# Chapter 7

# In Vitro Analysis of Ribonucleoprotein Complex Remodeling and Disassembly

**Hua-Lin Zhou and Hua Lou**

## Abstract

Ribonucleoprotein (RNP) complexes play essential roles in gene expression. Their assembly and disassembly control the fate of mRNA molecules. Here, we describe a method that examines the remodeling and disassembly of RNPs. One unique aspect of this method is that the RNA-binding proteins (RBPs) of interest are produced in HeLa cells with or without the desired modification and the RNP is assembled in cellular extracts with synthetic RNA oligonucleotides. We use this method to investigate how ubiquitination of an RBP affects its ability to bind its RNA target.

**Key words** RBP, RNP assembly/disassembly, Ubiquitination

## 1 Introduction

Ribonucleoprotein (RNP) complexes form when RNA-binding proteins (RBPs) interact with their cognate target sequences on RNA. RNPs play essential roles in gene expression, as they control many aspects of the life of an mRNA from its birth to maturation, localization, translation, and turnover [1, 2]. Remarkably, RNPs are assembled and disassembled in a highly dynamic and orderly manner, because a different array of RBPs is required to be loaded onto pre-mRNA/mRNA during every step of post-transcriptional mRNA metabolism. Thus, the precise regulation of RNP remodeling is essential in ensuring appropriate gene expression both spatially and temporally, and failure to remodel RNP complexes leads to disruption of downstream events such as mRNA export, translation, and decay.

In order to understand the mechanisms by which RNP remodeling is regulated, we developed an in vitro assay of RNP remodeling [3]. Using this assay, we investigated how a specific form of post-translational modification, ubiquitination, affects the ability of the RBP HuR to interact with its RNA target. To ensure that

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the HuR protein is ubiquitinated in its native form, we used proteins prepared from transfected HeLa cells.

Figure 1 illustrates the general procedure of this assay. We first transfected HeLa cells with Myc-tagged HuR and HA-tagged ubiquitin. Forty-eight hours post-transfection, we lysed the cells and prepared total protein lysate. We then assembled RNP complexes on RNA oligonucleotides, either with or without biotin, that contain the HuR target sequence on p21 3′-UTR by incubating the protein lysate with the RNA. The assembled RNP complexes contain both ubiquitinated and non-ubiquitinated Myc-HuR. The central question of this particular study is how ubiquitination of HuR affects its ability to interact with its target RNA. We answered this question by incubating the RNP complexes with recombinant UBXD8 and p97, the complex-remodeling machine known to recognize ubiquitinated protein substrate and extract it from a macromolecular complex [4].

Following the incubation, we tested if the HuR-RNA-containing RNP is remodeled to release RNA from ubiquitinated HuR by conducting the following two assays. The first assay is the RBP-releasing assay. As shown in Fig. 1a, in this assay, biotinylated RNA oligonucleotides were used and the RNP complexes were collected using the streptavidin beads. We examined if ubiquitinated HuR is released from the RNP after incubation with UBXD8-p97 by separating the supernatant from the beads that contains biotinylated RNA and proteins assembled on the RNA, and conducting western blot analysis using the anti-Myc antibody. An increase in the level of ubiquitinated HuR in the supernatant after the incubation with UBXD8-p97 is an indication that ubiquitination of HuR reduces its ability to remain interacting with its target RNA. The second assay is the RNA-releasing assay. As shown in Fig. 1b, in this assay, RNA oligonucleotides without biotin were used and the RNP complexes were collected using anti-Myc agarose beads. We examined the level of RNA oligonucleotides that is complexed with HuR when ubiquitination of HuR is reduced. In this assay, following the incubation of p97 and UBXD8, we isolated RNA from the supernatant, labeled RNA with $^{32}$P and detected it by electrophoresis followed by phosphoimaging.

The methods described here can be adapted to examine how RNP remodeling is affected by changes of RNA sequence or structure, as well as changes of RBP sequence, structure or post-translational modifications.

## 2 Materials

1. *For cell transfection:* HeLa cells, DMEM medium, fetal bovine serum (Life Technologies), plasmid DNA Myc-HuR and HA-Ub, PolyJet (SignaGen Laboratories); for protein

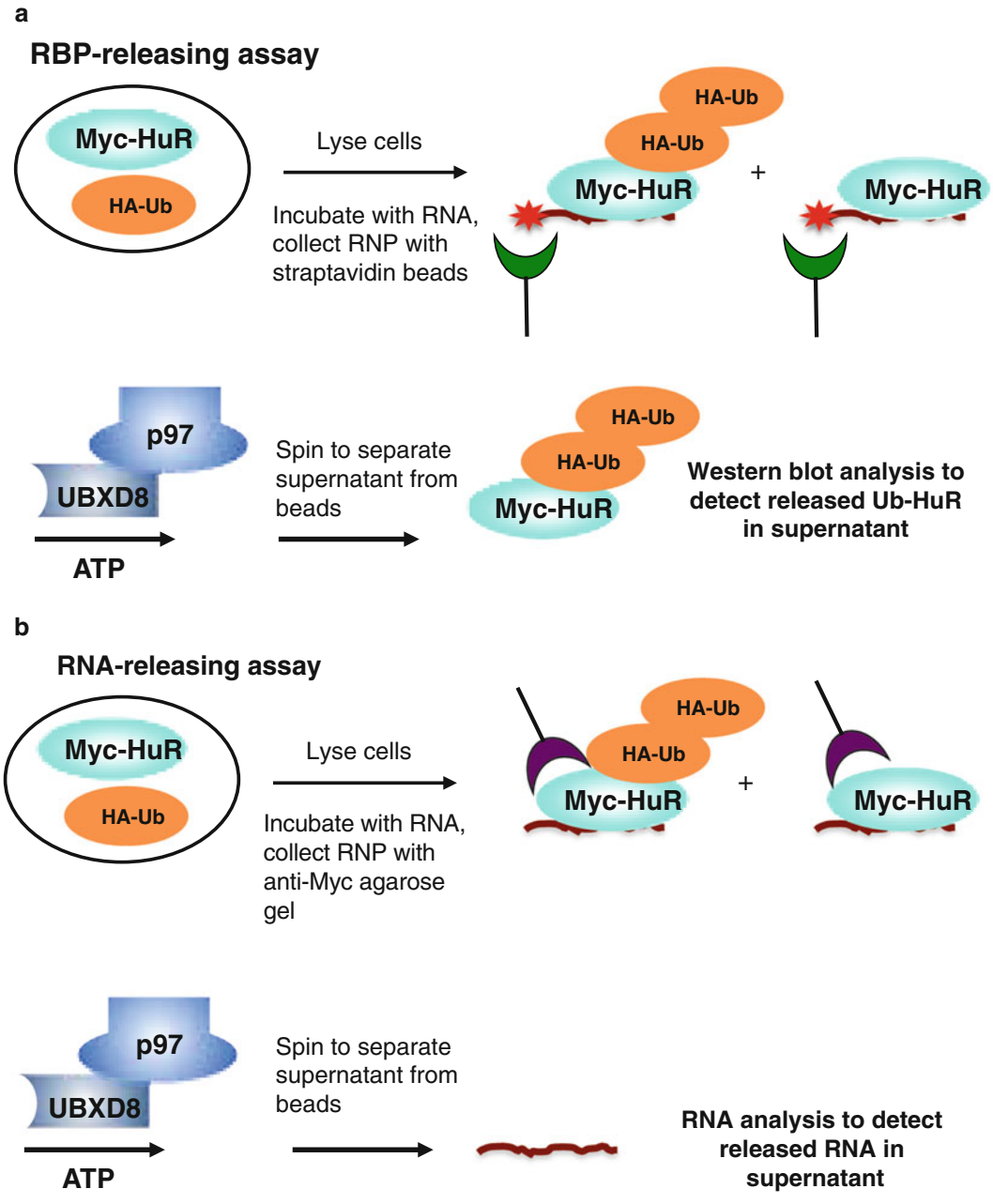


**Fig. 1** Diagrams depicting the RNP remodeling assays. (**a**) RBP-releasing assay. HeLa cells are transfected with Myc-HuR and HA-Ub. The protein lysate is incubated with biotinylated p21 RNA. The RNP complexes are collected using streptavidin beads. The RNP complexes are then incubated with recombinant UBXD8 and p97. The bound and released Myc-HuR is detected by western blot analysis using anti-Myc antibodies; (**b**) RNA-releasing assay. HeLa cells are transfected with Myc-HuR and HA-Ub. The protein lysate is incubated with p21 RNA that is not biotinylated. The RNP complexes are collected with anti-c-Myc agarose affinity gel. The RNP complex was then incubated with recombinant UBXD8 and p97. The bound and released p21 RNA is $^{32}$P-labeled, purified, run on a denaturing acrylamide gel and detected by phosphoimaging

extraction: proteinase inhibitor cocktail, dialysis cassette, protein concentration assay reagent; for RNP assay: RNA oligos (Dharmacon), TE, glycogen, glycerol, RNaseOUT, Streptavidin beads, anti-c-myc agarose, UBXD8 protein, p97 protein, ADP, AMP-PNP, gradient gel, SDS loading dye, TRIzol, $^{32}$P-ATP, polynucleotide kinase.

2. *Cold lysis buffer:* 1 mM PMSF, 20 units/ml RNaseOut, 10 mM Tris–HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.1 mM DTT, 0.5 % NP40.
3. *Dialysis buffer:* 20 % glycerol, 20 mM HEPES, pH 7.6, 1 mM EDTA, 100 mM KCl, 2 mM DTT, 0.1 mM PMSF.
4. *10X Binding buffer:* 200 mM HEPES, pH 7.5, 1 M NaCl, 30 mM $MgCl_2$.
5. *1X Wash buffer:* 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.5 M NaCl.
6. *10X Reaction buffer:* 200 mM HEPES, pH 7.5, 1 M NaCl, 30 mM $MgCl_2$.
7. *Elution buffer:* 10 mM Tris–HCl, pH 6.0, 2 M NaCl, 1 mM EDTA, 0.5 M $MgCl_2$.
8. Nutator-mixer.

## 3 Methods

### 3.1 Cell Culture and Transfection

1. Split 1 plate (10 cm) of HeLa cells that is 90 % confluent into 20 plates (10 cm) in DMEM complete medium containing 10 % Fetal Bovine Serum (FBS) and 1 % Penicillin-Streptomycin. After 2 days, the plates should be approximately 70 % confluent. At this point, transfection HeLa cells using PolyJet by following manufacturer's instructions (*see* **Note 1**). The procedure is briefly described below.
2. Feed cells with 10 ml of fresh DMEM 30–60 min before transfection.
3. For each plate, dilute 8 μg of plasmid DNA into 200 μl of serum-free DMEM. Gently pipette up and down to mix.
4. For each plate, dilute 16 μl of PolyJet™ reagent into 200 μl of serum-free DMEM. Gently pipette up and down 3–4 times to mix.
5. Add the diluted PolyJet™ reagent to the diluted DNA solution. Immediately pipette up and down 3–4 times to mix.
6. Incubate for 10–15 min at room temperature to allow PolyJet™/DNA complexes to form.
7. Add the 400 μl PolyJet™/DNA mixture drop-wise onto the medium in each plate and gently swirl the plate.

8. Remove PolyJet™/DNA complex-containing medium and replace with fresh DMEM-containing 10 % FBS 17–18 h post transfection.

### 3.2 Cell Lysis

1. Two days post transfection, wash cells on the plate with cold 1XPBS twice. Scrape cells in 2 ml cold 1XPBS. Move all of cells to a 50 ml conical tube. Collect cells by centrifugation for 1 min at 2000 × *g* at 4 °C. Remove as much supernatant as possible.
2. To the cell pellet, add 1 ml lysis buffer, which is supplemented with proteinase inhibitor cocktail immediately before use (*see* **Note 2**). Rock the tube on a Nutator mixer for 10 min at 4 °C. Centrifuge for 5 min at 10,000 × *g* at 4 °C. Transfer the supernatant to a 1.5 ml tube.
3. Add 500 μl lysis buffer with proteinase inhibitor cocktail to the pellet, resuspend and rock for 5 min at 4 °C. Centrifuge for 5 min at 10,000 × *g* at 4 °C. Remove supernatant and combine with that from **step 2**. There should be 1.5 ml of total supernatant.
4. Dialyze the supernatant in the dialysis buffer for 4 h at 4 °C with Slide-A-Lyzer Dialysis Cassette. Change to fresh dialysis buffer after 2 h.
5. Measure protein concentration with the Bio-Rad Protein Assay Dye Reagent Concentrate. Dilute the protein lysate to 18 μg/μl and aliquot the lysate into 0.5 ml tube, 50 μl/tube. Flash freeze the aliquots in liquid nitrogen, and store them at −80 °C for future use (*see* **Note 3**).

### 3.3 RNA Oligonucleotides

1. Order wild type and mutant RNA oligonucleotides. In our case, the RNAs are 42 nucleotides in length and contain sequences of the p21 3′-untranslated region that have either wild type or mutated HuR binding sites (wild type: UCUUAAUUAUUAUUUGUGUUUUAAUUUAAACAC CUCCUCAUG; mutant: UCGGACGUGGGAGUCGUG CGGUAGGGUGCACACCUCCUCACG). The RNAs used for RBP-releasing assay are biotinylated at their 5′ end. The RNAs used for RNA-releasing assay have a 2′-bis(2-acetoxyethoxy)methyl (ACE) protection instead of biotin at their 5′ ends. Order the oligonucleotides at the 0.01 μmol scale. Dissolve the oligonucleotides in 2 ml TE buffer to make 5 μM stock, aliquot RNA in 50 μl/tube and store at −80 °C. These RNA oligonucleotides are named as WT-BIO and Mut-BIO (for RBP-releasing assay) or WT-w/o-BIO and Mut-w/o-BIO (for RNA-releasing assay).
2. Deprotection of RNA oligonucleotides. The RNA oligonucleotides that come with the proprietary 2′-ACE protection are

deprotected before use. We use a modified version of the deprotection protocol from Dharmacon, briefly described as the following:

Precipitate RNA from one 50 µl aliquot. To the aliquot, add 2 µl glycogen (30 mg/ml), and 100 µl 100 % ethanol. Incubate at −80 °C for at least 30 min. Spin for 15 min at 14,000×*g* in cold room. Remove supernatant and wash pellet with 1 ml 100 % ethanol, spin for 1 min at 14,000×*g*, remove ethanol and wash with ethanol once. Air-dry pellet.

Resuspend RNA in 100 µl 2′-deprotection buffer (supplied with oligonucleotide). Vortex for 10 s and centrifuge for 10 s. Incubate at 60 °C for 30 min. Add 350 µl TE, 2.5 µl glycogen (30 mg/ml) and 1 ml ethanol. Incubate at −80 °C for 30 min. Spin down for 15 min at 14,000×*g* in cold room, wash 2X with 1 ml ethanol. Air-dry pellet. Resuspend RNA in 40 µl TE and store at −80 °C.

### 3.4 RNP Formation

#### 3.4.1 Formation of RNP Complex for Protein-Releasing Assay

1. Dilute 5 µM WT-BIO or Mut-BIO to 500 nM by mixing 2 µl RNA stock with 18 µl TE. Heat the 20 µl WT-BIO (500 nM) or Mut-BIO (500 nM) to 70 °C for 5 min (*see* **Note 4**). Cool down to room temperature. Set up the following reaction:
   - 50 µl cell extract.
   - 10 µl biotinylated RNA oligonucleotide (500 nM).
   - 50 µl 10X binding buffer.
   - 100 µl 50 % w/v glycerol.
   - 25 µl RNaseOUT.
   - 50 µl 10X proteinase cocktail inhibitor.
   - Up to 500 µl $ddH_2O$.
2. Incubate for 3 h at 4 °C with gentle rocking on a Nutator.
3. Wash streptavidin agarose beads (~100 µl/sample 1:1 slurry). Add 1 ml 1X wash buffer to 100 µl beads (1:1 slurry) in a 1.5 ml microfuge tube (*see* **Note 5**). Spin down for 1 min at 1000×*g*. Remove supernatant. Repeat washing for five times.
4. Add streptavidin agarose beads into reaction sample. Incubate beads with RNP for 2 h at 4 °C with gentle rocking on a Nutator to form the beads-RNP.
5. Purification of RNP. Add 1 ml wash buffer to beads and rock for 5 min in cold room. Spin down for 1 min at 1000×*g* at 4 °C. Remove supernatant. Repeat washing five times. For the last wash, use 1X reaction buffer instead of washing buffer. After the last wash, remove supernatant as much as possible with a 30G needle. Save a small fraction of the supernatant after the last wash as the unbound fraction.
6. Resuspend beads with 100 µl 1X reaction buffer and store at 4 °C for future use. The resulting RNA/RBP complexes can be

used for the RNP remodeling assay. To detect if RNP complex is formed on the streptavidin agarose beads, 5 μl beads are removed and incubated with 100 μl of elution buffer at 65 °C for 5 min. Spin down for 1 min at 1000 × *g* and remove supernatant to new 1.5 ml tube. Western blot analysis is carried out to detect the presence of the RBP of interest in the RNA/RBP complex.

*3.4.2 Formation of RNP Complex for RNA-Releasing Assay*

1. Dilute WT-w/o-BIO or Mut-w/o-BIO RNA oligonucleotide to 50 μM with TE (*see* **Note 4**). Incubate 20 μl diluted RNA at 70 °C for 5 min. Cool down at room temperature. Set up the following reaction:
   - 50 μl cell extract.
   - 10 μl RNA (50 μM).
   - 50 μl 10X binding buffer.
   - 100 μl 50 % w/v glycerol.
   - 25 μl RNaseOUT.
   - 50 μl 10X proteinase cocktail inhibitor.
   - Up to 500 μl $ddH_2O$.
2. Incubate for 3 h at 4 °C with gentle rocking on a Nutator.
3. Wash Anti-c-Myc Agarose Affinity Gel antibody (~100 μl/sample 1:1 slurry) (*see* **Note 5**). Add 1 ml 1X wash buffer to 100 μl beads in a 1.5 ml microfuge tube. Spin down for 1 min at 1000 × *g*. Remove supernatant. Repeat washing five times. In our experiment, as over-expressed RBP is Myc-tagged, we use the commercial Anti-c-Myc Agarose Affinity Gel antibody.
4. Same as the **steps 4** and **5** in preparation of RNP complex for the protein-releasing assay.

### *3.5 Releasing Assay*

*3.5.1 RBP-Releasing Assay*

1. Resuspend the RNP complex prepared for the protein-releasing assay with a 200 μl cut-tip (tip cut to create bigger opening). Set up the following reaction:
   - 25 μl beads-RNP.
   - 25 μl 10X binding buffer.
   - 12.5 μl ATP (5 mM).
   - 5 μl recombinant p97 (ATPase) protein (12.5 μM) (*see* **Note 6**).
   - 5 μl UBXD8 (co-factor) protein (12.5 μM) (*see* **Note 6**).
   - 50 μl 50 % w/v glycerol.
   - 12.5 μl RNaseOUT.
   - 25 μl 10X proteinase cocktail inhibitor.
   - $ddH_2O$ up to 250 μl.

2. Incubate the reaction mixture at 30 °C. At 0, 15, 30, and 60 min time points, resuspend the reaction solution and remove 50 μl of the mixture including beads to a new 1.5 ml tube.
3. Spin for 1 min at 1000 × *g* and remove 40 μl supernatant containing released RBP into a new 1.5 ml tube (*see* **Note 7**). To examine the amount of RBP still bound to RNA after the releasing assay, spin for 1 min at 1000 × *g* again and remove supernatant as much as possible with a 30G needle (*see* **Note 8**). Elude the bound RBP with 200 μl of elution buffer at 65 °C for 5 min.
4. To examine if the RNP remodeling is dependent on ATP, ADP. or AMP-PNP, an ATP analog can be used to replace ATP in the RBP-releasing reaction.
5. Run 20 μl released RBP and 20 μl bound RBP on a 4–15 % gradient Criterion Tris–HCl Gel. Add 6.6 μl 4x SDS loading dye, heat at 95 °C for 5 min and load the samples on the gel.
6. Detect the released RBP and bound RBP by western blot analysis using an appropriate antibody (*see* **Note 9**). Anti-Myc antibody was used in our assay. Quantification of the releasing assay was performed using the ImageJ software on the GE Healthcare Typhoon Trio Variable Mode Imager. The released RBP was normalized to the bound RBP.

#### *3.5.2 RNA-Releasing Assay*

1. Resuspend RNP complex prepared in RNP complex for RNA releasing with a 200 μl cut tip. Set up the following reaction:
   - 25 μl Beads-RNP.
   - 25 μl 10X binding buffer.
   - 12.5 μl ATP (5 mM).
   - 5 μl recombinant p97 (ATPase) protein (12.5 μM).
   - 5 μl UBXD8 (co-factor) protein (12.5 μM).
   - 50 μl 50 % w/v glycerol.
   - 12.5 μl RNaseOUT (Life Technologies, cat# 10777-019).
   - 25 μl 10X proteinase cocktail inhibitor.
   - $ddH_2O$ up to 250 μl.
2. Incubate the reaction mixture at 30 °C. At 0, 15, 30. and 60 min time points, resuspend the reaction solution and remove 50 μl to a new 1.5 ml tube.
3. Spin for 1 min at 1000 × *g* and remove 40 μl supernatant containing released RNA into a new 1.5 ml tube. To examine the amount of RNA still bound to the RBP after the releasing assay, spin for 1 min at 1000 × *g* again and remove supernatant as much as possible with a 30G needle (*see* **Note 8**). Elute the bound RNA with 1 ml TRIzol.

4. Isolate the released and bound RNA following the TRIzol protocol. At the last step, dissolve the released RNA pellet in 10 μl TE and the bound RNA pellet in 50 μl TE.
5. Label the 5′-end of the RNA oligonucleotides with γ-$^{32}$P-ATP using T4 polynucleotide kinase. Set up the following reaction:
   - 5 μl RNA.
   - 3 μl 10X PNK buffer.
   - 1 μl T4 PNK.
   - 1 μl γ$^{32}$P-ATP (ICN Biomedical, cat# 38101X, 10 μCi/μl).
   - Up to 30 μl with dd$H_2O$.
   - Incubate for 30 min at 37 °C.
6. Add 370 μl TE and 400 μl phenol-chloroform (1:1) into the reaction tube, vortex, and spin for 5 min at 14,000 × *g*. Remove supernatant to a new tube. Add 2.5 μl glycogen and 1 ml ethanol. Incubate at–80 °C for 1 h. Spin for 30 min at 14,000 × *g* at 4 °C. Wash 2X with 1 ml ethanol. Air-dry.
7. Dissolve RNA pellet in 10 μl TE. Separate RNA on a denaturing 10 % polyacrylamide gel. The GE Healthcare Typhoon Trio Variable Mode Imager is used to calculate the released RNA amount.

## 4 Notes

1. High transfection efficiency is important for this assay. Use DMEM but not Opti-MEM to dilute PolyJet™ reagent and DNA. Do not vortex the mixture. Examine the RBP expression in the transfected cell by western blot analysis.
2. Lyse cells without SDS. In order to obtain high concentrations of cell extract, adjust the amounts of lysis buffer.
3. If post-translational modification of RBP is important for releasing, examine the post-translational modification of the RBP in the cell extract by western blot analysis as a control.
4. To reduce the potential RNP reassembly after the RBP-releasing assay, try not to use more RNA than RBP in this assay. Likewise, to reduce the potential RNP reassembly after the RNA-releasing assay, try not to use more RBP than RNA in this assay.
5. To reduce nonspecific binding, do not use too much beads and wash beads-RNP more than six times to remove nonspecific binding.
6. Carefully purify the recombinant ATPase and its co-factor protein to ensure that these recombinant proteins have activity.

7. After the beads-RNP disassociation reaction, remove up to only 2/3 supernatant to new tube to prevent from taking any beads.
8. 30G needles are used to remove as much as supernatant without accidentally taking any beads.
9. Gradient gels are used to better separate ubiquitinated proteins.

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