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Protocol for RNA binding proteins pulldown

RNA templates.

1) RNA templates can be obtained in the following ways:

a) use commercial suppliers to synthesize them. This is generally the best course of action for sequences less than 50 nucleotides in length (Biosyn, Sigma-Aldrich).

b) cloning the sequence of interest in a pBluescript KS+ plasmid (Stratagene) or any other plasmid that contains a T7 promoter. In this case, it is advisable to make sure that the 5' end of the sequence to be transcribed is placed as near as possible to the end of the T7 promoter and that a suitable restriction enzyme to linearize the plasmid is present at its 3' end. This is to minimize the length of plasmid-related RNA that will eventually be transcribed together with the sequence of interest. In this case, it is important to choose an enzyme that cleaves efficiently and leaves a 5' overhang (ie. BamHI, HindIII, XbaI).

c) amplifying the sequence of interest using a forward primer carrying a T7 polymerase target sequence at the 5' end and 12-15 complementary nucleotides at the 3' end (5'-taatacgaactcactatagg(n)₁₂₋₁₅-3') and a reverse primer carrying 12-15 nt of the target sequence.

2) Products from steps (b) and (c) should be purified by phenol/chloroform extraction, precipitated using standard protocols (1/10 3M NaAc, 2.5 vol ethanol, -20°C for 1 hr), and resuspended in RNase-free water to a concentration of approximately (~1 µg/µl).

3) Approximately 2µg of linearized plasmids/amplified products are transcribed using T7 RNA Polymerase (Stratagene) in the presence of transcription buffer (350 mM HEPES,

pH=7.5, 30 mM MgCl₂, 2 mM spermidine, and 40 mM dithiothreitol), 40 units of RNasin, 7 mM each of the four NTPs, and 60 units of T7 polymerase (1.5 units/μg).

4) In general, one should perform three 40μl reactions for each RNA of interest, placing in each 1.5 ml Eppendorf tube 2μg of linearized plasmids/amplified products.

5) Following incubation for 2 h at 37 °C, the reactions are pooled, purified by a cycle of acid phenol/chloroform extraction, precipitated according to standard protocols, and resuspended in 40 μl RNase-free water. Usually, this approach yields the desired 15μg of transcribed RNA for the following steps (see Section 3.2). It is strongly recommended to check their production/integrity on a standard agarose gel.

Loading the beads with RNA.

1) The 500 pmoles of T7-transcribed RNA (approx. 15 μg of a 100mer RNA) previously dissolved in 40 μl of water are placed in a 1.5 ml Eppendorf test tube.

2) To each sample, add 360 μl of a 5 mM Sodium m-Periodate (Sigma, #S11448) solution in 0.1 mM NaOAc pH=5.0 (to prepare 50ml of this reagent dissolve 53mg of Sodium m-Periodate in 0.1M NaOAc pH=5). This reagent has to be prepared fresh each time.

3) This 400 μl reaction mix is incubated for 1 hour in the dark (each test tube wrapped in aluminium foil) at room temperature in a rotator wheel.

4) Each RNA is then ethanol precipitated according to standard protocols, washed once with EtOH 70%, and resuspended in 100 μl of 0.1 M NaOAc, pH 5.0. Be careful not to lose the very small pellet!

5) in the meantime, take 100 μl of adipic acid dehydrazide agarose bead 50% slurry (Sigma, #A0802) for each RNA sample to be conjugated and place them in a 15 ml

Falcon tube. Wash the beads four times with 10 ml of 0.1 M NaOAc pH 5.0. Each time spinning down at 3000 rpm for 5 minutes in a clinical centrifuge (we use a 581R, Eppendorf).

6) After the final wash, resuspend the beads pellet at the bottom of the 10 ml tube calculating 300 μ l of 0,1 M NaOAc pH= 5.0 for each RNA sample prepared in step 4.

7) After mixing well, take separate 300 μ l aliquots and add them to the 100 μ l of periodate-treated RNA from step 4.

8) incubate overnight in the dark at 4°C the resulting 400 μ l mix on a rotator (each test tube wrapped in aluminium foil).

Incubation with protein mix (Buffer A).

1) Pull down the beads incubated overnight at 4000 rpm for 5 min using a bench top Eppendorf minifuge (from now on we always use a Centrifuge 5415 D). RNA loaded beads will often tend to cling to the side of the test tube, shake them off until they collect at the bottom of the tube by tapping gently the eppendorf tube on the side of the rack.

2) Throw out the supernatant and wash the RNA loaded beads twice with NaCl 2M. Then, spin down at 4000 rpm for 5 min in Eppendorf minifuge.

3) Wash the beads three times with 1.0 ml of Sol.D 1X (20 mM Hepes pH=7.9, 100 mM KCl, 0.2 mM EDTA pH=8.0, 100 mM DTT, 6% v/v Glycerol), spinning down at 4000 rpm for 5 min and discarding supernatant each time.

4) During the last spin down described in step 3 prepare the following 500 μ l mix for each RNA sample to be tested:

a) 50 μ l Sol.D 10X (200 mM Hepes pH=7.9, 2 mM EDTA pH=8.0, 1M DTT, 60% v/v Glycerol).

- b) 50 μ l KCl 1M (add separately).
- c) 100 μ l NE (approx. 10-15 μ g/ μ l) or any other protein mix of interest.
- d) 300 μ l H₂O.
- e) Heparin (200 μ g/ μ l stock) to desired final concentration (0.5-2.5-5.0 μ g/ μ l of the final volume).

5) Add 500 μ l Nuclear Extract/Protein mix to the individual eppendorfs and mix gently by manually shaking the tube.

6) Incubate on a rotor for 30 min. at RT.

7) Spin down the beads at 4000 rpm with an Eppendorf minifuge and remove as much protein mix as possible.

8) Wash the beads 4 times with 1.5 ml of Sol.D 1X by incubating them each time for 5 min on a rotating wheel at room temperature, each time spinning them down with an Eppendorf minifuge at 4000 rpm to remove the supernatant.

9) add 50 μ l of SDS loading buffer, denature, and load sample on a SDS-PAGE gel (for loading, it is recommended to use a glass Hamilton syringe in order to avoid loading the beads in the well).

Incubation with protein mix (Buffer B).

1) Pull down the beads incubated overnight at 4000 rpm for 5 min using an Eppendorf minifuge. RNA loaded beads will sometimes tend to cling to the side of the test tube, to shake them off gently tap the eppendorf tube on the rack side.

2) Throw out the supernatant and wash the RNA loaded beads twice with NaCl 2M. Then, spin down at 4000 rpm for 5 min in Eppendorf minifuge.

- 3) Wash the beads three times with 1.0 ml of Buffer B (5 mM HEPES pH= 7.9, 1 mM MgCl₂, 0.8 mM Magnesium acetate), spinning down at 4000 rpm for 5 min and discarding supernatant each time.
- 4) During the last spin down described in step 3 prepare the following 500 µl mix for each RNA sample to be tested:
 - a) 50 µl Binding Buffer 10X (50mM Hepes pH=7.9, 10mM MgCl₂, 8mM Mg Acetate, 5.2mM DTT, 7.5mM GTP, 10mM ATP, and 38% v/v Glycerol).
 - b) 100 µl NE (approx. 10-15 µg/µl) or any other protein mix of interest.
 - c) 350 µl H₂O.
 - d) Heparin (200 µg/µl stock) to desired final concentration (0.5-2.5-5.0 µg/µl of the final volume).
- 5) Add 500 µl Nuclear Extract/Protein mix to the individual eppendorfs and mix gently by manually shaking the tube.
- 6) Incubate on a rotor for 30 min. at RT.
- 7) Spin down the beads at 4000 rpm with an Eppendorf minifuge and remove as much protein mix as possible.
- 8) Wash the beads 4 times with 1.5 ml of Buffer B (5 mM HEPES pH= 7.9, 1 mM MgCl₂, 0.8 mM Magnesium acetate) by incubating them each time for 5 min on a rotating wheel at room temperature, each time spinning them down with an Eppendorf minifuge at 4000 rpm to remove the supernatant.
- 9) add 50 µl of SDS loading buffer, denature, and load sample on a SDS-PAGE gel (for loading, it is recommended to use a glass Hamilton syringe in order to avoid loading the beads in the well).

Troubleshooting.

Problem	Reason+Solution
Too strong protein binding signals or background in beads too high.	<ul style="list-style-type: none">• Increase Heparin concentration added to the protein mix.• Shorten size of RNA targets bound to the beads (ideal length is normally less than 200 nt.).• Make sure the protein mix added to the mix is NOT cloudy. If, after Heparin addition, the solution does not clear up to near transparency it is advisable to centrifuge briefly (approx. 5min. at 4000 rpm in a tabletop Eppendorf minifuge) and discard any eventual pellet.• Control (ie. empty) beads have the tendency to absorb high molecular weight proteins (>100 kDa in MW).
Too weak protein binding signals to beads.	<ul style="list-style-type: none">• Failure of synthesized/synthetic RNAs binding to beads. Use fresh reagents. If problem persists, binding reactions to beads can be followed using a radioactively labelled RNA on a small experimental scale.• Decrease Heparin concentration added to the protein mix.• Increase protein extract concentration added to the protein mix.• Use Binding Buffer B (section 3.3). This buffer tends to yield more protein signals than Sol.D (warning: it will also raise background binding levels especially with empty beads, if used as control).
Small or no differences detected in band intensities between different samples.	<ul style="list-style-type: none">• Increase the size of RNA sequence analyzed (max. length is >1000 nt. in length).• Compare RNA sequences that display <u>clear</u> functional differences (ie. gross deletion mutants etc.).• Pre-incubate the protein mix with semi-specific RNA competitors (in addition to Heparin).• Use a mass-spec compatible silver stain procedure to stain SDS-PAGE gels.