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Detection of RNA-Protein Interactions using Tethered RNA Affinity Capture

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Summary

Recent progress in large-scale nucleic acid analysis technology has revealed the presence of vast numbers of RNA species in cells, and extensive processing. To investigate the functions of these transcripts highly efficient methods are needed to analyze their interactions with RNA-binding proteins (RBPs), and to understand the binding mechanisms. Many methods have been described to identify RBPs, but none are wholly satisfactory, in part because RNAs are flexible macromolecules that adopt multiple conformations only some of which might bind to specific proteins. Here we describe a novel in vitro RNA-pull down assay using tRNA scaffolded Streptavidin Aptamer (tRSA), to identify transcript specific RNA binding protein from mammalian cell lysates. The tRNA scaffold functions to stabilize the structure of the aptamer and the attached RNA, increasing the efficiency of the affinity purification.

Keywords

RNA; pull down; aptamer; tRNA scaffold; RBP

1. Introduction

Technological innovation in the high throughput analysis of nucleic acids has revealed the existence of many classes of noncoding transcripts in addition to thousands of splice variants of mRNAs (1), (2), (3). A substantial fraction of the genome in mammals is transcribed as long non-coding RNAs, mostly of unknown function, and multiple families of small RNAs regulate gene expression. Many mRNAs are modified by post-transcriptional mechanisms such as alternative splicing and RNA editing, (4), (5), (6). It is likely that the functions of this universe of RNAs involve sequence- or structure-specific interactions with proteins, and robust and sensitive methods are required, therefore, to interrogate and understand these interactions.

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One of the simplest ways to analyze RNA - protein interaction is to attach a functional tag such as GST or His6 to a protein of interest and perform co-precipitations to identify associated RNAs. Conversely, it should be possible to attach a tag to a RNA of interest and pull down associated proteins. However, RNAs are dynamic molecules that do not always possess a stable tertiary structure, so effective attachment of a tag to a RNA of interest is not trivial.

RNA aptamers are RNA molecules that possess high affinity for specific molecules, through sequence specific interactions and tertiary structures that are generated by base pairing in molecules of the RNA. Aptamers have been developed empirically against a number of targets by selection from large libraries of random sequences, and they are promising candidates for RNA tags. However, as a consequence of various factors such as the composition of the solution, the presence of non-specific RNA binding proteins, and degradation, the affinity for the target molecule can be reduced to the point that affinity capture becomes very inefficient. To address these problems, many of the existing aptamer-based RNA pull-down assays require a special matrix, recombinant protein, or multi-step biochemical separation (7),(8),(9). An alternative approach is to stabilize the aptamer structure. Ponchon and Dardel demonstrated that endogenous L20 protein could be co-precipitated from bacterial lysate using a tRNA-scaffolded Sephadex aptamer-23SrRNA fusion (10). We reasoned that this tRNA scaffold technology might provide a great advantage for transcript-specific affinity purification.

By applying tRNA scaffold technology, we established an efficient RNA pull down method to detect transcript specific RNA binding protein from mammalian cell lysates (see Fig. 1) (11). In this chapter, we describe the detailed procedure for our assay.

2. Materials

Particular care must be taken with all reagents to prevent RNase contamination. Basically, all reagents should be treated with DEPC (0.1%, 1 hour~) followed by autoclaving, except for HEPES, DTT, and protein reagents. Molecular Biology grade reagents and weigh boats are usually RNase free, so these reagents only have to dissolve with DEPC-treated water or RNase-free water. Ideally, reagents for all RNA experiments should be kept on an isolated shelf, and dedicated pipets should be used. In addition, RNA solutions should not be repeatedly frozen and thawed.

2.1. Bait RNA production

1. Template DNA (Cut plasmid or PCR product) (see ^{Notes 1})
2. Ampliscribe T7 Flash Transcription Kit (Epicentre, cat#ASF3257)

¹In this pull-down assay, tRSA (tRNA scaffolded Streptavidin Aptamer) tagged RNA is employed as a bait RNA. Prior to production of bait RNA in vitro, the template DNA including promoter, tRSA, and RNA of interest must be constructed. We constructed a pcDNA3-tRSA-empty vector (Addgene Plasmid #32200) and positive control vector (pcDNA3-tRSA-G quartet, Addgene #32203), and both of them are available from Addgene. The tRSA tag is cloned in the EcoRI site of pcDNA3 (Invitrogen) (see Fig. 2). The RNA of interest should be cloned either upstream or downstream of the tRSA tag. A PCR product is preferable to employ as a DNA template for in vitro RNA production because we have found that transcription efficiency is usually higher than with cut plasmid. Prepared DNA template should be purified using Qiaquick PCR purification kit (Qiagen, cat#28104).

3. Phenol:Chloroform (Sigma, cat#P1944)
4. 3 M NaOAc (pH 5.2)
5. Ethanol

2.2 RNA agarose preparation

1. Streptavidin agarose (Pierce, cat#20349)
2. Annealing buffer (50 mM HEPES, 50 mM MgCl₂)
3. Lysis Buffer including RNase inhibitor: 10 mM HEPES pH7.0, 200 mM NaCl, 10 mM MgCl₂, 1% TritonX-100, 1 mM DTT (Dithiothreitol), protease inhibitors, and RNase inhibitor (Promega, cat#N2115). (see ^{Notes 2})
4. TRIzol (Life technologies, cat#15596-026)
5. 3 M NaOAc (pH 5.2)
6. Ethanol

2.3. RNA electrophoresis

1. 10x MOPS buffer: 0.4 M MOPS (pH 7.0), 0.1 M sodium acetate, 0.01 M EDTA (pH 8.0)
2. Formalin (37% Formaldehyde)
3. Agarose powder
4. Deionized water

2.4 Cell lysate preparation

1. Lysis Buffer: 10 mM HEPES pH 7.0, 200 mM NaCl, 10 mM MgCl₂, 1 % TritonX-100, 1 mM DTT (Dithiothreitol), and protease inhibitors.
2. White Egg Avidin (EMD chemicals, cat#189725)
3. Yeast RNA (Sigma, cat#R6750)
4. Streptavidin agarose (Pierce, cat#20349)

3. Methods

3.1 Preparation of bait RNA

1. Follow the manufacturer's protocol of Ampliscribe T7 Flash Transcription Kit.
2. Add 1 volume of Phenol:Chloroform to RNA solution, and vortex for few seconds.

²To avoid RNase contamination, premade solutions (5 M NaCl, 1 M MgCl₂ and 10 % TritonX-100) must be treated with 0.1 % DEPC (Diethyl Pyrocarbonate) or prepared with RNase-free water. Because DEPC is reactive to those reagents, 1 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and 1M DTT must be prepared with RNase-free guaranteed reagents and DEPC pre-treated water or RNase-free water.

3. Centrifuge (4 °C, 16000 × G, 5 min)
4. Transfer supernatant to new tube, add 1/10 volume of 3 M NaOAc (pH 5.2) and 2.5 volume of ethanol.
5. Slightly vortex mix and precipitate RNA at −20 °C for 30 min or more.
6. Centrifuge (4 °C, 16000 × G, 10 min).
7. Remove liquid phase, and rinse white pellet with 80 % ethanol
8. Add RNase free H₂O and quantitate RNA concentration by spectrometer.
9. Make aliquots and store at −80°C.

3.2 preparation of cell lysate

There are various ways to prepare cell lysates. The optimal condition of buffer, salt and detergent must be determined initially to maximize the solubilization of the target fraction. However, because Mg²⁺ plays an important role in the RNA folding, chelating reagents (e.g. EDTA) should not be added to the Lysis buffer (12). Here we describe the conditions to pull-down endogenous FMRP (Fragile X Mental Retardation Protein) from HEK293 cell lysate (13). Lysate should be kept at 4 °C or on ice throughout the assay.

1. Freshly maintain HEK293 cells on 10 cm dishes.
2. Add 2 ml ice cold lysis buffer, and collect cells using cell scraper. Transfer cell lysate to 15 ml conical tube.
3. Homogenize lysate by sonication (Sonics & Material, Vibra-Cell, Duty cycle 50 %, output 4, 10 sec × 3).
4. Centrifuge lysate (4 °C, 16000 × G, 10 min).
5. Transfer supernatant to new tube and quantitate protein concentration by measuring absorbance 280 nm.
6. To block endogenous biotinylated protein and non-specific RNA binding proteins, add white egg avidin (10 µg/mg protein), yeast RNA (0.5 mg/mg protein), and incubate at 4 °C for 30 min on rotator.
7. Preclear the lysate by adding 40 µl (bed volume) of streptavidin agarose, incubate at 4 °C for 30 min on rotator.
8. Centrifuge tube (4 °C, 16000 × G, 5 min), transfer supernatant to new tube, and add RNase inhibitor (200 U/ml).

3.3 Preparation of RNA agarose

1. Prepare 30 µg of bait RNA in 1x annealing buffer in 1.5 ml tube (50–100 µl). Bait RNA is heat denatured at 65 °C for 5 min. The tube is then kept at room temperature for 5 min, and finally transferred to ice.
2. Prepare 60 µl streptavidin agarose in 1.5 ml tube, and wash it twice with lysis buffer including RNase inhibitor.

3. Add bait RNA (from 3.3.1) on streptavidin agarose, and adjust volume 300 μ l by adding lysis buffer including RNase inhibitor. Incubate tube for 20 min at 4 °C on rotator.
4. Wash Streptavidin Agarose twice with lysis buffer. Half is applied for RNA pull-down, and half is treated with 200 μ l Trizol to analyze tethered RNA.
5. Vortex mix TRIZOL added tube, and centrifuge (16000 $\times$ G, 5 min).
6. Transfer supernatant new tube, and add 1/10 volume of 3 M NaOAc (pH5.2) and 2.5 volume of ethanol.
7. Vortex briefly and precipitate RNA at -20 °C for 30 min or more.
8. Centrifuge (4 °C, 16000 $\times$ G, 10 min).
9. Remove liquid phase, and rinse white pellet with 80 % ethanol.
10. Add RNase free H₂O and quantitate RNA concentration by spectrometry.
11. Analyze bait RNA extracted from RNA agarose by formaldehyde agarose gel electrophoresis. (see ^{Notes 3})

3.4 Pull down assay

1. Add precleared cell lysate on RNA beads. Incubate tube for 1–2 hours at 4 °C on rotator.
2. Wash RNA agarose with lysis buffer at least 5 times.
3. Add sample buffer to apply for SDS-PAGE.

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³Add agarose (1.5 gram), 10x MOPS buffer MOPS (10 ml) and 72 ml deionized Water to flask, and boil until the agarose is completely dissolved. Keep flask on a magnetic stirrer at room temperature. After the temperature has fallen to 60 °C, add 18 ml formalin (37 % formaldehyde solution) to the flask, mix it thoroughly, and pour formalin gel solution into a mold. This step must be done in a chemical fume hood.

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Figure 1.

Timeline for tRSA affinity purification assay. The in vitro synthesis of the bait RNA and attachment to agarose can be performed at the same time as the preparation and preclearing of cell lysates, so that neither has to be stored in the refrigerator prior to use.

Figure 2.

Structure of the tRSA. Top shows a diagram of the predicted secondary structure of the construct, with an RNA of interest attached to the 3' end of the tRNA scaffold. The sequence of the tRSA tag in the vector is shown with useful cloning sites. Expression is driven from the T7 promoter.