In vitro RNA-protein Binding Assay by UV Crosslinking

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[Abstract] Because covalent bond can form between RNA and its binding proteins after UV irradiation, UV cross-linking is widely used to identify the specific RNA binding proteins. This protocol is described in details as follows.

Materials and Reagents

1. Linearized DNA template for transcription of the RNA of interest
2. MAXIscript *in vitro* Transcription Kit (Applied Biosciences, catalog number: AM1308M) (This kit contain ATP, CTP, GTP, α-P32UTP and DNase I)
3. RNasin (Promega Corporation, catalog number: N2611)
4. RNase T1 (Applied Biosciences, catalog number: 10109193001)
5. 0.5 M EDTA (Life Technologies, Gibco®, catalog number: 15575-038)
6. ddH_2O (GeneMate Loyalty, catalog number: UPW-1000)
7. HEPES (Sigma-Aldrich, catalog number: H3375)
8. $MgCl_2$ (Sigma-Aldrich, catalog number: M-2393)
9. Glycerol (Sigma-Aldrich, catalog number: G7893-1L)
10. DTT (Sigma-Aldrich, catalog number: D9779-5G)
11. Tris-Base (Thermo Fisher Scientific, catalog number: BP152-1)
12. SDS (Sigma-Aldrich, catalog number: L-4390)
13. Bromphenol blue (Sigma-Aldrich, catalog number: B5525)
14. TEMED
15. Ammoniampersulfate
16. 5x binding buffer (see Recipes)
17. 2x SDS loading buffer (see Recipes)
18. 10x TGE buffer (see Recipes)
19. 6% TGE gel (see Recipes)

Equipment

1. SPIN-PureTM column (G-50) (PireBiotech SCW50-50 DEPC-water)

2. SDS-PAGE system (Bio-Rad, catalog number: 165-1802)
3. UV Stratalinker 1800 (Stratagene, catalog number: 474645)
4. Film processor (Konica Minolta, model: SRX-101A)
5. Centrifuges (Eppendorf, catalog number: 5810R)
6. Scintillation Counter (Beckman, catalog number: LS6500)
7. Bench top Radiation Shield with 6-1/4" base (Cole-Parmer, catalog number: WU-36218-00)
8. 37 °C water bath

Procedure

A. *In vitro* transcription of ^{32}P-labled RNA probes

1. DNA templates for synthesis of the RNA probe are generated by PCR using specific primers.
2. Thaw frozen reagents, place RNA polymerase on ice and vortex the 10x Transcription buffer and keep the buffer at room temperature. All reagents should be centrifuged briefly before opening.
3. Assemble transcription reaction at room temperature as the following sequence:
   1 µg DNA template
   ddH_2O to 20 µl
   2 µl 10x transcription buffer (after addition of water and DNA)
   1 µl 10 mM ATP
   1 µl 10 mM CTP
   1 µl 10 mM GTP
   5 µl [α-^{32}P]UTP
   2 µl T7 enzyme mix
   2 µl RNasin (40 U/µl)
   Mix thoroughly.
4. Incubate 1 h at 37 °C.
5. Add 1 µl of 5 mg/ml DNase I to digest the rest template DNA and incubate 10 min in 37 °C water bath.
6. Add 1 µl of 0.5 M EDTA, mix and add 78 µl ddH_2O to adjust final volume to 100 µl and mix.

B. Purification of ^{32}P-labled RNA probes with SPIN-Pure column (G-50)

1. Allow a minimum of 30 min of room temperature to warm the columns before the following steps.
2. Gently invert the column several times to suspend the column buffer (DEPC-Water).

3. Remove the top cap from the column, and then remove the bottom tip.
4. Allow the column buffer to drain by gravity before proceeding.
5. Place this column/tube apparatus into an adaptor tube. Centrifuge at 1,100 *x g* for 2 min at room temperature.
6. Repeat centrifugation again to let the column dry completely and discard the collection tube and the eluted buffer.
7. Put the column in a second collection tube in upright position and apply the RNA sample (20 to 50 µl) to the center of the column gel very slowly and carefully (the rest RNA sample can be stored at -80 °C for one week).
8. Centrifuge at 1,100 *x g* for 4 min. The purified ^{32}P-labled RNA probe is collected in the bottom of the collection tube. Discard the spin column and continue with the following procedure.
9. Pick 1 µl of the RNA sample and count the cpm value.

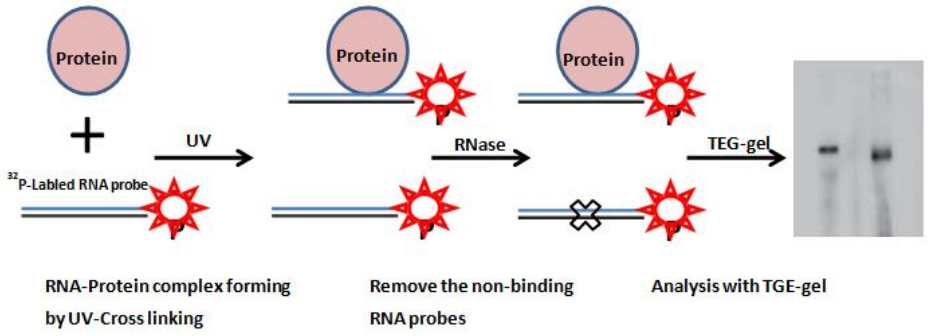
C. RNA and protein binding reaction

1. Prepare the following mixture on ice:
   ^{32}P-labled RNA probe (2 x 10^5 cpm)
   1 µl 12.5 mM ATP
   3µl 5x binding buffer
   3 µl 0.5 M KCl
   Protein (100 ng) (this protein can be a GST fusion protein purified according to the protocol prepared by GE Healthcare Life Sciences)
   H_2O to final volume of 15 µl.
2. Mix gently by pipetting up and down for 10 times, avoiding vortex.
3. Place the uncovered tube containing the reaction mixture on ice, directly underneath the bulb (about 10 cm from the surface) of a 254-nm UV light source, irradiation with 4 x 10^5 µJ/cm^2 energy.
4. Incubate at room temperature for 30 min then keep on ice for another 3 h.

D. Remove the non-incorporated ^{32}P

1. Add 1 µl of RNase T1 (1 U/µl) to each tube and incubate for additional 10 min at 37 °C to degrade the free RNA.
2. Add an equal volume (16 µl) of 2x SDS loading dye andseparate the sample in TGE-gel or SDS-PAGE (no boiling need).
3. Autoradiography for 12 h to 7 days for visualization.

Principle Figure



Recipes

1. 5x binding buffer
   50 mM HEPES (pH 7.2)
   15 mM $MgCl_2$
   25% glycerol
   5 mM DTT
   Stored at -20 °C
2. 2x SDS loading buffer
   125 mM Tris-Cl (pH 7.6)
   20% (v/v) glycerol
   4% (w/v) SDS
   0.008% (w/v) bromphenol blue
   20 mM DTT
3. 10x TGE buffer
   Tris 100 mM
   Glycine 1 M
   EDTA 10 mM
   pH 8.3
4. 6% TGE gel
   6 ml 30% acrylamide
   5 ml 10x TGE buffer
   35 ml H_2O
   300 µl ammoniampersulfate
   22 µl TEMED
   Notes:
   a. *Avoiding RNase contamination is the key for successful.*

b. *As ^{32}P is radioactive, this experiment should be performed under the protection of Benchtop Radiation Shield.*

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References

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