains its export activity (Zhou et al., 2000).

Critical Parameters
The efficacy of formation of the RNA-protein complex of interest is a major factor in determining the final yield of the purified complexes. In the case of spliceosome purification, nuclear extract is of great importance. High-quality nuclear extract allows higher efficiency of spliceosome assembly and less RNA degradation. Another important factor is the quality of the MS2-MBP protein. Purification of MS2-MBP via a heparin column is critical for improving the binding efficiency of MS2-MBP to RNA and the final yield of the complexes. The last critical parameter is the negative control of the experiment. Generally, a similar RNA with a mutation in a critical functional site should be used in the same purification procedure to identify nonspecifically bound proteins.

Troubleshooting
The most common problem is low yield of purified RNA-protein complexes in the elution. This can be caused by insufficient binding of MS2-MBP to the RNA, inefficient binding of RNA-protein complexes to the amylose resin, poor elution, or simply inefficient formation of RNA-protein complexes. Any degradation of the RNAs or proteins will reduce or eliminate the yield as well. Thus, it is critical to perform stepwise assays to make sure that every step is working. For example, the MS2-MBP protein should be checked by SDS-PAGE to make sure it is purified and intact. A mobility gel-shift assay is necessary to optimize the binding of MS2-MBP to RNA. The RNA integrity should be monitored from the beginning of preparation of RNA, after RNA is bound by MS2-MBP, after RNA/protein complex formation, and during gel filtration and binding to the amylose beads. It is suggested that an aliquot of the reaction be saved at every step for RNA and protein quality assurance. The binding efficiency of the complex to the amylose beads and the elution efficiency of the complex can be monitored by comparing total radioactivity before and after the binding or elution.

Anticipated Results
In the case of spliceosome purification, typically about 30% of the spliceosomes will bind to the amylose resin and 80% of them will be eluted. The limiting factor in most instances is the efficiency of spliceosome assembly, which mainly depends on the quality of the nuclear extract and the sequences of the pre-mRNA substrate.

Time Considerations
Purification of MS2-MBP and preparation of substrate RNA takes one day. Assembly and purification of spliceosomes will take another
day. The time-consuming part of this protocol is the gel-filtration step, which takes ~14 hr, and it is best to schedule it to run overnight.

**Literature Cited**


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