Experimental Protocol

Identification of Direct RNA-binding Proteins using RNA Antisense Purification with Mass Spectrometry (RAP-MS)

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Abstract:

RNA antisense purification with mass spectrometry (RAP-MS) is a method for identification of direct RNA-protein interactions present in living cells. This protocol describes the experiments performed in McHugh et al., 2015 for the purification of proteins that bind directly to long non-coding RNAs. Experiments can be performed using whole cell lysate or nuclear extract, and yield a list of proteins that bind the target RNA in living cells.

Please cite: McHugh et al. *Nature* 2015 doi:/10.1038/nature14443

For the most updated protocols and information on the RAP-MS method, please visit the Guttman Laboratory website at http://lncRNA.caltech.edu.

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1 Materials

1.1 Equipment

Sonicator (Branson Sonifier Model 102C with microtip)

Thermomixer (Eppendorf Thermomixer C)

Real-Time PCR instrument (Roche LightCycler 480)

Magnetic rack for Eppendorf tubes (Life Technologies Dynamag-2 #12321D)

Magnetic rack for 15 mL tubes (Life Technologies Dynamag-15 #12301D)

Magnetic rack for 96 well plates (Life Technologies Dynamag-96 #12331D)

Refrigerated microcentrifuge (Eppendorf 5424R)

Refrigerated tabletop centrifuge (Beckman Allegra 25R or Allegra X-14R)

Spectrophotometer or plate reader (Bioteck Cytation3)

Glass dounce homogenizer (Kimble Chase, Kontes 2 mL size #885300-0002 or 15 mL size #885300-0015 as needed)

UV Crosslinker with 254 nm bulbs (Spectrolinker XL-1500)

Freezer at -20 ˚C (Frigidaire)

Freezer at -80 ˚C (New Brunswick U410)

Speed-Vac vacuum lyophilizer (Labconco CentriVap)

1.2 SILAC Medium Recipes

<u>Heavy ES Cell SILAC Medium</u>

Custom DMEM/F-12 without lysine and arginine (Dundee Cell Products)

0.398 mM heavy arginine (Sigma #608033-500MG)

0.798 mM heavy lysine (Cambridge Isotope Laboratories #CNLM-291-H-0.25G)

0.2 mg/ml proline (Sigma #P5607-25G)

0.5X B-27 supplement (Life Technologies #17504-044)

1X N2 supplement (Life Technologies #17502-048)

2 mg/ml bovine insulin (Sigma #I1882-100MG)

1.37 μg/ml progesterone (Sigma #P0130-25G)

5 mg/ml BSA Fraction V (Life Technologies #15260037)

0.1 mM 2-mercaptoethanol (Sigma #M3148-25ML)

5 ng/ml murine LIF (GlobalStem #GSR-7001)

0.1 μM PD0325901 inhibitor (SelleckChem #S1036)

0.3 μM CHIR99021 inhibitor (SelleckChem #S2924)

Light ES Cell SILAC Medium

Custom DMEM/F-12 without lysine and arginine

0.398 mM light arginine (Sigma #A8094-25G)

0.798 mM light lysine (Sigma #L5501-25G)

0.2 mg/ml proline

0.5X B-27 supplement

1X N2 supplement

2 mg/ml bovine insulin

1.37 μg/ml progesterone

5 mg/ml BSA Fraction V

0.1 mM 2-mercaptoethanol

5 ng/ml murine LIF

0.1 μM PD0325901 inhibitor

0.3 μM CHIR99021 inhibitor

1.3 Buffer Recipes

For cell harvesting and crosslinking:

1X Phosphate Buffered Saline (Life Technologies #10010-023)

For whole cell lysate preparation:

Total Cell Lysis Buffer

10 mM Tris-HCl pH 7.5 (Life Technologies #15567-027)

500 mM LiCl (Sigma #L7026)

0.5% dodecyl maltoside (DDM, Sigma #D4641)

0.2% sodium dodecyl sulfate (SDS, Ambion #AM9820)

0.1% sodium deoxycholate (Sigma #06750)

200X DNase Salt Solution

500 mM $MgCl_2$ (Life Technologies #AM9530G)

100 mM $CaCl_2$ (Sigma #21115)

1.5X Hybridization Buffer

15 mM Tris-HCl pH 7.5

7.5 mM EDTA (Life Technologies #15575-020)

750 mM LiCl

0.75% DDM

0.3% SDS

0.15% sodium deoxycholate

6 M urea (Sigma #U0631-500G)

3.75 mM Tris(2-carboxyethyl)phosphine (TCEP, Sigma #646547)

For nuclear lysate preparation:

Cell Lysis Buffer (Nuclear I)

10 mM HEPES pH 7.4 (Teknova #H1030)

20 mM KCl (Ambion #AM9640G)

1.5 mM $MgCl_2$

0.5 mM EDTA

1 mM TCEP

0.5 mM phenylmethylsulfonyl fluoride (PMSF, Sigma #93482)

Cell Lysis Buffer (Nuclear I with 0.1% DDM)

10 mM HEPES pH 7.4

20 mM KCl

1.5 mM $MgCl_2$

0.5 mM EDTA

1 mM TCEP

0.5 mM PMSF

0.1% DDM

Cell Lysis Buffer (Nuclear II)

20 mM Tris-HCl pH 7.5

50 mM KCl

1.5 mM MgCl2

2 mM TCEP

0.5 mM PMSF

0.4% sodium doxycholate

1% DDM

0.1% N-lauroylsarcosine (NLS, Sigma #L7414)

2X Hybridization Buffer

20 mM Tris-HCl pH 7.5

10 mM EDTA

1 M LiCl

1.0% DDM

0.4% SDS

0.2% sodium deoxycholate

8 M urea

5 mM TCEP

For RAP captures:

<u>1X Hybridization Buffer</u>

10 mM Tris-HCl pH 7.5

5 mM EDTA

500 mM LiCl

0.5% DDM

0.2% SDS

0.1% sodium deoxycholate

4 M urea

2.5 mM TCEP

<u>Benzonase Elution Buffer</u>

20 mM Tris-HCl pH 8.0 (Life Technologies #15568-025)

0.05% NLS

2 mM $MgCl_2$

0.5 mM TCEP

<u>NLS Elution Buffer</u>

20 mM Tris-HCl pH 8.0

10 mM EDTA

2% NLS

2.5 mM TCEP

1.4 Additional Materials and Reagents

Custom biotinylated oligonucleotide probes (Integrated DNA Technologies)

Cell lifters (Corning #3008)

Murine RNase Inhibitor (New England Biolabs #M0314L)

Liquid nitrogen

Turbo DNase, 2U/µl (Ambion #AM2238)

Microcentrifuge tubes (VWR #87003-294)

15 ml conical tubes (Thermo Fisher Scientific #339650)

PCR strip tubes (USA Scientific #1402-4700)

0.5 M EGTA (BioPlus #40520008-2)

Streptavidin-coated magnetic beads (Life Technologies, Dynabeads MyOne Streptavidin C1 #65002)

Protease Inhibitor Cocktail Set III, EDTA free (EMD Millipore #539134-SET)

Benzonase nuclease (EMD Millipore #71206)

Proteinase K (New England Biolabs #P8107S)

Trichloroacetic acid (Ricca Chemical Company #8693-4)

Acetone (Macron #2440-02)

SILANE beads (Invitrogen, Dynabeads MyOne #37002D)

RLT Buffer (Qiagen #79216)

100% ethanol (Koptec #V1016)

UltraPure distilled water (Life Technologies #10977-015)

HiPPR detergent removal columns (Life Technologies #88306)

Trypsin (Promega #V5111)

Endopeptidase Lys-C (Wako #125-0506)

$CaCl_2$ (Sigma #C5080-500G)

100 mM Tris-HCl pH 8.5 (MP Biomedicals #819620)

Formic acid (Fluka #94318-250ML-F)

Acetonitrile (Sigma #34851-4L)

2 Probe Generation

2.1 Oligonucleotide Design and Synthesis

1. Design 90-nucleotide oligos that sequentially cover the target RNA sequence without overlapping. To avoid off-target hybridization, use BLAST to remove sequences that contain a perfect 30 base pair match or an imperfect (90%) identity 60 base pair match with another transcript or genomic region.

Compare the oligos to RepeatMasker annotations and remove probes that contain more than 30 bases that overlap with a repeat annotation.

2. Order oligos with 5’ biotin standard modification from an oligonucleotide synthesis company such as Integrated DNA Technologies. Request individual probes be resuspended at 500 micromolar or 1 millimolar concentration depending on the synthesis scale.

2.2 Preparation of Probe Stocks

1. Dilute probe stocks 1:100 from 96-well plates or individual tubes into UltraPure water. Mix all individual probes together to create a probe stock to cover the length of the target RNA.
2. Make several aliquots of probe stock mixtures and store at -20 ˚C. Avoid multiple freeze-thaw cycles.

3 Lysate Preparation

3.1 SILAC Cell Culture

1. Initiate culture of cell line of interest.
2. Split into two parallel cultures in Heavy and Light SILAC Medium. Sample SILAC media recipes for mouse ES cell culture are given here, but SILAC medium should be adapted to fit the requirements of the desired cell line.
3. Grow cells for at least 3 passages in SILAC Medium.
4. If desired, test SILAC incorporation using mass spectrometry. Incorporation should be >95% for peptides from strain grown in Heavy SILAC Medium.

3.2 Cell Harvesting and Crosslinking

1. Grow adherent cells on 15 cm tissue culture plate.
2. Remove medium from plate and replace with 10 ml ice-cold PBS.

3. Rock gently for 10 seconds then remove PBS wash.
4. Add 10 ml ice-cold PBS to plate.
5. UV crosslink plate of cells in Spectrolinker at 254 nm wavelength with 0.8 J/cm^2 (instrument setting: 8000 x 100 uJ/cm^2).
6. Remove plate of cells from crosslinker and place on ice.
7. Scrape cells from plate using cell lifter and transfer to sterile 15 ml tube.
8. Centrifuge at 1000*g* for 5 minutes at 4 ˚C to pellet cells.
9. Remove supernatant and resuspend cells in 1 ml cold PBS, pipetting gently to break up pellet. Add cold PBS as needed to resuspend to 20 million or 50 million cells per 1 ml of buffer.
10. Aliquot 1 ml of PBS/cell mixture into microcentrifuge tube and centrifuge at 1000*g* for 5 minutes at 4 ˚C.
11. Remove supernatant by aspiration or pipetting.
12. Flash freeze pellets in liquid nitrogen and store at – 80 ˚C.

3.3 Cell Lysis

For preparation of whole cell lysate from 20 million cells:

1. Chill all buffers and keep cells/lysate on ice unless otherwise specified.
2. Resuspend each frozen cell pellet by pipetting up and down in 900 μl cold Total Cell Lysis Buffer.
3. Add 1X protease inhibitor cocktail (4.6 μl) and 920 U of murine RNase inhibitor (23 μl).
4. Incubate sample on ice for 10 minutes. During this time, pass cell sample 3 – 5 times through a 26-gauge needle attached to a 1 ml syringe.
5. Sonicate samples with a microtip set at 5 watts power for a total of 30 seconds in intermittent pulses (0.7 seconds on, 1.3 seconds off).
6. Add 1X DNase salt stock (4.8 μl) and 20 U TurboDNase (15 μl).
7. Incubate for 10 minutes at 37 ˚C.

8. Return sample to ice and immediately add 10 mM EDTA (19.6 μl), 5 mM EGTA (9.8 μl), and 2.5 mM TCEP (4.9 μl).
9. Mix lysate with 2X the sample volume of 1.5X Hybridization Buffer (split lysate into two microfuge tubes containing 490 μl each, then add 980 μl of 1.5X Hybridization Buffer to each tube).
10. Incubate for 10 minutes on ice.
11. Centrifuge at 16,000*g* for 10 minutes at 4 ˚C.
12. Transfer supernatant to fresh tube and flash freeze in liquid nitrogen.
13. Store lysate at -80 ˚C.

For preparation of nuclear lysate from 50 million cells:

1. Resuspend 50 million cell pellet in 1 ml of Cell Lysis Buffer (Nuclear I).
2. Centrifuge at 3,300*g* for 10 minutes at 4 ˚C.
3. Discard supernatant and resuspend cell pellet in 1 ml of Cell Lysis Buffer (Nuclear I with 0.01% DDM).
4. Incubate for 10 minutes on ice.
5. Transfer sample to a dounce tissue homogenizer and use the B (small clearance) pestle 20 times to break cells.
6. Transfer sample back to microcentrifuge tube.
7. Pellet nuclei by centrifugation at 3,300*g* for 10 minutes at 4 ˚C.
8. Discard supernatant and resuspend pellet in 580 μl of Cell Lysis Buffer (Nuclear II).
9. Incubate for 10 minutes on ice.
10. Sonicate with microtip using 5 watts of power (25% duty) for 60 seconds total in pulses of 0.7 seconds on, followed by 3.3 seconds off.
11. Add 1X DNase salt solution (3.75 μl) and 330 U TurboDNase (165 μl).
12. Incubate for 12 minutes at 37 ˚C.
13. Mix lysate with equal volume of 2X Hybridization Buffer (750 μl).
14. Centrifuge at 16,000*g* for 10 minutes at 4 ˚C.
15. Transfer supernatant to fresh tube and flash freeze in liquid nitrogen.

16. Store lysate at – 80 ˚C.

4 RAP-MS Captures

Note: Volumes listed are for 200 million cell sample per capture. Captures can be scaled up or down depending on the abundance of the target RNA.

4.1 Pre-clearing Lysate

1. Warm frozen aliquots of lysate to 37 ˚C using a thermomixer.
2. Pool samples to one tube.
3. Transfer 1.2 ml of streptavidin-coated magnetic beads per 200 million cell sample into a fresh microfuge tube.
4. Separate on magnetic rack and remove storage buffer from beads.
5. Resuspend beads in 1 ml of 10 mM Tris-HCl pH 7.5.
6. Separate on magnetic rack and remove supernatant.
7. Repeat washes for a total of 4 washes in Tris, and 2 washes in 1X Hybridization Buffer.
8. Magnetically separate and remove last wash from beads.
9. Resuspend beads in lysate by pipetting gently.
10. Incubate for 30 minutes at 37 ˚C with intermittent mixing at 1100 rpm on thermomixer (30 seconds shaking, 30 seconds off).
11. Magnetically separate beads and transfer supernatant to fresh tubes. Repeat this step to transfer lysate to fresh tubes a second time.
12. Remove sample of 100,000 cells worth of lysate and transfer to PCR strip tube. This is the RNA input sample.

4.2 Hybridization, Capture and Protein Elution

1. Denature appropriate quantity of probe by heating at 85 ˚C for 3 minutes, then place on ice. (Example: 20 ug probe per 200 million cells for Xist.)

2. Mix lysate and probe.
3. Incubate for 2 hours at 67 ˚C with intermittent mixing at 1100 rpm on thermomixer (30 seconds shaking, 30 seconds off).
4. During the 2 hour incubation, prepare streptavidin beads as previously described (4 washes with 10 mM Tris-HCl pH 7.5, 2 washes with 1X Hybridization Buffer).
5. Magnetically separate beads and remove final wash from beads.
6. At the end of the 2 hour incubation, remove sample of 100,000 cells worth of lysate and transfer to PCR strip tube. This is the RNA input + probe sample.
7. Resuspend beads in lysate.
8. Incubate for 30 minutes at 67 ˚C with intermittent mixing at 1100 rpm on thermomixer (30 seconds shaking, 30 seconds off).
9. Magnetically separate beads and remove supernatant. Take sample of 100,000 cells worth of supernatant and transfer to PCR strip tube. This is the RNA flow-through sample.
10. Wash beads 3 to 6 times, with at least one bead volume of 1X Hybridization Buffer per wash. Incubate each wash for 5 minutes at 67 ˚C.
11. Remove a sample of beads between 0.5% and 1% of the total volume and transfer to a PCR strip tube. This is the RNA elution sample.
12. Magnetically separate remaining beads and remove supernatant.
13. Resuspend beads in 1 ml of Benzonase Elution Buffer.
14. Add 125 U of benzonase non-specific nuclease to sample.
15. Incubate for 2 hours at 37 ˚C with intermittent mixing at 1100 rpm on thermomixer (30 seconds shaking, 30 seconds off).
16. Magnetically separate beads and transfer supernatant to a fresh microcentrifuge tube. Repeat this step for a total of 6 transfers to fresh tubes to remove all traces of streptavidin beads. This is the protein elution sample.

4.3 RNA Elution and Analysis

1. Take the RNA elution sample of beads from previous Step 11 and separate on magnetic rack.

2. Remove supernatant and resuspend beads in 20 μl of NLS Elution Buffer.
3. Heat samples for 2 minutes at 95 ˚C.
4. Magnetically separate and transfer supernatant containing eluted RNA to a fresh PCR strip tube.
5. Take the previously collected samples (RNA input, input + probe, and flow-through) and dilute to each sample to 20 μl total volume with NLS Elution Buffer.
6. Add 1 mg/ml Proteinase K to each sample.
7. Incubate for 1 hour at 52-55 ˚C.

Note: At this point, RNA samples can be frozen at -20 ˚C for short term storage or at -80 ˚C for long term storage.

8. Perform SILANE bead cleanup using the following steps (for a 20 μl sample):
    a. Aliquot 20 μl of beads per sample into PCR strip tubes.
    b. Magnetically separate beads and remove storage buffer.
    c. Resuspend beads in 60 μl RLT Buffer.
    d. Transfer beads to 20 μl sample and mix well. Add 120 μl 100% ethanol.
    e. Wait 2 minutes for sample to bind beads.
    f. Wash beads 2 times with 150 μl of 70% ethanol.
    g. Remove supernatant and allow to air dry approximately 5 minutes.
    h. Elute RNA in desired buffer
9. In this case, elute RNA samples by adding 26 μl of 1X TurboDNase Buffer
10. Leave beads in tube. Add 1 μl of murine RNase inhibitor and 3 μl of TurboDNase to each sample (30 μl total reaction volume).
11. Incubate for 20 minutes at 37 ˚C.
12. Perform a second SILANE cleanup using beads already in the tube
    a. Add 90 μl RLT Buffer to each 30 μl sample.
    b. Add 180 μl of 100% ethanol and mix well.
    c. Wait 2 minutes for sample to bind beads.
    d. Wash beads 2 times with 70% ethanol.

   e. Remove supernatant and allow beads to air dry approximately 5 minutes.
   f. Elute in 10 μl of UltraPure water.

13. Analyze RNA samples using Agilent Bioanalyzer or by qPCR.

4.4 Protein Precipitation

1. Add 10% final concentration of tricholoroacetic acid (TCA) to protein elution sample.
2. Incubate at 4 ˚C overnight.
3. Centrifuge at 16,000*g* for 30 minutes to pellet protein.
4. Remove supernatant and replace with 1 ml of cold acetone.
5. Centrifuge at 16,000*g* for 15 minutes.
6. Remove supernatant and allow pellet to dry in fume hood.
7. Store protein elution sample at -20 ˚C.

5 Mass Spectrometry Sample Preparation

5.1 In-solution Digest of Isolated Proteins

1. Resuspend protein elution sample in 40 μl of freshly prepared 8 M urea dissolved in 100 mM Tris-HCl pH 8.5.
2. Add 3 mM TCEP and incubate 20 minutes at room temperature.
3. Add 11 mM freshly prepared iodoacetamide and incubate for 15 minutes at room temperature in the dark.
4. Digest samples with 0.1 ug endoproteinase Lys-C for 4 hours at room temperature.
5. Dilute samples to final concentration of 2M urea by adding appropriate volume of 100 mM Tris-HCl pH 8.5.
6. Add 1 mM $CaCl_2$ to sample.
7. Digest with 0.1 to 0.5 grams of trypsin overnight at room temperature.

5.2 Peptide Purification

1. Remove detergent from protein samples using HiPPR columns according to manufacturer's instructions.
2. Add 5% formic acid and centrifuge for 1 minute at 16,000*g*.
3. Desalt using Michrom Bioresources peptide MicroTrap column or equivalent. For MicroTrap column, purify peptides using an Agilent 1200 HPLC or equivalent system, with Buffer A (0.2% formic acid) and Buffer B (100% acetonitrile). Alternatively, purify peptides using C18 Stage Tips.
4. Collect fractions containing peptides.
5. Lyophilize peptides in SpeedVac.
6. Store samples at -20 ˚C until ready for mass spectrometry.
7. Resuspend samples in 0.2% formic acid and 5% acetonitrile.

Note: At the end of this protocol, samples are ready for LC-MS measurements using the desired instrument. For example, a nanoflow LC system (Proxeon EASy-nLC1000) coupled to a hybrid linear ion trap Obritrap Elite mass spectrometer (Thermo Fisher Scientific) was used for the experiments described in McHugh et al., 2015. Peptide searches can be performed using MaxQuant version 1.5.0.30 or other desired analysis software.

For more information on mass spectrometry analysis, please visit the Caltech Proteome Exploration Laboratory website at http://pel.caltech.edu.