Protocol for Ordered Two-Template Relay Ribosome Profiling with P1 Nuclease (OTTR-RP)
From Ferguson *et al. Nature Methods* (2023)

- The detailed cell lysis protocol below is for cultured human cells.
- For budding yeast, perform cell lysis as in McGlincy & Ingolia *Methods* (2017).

- Major equipment
  - Vertical nucleic acid electrophoresis system
  - (recommended) Depending on OTTR primers, either Cy5 or IR800 gel scanner
  - Printer for paper copy of size selection gel images
  - Refrigerated microcentrifuge, *e.g.* Eppendorf 5430R
  - GloMax-Multi Jr Detection System (Promega) for RNA quantification
  - Optima TLX Ultracentrifuge
  - TLA100.3 or TLA110 rotor (Beckman Coulter)
  - (optional) Dark Reader Blue-Box transilluminator (Clare Chemical Research)
  - Nanodrop spectrophotometer
  - PCR machine

- Main consumables
  - Minicell Borosilicate Glass Cuvettes (Promega)
  - 13 × 51 mm polycarbonate ultracentrifuge tubes (Beckman Coulter)
  - (optional) 12% or 15% 1X TBE denaturing urea polyacrylamide gels
  - 8% 1X TBE denaturing urea polyacrylamide gels
  - 9% 1X TBE native polyacrylamide gels
  - 0.2, 0.5, and 1.5 mL tubes, sterile and RNase/DNase-free

- Main reagents and kits
  - (optional) Phenol:Chloroform:Isoamyl alcohol (25:24:1, PCI), pH 6.7
  - TRIzol (Invitrogen, 15596018)
  - Nuclease P1, 100 U/µL (New England BioLabs, M0660S)
  - RNase A (Sigma, R6513)
  - (optional) Thermostable RNase H (NEB, M0523S)
  - (optional) Proteinase K (NEB, P8107S)
  - Turbo DNase I, 2 U/µL (Invitrogen, AM2238)
  - 20 mg/mL glycogen, RNA grade (Invitrogen, R0551)
  - iTaq™ Universal SYBR® Green Supermix (Bio-Rad, 1725120)
  - 100% and 70% ethanol, molecular biology grade
  - 100% isopropanol, molecular biology grade
  - Direct-zol RNA Miniprep column kit (Zymo Research, R2052)
  - SYBR Gold (Invitrogen, S11494)
  - RNA Clean & Concentrator-5 (Zymo Research, R1014)
  - DNA Clean & Concentrator-5 (Zymo Research, D4014)
  - (optional) Oligo Clean & Concentrator (Zymo Research, D4061)

- NEBNext® Library Quant DNA Standards (NEB, E7642S)
- OTTR library generation kit (Karnateq)
- Quant-iT RiboGreen RNA Assay kit (Invitrogen, R11490)
- (for PCR of cDNA) Q5 polymerase + buffer (NEB, M0491)
- (for PCR of cDNA) dNTP

- Main RNase-free buffer stocks
  - 5 M NaCl (Invitrogen, AM9760G)
  - 3 M sodium acetate, pH 5.5 (Invitrogen, AM9740)
  - 1 M $MgCl_2$ (Invitrogen, AM9530G)
  - 0.5 M EDTA (Invitrogen, AM9260G)
  - 1 M Tris-HCl, pH 7.0 (Invitrogen, AM9850G)
  - 1 M Tris-HCl, pH 8.0 (Invitrogen, AM9855G)
  - 1 M Tris-HCl, pH 7.4 (bioWORLD, 21420063-1)
  - 1 M Bis-Tris, pH 6.0 (bioWORLD, 40121185-1)
  - 7.5 M ammonium acetate (Sigma-Aldrich, A2706-100ML)
  - 10% SDS (Promega, V6551)
  - 10X TBE (Promega, V4251)
  - Triton X-100 molecular biology grade (Calbiochem, 648466)
  - DTT (Invitrogen, 15508013) for 1M stock. Aliquot and store frozen.
  - 10X PBS (Invitrogen, AM9624)
  - Sucrose (Thermo, 177140010)
  - Nuclease-free water

- Buffer recipes
  - Polysome buffer
    - 20 mM Tris, pH 7.4
    - 150 mM NaCl
    - 5 mM $MgCl_2$
    - 1 mM DTT

  - Polysome buffer with sucrose
    - 20 mM Tris, pH 7.4
    - 150 mM NaCl
    - 5 mM $MgCl_2$
    - 1 M sucrose
    - 1 mM DTT

  - 2X Bromophenol blue formamide loading dye
    - 95% formamide
    - 5 mM EDTA (pH 8.0)
    - 0.05% (w/v) bromophenol blue

  - 2X Xylene cyanol formamide loading dye
    - 95% formamide
    - 5 mM EDTA (pH 8.0)
    - 0.05% (w/v) xylene cyanol

- RNA elution buffer
  - 300 mM NaCl
  - 10 mM Tris pH 7.0
  - 1 mM EDTA
  - 0.25% v/v SDS

- cDNA elution buffer
  - 300 mM NaCl
  - 10 mM Tris pH 8.0
  - 1 mM EDTA

- cDNA stop solution
  - 50 mM Tris pH ~7.4
  - 20 mM EDTA
  - 0.2% SDS

- Oligonucleotides synthesized by Integrated DNA Technologies
  - 30 nt RNA control (can be without 5′PO_4)
    - 5′PO_4-rNrGrArCrArGrArCrUrGrArCrUrArCrUrCrACrArCrGrArArCrArGrGrArN-3′OH
    - RNA size selection: 1 µL of 1 µM stock + 9 µL of 2X Bromophenol blue formamide loading dye
    - Input for OTTR cDNA synthesis control: 9 µL of 100 nM stock, *i.e.* 0.9 pmoles maximum.
      - (optional, for disome profiling) Increasing the amount of template input above 0.9 pmoles, *e.g.* 9 µL of 200 nM (or 400 nM) stock, *i.e.* 1.8 pmoles (or 3.6 pmoles), will yield two distinct OTTR cDNA products. One will be equivalent to a 30 nt template (74 nt + 30 nt) and the other equivalent to a 60 nt template (74 nt + 30 nt + 30 nt), due to concatemerization of the input template during cDNA synthesis.

  - 40 nt RNA control (can be without 5′PO_4)
    - 5′PO_4-rNrArGrUrCrGrUrCrUrCrArUrCrArGrGrUrCrUrCrUrCrArCrUrCrArCrUrArCrArCrArCrUrCrUrCrN-3′OH
    - RNA size selection: 1 µL of 1 µM stock + 9 µL of 2X Bromophenol blue formamide loading dye
    - Input for OTTR cDNA synthesis control: 9 µL of 100 nM stock, *i.e.* 0.9 pmoles maximum.
      - (optional, for monosome/ribosome profiling) A single cDNA synthesis control using 9 µL of a 1:1 pool of both the 100 nM upper (40 nt) marker and 100 nM lower (30 nt) marker stocks can be used instead. This will yield two OTTR cDNA products equivalent to a 40 nt and 30 nt template (74 nt + 40 nt and 74 nt + 30 nt).
      - (optional, for disome profiling) Increasing the amount of template input above 0.9 pmoles, *e.g.*, 9 µL of 200 nM (or 400 nM) stock, *i.e.* 1.8 (or 3.6) pmoles, will yield two full length OTTR cDNA products. One will be equivalent to a 40 nt template (74 nt + 40 nt) and the other equivalent to a 80 nt template (74 nt + 40 nt + 40 nt).

  - cDNA qPCR quantitation primers and control oligo [**1**]
    - Forward, NI-827: 5′OH-CTCTTTCCCTACACGACGCTC-3′OH
    - Reverse, NI-828: 5′OH-GTGACTGGAGTTCAGACGTGTG-3′OH
    - Control, NI-803: 5′OH-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCAGCATGNNNNNTCGCATTACCCTGTTATCCCTAACATNNAGATCGGAAGAGCGTCGTGTAGGGAAAGAG-3′OH (The degenerate bases can be swapped for a specific sequence to reduce cost.)

- Example multiplexing primers [**2**]
  - I5 primer: 5′OH-AATGATACGGCGACCACCGAGATCTACAC[i5]ACACTCTTTCCCTACACGAC-3′OH
  - I7 primer: 5′OH-CAAGCAGAAGACGGCATACGAGAT[i7]GTGACTGGAGTTCAGACGTG-3′OH

- cDNA 5′ and 3′ adapter oligonucleotides are provided with the OTTR kit (Karnateq)
  - Custom adaptor sequences can be used by substitution of kit Buffer 4B. Updated information and additional protocols can be downloaded from the Karnateq website.
  - cDNA strand 5′ adapter is Illumina R2 sequence provided as an annealed primer duplex.
    - In published work, the cDNA 5′ adaptor primer was a mix of duplexes with a 3’ overhang of +1T (80%) or +1C (20%). These primers have 5’ Cy5.
    - In new v1.1 OTTR kits, only a +1T overhang primer is used. The primer has 5’ IR800.
  - cDNA strand 3′ adapter has a degenerate sequence to create a unique molecular identifier (UMI) followed by the complement of the Illumina R1 sequence. The 3’ adaptor template has a 5’ Cy5.
    - In published work, the UMI was 3′CYNNNNN.
    - In new v1.1 OTTR kits, the UMI is 3′CRNNNNN.

Adherent cell lysis and lysate management

Cell lysis should be performed rapidly and consistently. Differences in metabolism, temperature changes, O_2 and CO_2 saturation, and time can yield profound differences in ribosome occupancy profiles. The below steps are designed to expedite cell lysis in near-freezing conditions. To measure ribosome occupancy profiles from actively dividing cells, we recommend lysing before cell confluency exceeds 70%.

1. The day before cell lysis, prepare 1X PBS and store at 4 °C overnight.
2. 8 hours before cell lysis, refeed the cells with pre-warmed media. Simply aspirating and replacing the media without washing the cells will suffice.
3. Prepare 1X polysome buffer supplemented with 1% (v/v) Triton X-100 and 25 U/mL Turbo DNase I.
4. Fill three buckets with ice.
5. For the first bucket place the pre-labeled tubes, 1X PBS, and polysome lysis buffer on ice.
    a. *e.g.,* 6 – 7 pre-labeled tubes would suffice for a 15 cm dish.

Table 1: Description of each pre-labeled tube.

Tube 1	Initial tube lysate is moved to. (Step 12)	Save pellet and 10% of lysate volume
Tube 2	Tube which clarified lysate is transferred to (Step 15)	Save at least 10 µL, to be thawed later for RNA quantification
Tube 3	(optional) Aliquot for RNA-seq	50 – 100 µL
Tube 4	Aliquot for ribosome profiling	200 – 300 µL
Tube 5	Aliquot for ribosome profiling	200 – 300 µL
Tube 6	Aliquot for ribosome profiling	200 – 300 µL
Tube 7	Aliquot for ribosome profiling	200 – 300 µL

6. For the second bucket, sprinkle ~1 – 3 grams of NaCl over the ice and mix the top layer.
7. For the second and third ice buckets, deform the ice such that a cell culture dish can rest at a slight angle and the ice is evenly in contact with the surface of the dish.
8. For each cell culture dish, remove it from the incubator and either rapidly aspirate or immediately dump the cell media into a waste disposal bucket. Immediately pour 10 mL of ice-cold 1X PBS into the cell culture dish, taking care to avoid directly pouring the PBS directly onto the cells but instead on the wall of the dish. Place the cell culture dish on the salted ice, rotating the bottom of

the dish to ensure the entire surface has contact with the ice. Tilt the cell culture dish and completely aspirate the ice-cold 1X PBS.

9. Leave the cell culture dish slightly tilted (on ice) for ~15 seconds to allow the residual ice-cold 1X PBS to collect. Aspirate any remaining 1X PBS.
10. Add Polysome buffer dropwise to the entire cell culture dish. For a 10 cm dish, add 400 µL; for a 15 cm dish, add 600 µL.
11. Transfer the dish to the salt-free third ice bucket (to avoid allowing lysate to freeze).
    a. Lysate freezing at this point would likely jeopardize the experiment.
12. Using a cell scraper, slosh the lysis buffer around the dish and completely detach the cells. The volume will roughly double after lysis. Transfer lysate to a pre-labeled tube.
13. Incubate the cell lysate on the salt-free ice for at least 30 minutes to allow for DNA digestion. This is roughly the time it takes for lysis of 6 cell culture dishes.
14. Clarify the cell lysate by centrifuging at 4 °C at 20,000 × g for 10 minutes.
15. Transfer the cell lysate to a pre-chilled and pre-labeled 1.5 mL tube. The insoluble debris should be entirely avoided so we recommend leaving behind 10% of liquid volume with the insoluble debris pellet. If the insoluble debris pellet was at all disturbed during lysate transfer, the centrifugation can be repeated on the incompletely clarified lysate.
16. Aliquot 50 µL of lysate to one tube for RNA-seq and 10 µL of lysate to another tube for total RNA quantification. For the remaining lysate, make aliquots of 200 – 300 µL for ribosome profiling (ideally, at least 2 to 3 aliquots).
17. Snap-freeze lysate in liquid nitrogen and store at -80 °C.

Total extract RNA quantification

Total RNA quantification is a critical step before nuclease digestion. Users should be aware different methods may yield inconsistent results. We rely on the Quant-iT RiboGreen RNA kit to assay total RNA relative to the kit's rRNA reference standard.

1. Turn on the GloMax-Multi Jr Detection System and leave it on for 5 minutes before making measurements.
2. Thaw the 10 µL lysate aliquot reserved for RNA quantification.
3. Prepare 1X TE from 20X TE and nuclease free water. This will be used for both lysate dilution, 4X dye recipe, and assay recipe. Prepare one stock for the entire experiment to ensure consistency.
    a. Formula for estimating how much 1X TE to prepare: ***600 µL + (500 µL X n)***, where ***600 µL*** is the volume for the standards, where ***500 µL*** is the volume needed per sample, and where ***n*** is the number of samples. This estimate includes the volume of 1X TE needed to make the dye-mix in the next step.
4. Prepare 4X dye-mix in 1X TE from the 200X dye-mix. Prepare one stock for the entire experiment to ensure consistency.
    a. Formula for estimating how much 4X dye-mix to prepare: ***50 µL X (7 + n)***, where ***n*** is the number of samples.
5. Prepare 100 µL of 2 µg/mL of ribosomal RNA standard in 1X TE.
    a. 2 µL 100 µg/mL rRNA + 98 µL 1X TE
6. For the standard curve, combine the following:

1X TE (µL)	**2 µg/mL stock (µL)**	**Dye-mix (µL)**	**ng of RNA (µL)**	**Concentration (ng/µL)**
0	50	50	100	*1*
25	25	50	50	*0.5*
45	5	50	10	*0.1*
49	1	50	2	*0.02*
50	0	50	0	*0*

7. Dilute samples 1:100 in 1X TE buffer
    a. 2 µL lysate + 198 µL 1X TE

8. Dilute the samples further to 1:500 in 1X TE buffer
    a. 20 µL 1:100 lysate + 80 µL 1X TE
9. For the diluted samples, combine the following:

Sample (µL)	**Dye-mix (µL)**	**1X TE (µL)**	**Initial dilution factor**	**Final dilution factor**
10	50	40	1:100	*1000*
10	50	40	1:500	*5000*

10. Once combined, incubate the standards and samples for at least 5 minutes before measuring.
11. Transfer the 100 µL assay volume to a Promega Minicell Borosilicate Glass Cuvettes.
12. Operate the GloMax-Multi Jr Detection System in “Blue” mode. This requires using the Fluorometer Blue filter for the cuvettes.
13. Measure the raw fluorescence value, recording the FSU values for each sample and standard.
14. Using a standard curve from the diluted standard, compute the concentration of the samples using the measurements taken for the 1:1000 and 1:5000 dilution.
    a. If the FSU values of the diluted samples do not fall within range of the standard curve, repeat the assay with a better dilution strategy.

P1 nuclease digestion and ribosome pelleting by sucrose cushion

For yeast cell lysate we have used 300 – 600 U of P1 nuclease with 30 µg of lysate (*i.e.*, 10 – 20 U/µg), and for human cell lysate we have used 450 – 900 U of P1 nuclease with 30 µg of lysate (*i.e.*, 15 – 30 U/µg). It may be necessary to test optimal conditions for other lysates by titration of nuclease. Adjusting the lysate pH to 6.5 is required before P1 nuclease digestion. Digestion temperature can be tuned to improve polysome collapse efficiency, for example we find 30 °C ideal for yeast lysate and 37 °C ideal for human lysate.

1. Using the previously determined RNA concentration, estimate the total RNA in one 200 µL aliquot for each sample. We routinely digest 200 – 300 µL of lysate.

Table 1: Example for defining how much P1 nuclease to use.

Sample Name	Sample volume (µL)	RNA concentration (µg/µL)	RNA mass (µg)	Digestion ratio (U/µg)	100 U/µL P1 nuclease volume (µL)
A	200	0.1	20	15	*3*
B	200	0.15	30	15	*4.5*
C	200	0.2	40	15	*6*

2. Thaw lysate from -80 °C on ice for one hour or until thawed.

3. Confirm the incubator/room/heating block/thermomixer is set to the appropriate temperature for digestion. Pre-chill the ultracentrifuge to 4 °C, and confirm the rotor for ultracentrifugation has been stored overnight at 4 °C.

4. While the lysate thaws, prepare a polysome buffered 1 M sucrose solution.

    a. The sucrose should dissolve within ~ 15 minutes. Filter-sterilize the solution using a 0.22 µM filter and syringe. Polysome buffer contains DTT and should be made fresh each day.

5. For every 100 µL of lysate add 7 µL of 300 mM Bis-Tris pH 6.0 (pH should decrease to ~ pH 6.5). Estimate the total amount of RNA, and add P1 nuclease based on that.

Table 2: Example of P1 nuclease digestions.

Sample Name	Sample volume (µL)	300 mM Bis-Tris pH 6.0 volume (µL)	100 U/µL P1 nuclease (µL)	Final volume (µL)
A	200	14	3	217
B	200	14	4.5	218.5
C	200	14	6	220

6. For yeast cell lysate, digest samples at 30 °C for 1 hour; for human cell lysate, digest samples at 37 °C for 1 hour.
    a. **Option 1:** Place the tubes on a tube rotator in an incubator at the appropriate temperature.
    b. **Option 2:** Place the tubes in a pre-warmed thermomixer and set it to 250 RPM.
    c. **Option 3:** Place the tubes in a heating block and agitate the tubes every ~5 – 10 minutes by flicking.
7. After digestion, transfer sample tubes to a bucket of ice.
    a. Temperature < 25 °C greatly reduces P1 nuclease activity.
    b. Lysate turbidity may increase after nuclease digestion. This is due to proteins aggregating at pH 6.5 during incubation at temperature greater than 4 °C. This occurs regardless of P1 nuclease addition and does not impact the success of the experiment.
8. Label the polycarbonate tubes used in ultracentrifugation with the sample name, and also circle the bottom of the tube where one expects the pellet to form. Alternatively, after the tube is balanced and loaded into the rotor, mark the rim with a dot where the outer edge of the tube lies.
9. Before adding anything to the polycarbonate tubes, deform the ice so each tube is resting vertically and not leaning.
10. Prepare sucrose cushion by either over-laying or under-laying the sample.
    a. **Over-layering:** Pipette 900 µL of 1 M sucrose in polysome buffer into polycarbonate tube. Using a wide-mouth 1 mL pipette tip, gently dispense the lysate onto the very top of the sucrose solution.
    b. **Under-layering:** Pipette the lysate volume into the very bottom of the polycarbonate tube. Using a 1 mL pipette tip, collect 900 µL of 1 M sucrose in polysome buffer and gently plunge the pipette tip into the very bottom of the tube. Gently dispense the sucrose underneath the lysate. As carefully and vertically as possible, lift the pipette out of the cushion.
    c. New users should practice this technique several times using 200 µL of 1X polysome buffer instead of an actual sample.
11. After preparing the cushions, balance pairs of polycarbonate tubes by adding additional volumes of 1X polysome buffer to the sample layer of the lighter tube. After each pair is balanced, load the tubes into opposite wells of the rotor. Always strictly adhere to manufacturer specifications for using your rotor.
    a. It is easiest to place 1 tube on the scale, tare the scale, and then measure the opposite tube. Identify which of the two tubes was lightest, add 1X polysome buffer to the lighter tube, and remeasure both weights to confirm balancing was successful.
    b. After a pair of polycarbonate tubes are balanced, place them in opposing wells of the centrifuge rotor (which should still be stored in a fridge at this time). Remember to load the tubes so the pellet forms on the demarcated spot.
    c. Focus on slow movements, and precise and accurate balancing. This step should not be rushed.

12. After all tubes are balanced, remove the rotor from the fridge and carefully transfer to the ultracentrifuge. If using Optima TLX Ultracentrifuge and either TLA100.3 or TLA110 rotor, centrifuge at 4 °C at 100,000 RPM for 1 hour.

Ribosome pellet resuspension and mirRICH size selection

Ribosome pellets need to be completely resuspended. Failure to do so will result in clumps of debris once TriZol is added to the sample, and RNA recovery will not be limited.

1. Following ultracentrifugation, aspirate the sucrose solution entirely. Special care must be taken to avoid disturbing the transparent pellet.
2. Add 30 µL of nuclease-free water to the pellet. Using the same pipette tip, scratch the pellet from the wall of the tube. After scratching, gently pipette the slurry to encourage the pellet to break apart. Avoid producing bubbles.
3. To further encourage resuspension, let the pellet continue to dissolve for at least 10 minutes while on ice. In the meantime, continue processing the other samples.
4. Once all samples have incubated for 10 minutes or more, check that no clumps are visible. Gentle pipetting may be needed to disrupt any remaining clumps.
5. Transfer the 30 µL of sample to a labeled tube containing 300 µL of TriZol reagent. Vortex the samples for 1 minute then incubate at room temperature for 10 minutes. No clumps should be visible. If the sample remains turbid, more TriZol reagent is necessary. Add ~150 µL more, vortex, and incubate for another ~5 minutes to improve turbidity.

If you want to use denaturing urea PAGE for size selection of RNA footprints, continue by using the Direct-Zol RNA purification protocol and elute with 100 µL. Then follow the protocol for **General RNA precipitation** followed by **RNA size selection, overnight elution, and precipitation**.

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6. Add 60 µL of 100% chloroform. Vortex for 1 minute. Incubate at room temperature for 5 minutes. The ratio of chloroform is 0.2X of the final volume of TriZol reagent used.
7. Separate aqueous from organic and interphase by centrifugation at 4 °C at 20,000 × g for 15 minutes.
8. Transfer the entire aqueous phase to a new tube. Repeat the centrifuging and transfer steps if there is any concern that some organic phase was transferred. Add 1 µL of 20 mg/mL glycogen. Vortex well.
9. Add 150 µL of 100% isopropanol to the aqueous fraction. Vortex well. Incubate on ice for 15 minutes.
    a. The 100% isopropanol is 0.5X of the final volume of TriZol reagent used. If more than 300 µL of TriZol reagent was used, adjust the 100% isopropanol volume accordingly.
    b. Incubating on ice enhances co-precipitation of the guanidinium isothiocyanate from TriZol, which is desirable in this case as it improves the reproducibility of mirRICH small RNA enrichment.
10. Pellet RNA by centrifuging at 4 °C at 20,000 × g for 15 minutes. Aspirate pellet completely.

11. Centrifuge at room temperature at 10,000 × g for 1 minute. Using a 10 µL pipette tip, remove any remaining liquid from the pellet. It is crucial to completely remove all liquid before air drying.
12. Air dry the pellet with the lids open for 2 hours. Loosely cover the tubes with a sheet of aluminum foil to protect from dust.
13. Add 10 µL of nuclease-free water to the air dried pellet. Vortex, and briefly centrifuge to collect the liquid at the bottom of the tube. Elute RNA from the pellet for exactly 5 minutes at room temperature.
    a. We recommend processing ~6 samples at a time to avoid eluting RNA for too long.
14. After incubation, vortex again, and briefly centrifuge to collect the liquid at the bottom of the tube. Remove 9 µL of eluate, leaving behind 1 µL and the remaining pellet.
    a. Store the remaining ~1 µL of eluent and pellet at -80 °C until the success of mirRICH has been assessed.
15. Perform RNA Clean and Concentrate (see manufacturer's protocol) on the eluate to remove excess guanidinium isothiocyanate. Include an extra wash step and a penultimate "dry spin" to remove residual ethanol before eluting with 25 µL of nuclease-free water. Store at -80 °C or proceed.
16. Quantify the mirRICH RNA yield using a Nanodrop spectrophotometer.
    a. In yeast and human cell extracts, when mirRICH was successful, the yield of RNA was roughly ~1/30th the input (*e.g.*, a 30 µg digest will yield ~1 µg of mirRICH RNA).
    b. If mirRICH RNA yield exceeds expectation, test whether the size selection was effective. One way to do this is by resolving ~50 – 100 ng of the mirRICH RNA on a 12% denaturing urea polyacrylamide gel. mirRICH should nearly eliminate all RNA > ~ 300 nt. See Extended Data 6a as an example of what to expect.
    c. If mirRICH failed to size-enrich small RNA, combine the remaining mirRICH RNA and the corresponding airdried RNA pellet that was stored at -80 °C, and adjust the volume to 100 µL with nuclease-free water. Incubate and occasionally vortex the sample until the RNA pellet is resuspended and proceed with **general RNA precipitation.** Resuspend precipitated total RNA pellet in 5 µL of 2X formamide loading dye and proceed with **RNA size selection, overnight elution, and precipitation.**
    d. The Nanodrop spectrophotometer trace can be interpreted to determine whether any contaminants (*i.e.*, phenol, guanidinium thiocyanate, or chloroform) are present. Residual phenol has a peak at ~220 nm and ~270 nm. Residual guanidinium thiocyanate has a peak at ~250 nm. Residual chloroform has a peak at ~225 nm. If detected, additional RNA clean-up is required. Follow steps for **general RNA precipitation**. If mirRICH RNA yield was < 4.5 ng/µL, concentrate to 10 µL instead of 25 µL of nuclease-free water.

(recurring method) General RNA precipitation

The steps described below describe RNA precipitation. To avoid losing an RNA pellet we suggest using generic autoclaved/sterile RNase-free and DNase-free 1.5 mL tubes instead of low-binding or siliconized 1.5 mL tubes, and to use RNase-free glycogen instead of GlycoBlue as precipitation carrier. If working with low-input starting material (*e.g.,* < 1 µg lysate was digested) low-binding / siliconized tubes may increase yield, but be aware the pellet will likely readily detach from the tube wall, thus requiring gentler aspiration.

1. Adjust the RNA volume to 100 µL.
2. Add 1 µL of 20 mg/mL glycogen and briefly vortex.
3. Add 10 µL of 3 M sodium acetate pH 5.5 and briefly vortex.
    a. Note: If SDS is present in the sample, use 10 µL of 5 M sodium chloride, instead.
4. Add 300 µL 100 % ethanol and briefly vortex.
5. Incubate at -80 °C for one hour.
    a. Alternatively, either flash-freeze in liquid nitrogen or incubate for 5 minutes on dry-ice
6. Pellet the RNA by centrifugation at 4 °C at 20,000 × g for 15 minutes.
7. Aspirate 90% of the solution and add 1 mL of -20 °C chilled 70% ethanol and briefly vortex, ideally until pellet detaches.
8. Incubate at -20 °C for 15 minutes. Pellet the RNA by centrifugation at 4 °C at 20,000 × g for 15 minutes.
9. (optional) Aspirate 90% of the solution and wash again by adding 1 mL of -20 °C chilled 70% ethanol and briefly vortexing. Again, incubate at -20 °C for 15 minutes and pellet the RNA by centrifugation at 4 °C at 20,000 × g for 15 minutes.
10. Using a 1 mL pipette, remove nearly all of the solution leaving ~100 µL.
11. Carefully remove the remaining liquid using a 200 µL pipette.
12. Briefly spin the tubes to collect any remaining liquid to the bottom of the tube, and carefully remove the remaining liquid using a 10-20 µL pipette.
13. Air dry, ideally for no more than 10 minutes, to evaporate any remaining 70% ethanol.
14. Resuspend in 5 – 25 µL of nuclease free water.
    a. If proceeding to **RNA size selection, overnight elution, and precipitation,** resuspend pellets in 5 µL of 2X formamide loading dye.
15. Allow 10 minutes for resuspension, and then vortex and briefly spin to collect the liquid at the bottom of the tube.
16. Store at -80 °C or proceed.

(alternative to mirRICH) RNA size selection, overnight elution, and precipitation

Gel-based size selection for P1 nuclease RPFs is an alternative to mirRICH. The RNA size selection markers will likely migrate slightly faster than sample RNAs, because sample RNAs have residual salt carried over from RNA purification steps, whereas RNA size selection markers do not. For P1 nuclease RPFs we use 30 and 40 or 45 nt RNA markers. See additional notes at the end of this protocol if using RNase I.

1. Resuspend precipitated RNA in 5 μL 2X bromophenol blue formamide loading dye.
2. Prepare 30 and 40 nt markers for size selection in 2X bromophenol blue formamide loading dye as described in the **Oligos** section.
3. Using RNase-free 1X TBE and electrophoresis equipment, set up a 12% or 15% denaturing urea polyacrylamide gel. Clear the wells by flow of 1X TBE using a needle and syringe.
    a. 12% denaturing urea polyacrylamide gel makes it easier to select RNA from 45 to 80 nt.
    b. 15% denaturing urea polyacrylamide gel makes it easier to select RNA from 30 to 45 nt.
4. Pre-run the gel for 15 minutes at 200 V.
5. Denature RNA samples at 85 °C for 3 minutes. Immediately place samples on ice.
6. After pre-running, wash the wells once more, and load the no-sample wells with 5 μL 2X formamide loading dye as well as the RNA size selection marker. Blank wells of just formamide loading dye are ideally included as the first two and final two lanes of a gel, between marker and samples, and between individual samples. At the very least, there should be a blank well between unrelated (*i.e.*, not replicates) samples.
7. Run the gel until the bromophenol blue dye front reaches the bottom of the gel.
8. After running the gel, disassemble it and transfer to Saran Wrap.
9. Stain and image RNA. The protocol for using SYBR Gold is described below. Combine 5 μL of SYBR Gold nucleic acid stain with 5 – 10 mL of 1X TBE and cover the gel with this buffer for no more than 3 minutes. Agitate the gel while it stains by gentle shaking. Rinse briefly with 1X TBE and remove as much residual buffer from the gel as possible before proceeding. Transfer gel to a new and dry Saran Wrap. Consider imaging the gel before (and after) excision of RNA gel slices. Transfer gel to the scanner and capture the Cy2 spectra by using an emission filter of 515 – 535 nm. Transfer gel to a Blue-Box transilluminator to visualize the RNA. Excise the RNA by cutting above and below the lower and upper markers. Use either a razor blade or scalpel. Do not use the edge of a blade for more than one sample.
10. Transfer gel slice to a pre-labeled tube using the blade used for slice excision. Using a 1 mL pipette tip, crush the gel slice against the wall of the tube.
11. After crushing, add 500 μL of RNA elution buffer.
12. Elute RNA by placing the tube on a rotator overnight at room temperature.
13. After elution, centrifuge the tube for 10 minutes at max speed.
14. Transfer 450 μL of eluate to a new tube. If necessary, centrifuge and transfer again to completely remove residual gel fragments.

15. Add 1 µL of 20 mg/mL glycogen, vortex, and 3 volumes of 100% ethanol. Split across two tubes if necessary.
    a. After adding ethanol and vortexing, any remaining gel fragments will immediately become opaque and flocculate. Typically, a pipette tip can be used to fish these bits out before centrifuging
16. Incubate at -80 °C for one hour.
    a. Alternatively, either flash-freeze in liquid nitrogen or incubate for 5 minutes on dry-ice
17. Pellet the RNA by centrifugation at 4 °C at 20,000 × g for 15 minutes.
18. Aspirate 90% of the solution and add 1 mL of -20 °C chilled 70% ethanol and briefly vortex, ideally until pellet detaches.
19. Incubate at -20 °C for 15 minutes. Pellet the RNA by centrifugation at 4 °C at 20,000 × g for 15 minutes.
20. (optional) Aspirate 90% of the solution and wash again by adding 1 mL of -20 °C chilled 70% ethanol and briefly vortexing. Again, incubate at -20 °C for 15 minutes and pellet the RNA by centrifugation at 4 °C at 20,000 × g for 15 minutes.
21. Using a 1 mL pipette, remove nearly all of the solution leaving ~100 µL.
22. Carefully remove the remaining liquid using a 200 µL pipette.
23. Briefly spin the tubes to collect any remaining liquid to the bottom of the tube, and carefully remove the remaining liquid using a 10 – 20 µL pipette.
24. Air dry, ideally for no more than 10 minutes, to evaporate any remaining 70% ethanol.
25. Resuspend RNA pellet(s) in 15 µL of nuclease-free water and measure RNA concentration, for example by Nanodrop spectrophotometer.

Simultaneous cDNA synthesis and adapter incorporation by OTTR

OTTR is a single-tube workflow suitable for any small RNA library generation [**2**]. Phenol, alcohols, SDS, or chelating agents carried over from RNA purification will be inhibitory. We recommend using a P2 pipette for nearly all steps of OTTR. We also recommend using 0.5 mL tubes for OTTR as they are generally more sturdy than 0.2 mL tubes and the lids are more reliable.

1. Dilute up to 20 ng of mirRICH RNA OR between 20 pg and 10 ng of size-selected RPFs (*i.e.,* 30 – 40 nt) to 9 µL with nuclease-free water. Do not start with more than 9 µL of input nucleic acid per reaction. If you exceed 0.9 pmoles of RNA, the number of small RNA molecules will exceed cDNA synthesis capacity and result in biased capture of RPFs based on 3′ ends, and potentially, cDNA concatemerization.
    a. (optional) 20 ng of mirRICH RNA should be below the 0.9 pmoles input limit of OTTR. High-resolution electrophoretic analysis tools such as the Agilent TapeStation or Bioanalyzer, can provide molar RNA concentration estimates.
    b. OTTR v1.1 kits use 10 rather than 9 µL input RNA volume.
2. (this is a control that should be repeated with each reaction set) In parallel, prepare an extra OTTR library synthesis reaction to be used as a cDNA size selection marker. Recommended control oligos and instructions for how to use them to generate cDNA size selection markers appropriate for monosome and disome profiling is described in the **Oligos** section.
3. Add 2 µL Buffer 1A. Check the pipet tip used to withdraw from the Buffer stock tube for unwanted carry over of excess liquid.
    a. Note, Pre-warming Buffer 1A to room temperature can make it easier to pipet.
4. Add 1 µL Buffer 1B.
    a. Original Buffer 1B was 28 mM $MnCl_2$ and 3.5 mM ddATP. OTTR v1.1 kits have Buffer 1B2 with 28 mM $MnCl_2$, 3.5 mM ddATP, 10 mM sodium acetate pH 5.5, and 28 mM ammonium sulfate. The additives reduce the manganese oxidation that can occur with many freeze and thaw cycles.
5. Due to the viscosity of the reaction mix, we recommend vortexing aggressively before adding Tail Pol in the next step.
6. Add 1 µL of Tail Pol. Gently vortex (50 – 60% power), or flick the tube for 5 seconds, or pipette the entire 50% of the volume 20 times.
7. Incubate at 30 °C for 1 hour 30 minutes.
8. Add 1 µL of Buffer 1C. Gently vortex or flick the tube for 5 seconds.
    a. In published work, Buffer 1C was used to chase the ddATP labeling with ddGTP. OTTR v1.1 kits use only ddATP tailing. Instead of Buffer 1C, input volume is 10 rather than 9 µL.
9. Incubate at 30 °C for 30 minutes.
10. Make a 1:1 mastermix of Buffer 2 and Phosphatase.
11. Add 2 µL of the Buffer 2 and Phosphatase mastermix. Gently vortex for 5 seconds.
12. Incubate at 37 °C for 15 minutes.

13. Add 1 µL of Buffer 3. Vortex for 10 seconds.
    a. Note, Buffer 3 contains EGTA, which will protect RNA from divalent cations in the subsequent heating step.
14. Incubate at 65 °C for 3 minutes.
15. Place on ice and let the reactions cool for 5 minutes.
16. Pre-warm Buffer 4A to room temperature. Using a 20 µL filter-tipped pipette, resuspend the precipitated dNTPs at the bottom of Buffer 4A until uniformly distributed. This is required after each freeze-thaw cycle.
17. Add 1 µL of Buffer 4A. Vortex for 10 seconds. DO NOT make a master-mix of Buffer 4A with either Buffer 4B or Relay Pol.
18. Add 1 µL of Buffer 4B. OK to make a 1:1 master-mix of Buffer 4B and Relay Pol.
    a. In published work, Buffer 4B contains both +1T and +1C primer duplexes. OTTR v1.1 kits use Buffer 4B2 with only a +1T primer duplex.
19. Add 1 µL of Relay Pol. Gently vortex (50 – 60% power), or flick the tube for 5 seconds, or pipette the entire 50% of the volume 20 times.
20. Incubate at 37 °C for 30 minutes.
21. Incubate at 70 °C for 5 minutes.
22. If intending to perform cDNA clean up by PCI (see below, Option 1).
    a. Add 1 µL of RNase A. (optional: Including 1 µL of thermostable RNase H may improve purification of longer cDNAs.) Gently vortex or flick the tube for 5 seconds.
    b. Incubate at 50 °C for 10 minutes.
    c. Add 80 µL of cDNA stop solution. Gently vortex or flick the tube for 5 seconds. (optional: Including 1 µL of proteinase K and incubating again at 50 °C for 10 minutes can also improve purification.)
    d. Incubate at 95 °C for 5 minutes.
23. If intending to perform cDNA clean up by spin column (see below, Option 2)
    a. Add 1 µL of RNase A and 1 µL of thermostable RNase H. Gently vortex or flick the tube for 5 seconds.
    b. Incubate at 50 °C for 10 minutes.
    c. Determine the total volume of cDNA stop solution needed for all OTTR reactions and dilute that with one volume of water to reduce the cDNA stop solution SDS concentration to 0.1% (v/v), the maximum tolerated amount of SDS for Oligo Clean & Concentrator workflow. Add 30 µL of diluted cDNA stop solution and 1 µL of proteinase K. Gently vortex or flick the tube for 5 seconds.
    d. Incubate at 50 °C for 10 minutes. The reaction can be terminated at 95 °C for 5 minutes.

24. Cool the samples to room temperature, but avoid placing the samples on ice to prevent SDS precipitation. Library preparation can be paused at this step by storage at -20 °C. When resuming, dissolve the SDS at 50 °C for ~5 minutes or longer if necessary.

cDNA purification and size selection from OTTR

cDNA clean-up requires complete disruption of the stable enzyme-nucleic acid interaction and removal of unwanted nucleic acids. Particularly with low input OTTR reactions, ~75 nt "adapter dimer" cDNA will contaminate libraries and be amplified preferentially during library multiplexing PCR. We use an 8% denaturing urea polyacrylamide gel to select our desired products (monosome-sized cDNA library of 105 – 120 nt and/or sub-disome and disome-sized cDNA library of 121 – 155 nt). With an 8% gel, in our experience, adapter dimer and xylene cyanol migrate equivalently, providing a visual clue where the adapter dimer is on the gel. After electrophoresis, prior to imaging, we recommend cutting off the adapter dimer/xylene cyanol dye front part of the gel, especially if users intend to directly stain the gel with SYBR Gold before imagining (we recommend direct imaging for the primer 5′ dye instead, to limit nucleic acid diffusion in the gel).

<u>cDNA purification option 1: PCI organic extraction</u>

1. Transfer the 100 µL of OTTR reaction + cDNA stop solution to a new tube.
2. (optional) Add vacuum grease to fill the lid of the tube. This will aid in keeping the aqueous phase separate from the organic phase in a subsequent step.
3. In a chemical hood, add 100 µL of PCI solution to the sample. Vortex for 10 seconds.
    a. PCI is an organic solution that is buffered and stored with an aqueous layer. We recommend taking an aliquot of buffered PCI into a working stock in 50 mL conical. Before use, when removing PCI from the refrigerator, do not shake the bottle to avoid mixing the transparent aqueous (top) and organic (bottom) phases. Take PCI from the organic (bottom) phase.
4. Centrifuge for 5 minutes at 16,000 × g.
5. In a chemical hood, transfer the ~100 µL aqueous phase to a new tube.
6. Add 1 µL of 20 mg/mL glycogen and briefly vortex.
7. Add 10 µL of 7.5 M ammonium acetate and briefly vortex
8. Add 300 µL of 100% ethanol and briefly vortex.
9. Incubate at -20 °C for 30 minutes.
10. Pellet the cDNA by centrifugation at 4 °C at 20,000 × g for 15 minutes.
11. Aspirate 90% of the solution and add 1 mL of -20 °C chilled 70% ethanol and briefly vortex, ideally until pellet detaches.
12. Incubate at -20 °C for 15 minutes. Pellet the cDNA by centrifugation at 4 °C at 20,000 × g for 15 minutes.
13. Using a 1 mL pipette, remove nearly all of the solution leaving ~100 µL.
14. Carefully remove the remaining liquid using a 200 µL pipette.
15. Briefly spin the tubes to collect any remaining liquid to the bottom of the tube, and carefully remove the remaining liquid using a 10-20 µL pipette.

25. Air dry, ideally for no more than 10 minutes, to evaporate any remaining 70% ethanol.
26. Resuspend pellet in 5 µL 2X bromophenol blue formamide loading dye. Incubate at room temperature for at least 10 minutes, vortex briefly, and spin to collect the liquid at the bottom of the tube.

<u>cDNA purification option 2: Oligo Clean & Concentrate</u>

1. Transfer the 50 µL of OTTR reaction + diluted cDNA stop solution to a new tube.
2. Add 100 µL of Oligo Binding Buffer.
3. Add 400 µL of 100% ethanol. Mix well by vortex.
4. Transfer to Zymo-Spin™ IC Column. Centrifuge at 10,000 × g for 1 minute.
5. Add 750 µL of DNA Wash Buffer to the column and centrifuge at 10,000 × g for 1 minute.
6. (optional) Repeat the preceding step.
7. Transfer Zymo-Spin™ IC Column to a clean collection tube and centrifuge at 10,000 × g for 1 minute to remove any residual buffer.
8. Pre-heat nuclease-free water to 95 °C.
9. Dispense 6 µL of pre-heated nuclease-free water and centrifuge at 12,500 × g for 2 minutes.
10. Add 5 µL of 2X bromophenol blue formamide loading dye to the eluent.

<u>cDNA size selection by gel purification</u>

1. Denature cDNA at 95 °C for 5 minutes and immediately return to ice.
2. Pre-run an 8% denaturing urea polyacrylamide gel for 15 minutes at 200 V.
3. Rinse the wells of the gel using a needle and syringe.
4. Load the gel. Ideally, load the 30+40 nt oligonucleotide control cDNA library in the middle lane with a blank well of bromophenol blue formamide load dye on either side. It is best not to load samples on the first two or final two lanes and to include a blank well between libraries that are not biological replicates. One of the blank wells should be loaded with 1:20 xylene-cyanol:bromophenol blue formamide loading dye as a marker.
5. Run the gel until the xylene cyanol dye front is near the bottom of the gel. In a 1X TBE 8% denaturing urea polyacrylamide gel, the xylene cyanol migrates at roughly the same rate as the adapter dimer cDNA.
6. Disassemble gel and transfer to saran wrap.
7. Carefully cut the gel above the xylene cyanol dye front and discard the bottom portion of the gel. This will reduce adapter dimer cDNA contamination of desired cDNA.
8. There are several options for detecting the cDNA, depending on which fluorescent dye is present at a primer 5′ end (original OTTR primers have 5′ Cy5, others have IR800).

a. **Direct Cy5 detection**: Transfer gel to the scanner and capture the Cy5 spectra by using an emission filter of 655 – 685 nm. Warning: xylene cyanol remaining on the gel will also be detected in this emission spectra.

b. **Direct IR800 detection**: Transfer gel to the scanner and capture the IR-long spectra by using an emission filter of 810 – 840 nm.

c. **SYBR Gold nucleic acid staining**: Combine 5 µL of SYBR Gold nucleic acid stain with 5 – 10 mL of 1X TBE and cover the gel with this buffer for no more than 3 minutes. Agitate the gel while it stains by gentle shaking. Rinse briefly with 1X TBE and remove as much residual buffer from the gel as possible before proceeding. Transfer gel to the scanner and capture the Cy2 spectra by using an emission filter of 515 – 535 nm.

9. Save the gel scan (.gel on Typhoon) and open it in ImageJ. Print the image at its actual size with signal adjusted so that it is easy to see cDNA products (“File” > “Page Setup” > “Print actual size”). Select “Print,” and save as a PDF file.

10. Transfer the gel on Saran Wrap to the printed image and position the gel so it is perfectly aligned with the image.

11. Using a clean razor blade, excise the cDNA by cutting below the 30 nt cDNA control and above the 40 nt cDNA control band. Use a clean edge of the blade for each cDNA sample. Transfer gel slice to a pre-labeled tube.

12. Also excise the cDNA bands for the 30 nt and 40 nt cDNA controls, place them in separate tubes, and process them in parallel with your samples.

13. Crush the gel slices with a clean 1 mL pipette tip.

14. Add 500 µL of cDNA elution buffer. Incubate at 70 °C for 1 hour to elute cDNA.

15. After elution, centrifuge for 10 minutes at max speed.

16. Transfer 450 µL of eluate to a new tube. If necessary, centrifuge and transfer again to completely remove residual gel fragments.

17. Add 1 µL of 20 mg/mL glycogen, vortex, and 3 volumes of 100% ethanol. Split across two tubes if necessary. After adding ethanol and vortexing, any remaining gel fragments will immediately become opaque and flocculate. Typically, a pipette tip can be used to fish these bits out before centrifuging.

18. Incubate at -20 °C for 30 minutes.

19. Pellet the cDNA by centrifugation at 4 °C at 20,000 × g for 15 minutes.

20. Aspirate 90% of the solution and add 1 mL of -20 °C chilled 70% ethanol and briefly vortex, ideally until pellet detaches.

21. Incubate at -20 °C for 15 minutes. Pellet the cDNA by centrifugation at 4 °C at 20,000 × g for 15 minutes.

22. Using a 1 mL pipette, remove nearly all of the solution leaving ~100 µL.

23. Carefully remove the remaining liquid using a 200 µL pipette.

24. Briefly spin the tubes to collect any remaining liquid to the bottom of the tube, and carefully remove the remaining liquid using a 10-20 µL pipette.
25. Air dry, ideally for no more than 10 minutes, to evaporate any remaining 70% ethanol.
26. Resuspend cDNA pellet in 25 – 35 µL of nuclease-free water.

cDNA quantification by qPCR

We rely on a previous protocol (section 5.8, [**1**]) to perform cDNA qPCR.

1. Prepare serial dilution of the standard curve oligo NI-803. To make a 10 nM working solution, dilute 2 µl of 1 µM stock solution in 198 µl of water. Next, take 10.2 µl of the 10 nM working solution and dilute it with 89.8 µl of water to create a 1.02 nM stock solution. Next, produce several dilutions starting with 20 µl of 1.02 nM stock solution and add it to 60 µl of water to create a 1:4 dilution (256 pM). Repeat this process by using 20 µl of the previous dilution and adding it to 60 µl of water to obtain a 1:16 dilution (64 pM), and so on for a 1:64 dilution (16 pM) and a 1:256 dilution (4 pM).
2. Prepare cDNA qPCR master mix. Dispense 19 µL of master mix into each well for qPCR.
    a. One 20 µL reaction is 1 µL of cDNA plus 10 µL iTaq™ Universal SYBR® Green Supermix, 1 µL 10 µM NI827, 1 µL 10 µM NI828, and 7 µL nuclease-free water.
    b. Prepare enough master mix for the samples, the 30 and 40 nt control cDNA, and the serially diluted standard.
3. Add 1 µL of serial diluted standard or sample. We recommend performing duplicate qPCR reactions for the standard curve, but we routinely rely on a single replicate of our experimental samples to confirm cDNA concentrations.
4. Perform qPCR amplification with the following cycling parameters:
    a. 95°C, 15 min
        i. 94°C, 10 sec
        ii. 54°C, 20 sec
        iii. 72°C, 30 sec (measurement)
        iv. Repeat 40 times.
    b. 72°C, 2 min
5. Compute a linear regression of the pM of the standards and Ct values.
6. Compute the pM of the experimental samples.
7. Divide the concentration of the experimental samples by 2, this is approximately the concentration of the experimental sample if 100% of the material was sacrificed for a 50 µL Q5 PCR reaction.
8. Using Table 15 from [**2**] (reproduced below), identify the number of PCR cycles necessary to amplify the cDNA. Use less or more cDNA so each sample can be amplified with the same number of cycles.

Template concentration (pM) in 50 µL PCR reaction	**PCR Cycles**

800	*7*
400	*8*
200	*9*
100	*10*
50	*11*
25	*12*
12.5	*13*
6.3	*14*
3.1	*15*
1.6	*16*

Library multiplexing PCR and native polyacrylamide gel size selection

Ideally, all cDNAs within a cohort of libraries will be amplified the same number of cycles (see previous protocol for calculations). When possible, do not use more than 14 cycles to amplify a given library. Template should be no more than 10% volume of the PCR reaction to avoid contaminant carry over. Select a unique i5 and i7 index for each library.

1. Combine cDNA and nuclease-free water to 36.5 μL. We recommend parallel PCR amplification of cDNA from the 30 nt and 40 nt RNA controls in two separate reactions, for use in native polyacrylamide gel size selection of the desired library PCR products.
2. Add 1 μL 25 μM i5 primer. See **Oligos** section for sequence.
3. Add 1 μL 25 μM i7 primer. See **Oligos** section for sequence.
4. Add 1 μL 10 μM dNTPs.
5. Add 10 μL 5X Q5 reaction buffer (NEB).
6. Add 0.5 μL of Q5 polymerase (NEB).
7. Pre-heat thermocycler to 95 °C.
8. Perform PCR with the following cycling parameters, where X is cycle number:
    a. 98°C, 1 min
        i. 98°C, 20 sec
        ii. 65°C, 20 sec
        iii. 72°C, 10 sec
        iv. Repeat X times.
    b. 72°C, 2 min
9. Transfer 50 μL reaction to a 1.5 mL tube and add 50 μL nuclease-free water, 1 μL 20 mg/ml glycogen, 10 μL 7.5 M ammonium acetate, 100 μL 100% isopropanol.
10. Incubate at -20 °C for 30 minutes and pellet the DNA by centrifuging for 15 minutes at 20,000 × g.
11. Wash the pellet once by adding 1 mL of 75% ethanol and vortexing until the pellet detaches. Incubate at -20 °C for 15 minutes, and pellet the DNA by centrifuging for 15 minutes at 20,000 × g.
12. Aspirate the liquid from the pellet and let it air dry for 10 minutes.
13. Dilute 6X loading dye to 2X loading dye and resuspend DNA pellet in 5 μL.
14. Pre-run 9% native polyacrylamide gel for 15 minutes at 100 V.
15. Rinse the wells with 1X TBE before adding the DNA samples. Do not load samples to the very first or last lanes. In the 2nd lane, add either a 100 bp or 50 bp ladder. In the 3rd and 4th lanes,

load the PCR product from the 30 and 40 nt controls. Skip the 5th lane, then add experiment samples from the 6th to the penultimate lane.

16. Run the gel at 100 V until the bromophenol blue dye front reaches the bottom of the gel.
17. Disassemble the gel and transfer to saran wrap.
18. Combine 5 µL of SYBR Gold nucleic acid stain with 5 – 10 mL of 1X TBE and cover the gel with this buffer for no more than 3 minutes. Agitate the gel while it stains by gentle shaking. Rinse briefly with 1X TBE and remove as much residual buffer from the gel as possible before proceeding.
19. Transfer gel to a new and dry Saran Wrap. Consider imaging the gel before (and after) excision of RNA gel slices. Transfer gel to the scanner and capture the Cy2 spectra by using an emission filter of 515 – 535 nm.
20. Using a Blue-Box transilluminator, illuminate the gel and excise the DNA between the 30 and 40 nt control's PCR product.
21. Transfer the gel slice to a tube. Crush the gel with a clean 1 mL pipette tip.
22. Add 500 µL of DNA elution buffer. Incubate at room temperature overnight under gentle rotation.
23. After elution, centrifuge for 10 minutes at max speed.
24. Transfer 450 µL of eluate to a new tube. If necessary, centrifuge and transfer again to completely remove residual gel fragments.
25. Add 1 µL of 20 mg/mL glycogen, vortex, and 3 volumes of 100% ethanol. Split across two tubes if necessary.
    a. After adding ethanol and vortexing, any remaining gel fragments will immediately become opaque and flocculate. Typically, a pipette tip can be used to fish these bits out before centrifuging.
26. Pellet the DNA by centrifugation at 4 °C at 20,000 × g for 15 minutes.
27. Aspirate 90% of the solution and add 1 mL of -20 °C chilled 70% ethanol and briefly vortex, ideally until pellet detaches.
28. Incubate at -20 °C for 15 minutes. Pellet the DNA by centrifugation at 4 °C at 20,000 × g for 15 minutes.
29. Using a 1 mL pipette, remove nearly all of the solution leaving ~100 µL.
30. Carefully remove the remaining liquid using a 200 µL pipette.
31. Briefly spin the tubes to collect any remaining liquid to the bottom of the tube, and carefully remove the remaining liquid using a 10-20 µL pipette.
32. Air dry, ideally for no more than 10 minutes, to evaporate any remaining 70% ethanol.
33. Resuspend DNA pellet in 15 – 20 µL of nuclease-free water.

Library qPCR and pooling

The NEBNext® Library Quant DNA Standards are a reliable standard for measuring the relative molar abundances of individual libraries prepared from this workflow. The measurements made here enable equimolar pooling of libraries. We recommend users not donate more than 50% of a given library. This can make it possible to resubmit specific libraries for further sequencing if more depth is required.

1. For each library, prepare two 1.5 mL tubes. Low-binding tubes can be used here. To the first add 38 µL of 0.2% Tween-20, and to the second add 998 µL of 0.2% Tween-20.
    a. The first tube will be a 1:20 dilution, and the second tube will be a 1:500 of the 1:20 dilution, which is a 1:10,000 dilution.
2. Dilute the library 1:20 by combining 2 µL of the library with the 38 µL of 0.2% Tween-20. Vortex for 10 seconds.
3. Dilute the library further to a 1:10,000 by combining 2 µL of the 1:20 dilution with the 998 µL of 0.2% tween-20. Vortex for 10 seconds.
4. Prepare library qPCR master mix. Dispense 18 µL of master mix into each well for qPCR.
    a. One 20 µL reaction is 2 µL of diluted library DNA plus 10 µL iTaq™ Universal SYBR® Green Supermix, 0.2 µL 10 µM R1 primer, 0.2 µL 10 µM R2 primer, and 7.6 µL nuclease-free water.
    b. Prepare enough master mix for two replicates with all samples and at least one replicate of each of the standards and a no-template control.
5. Perform qPCR amplification with the following cycling parameters:
    a. 95°C, 1 min
        i. 95°C, 15 sec
        ii. 63°C, 45 sec (measurement)
        iii. Repeat 35 times.
6. Compute a linear regression with the log10(pM) (x-axis) concentration and the mean Ct value (y-axis) for each standard.
7. Use the linear regression to compute the pM concentration of the diluted libraries given the Ct.
8. Correct for the 1:10,000 dilution factor and convert pM to nM (*i.e.*, multiply by 1000).
9. Define the maximum volume of a sample you are willing to use for library pooling. (*e.g.*, no more than 66%).
10. Define the desired final concentration and volume of pooled libraries necessary for submission. Consult with your sequencing service before pooling.
11. Determine which library had the lowest yield. Calculate the number of fmoles available for pooling from the lowest yield library by multiplying the nM concentration by the volume defined in Step 9. This defines the number of fmoles/library used to pool them equimolarly together.

a. Multiple the fmoles/library defined in Step 11 by the total number of libraries, and divided that product by the desired volume defined in Step 10. Determine whether this quotient is above or below the desired concentration defined in Step 10.

12. For the remaining libraries, divide fmoles/library defined in Step 11 by the nM concentration of each library (defined in Step 8). The resulting quotient is the volume of library needed for pooling.

13. Pool all the libraries together given the calculated volumes needed in Step 12.

    a. See the example calculations table for six hypothetical libraries in the table below.

14. Perform DNA Clean & Concentrate, and elute DNA in enough water to meet the desired final concentration and volume defined in Step 10.

Table 1: Example pooling strategy for six hypothetical libraries.

Sample	Conc. (nM); Step 8	Max Volume to Pool (µL); Step 9	Minimum Conc. (nM); Step 11	Amount to Pool (fmoles/library); Step 11	Volume contributed to Pool (µL); Step 12
A1	2.4	18	1.6	28.8	12
A2	3.3	18	1.6	28.8	8.7
A3	1.6	18	1.6	28.8	18
B1	5.5	18	1.6	28.8	5.2
B2	6.3	18	1.6	28.8	4.6
B2	3.5	18	1.6	28.8	8.2

Note: nM = fmoles/µL.

Important notes to consider when modifying the above protocols

- Nuclease digestion optimization: We recommend users first perform polysome collapse efficiency assays of nuclease digestion conditions before proceeding with large scale experiments. Differences in cell types, cell culture, or tissue types may require optimizations. For example, 800 µg/mL of heparin has been included for splenic tissue ribosome profiling experiments to limit endogenous RNase A activity [**3**]. Briefly, from a single batch of lysate perform a series of P1 nuclease digestions from undigested to 2X the recommended digestion. Resolve the digested lysate through a 10 – 50% sucrose density gradient and measure the A_{260nm}. Compute the area under the curve (AUC) for the 80S fraction and do the same for the polysome fraction (*i.e.*, everything beyond the 80S peak). Compute the collapse efficiency as the ratio of AUC(80S)/AUC(polysome) for each condition. Compare the collapse efficiency of each digestion condition relative to the undigested control. Identify which digestion conditions maximize polysome collapse but do not decrease the 80S peak by over-digestion.

- RNA quantification differences: RNA quantification by alternative methods may not be directly comparable; we recommend recalibration of nuclease digestion conditions using polysome collapse efficiency as described above.

- Using RNase I instead of P1 nuclease: Several modifications to the protocol above are necessary in order to use *E. coli* RNase I.

    a. mirRICH is not advantageous for RNase I digests as the rRNA fragmentation is severe. Instead, a higher precision method for RNA size selection such as gel purification should be done.

    b. T4 PNK, as described in [**1**], should be used to convert the 2′-3′ cyclic monophosphate product of RNase I to a 3′-OH. We encourage users to clean up gel-purified size selected RNA samples using RNA Clean and Concentrate before T4 PNK treatment because borate inhibits T4 PNK.

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