Protocol for RNA Affinity Purification with raPOOLs

Materials provided:
raPOOL, 10 nmol (freeze-dried)
Negative control raPOOL, 10 nmol (freeze-dried)
Nuclease-free water

Product description:
raPOOLs are used for the pulldown of ribonucleic acids (RNA) and their associated proteins or nucleic acids (RNA/DNA). One raPOOL consists of 30 single-stranded 3'-biotinylated DNA oligonucleotides (20 base length) designed to hybridize along the entire length of the target RNA. The high-affinity biotin-streptavidin association allows the raPOOL-bound RNA and its associated proteins/nucleic acids to be purified with the use of streptavidin-conjugated magnetic beads.

The protocol below describes how to perform RNA affinity purification using raPOOLs.

Additional references:
Chu C, Zhang QC, da Rocha ST, Flynn RA, Bharadwaj M, Calabrese JM, Magnuson T, Heard E, Chang HY. Systematic discovery of Xist RNA binding proteins. Cell. 2015, 161(2):404-16.

McHugh CA, Chen CK, Chow A, Surka CF, Tran C, McDonel P, Pandya-Jones A, Blanco M, Burghard C, Moradian A, Sweredoski MJ, Shishkin AA, Su J, Lander ES, Hess S, Plath K, Guttman M. The Xist lncRNA interacts directly with SHARP to silence transcription through HDAC3. Nature. 2015, 521(7551):232-6.

Leucci E, Vendramin R, Spinazzi M, Laurette P, Fiers M, Wouters J, Radaelli E, Eyckerman S, Leonelli C, Vanderheyden K, Rogiers A, Hermans E, Baatsen P, Aerts S, Amant F, Van Aelst S, van den Oord J, de Strooper B, Davidson I, Lafontaine DLJ, Gevaert K, Vandesompele J, Mestdagh P, and Marine JC. Melanoma addiction to the long non-coding RNA SAMMSON. Nature. 2016, 531, 518-22.

Roth A, Diederichs S. Molecular biology: Rap and chirp about X inactivation. Nature. 2015, 521(7551):170-1.

Other materials required for RNA affinity purification (not provided):
Cell culture reagents
Streptavidin-coated magnetic beads e.g. Dynabeads MyOne Streptavidin C1 (Thermo Fisher)
*Hybridization buffer – 750 mM NaCl, 1% SDS, 50 mM Tris-Cl pH 7.0, 1 mM EDTA, 15% formamide (store in the dark at 4 °C). Add protease and nuclease-inhibitors fresh before use.
*Lysis buffer – 50 mM Tris-Cl pH 7.0, 10 mM EDTA, 1% SDS with protease and nuclease inhibitors added in fresh before use.
*Wash buffer – 2X Sodium chloride and sodium citrate (SSC), 0.5% SDS, with protease and nuclease inhibitors added in fresh before use.
Sonicator
Cross-linking reagents – 3% fresh formaldehyde, 1.25 M glycine for quenching
Magnet
Temperature-controlled mixer
For RNA elution: TRIzol, Chloroform, Isopropanol, Glycogen (20 µg/µl), Proteinase K (20 mg/ml) and Proteinase K buffer – 1% SDS, 50 mM EDTA, 10 mM Tris-Cl pH 7.4
For DNA elution: RNase A (10 mg/ml), RNase H (10 U/µl), nucleic acid spin columns, Proteinase K (20 mg/ml) and Proteinase K buffer, DNA Elution Buffer – 50 mM NaHCOO3, 1% SDS
For Protein elution: Benzonase, Tricholoroacetic acid (TCA), cold acetone, Benzonase Elution Buffer – 20 mM Tris pH 8.0, 0.05% N-lauroylsarcosine, 2 mM $MgCl_2$, 0.5 mM TCEP (for analysis by mass spectrometry); 2X SDS Sample buffer (for analysis by western blot)

*Depending on your applications, buffer base, detergent, salt and pH may be altered.

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Protocol:

1. ***Preparation of lysate***
    a. Lysates may be derived from cells or tissues. For cellular lysates, 20 million cells are recommended per pulldown. However, this may be varied according to the copy number of your target RNA.
    b. Cross-linking. *Prior to lysis, samples may be subject to cross-linking with UV, formaldehyde or glutaraldehyde to preserve nucleic acid-protein interactions. RAP may also be performed without cross-linking, refer Leucci et al., 2016.*
        i. Trypsinize cells and wash with PBS.
        ii. Resuspend cells in 3% formaldehyde dissolved in PBS at 1 million cells per ml. Agitate at room temperature for 10 min. *Formaldehyde exposure may be increased to preserve more transient interactions.*
        iii. Quench cross-linking reaction by adding 0.1 vol of 1.25 M glycine and agitate for 5 min.
        iv. Spin cells down at 2000 g for 5 min at 4°C. Remove supernatant.
        v. Resuspend cells in cold PBS at 1 million cells per ml and centrifuge at 2000 g for 5 min at 4°C. Remove supernatant.
        vi. Resuspend 20 million cells in 1 ml cold PBS and spin in Eppendorf tubes at 2000 g, 3 min, 4°C.
        vii. Remove all PBS without disturbing the pellet and snap freeze cell pellets in liquid nitrogen. These pellets can be stored indefinitely at -80°C.
    c. Cell lysis.
        i. Thaw and resuspend cell pellets in 1 ml cold lysis buffer prior to sonication.
    d. Sonication. *It is recommended to optimize sonication time and intensity to ensure that the majority of DNA/RNA is fragmented*
        i. Perform sonication with the instrument of choice (e.g. Bioruptor® or Covaris), ensuring temperatures are kept below 10°C.
        ii. Collect 5-10 µl fractions over several time-points.
        iii. To these fractions, add 90 µl Proteinase K buffer and 5 µl Proteinase K. Incubate at 50°C for 45 min.
        iv. Extract DNA/RNA by spin-column purification or sodium chloride (0.2 M final concentration) and ethanol precipitation.
        v. Run DNA/RNA samples on a 1.5% agarose gel together with a ladder to analyze fragmentation. Choose the sonication conditions where majority of DNA/RNA are within 100-500 bp.
        vi. After sonication, centrifuge the cell lysate at 14 000 g for 10 min at 4°C and keep supernatant for further processing. These can be flash frozen and stored at -80°C.
2. ***Preparation of beads***
    a. Prepare beads fresh just before use. For pre-clearing of lysate, use 30 µl of beads per ml of lysate. For RNA affinity pulldown, use 100 µl of beads per ml of lysate.
    b. Wash beads 3 times with lysis buffer, using a magnet to separate the beads from buffer each time.
    c. Resuspend beads in original volume with lysis buffer.
3. ***Pre-clear lysate (optional)*** *It is recommended that lysates be pre-cleared with the washed magnetic beads to reduce non-specific binding.*
    a. Add 2 ml of hybridization buffer to 1 ml of lysate.
    b. Add 30 µl of washed beads.
    c. Incubate with agitation for 30 min at 37°C.
    d. Remove the beads with the magnet; lysate is now ready for hybridization. Note that it is important to keep a fraction of the pre-cleared lysate to serve as "input" control.
4. ***Resuspension of raPOOL***
    a. Resuspend 10 nmol of raPOOL in 100 µl nuclease-free water (final concentration: 100 pmol / µl).
5. ***Hybridization***
    a. Add raPOOL to lysate. The recommended amount is 100 pmol raPOOL per ml of lysate obtained from 20 million cells, though this can be varied depending on your target RNA.
    b. Hybridization is carried out for 4 h at 37°C with agitation.
6. ***Affinity purification***

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a. Add washed beads to raPOOL-treated lysate. The recommended amount of beads is 100 µl per 100 pmol of raPOOL.

b. Incubate with agitation for 30 min at 37°C.

c. Separate beads from lysate with the magnet. Keep lysate as "input after hybridization" to determine extent of target RNA depletion from the lysate.

d. Wash beads in 1 ml of 37°C pre-warmed wash buffer 5 times. During each wash, agitate the tube for 5 min at 37°C, then magnetically separate the lysate from beads, aliquoting the lysate into a clean tube each time.

e. In the last wash, resuspend beads well in 1 ml of wash buffer and aliquot into fractions for RNA, DNA and protein analysis.

7. Elution of RNA

a. Using the magnet to separate the beads, resuspend beads in 95 µl Proteinase K buffer. For input samples, dilute a 10 µl fraction with 85 µl of Proteinase K buffer.

b. Add 5 µl Proteinase K and incubate at 50°C for 45 min with agitation.

c. Add 1 ml of TRIzol and snap freeze or proceed on to RNA isolation.

d. Add 500 µl chloroform to TRIzol-treated samples and vortex for 10 s.

e. Incubate at room temperature for 10 min.

f. Spin at 14 000 g for 15 min at 4°C.

g. Pipette aqueous phase containing RNA carefully and dispense into a new tube.

h. Add 0.5 vol isopropanol and mix well.

i. Incubate for 10 min at room temperature

j. Centrifuge at 14 000 g for 30 min.

k. Remove supernatant and wash pellet with 80% EtOH.

l. Dry pellet and resuspend in nuclease-free water or Tris-based buffer.

8. Elution of DNA

a. Add 10 µl RNase A and 10 µl RNase H to 1 ml of DNA Elution Buffer and mix well.

b. Resuspend beads in 150 µl RNase-containing DNA elution buffer. Dilute 10 µl of input samples in 140 µl of the same. Incubate at 37°C for 30 min with agitation.

c. Separate lysates from beads and aliquot into fresh tubes.

d. Add another 150 µl of RNase-containing DNA elution buffer, resulting volume: 300 µl. Incubate at 37°C for 30 min with agitation.

e. Add 15 µl Proteinase K to each sample and incubate at 50°C for 45 min with agitation.

f. Purify DNA using a spin-column and elute in 30 µl nuclease-free water or Tris-based buffer.

9. Elution of proteins for analysis by mass spectrometry

a. Magnetically separate the beads from the wash buffer and resuspend in 1 ml Benzonase Elution Buffer.

b. Add 125 U benzonase non-specific nuclease and incubate for 2 h at 37°C, agitating at 1100 rpm, 30 s on and 30 s off.

c. Magnetically separate beads and transfer supernatant to a fresh tube. Repeat this step up to 6 times to completely eliminate all beads.

d. To precipitate proteins, add tricholoroacetic acid (TCA) to 10% final concentration.

e. Incubate at 4°C overnight.

f. Centrifuge at 16 000 g for 30 min to precipitate proteins.

g. Aspirate supernatant and replace with 1 ml cold acetone.

h. Centrifuge at 16 000 g for 15 min.

i. Aspirate supernatant and dry pellet in fume hood. Pellet can be stored at -20°C till processing for mass spectrometry analysis.

For elution of proteins for western blot analysis, beads may be resuspended in 2X SDS sample buffer and boiled at 95°C for 10 min. Centrifuge at low speed before loading supernatant into the SDS-PAGE gel.

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