

RNA-protein UV-crosslinking Assay

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[Abstract] RNA-protein interactions play a crucial role in every aspect of RNA metabolism, and also plays a major role in post-transcriptional gene regulation. RNA-binding proteins have been implicated in viral gene expression (Ray and Das, 2002) and microRNA-mediated gene regulation (Poria *et al.*, 2016). Here we have described the protocol which (1) covalently links transiently interacting RNA-protein complexes by UV crosslinking, (2) removes the unprotected RNA by RNase digestion and (3) detects the RNA-protein complexes by SDS-PAGE analysis. This protocol provides a rapid and reliable means to directly assay RNA-protein interactions and their kinetics using purified proteins and also help in identifying novel RNA-protein interactions

Keywords: RNA-protein interaction, UV-crosslinking, RNA-binding proteins

[Background] RNA-protein interactions are mediated by transient non-covalent interactions such as electrostatic interactions and hydrogen bonds between specific residues in RNA and protein molecules. Short wave UV radiation can induce covalent bond formation between two closely placed aromatic rings. Aromatic ring structures are found in several amino acids in proteins and in nitrogenous bases in nucleic acids. Therefore, UV irradiation is used to covalently link RNA and interacting proteins, whereby the RNA-protein complex can be further analysed by SDS-Polyacrylamide gel electrophoresis. This protocol describes a simple and rapid assay system that can assay RNA-protein interactions and their binding kinetics *in vitro*. Also, mass spectrometric analysis of the fluorescently-labeled RNA-protein complexes obtained by this method can lead to identification of novel RNA-protein interactions.

Materials and Reagents

1. 1.5 ml RNase, DNase free microcentrifuge tube (Corning, Axygen[®], catalog number: MCT-150-C or equivalent)
2. 96-well round-bottomed plate (Greiner Bio one International, catalog number: 650101)
3. Agarose (Lonza, catalog number: 50004)
4. Transcription Kit (MAXIscript[®] Kit) (Thermo Fisher Scientific, Invitrogen[™], catalog number: AM1312 or any equivalent)
5. 10 μ Ci/ μ l α -P³² UTP (BRIT, catalog number: PLC 108 or PerkinElmer, catalog number: BLU007H250UC) (Alternatively, Cy5-UTP can be used to generate fluorescently labelled RNA [GE Healthcare, catalog number: PA55026])
6. DNase I (optional) (Thermo Fisher Scientific, Thermo Scientific[™], catalog number: EN0521)

7. 100% ethanol (EMD Millipore, catalog number: 100983)
8. Nuclease free water
9. Ammonium acetate (Thermo Fisher Scientific, Affymetrix, catalog number: 75901)
10. Glycerol (Thermo Fisher Scientific, Invitrogen™, catalog number: 15514)
11. Urea-polyacrylamide gel
12. Yeast tRNA (Sigma-Aldrich, catalog number: R8759)
13. RNase inhibitor (Thermo Fisher Scientific, Thermo Scientific™, catalog number: EO0381)
14. RNase A (Sigma-Aldrich, catalog number: R6513)
15. 10% SOD-PAGE
16. Pre-stained protein markers or radiolabeled protein markers
17. HEPES (pH 7.4) (Thermo Fisher Scientific, Affymetrix, catalog number: 16926)
18. Potassium chloride (KCl) (AMRESCO, catalog number: 0395)
19. Magnesium chloride hexahydrate (MgCl₂·6H₂O) (AMRESCO, catalog number: 0288)
20. Dithiothreitol (DTT) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: R0861)
21. EDTA (AMRESCO, catalog number: 0105)
22. ATP (Sigma-Aldrich, catalog number: A8937)
23. SDS
24. Tris-Cl (pH 6.8)
25. Bromophenol blue
26. 2x RNA binding buffer (see Recipes)
27. 2x denaturing protein loading buffer (see Recipes)

Equipment

1. Refrigerated centrifuge (Eppendorf, model: 5418 R)
2. UV cross-linker or UV torch with 254 nm wavelength UV (UVP, model: CL1000)
3. Vertical gel electrophoretic system (Bio-Rad Laboratories, model: Mini-PROTEAN Tetra Cell, catalog number: 1658000EDU)
4. Scintillation counter (Hidex, model: Triathler or any equivalent model)
5. Phosphorimager (GE Healthcare, model: Typhoon Trio or any equivalent model)

Procedure

A. Template preparation for *in vitro* transcription

1. Clone the DNA template encoding the RNA of interest derived by a T7 promoter in a plasmid vector and linearize the plasmid at a site downstream of the DNA template using specific restriction enzyme.
2. Run the linearized plasmid DNA on a 0.8% agarose gel, excise the band and extract the linearized DNA for *in vitro* transcription.

- Alternatively use a T7 promoter-adapter containing forward primer and gene specific reverse primer to amplify target DNA incorporating T7 promoter for *in vitro* transcription (Figure 1).

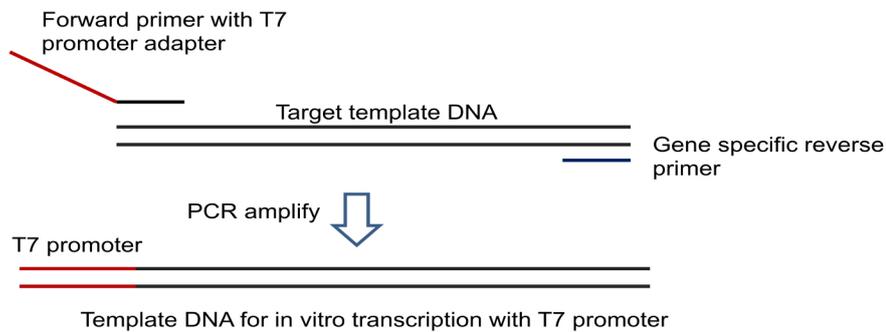


Figure 1. Preparation of template DNA for transcription using T7 promoter

- For oligo driven transcription of short RNAs, commercially synthesized template DNA oligo with T7 promoter site is annealed with a T7 promoter-adapter oligo to generate the template for *in vitro* transcription (Figure 2).

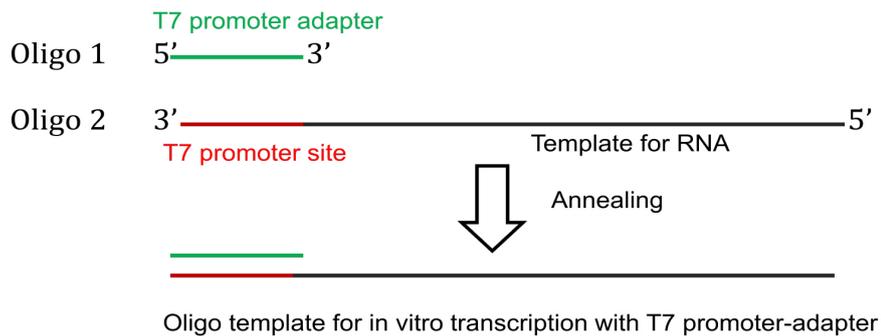


Figure 2. Preparation of DNA template for oligo-driven transcription

B. P³²UTP body-labelled RNA preparation (proper biosafety protocol should be followed)

- Setup the following *in vitro* transcription reaction using α -P³²UTP as radiolabel

| | |
|-------------------------------|-------------------------|
| 10x transcription buffer | 2 μ l |
| 10 mM ATP | 1 μ l |
| 10 mM CTP | 1 μ l |
| 10 mM GTP | 1 μ l |
| 100 μ M UTP | 1 μ l |
| α -P ³² UTP | 2 μ l |
| Linearized DNA template | 10 μ l (~1 μ g) |
| T7 RNA polymerase | 2 μ l |

Incubate for 1 h 30 min at 37 °C.

Add 1 μ l DNase I (optional) to remove the DNA template. Incubate at 37 °C for 15 min.

2. Remove unincorporated nucleotides either by ethanol precipitation or column purification. For ethanol precipitation, add 29 μ l nuclease free water to the transcription reaction to bring the volume to 50 μ l. Add 5 μ l 5 M ammonium acetate, 2 μ g glycogen and 2 volumes of 100% ethanol, and precipitate at -80 °C for at least 2 h. Spin for 20 min at maximum speed in a 4 °C centrifuge and wash the pellet once with cold 70% ethanol before drying.
3. Dissolve the dried RNA pellet in 20 μ l nuclease free water. Take 1 μ l of labelled RNA and count the specific activity with a scintillation counter or run on an 8 M urea-polyacrylamide gel at the percentage appropriate for the size of the RNA.

C. RNA-protein binding and UV crosslinking

1. Setup the following binding reaction for each sample in 1.5 ml microcentrifuge tubes on ice

| | |
|---------------------------------------|-------------------------------------|
| 2x RNA binding buffer (with 3 mM ATP) | 6 μ l |
| 10 mg/ml yeast tRNA | 1 μ l |
| Radiolabeled RNA | x μ l (~100,000 cpm) |
| RNase inhibitor (40 U/ μ l) | 0.5 μ l |
| Purified protein/cell lysate | x μ l (minimum 50 ng) |
| Nuclease free water | to make up the volume to 12 μ l |

2. Incubate on ice for 30 min.
3. Carefully transfer to a precooled 96-well round-bottomed plate placed on ice.
4. Place the plate containing the reaction mixture under the UV light source of UV crosslinker or UV torch and apply 500 mJ/cm² radiation for 10 min on ice.
5. Transfer the reaction to 1.5 ml tubes.
6. Add 2 μ l RNase A (10 μ g/ μ l) and incubate the tubes for 30 min at 37 °C.

D. Separation of RNA-protein complex and visualization (Figure 3)

1. Add 13 μ l of 2x denaturing protein loading buffer.
2. Boil the samples for 5 min at 100 °C and resolve in 10% SOD-PAGE with pre-stained protein markers or radiolabeled protein markers.
3. Dry the gel and expose overnight to a phosphorimager screen.
4. Scan the screen in a phosphorimager.
5. Alternatively expose the dried gel to X-ray film for 24 h and develop the film.

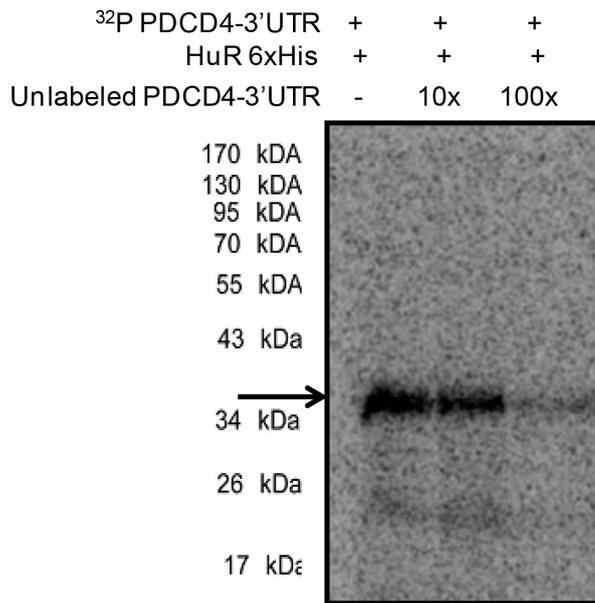


Figure 3. UV-crosslinking of ³²P labelled PDCD4-3'UTR RNA with 500 ng of purified 6x His-tagged HuR protein (lane 1) and in the presence of 10x (lane 2) and 100x unlabelled PDCD4 3'UTR RNA. RNA-protein complexes were digested with RNase A and resolved in 10% SDS-PAGE and exposed for phosphorimaging. The HuR-³²P PDCD4-3'UTR RNA complexes are indicated by the arrow.

Recipes

- 2x RNA binding buffer
 - 10 mM HEPES (pH 7.4)
 - 50 mM KCl
 - 4 mM MgCl₂
 - 4 mM dithiothreitol (DTT)
 - 0.2 mM EDTA
 - 7.6% glycerol
 - 3 mM ATP
- 2x denaturing protein loading buffer
 - 4 ml 10% (w/v) SDS (final concentration 4%)
 - 2 ml glycerol (final concentration 20%)
 - 1.2 ml 1 M Tris-Cl (pH 6.8) (120 mM)
 - 2.8 ml DEPC-H₂O (to make volume upto 10 ml)
 - Add bromophenol blue to a final concentration of 0.02% (w/v)

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References

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