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**Lysate Preparation**

Polysome Lysis Buffer:

10 ml of polysome buffer (20 mM Tris, Ph= 7.4, 250mM NaCl, 15mM MgCl2)

100 μL of cycloheximide (10 mg/ml)

50 μL Triton-X

10 μL 1 M DTT

For 1 ml of Polysome Lysis Buffer: (Add before lysis)

1 ml polysome lysis buffer

10 μL of 1000X protease inhibitor

12 μL of TurboDNase

10 μL of Rnasin

This protocol does not call for pretreatment of mammalian cells with cycloheximide. See: Ingolia et al. Nature Protocols Vol 7, No. 8, 2012 pg. 1534.

Cell lysis:

1. Aspirate medium from the 15 cm dish and place dish on ice.
2. Rinse dish with 25 ml ice-cold PBS supplemented with 250 μL of cycloheximide.
3. Aspirate the PBS and repeat wash. Make sure to remove all the PBS.
4. Drip on the dish 800 μL of the polysome lysis buffer (no RNase inhibitor if this lysate will be digested).
5. Scrape the cells off the plate with a cell scraper and mix well with the lysis buffer, keeping the cells and the plate cold in ice. Mix the suspension several times with a pipette.
6. Pass lysate five times through a 23G needle to homogenize.
7. Clarify the lysate: centrifuge at max. speed at 4oC for 10 minutes.
8. Transfer the supernatant to a new tube.
9. Dilute the lysate 1:10 and measure the absorbance.
	1. For the polysome profiles on sucrose gradients usually I load at lest 10 OD 260 units (i.e. 800 μL of a lysate with an absorbance at 260 of 1.3 when diluted ten-fold).
	2. Use the same amount of lysate for the pulldown.
10. Snap freeze the lysate in liquid nitrogen. Store at -80C.

Brain Tissue Lysis Protocol

1. Dissect right frontal cortex free of white matter, and determine the exact wet mass in a microcentrifuge tube. (Right frontal cortex of P35 mice brain (~25mg) . [Note: This step always on dry ice].
2. Rinse homogenizer with RNAse free water (3/4 times) – keep it at cold room.
3. Make fresh Polysome Lysis Buffer –keep it cold.
4. Homogenize ~25mg brain tissue with 1ml polysome lysis buffer using dounce homogenizer (20 strokes with A, 20 strokes with B)
5. Pass the lysate through 23 G needle for ~5 times.
6. Clarify the lysate: centrifuge at max. speed at 4oC for 10 minutes.
7. Transfer the supernatant to a new tube.
8. Dilute the lysate 1:10 and measure the absorbance.
9. Extract total RNA using Qiagen Rneasy column – measure total RNA concentration using qubit and Bioanalyzer.

**POLYSOME PROFILING**

**Useful Links :**

**http://bartellab.wi.mit.edu/protocols/mRNAseq\_ribosomeProfiling\_protocol.pdf**

[**https://www.jove.com/video/1948/eukaryotic-polyribosome-profile-analysis**](https://www.jove.com/video/1948/eukaryotic-polyribosome-profile-analysis)

**https://www.jove.com/video/51455/polysome-fractionation-analysis-mammalian-translatomes-on-genome-wide**

**Aug 2015**

**Preparation of 15-50% sucrose gradients**

* Prepare sucrose gradients one day before use to allow gradients to become continuous. Make sterile15% and 50% sucrose solutions in polysome lysis buffer and store at 4°C .
* In order to pour the gradients, use 5ml pipettes. Add 5.5 mL of 15% sucrose to the bottom of an ultracentrifuge tube. Next add 5.5 mL of 50% sucrose solution under the 5% solution by placing the pipette tip near bottom of tube. We should observe clear separation of layers, indicating that minimal mixing has occurred. Store gradients at 4°C overnight in a flat horizontal position.

**Preparation of lysates from mammalian cells**

* This protocol does not call for pretreatment of mammalian cells with cycloheximide. See: Ingolia et al. Nature Protocols Vol 7, No. 8, 2012 pg. 1534.

Cell lysis:

* Aspirate medium from the 15 cm dish and place dish on ice.
* Rinse dish with 25 ml ice-cold PBS supplemented with 250 μL of cycloheximide.
* Aspirate the PBS and repeat wash. Make sure to remove all the PBS.
* Drip on the dish 800 μL of the polysome lysis buffer (no Superasin inhibitor if this lysate will be digested).
* Scrape the cells off the plate with a cell scraper and mix well with the lysis buffer, keeping the cells and the plate cold in ice. Mix the suspension several times with a pipette.
* Pass lysate five times through a 23G needle to homogenize.
* Clarify the lysate: centrifuge at max. speed at 4oC for 10 minutes.
* Transfer the supernatant to a new tube.
* Dilute the lysate 1:10 and measure the absorbance.
* For the polysome profiles on sucrose gradients usually I load at least 10 OD 260 units (i.e. 800 μL of a lysate with an absorbance at 260 of 1.3 when diluted ten-fold).
* Use the same amount of lysate for the pulldown.
	+ 10. Snap freeze the lysate in liquid nitrogen. Store at -80C.

 **Centrifugation of gradients**

* Thaw polyribosome lysates on ice.
* Using a pipette with the tip placed against the wall of the tube near the surface of the gradient, gently layer 10 OD260 units of the lysate on top of the gradient. ( For a 35 mL gradient 500 μl of lysate is typically loaded. Lysis buffer can be used to assure all gradients are loaded with an equal volume. Lower amounts of material yield better resolution.) Centrifuge the gradients at 35,000 rpm in a SW 40 for 3.5 hr at 4°C.

**Collection of data and fractions**

[**https://www.jove.com/video/1948/eukaryotic-polyribosome-profile-analysis**](https://www.jove.com/video/1948/eukaryotic-polyribosome-profile-analysis)

* Approximately 30 min prior to the completion of the spin attach switch on the Isco Model UA-5 Absorbance/Fluorescence Monitor. Profiles can then be collected in real time and saved for later annotation.
* Fill the syringe pump with 50mL of 80% sucrose with coomasie dye using the reverse flow, rapid setting. Make sure that there are no air bubbles present. Connect the hose from the syringe to the tube piercer and briefly turn on the flow of Fluorinert at 3 mL/min in the forward direction to clear the hose of any air bubbles.
* Carefully remove the centrifuge tubes containing the sucrose gradient from centrifuge rotor and place them on ice.
* To analyze cell extracts, place the gradient tube on the tube piercer. Connect the top of the tube to the outlet and secure. Raise the bottom stage to the centrifuge tube so that the needle pierces the bottom of the tube. Once the needle has protruded through the bottom of the centrifuge tube, begin the flow of in the forward direction at 6mL/min.
* The monitor will record the OD254 starting with the free material followed by 40S ribosomal subunit detection and continuing through to polyribosome complexes. When the end of the polyribosome profile has been reached, turn off the flow of to the centrifuge tube and stop the chart movement of the absorbance/fluorescence monitor.

**Polysome IP**

100 uL of lysate is used as input (total RNA) from which RNA was extracted using the RNeasy Mini kit (Qiagen). The remaining lysate (~600ul) was used for indirect IP of polysomes, we coupled 15uL of rabbit polyclonal anti-HA (abcam, Cat no: ab9110) to lysate with rotation at 4C for 4 hours. We use 300uL of protein G Dynabeads (30mg/mL, Life Technologies) and wash them with 600 uL polysome buffer (3-Times). The conjugated lysate was then added to washed protein G-coated dynabeads and incubated with rotation at 4C overnight. Beads were then washed with polysome buffer (3-times). RNA was extracted from magnetic beads with polysome release buffer (20 mM Tris-HCl pH 7.3, 250 mM NaCl, 0.5 % Triton X-100, 50 mM EDTA) four times for 5 minutes each (140 μL × 4) at 4 deg. RNA from the pooled supernatants (560 μL) was then extracted with the RNeasy Mini Kit (Qiagen) and RNA integrity was assessed using a Bioanalyzer (Agilent

Sims Lab September 20, 2015

**Ribosome Profiling Library Construction Utilizing Clontech Template Switching and Poly-A Tailing**

Polysome Lysis Buffer (w/o enzyme):

10 ml polysome buffer

100 μL cycloheximide (10 mg/ml)

50 μL Triton-X

10 μL 1 M DTT

Polysome Buffer:

20 mM Tris-HCL pH 7.3

250 mM NaCl

15 mM MgCl2

RNA Gel Extraction Buffer:

300 mM sodium acetate (pH 5.5)

1 mM EDTA

For 1 ml of Polysome Lysis Buffer:

1 ml polysome lysis buffer (w/o enzyme)

10 μL 1000X protease inhibitor

10 μL RNAsin

10 μL TurboDNAse

This protocol has been adapted from Ingolia et al. Nature Protocols Vol 7, No. 8, 2012 and the Clontech SMARTer smallRNA-Seq library Preparation manual.

Cell lysis[[1]](#footnote-1):

1. Aspirate media from 10 cm 70% confluent cell-culture dish and place on ice.
2. Rinse with 10 ml ice-cold PBS supplemented with 100 μL of cycloheximide (10mg/mL).
3. Aspirate PBS.
4. Add 800 μL of polysome lysis buffer to dish.
5. Scrape the cells off the plate with a cell scraper and mix well with the lysis buffer, keeping the cells and the plate cold in ice. Mix the suspension several times with a pipette.
6. Pass lysate five to ten times through a 23G needle to homogenize.
7. Clarify the lysate: centrifuge at max. speed at 4oC for 10 minutes.
8. Transfer the supernatant to a new tube.
9. Snap freeze the lysate in liquid nitrogen. Store at -80C.

Sucrose Gradient:

1. Digest 400 μL lysate by addition of 8 μL RNAse I (100U/μL). Let sit for 30 minutes at room temperature with gentle agitation.
2. Prepare solutions of 15% and 50% sucrose in polysome buffer. Add 6 mL 15% to a 15mL ultracentrifuge tube. Carefully layer 6 mL 50% sucrose underneath the 15% layer. Finally, layer 400 μL of digested lysate on top of the 15% sucrose layer.
3. Spin at 4oC for 3.5 hours at 37,000 RPM.
4. Using a sucrose gradient fractionator, isolate the 80S ribosomal peak. We usually find this between our 9th and 12th fraction with a flow rate of 3 ml/min and fraction size of ~ 500 uL.

Phenol-Chloroform Extraction:

1. Supplement the fraction containing the 80S subunit with SDS to a concentration of 1%. Heat to 65oC.
2. Add 600 μl (1 volume) of hot acid-phenol and incubate 5 minutes at 65oC with frequent vortexing.
3. To a add 200 μL of chloroform. Spin at max. speed for 5 minutes.
4. Recover the aqueous phase and add 600 μL of acid phenol. Vortex to mix and transfer to a new tube. Add 200 μL of chloroform. Spin for 5 minutes.
5. To the aqueous phase add 500 μL of chloroform and mix with a pipette.
6. Recover the aqueous phase and add 56 μL of 3 M NaOAc, 556 μL of isopropanol and 2 μL of glycoblue. Precipitate overnight at -20oC.
7. Spin at max. speed at 4oC for 30 minutes. Remove the supernatant and dry the pellet for 5-10 minutes. Resuspend in 5 μL of water.

Size-Selection

1. Add 5 μL of 2x denaturing sample buffer to each RNA sample. Prepare a control oligo sample (markers from Ingolia et al).
	1. Oligo sample:

1 μL of 10 μM lower maker oligo

1 μL of 10 μM upper marker oligo

8 μL of water

10 μL of 2x denaturing sample buffer

1. Denature the samples for 90 secs at 80C.
2. Pre run a 15% polyacrylamide TBE-urea gel at 200 V for 15 min in TBE.
3. Load the samples on a 15% polyacrylamide TBE-urea gel with control oligo sample on either side of the RNA samples.
4. Separate by electrophoresis until the dye reaches the bottom of the gel.
5. Stain the gel with SYBR gold in TBE running buffer.
6. Visualize the gel and excise the 26nt to 34nt region demarcated by the marker oligos from each footprint sample. Place each excised gel slice in a clean nonstick RNase-free 0.5 ml microfuge tube.
7. Extract RNA from the polyacrylamide gel slices.
	1. Following disruption of polyacrylamide gel slices, add 400 μL of RNA gel extraction buffer supplemented with 4 uL SUPERaseIN and leave overnight at 4oC with gentle mixing.
	2. Cut the tip off of a 1,000 μL pipette tip and use to transfer all the liquid and gel slurry into a SPIN-X microfuge tube spin filter.
	3. Spin for 2 minutes at full speed. Transfer the eluate to a new non-stick RNase-free tube.
8. Precipitate RNA by adding 2 μL of GlycoBlue, mixing well, and adding 500 μL of isopropanol. Leave overnight at -20oC.
9. To recover the RNA, spin at max speed at 4oC for 30 minutes. Remove the supernatant and let the blue pellet air dry for 5-10 min.
10. Resuspend in 10 μL of water.

Dephosphorylation:

1. Add 33 μL of RNase-free water to the samples recovered from the gel (these should already be in 10 μL of water).
2. Denature samples for 90 s at 80oC.
3. Equilibrate to 37oC and set up the following reaction:

RNA sample 43 μL

T4 PNK buffer (10x) 5 μL

SUPERase-In (20 U/ul) 1 μL

T4 PNK (10 U/ul) 1 μL

1. Incubate for 1 hr at 37oC
2. Heat inactivate enzyme for 10 min at 70oC.
3. Precipitate RNA by adding 39 μL water, 1 μL glycoblue, 10 μL 3 M NaOAc, mix, transfer to a non-stick tube and add 150 μL of isopropanol.
4. Precipitate overnight at -80oC or on dry ice.
5. Resuspend in 6.5 μL of RNase-free water.

Poly-A Tailing[[2]](#footnote-2):

1. Determine concentration of input. We recommend the use of a BioAnalyzer smRNA chip.
2. Thaw samples and reagents for Polyadenylation and Reverse Transcription. Keep all reagents on ice.
3. Pre-cool thermocycler to 16oC.
4. Bring RNA to a volume of 5uL, diluted in water if necessary (keep cool).
5. Prepare Poly(A) Polymerase/RNase Inhibitor for all reactions, plus 10%. Each reaction requires 0.25μL Poly(A) Polymerase and 0.25 μL RNase Inhibitor. Keep cold! Prepare a minimum of 4 reactions due to the viscosity of the material and small amounts pipetted.
6. Prepare enough Polyadenylation Master Mix for all reactions plus 10%. Each reaction requires:

 Poly(A) Polymerase/ RNase Inihbitor Mix 0.5μL

 smRNA Mix 1 2.5 uL

1. Add 3 μL (4uL if additional ATP was added for high concentration samples) of the ice cold Polyandeylation Master Mix to each chilled RNA sample. Mix by pipetting up and down 5 times.
2. Incubate reaction for 5 minutes in thermocycler pre-cooled to 16 oC. Immediately transfer to ice and proceed to the next step within 5 minutes.

Reverse Transcription and Template Switching

1. Pre-heat thermocycler to 72 oC.
2. After allowing the previous reaction to cool to 4oC for 1 minute, add 1 μL smRNA SMART primer to each tube. Mix by pipetting up and down 5 times while the tubes remain on ice.
3. Incubate for 3 minutes at 72 oC, then immediately transfer to ice and let cool to 4 oC for 2 minutes.
4. During the previous incubation step, prepare enough Reverse Transcription Master Mix for all reactions plus 10%. Each reaction should contain:

 smRNA Mix 2 6.5uL

 RNase Inhibitor 0.5uL

 PrimeScript RT 2μL

1. Add 9 μL of Reverse Transcription Master Mix to each sample on ice, raising to a total volume of 20 uL.
2. Mix by pipetting up and down 5 times.
3. Place tubes in a thermocycler pre-heated to 42 oC. Run the following program.

42 oC 60 min

70 oC 10 min

4 oC Hold

rRNA Depletion

Heat a heat block to ~100oC and do the following:

1. Combine in PCR tube:

RT reaction 10 ul

Subtraction oligo pool 2 ul

1. Heat the mix for 90 s at 100oC in PCR machine. Then place in the hot heat block, and place the block on the bench and allow to cool (annealing). Once the temperature of the block reaches 37oC, place the tubes back in the PCR machine at 37oC and incubate for 15 min.
2. Prepare beads**:**
	1. Vortex the MyOne Streptavidin C1 DynaBeads to resuspend. Transfer to one tube 25 μL and to another 12.5 μL (one set for each sample).
	2. Withdraw all the liquid and resuspend in 1 volume of 1x polysome buffer. Repeat two more times.
	3. Withdraw the final wash from the large bead aliquot and resuspend in 12μL of the subtraction reaction. Incubate at 37oC for 15 minutes.
	4. Use magnets to hold the beads and recover the depleted library. Remove the final wash buffer from the smaller aliquot of beads and resuspend with the depleted library. Incubate at 37oC for another 15 min.
	5. Remove beads and transfer depleted library to new tube (~ 10μL due to liquid loss from beads).

Library Amplification by PCR

1. Preheat thermocyclyer to 98oC.
2. Prepare enough PCR Master Mix for all samples plus 10%:

RNase-free water 12uL

2X SeqAmp Buffer 25uL

SeqAmp Polymerase 1uL

1. Add 38μL to the depleted library recovered from the previous reaction. Additionally add 1 μL of each Forward and Reverse Primer[[3]](#footnote-3) (ensure primer combinations can be used for multiplexing!).
2. Pipette up and down 5 times to mix.
3. Place tubes in pre-heated PCR thermocycler and run the following reaction:

98oC 1 min

12-18 Cycles[[4]](#footnote-4)

 98oC 10 sec

 60 oC 5 sec

 68oC 10 Sec

4oC Hold

Purification of Libraries

1. Allow AMPure XP Beads to come to room temperature.
2. Mix 72μL AMPure XP beads and with 40 μL of each PCR reaction. (If using 25ul PCR rxn, change the vol of AMPure accordingly).
3. Incubate at room temperature for 5 minutes.
4. Apply to the magnetic rack for 5 min or more, until supernatant is clear.
5. Remove supernatant.
6. Without disturbing the beads, wash with 200 μL of freshly prepared 70% ethanol.
7. Wait for 15 seconds, then remove the ethanol and add an additional 200μL 70% ethanol.
8. Remove the ethanol and wait 3-7 minutes until the pellets are dry. Once dry add 20 μL RNAse-free water[[5]](#footnote-5).
9. Wait for 5 minutes, then apply magnet to solution to remove the beads.
10. Transfer supernatant to a new .2mL tube.
11. Repeat steps 2-10 with the following alterations:
	1. In Step 2, use a ratio of 1.2X beads: solution. If 20μL supernatant was recovered in Step 10, use 24 μL beads. Do not use NP40.
	2. In Step 8, resuspend in 12 μL water.

Validation of Library:

 We have used the Qubit dsDNA HS kit in order to quantify libraries prior to sequencing. Additionally, we evaluate libraries with the Agilent BioAnalyzer HS DNA chip. Some libraries require an additional round of 1.2X Ampure beads in order to remove primers and reduce the amount of empty adapter. Our product peak appears at 166 bases with a second peak appearing at 145 bases which is due to an artefact of the template switching reaction. The peak at 145 bases is more pronounced for low input libraries, but can be significantly reduced with Ampure beads. We see evidence of some GC-rich unmappable sequence reads in our raw data that we believe to be attributable to this peak. Although we are uncertain of the origin of these background reads, it hasn’t occurred at a rate >15% of the raw data. The base size depends on which sequencing sets are used (high-throughput vs low-throughput). The high-throughput indexes will generate libraries with a peak size of 186 nucleotides.

Bioinformatics[[6]](#footnote-6):

1. Sequencing was performed on an Illumina NextSeq with a high-output kit. Read 1 length was 50 bases, read 2 length (index) was 6 bases. 5-15% PhiX was added to ensure sequencing proceeded through the Poly-A tail. Demultiplexing was performed based on read 2 prior to downloading files from BaseSpace.
2. The first 5 and last 20 bases from each read were removed with fastx\_trimmer.
3. Stretches of ‘AAAAAAAA’ were removed from the 3’ end of each read with fastx\_clipper.
4. Reads mapping to rRNA were removed by mapping to a rRNA reference with Bowtie2.
5. Remaining reads were mapped to the genome and transcriptome with Tophat2.
6. Following mapping, read counts were performed with either HTSeq or featureCounts.
1. Although this lysis protocol is written for cell-culture samples, we have also had success creating libraries isolated from tissue. [↑](#footnote-ref-1)
2. The ensuing steps utilize enzymes which are not thermostable; ensure they are kept on ice at all times. The amount of input during this step is critical to proper library generation. The range given by Clontech is 0.1-100 ng. If using greater than 5 ng page purified footprints or 25 ng total RNA input, 1 uL 10mM ATP must be added to the Polyadenylation master mix. [↑](#footnote-ref-2)
3. During our trial of the smRNA kit, we have utilized the LT indexes. [↑](#footnote-ref-3)
4. The Clontech kit recommends 12-18 PCR cycles. We have not exceeded 16 cycles even for our very low input samples. [↑](#footnote-ref-4)
5. . Do not over-dry (the beads should go into solution with one or two pipetting movements) [↑](#footnote-ref-5)
6. Due to the unique library construction scheme employed, a number of changes were made to our traditional ribosome profiling bioinformatics pipeline. [↑](#footnote-ref-6)