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Identification of RNA-binding Proteins

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[Abstract] This protocol describes the extraction of RNA-binding proteins (RBPs) from cell lysates. In order to pull down target RBPs, 5-bromo-UTP (BrUTP)-incorporated RNA probes are used, which are generated by *in vitro* transcription. The schematic diagram (Flowchart) with procedure is indicated (Figure1 and Figure 2).

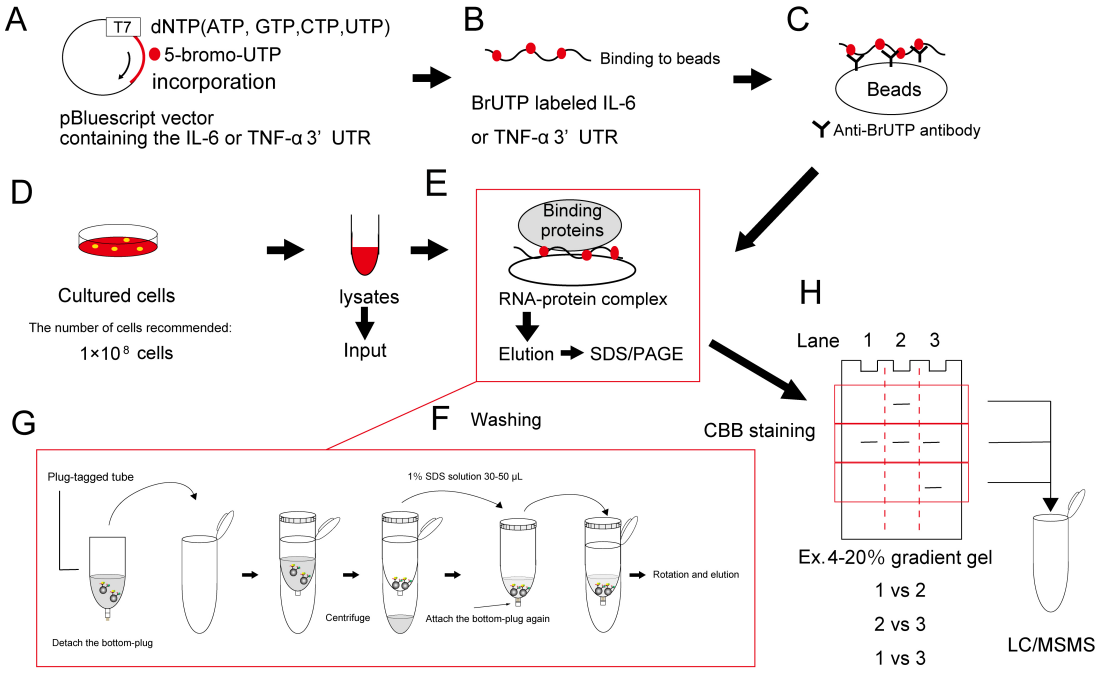

Figure 1. Schematic diagram of procedure (A-H). Flow chart of experimental procedure is indicated at A-H.

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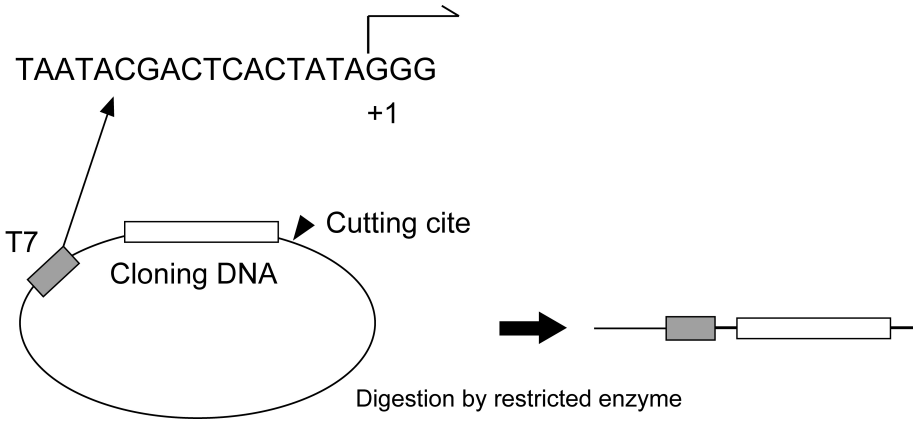

Figure 2. Linearization of plasmids by restricted enzyme. The plasmid is cut at restriction sites adjacent to its cloning element.

Materials and Reagents

1. Microcentrifuge tubes (1.5 to 2.0 ml)
2. Desalting MobiSpin columns (MoBiTec, catalog number: M105035F)
3. DNA template (*e.g.,* pBluescript vector encoding the target sequences such as non-coding elements IL-6 3'UTR and TNF-α 3'UTR)
4. Reagents for *in vitro* transcription kits (TAKARA BIO, catalog number: 6140)
5. 5-bromouridine 5'-triphosphate (sodium salt) (Cayman Chemical, catalog number: 18140)
6. 50 mM ATP solution
7. 50 mM GTP solution
8. 50 mM CTP solution
9. 50 mM UTP solution
10. RNaseOUT™ recombinant ribonuclease inhibitor (Thermo Fisher Scientific, Invitrogen™, catalog number: 10777-019)
11. T7 RNA polymerase
12. DNase I (RNase free) (New England Biolabs, catalog number: M0303S)
13. Protease inhibitor cocktail (Sigma-Aldrich, catalog number: MSSAFE)
14. Protease inhibitor cocktail (NACALAI TESQUE, catalog number: 04080)
15. TRIzol® Reagent (Thermo Fisher Scientific, Ambion™, catalog number: 15596-026)
16. Anti-BrdU antibody (IIB5) (Abcam, catalog number: ab8152)
17. Protein G Sepharose 4 fast flow (GE Healthcare, catalog number: 17061801)
18. Nuclease-free PBS (NACALAI TESQUE, catalog number: 14249)
19. Nuclease-free water (Thermo Fisher Scientific, Ambion™, catalog number: 4387936)
20. Dithiothreitol (DTT) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: R0861)
21. Chloroform (NACALAI TESQUE, catalog number: 08401-65)
22. DEPC water (Thermo Fisher Scientific, Ambion™, catalog number: AM9916)
23. 96% ethanol or 70% ethanol (NACALAI TESQUE, catalog number: 09666-85)

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24. NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific, catalog number: 78833)
25. 0.25% trypsin-EDTA solution (Thermo Fisher Scientific, catalog number: 25200056)
26. 1 M Tris-HCl, pH 7.4 (NACALAI TESQUE, Gibco®, catalog number: 35436-01)
27. 5 M NaCl (NACALAI TESQUE, catalog number: 31320-05)
28. 0.1 M spermidine (Sigma-Aldrich, catalog number: S2626-1G)
29. 10% Nonidet(R) P-40 (NACALAI TESQUE, catalog number: 25223-04)
30. 0.5 M EDTA (NACALAI TESQUE, catalog number: 06894-14)
31. 1 M $MgCl_2$ (NACALAI TESQUE, catalog number: 20942-34)
32. 1% Tween 20 (NACALAI TESQUE, catalog number: 35624-15)
33. PBS, pH 7.0 (NACALAI TESQUE, catalog number: 14249-24)
34. 1% SDS (NACALAI TESQUE, catalog number: 30562-04)
35. Coomassie brilliant blue (CBB) (Thermo Fisher Scientific, catalog number: 20278)
36. 10x transcription buffer (see Recipes)
37. Bead washing buffer (see Recipes)
38. RNA-binding buffer (see Recipes)
39. RNA-protein wash buffer (see Recipes)
40. Lysis buffer (see Recipes)
41. Elution buffer (see Recipes)

Equipment

1. MALDI-QIT-TOF (Shimadzu Europa GmbH, model: AXIMA Resonance)
2. Microcentrifuge capable of reaching up to 16,000 *x g*
3. Vortex mixer
4. Centrifuge capable of reaching up to 2,000 *x g*

Procedure

A. *In vitro* transcription of 5-bromo-UTP (BrUTP)-incorporated RNA probes

Note: DNA template such as pBluescript vector encoding the target sequences should be made linear DNA by restriction enzyme digestion and purified by phenol/chloroform extraction and ethanol precipitation (Figure 2).

1. Preparation of DNA template such as plasmids, PCR-generated or synthetic oligonucleotides.
2. *In vitro* transcription reaction:

   20 ng-1 µg DNA template

   2 µl 10x transcription buffer

   2 µl 50 mM ATP solution

   2 µl 50 mM GTP solution

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2 µl 50 mM CTP solution
1 µl 50 mM UTP solution
1 µl 50 mM 5-bromo-UTP (BrUTP) solution
0.5 µl RNase inhibitor
2 µl T7 RNA polymerase
X µl RNase free ddH_2O (up to total 20 µl)
Mix by pipetting, and centrifuge.

3. Incubate for 2 h at 42 °C.
4. Add 10-20 U DNase to 20 µl total solution to digest any DNA. After mixing, incubate for 1-2 h at 37 °C.
5. The RNA probes are purified using Trizol following standard protocol.
   Optional: If you want to confirm the size of RNA transcripts, agarose gel electrophoresis of RNA in formaldehyde will be performed following standard protocol.

B. Conjugation of anti-BrdU antibodies with protein G beads
   1. Wash protein G agarose beads three times with an equal volume of nuclease-free PBS (centrifuge at 2,000 *x g* for 1 min at 4 °C).
   2. Apply 100 µl of the solution (50% beads made up with PBS) to 1.5 ml new microcentrifuge tubes.
   3. Add 500 µl of bead wash buffer to each tube.
   4. Add 50 µl of anti-BrUTP m Ab to each tube.
   5. Incubate the tubes while rotating for at least 1 h at 4 °C. The sample can be incubated at 4 °C overnight.
   6. Wash antibody-conjugated beads once with 1 ml of bead wash buffer (centrifuge at 2,000 *x g* for 1 min at 4 °C).
   7. Discard the supernatants carefully.

C. Binding of BrUTP-labeled RNA to antibody-conjugated beads
   1. Resuspend the antibody-conjugated beads with 500 µl of bead wash buffer.
   2. Add 50 pmol of prepared BrUTP-labeled RNA (prepared in step A), and RNase inhibitor.
   3. Incubate while rotating for 2 to 3 h at 4 °C.
   4. Centrifuge at 2,000 *x g* for 1 min at 4 °C.
   5. Discard supernatant and wash BrUTP labeled RNA bound to the antibody-conjugated beads with 500 µl of bead wash buffer (centrifuge at 2,000 *x g* for 1 min at 4 °C) (To step E).

D. Pre-clearing the protein extraction
   Optional: Cytoplasmic and nuclear protein extract can be separated *using NE-PER Nuclear and cytoplasmic extraction reagents. Protein extracts were prepared from at least 1.0 x 10^8 cells.*
   1. The preparation of the protein extracts

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a. Collect the cultured cells into a 1.5 ml new microcentrifuge tube (if dissociation is necessary, Trypsin-EDTA solution can be used).
b. Centrifuge the tube at 2,000 *x g* for 3 min at 4 °C.
c. Discard the supernatants carefully.
d. Transfer 1 ml lysis buffer into the tube with cell pellet, and resuspend the cells on ice.
e. Put the tube on ice for 10 min.
f. Centrifuge the tube at 16,000 *x g* for 10 min at 4 °C.
g. Transfer the supernatant (protein extract) carefully into a 1.5 ml new microcentrifuge tube.
h. Store 50 µl protein extract as input.

2. The preparation of protein G beads
   a. Wash 50 µl protein G agarose beads three times with an equal volume of nuclease-free PBS (centrifuge at 2,000 *x g* for 1min at 4 °C). Discard supernatant gently.
   b. Apply 100 µl of the solution (50% beads made up with PBS) to 1.5 ml new microcentrifuge tubes (50 µl beads per sample).
   c. Add 500 µl of bead wash buffer to each tube.
   d. Centrifuge the tubes at 2,000 *x g* for 1 min at 4 °C.
   e. Discard the supernatant carefully.
3. The mixture of the protein extract and 50% bead solution
   a. Transfer the protein extract into the tube with protein G beads.
   b. Incubate the tube while rotating for 1 h at 4 °C.

E. Binding of proteins to BrUTP-labeled RNA-conjugated beads
1. Centrifuge the sample tubes containing the protein extract and protein G agarose beads at 2,000 *x g* for 2 min at 4 °C (save 10 µl of the protein extract as an input).
2. Transfer the protein extract (*optional: either purified cytoplasmic or nuclear protein extract*) into the tubes prepared in step C.
3. Incubate with rotation for 2 h at 4 °C.

F. Purification of the binding proteins to RNA-conjugated beads
1. After incubation, centrifuge the samples at 2,000 *x g* for 1 min at 4 °C. Discard the supernatant carefully.
2. The protein-RNA complex on the beads is washed three to four times with 1 ml RNA-binding buffer. Centrifuge at 2,000 *x g* for 2 min at 4 °C.

G. Elution of proteins binding to BrUTP-labeled RNA-conjugated beads
1. Resuspend the beads in 200 µl of nuclease-free PBS and transfer the slurry to a bottom-plugged spin column.
2. Detach the bottom plug from the spin column, and then put the column into a new centrifuge tube. Centrifuge the column at 1,000 *x g* for 30 sec at 4 °C.

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3. Attach the bottom plug to the spin column, and put the column into a new centrifuge tube. Add 50 µl of elution buffer into the column.
4. Incubate for 30 min at 4 °C with gentle shaking.
5. Detach the bottom plug from the spin column, and put the column into a new centrifuge tube.
6. Elute the protein-BrUTP-labeled RNA complexes by centrifugation.
7. For enrichment of purified proteins, repeat elution steps G3-6, and collect the eluates into a new tube.

H. Detection of RNA-binding proteins by LC/MS/MS
1. The eluted samples are subjected to SDS-PAGE, followed by CBB staining.
2. Cut the gel on the several compartments, in which some bands were detected.
3. The samples cut are eluted, and then analyzed by LC/MS/MS.
4. Target RNA-binding proteins are confirmed by Western blotting (After step G).

Recipes

1. 10x transcription buffer
   0.4 M Tris-HCl, pH 7.4
   100 mM $MgCl_2$
   0.5 M NaCl
   0.1 M spermidine
2. Bead washing buffer
   20 mM Tris-HCl, pH 7.4
   137 mM NaCl
   1% NP-40
   2 mM EDTA
   1.5 mM DTT
3. RNA-binding buffer
   0.2 M Tris-HCl, pH 7.4
   0.5 M NaCl
   20 mM $MgCl_2$
   1% Tween 20
4. RNA-protein wash buffer
   20 mM Tris-HCl, pH 7.4
   10 mM NaCl
   1% Tween 20
5. Lysis buffer
   50 mM Tris-HCl, pH 7.4
   150 mM NaCl

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1% NP-40
1% protease inhibitor cocktail
1 mM DTT

6. Elution buffer
   PBS (pH 7.0)
   1% SDS

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