Isolation of metabolites and RNA from liquid cultures

Batch sizes: Each lab will prepare replicate samples of each wild strain. Each iteration of the preparation will contain 48 wild strains. These strains will be randomized in each prep set, and the sets will be random for each lab. However, each set will contain four reference strains. At 44 unique strains per set, it will take 15 iterations to complete three replicates of 220 strains in each lab.

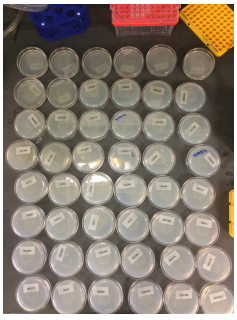
Naming conventions and labeling: We should use printed labels for worm plates, RNA tubes, metabolite tubes, etc. For worm plates, my lab uses clear, matte printed labels (cat #OL1930CX from onlinelabels.com) so that the labels do not interfere with worm picking. For RNA and metabolites, we should use cold-resistant labels (e.g. 15-930-A from Fisher or 9138-6000 from USA Scientific for Dymo printing). For naming strains and samples, we should use strain names as designated to match genotypes in CeNDR.

YYYYMMDD_CB4856_1_RNA = The first replicate of CB4856 for the RNA extraction
YYYYMMDD_CB4856_1_sup = The first replicate of supernatant from the CB4856 growth for the metabolite extraction
YYYYMMDD_CB4856_1_ppt = The first replicate of animals of CB4856 for the metabolite extraction
YYYYMMDD_CB4856_1_GCMS = first replicate for GC-MS analysis

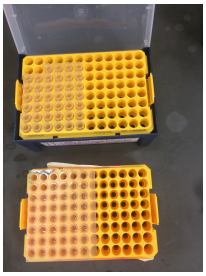
Walhout performs replicates 1-3 and Andersen performs replicates 4-6.

Strain maintenance from CeNDR to lab copies:
Strains will be shipped as frozen CeNDR sets to each lab. Clear labels for labeling the strains on arrival will be provided.

1. When you receive your strains, immediately remove the plate from the dry ice and place it in a rack to support 96-well plates.
2. Allow the material to thaw completely.
3. If possible, briefly spin down the plate to remove any liquid from the foil cover.
4. While the strains are thawing, label 48 6 cm plates with the provided labels on the bottom of the plate.
    - Array plates in a 6 x 8 grid to match the 48-well plate in which the strains arrived
    - Note that the labels are arrayed in (mostly) alphanumeric order. **Keep the plates in the above order as the strains are frozen in the plate in this order.** Wild strains all look identical to each other. Strain contamination is easy and all precautions to avoid mixing strains should be taken.
    - Place the plates with the bottom, labeled-side up.



5. Once the material is thawed, carefully remove the foil cover. Make sure that you do not jostle the tubes, because you do not want a strain from one well to contaminate a neighboring well.
    - To prevent strain contamination, it may help to first score the foil between the rows. Then, only peel off one row at a time. Alternatively, you can remove the entire foil cover and then tape over a row after you have pipetted out the worms from that row.
6. Starting with the upper-left, colored well, pipet 150 µl from the first well on to the first plate. To keep track of which wells you have pipetted from and which plates you have pipetted on to, use pipette tips in the same pattern as the 6 x 8 grid of the 48-well plate. When a plate has a strain on it, it will be lid-side up.
    - Pipet the material on an area of the plate that does not contain bacteria.
    - Keep the plate with the lid-side up until all the liquid is absorbed.



7. Pipet the material from the well to the right of the first well onto the next plate. Continue pipetting from each neighboring well on to the next plate in your alphanumeric-labeled array of plates. You can double-check the name of the strain with the well by consulting the Mapping Set grid provided.
8. You should see worms moving around ~10 minutes after all the liquid is absorbed.
9. Once the plates are dry, store the plates parafilmed and lid-side down at 20ºC.
10. After 3-4 days at 20ºC there should be gravid adults. We recommend cleaning the strain by the following method:
    - Pipet 15 µl of bleach solution (see recipe below) on the side of a 6 cm plate.
    - Place 10-20 gravid adults into the bleach spot.
    - Once the bleach is absorbed, incubate the plate at 20ºC.
    - The next day, pick 15-20 L1s from the bleach plate to a clean 6 cm plate. This plate is the master stock plate. Let this plate starve at 20ºC over the next week.

11. After one week, chunk a 1 cm square chunk to two labeled 6 cm plates and to one 10 cm plate. Let the 6 cm plates starve at 20ºC over the next four days. The 10 cm plate should be used to freeze a master copy of this wild strain in the Walhout and Schroeder labs. These 6 cm plates are the source plates for assay setup. Store them wrapped in parafilm at 15ºC. Every three months, one of these plates should be chunked to two more fresh plates, starved, and stored at 15ºC. After one year (or three chunks), discard these plates to prevent strain evolution.

NB: Strains should never be kept at temperatures warmer than 20ºC (*i.e.* avoid room temperature as much as possible).

NGMA plate setup:

1. Streak out project HB101 from frozen stock onto a fresh LB agar plate. Grow overnight at 37ºC.
2. The next day, pick a single colony to 5 mL of LB in a test tube and grow overnight at 37ºC. Because this strain is not under antibiotic selection, we highly recommend “inoculating” a test culture by touching a sterile inoculating loop to an empty area of your stock plate and dipping that loop into 5 ml of LB. Grow this culture along with your HB101 culture.
3. The next day, add 1 mL of overnight culture to 500 mL of LB. Grow this culture with shaking at 37ºC for approximately 4 hours until the OD_{600} = 0.5-0.6. .
4. Prepare 10 cm NGMA culture plates by seeding 1 mL of OD 0.6 HB101 onto each plate and drying at room temp for four days. NGMA plates should not be older than one month and stored at 4ºC after spotting and drying.

How to dissolve HB101 lysate:
We make 100 mg aliquots of lysate in 1.7 mL microfuge tubes. This aliquot can be easily resuspended in 1 mL of growth medium. The lysate should be stored at 4ºC and handled carefully (do not keep open, use sterile technique).
1. Weigh out 100 mg of lysate into a 1.7 mL microfuge tube. Vortex the capped tube to make sure the powdered lysate is not packed at the bottom.
2. Add 650 µL of growth media to the tube. Re-cap the tube.
3. Vortex by hand for one minute.
4. Using a microfuge rack, drag the tube back and forth across the top of the rack. This technique will disrupt troublesome clumps.
5. Repeat steps 3 and 4 until you can not see a pellet.
6. Put the tube on a bench top tube vortex set to run constantly. Vortex until all tubes have made it to step 5. I only do about 10 tubes at a time.
7. Add 300 µL of growth media and vortex for two minutes by hand or table-top tube vortex.
8. Centrifuge at 2000 rpm for 30 seconds.
9. Pipet the ~1 mL of 100 mg/mL lysate into your growth flask. If flasks are not prepared, pipet to a sterile storage tube (15 mL? tube) until ready. Be as careful as possible and use sterile technique.

Culture setup:
1. Based on the assay, choose one source plate for each strain from 15ºC.
2. Chunk a 1 cm chunk from these source plates to one 10 cm NGMA plate no more than two weeks before assay setup date and starve at 20ºC. This plate is the assay plate.
3. From these starved assay plates, chunk a 1 cm square chunk to each of ten 10 cm plates. The chunk should be placed at the edge of the bacterial lawn.
4. Incubate at 20ºC until just after the animals have cleared the bacterial lawn. This step usually takes 3-4 days. We need to watch the strains and prepare them when they clear the lawns of all 10 cm plates. Wild isolate strains grow at different rates.
5. Label three sterilized glass culture tubes for each of the strains.
6. Once the animals have cleared the bacterial lawns, wash all of the animals from one plate to the next for five plates by pouring 10 mL of sterile M9 from plate to plate. Then, pour from the final plate into a labeled glass culture tube. Repeat the procedure for a second 10 mL wash of all ten plates. NB: Do not prepare more than 10 strains a time. You want to ensure no one strain sits for much longer than others.
7. Go to bleach or settle procedure.

Bleach procedure:
1. Make bleach solution (recipe below) immediately before the bleach and store at 4ºC.
2. Centrifuge the 15 mL conicals at 1200 rpm for 1 min.

Comment [1]: Please comment on this thread for your own timing. We found that the four strains took 4-5 days. I don't think any strain will be ready in 3 days.

Comment [2]: 4 days here. Roughly 94 h post chunk

Comment [3]: I took pictures directly from the plates, with 8X, to check the stage of worms, eggs in the plate and burrowing.

Comment [4]: Higher magnifications may be better to see worm stage and burrowing. I will try next time and correct here.

3. Aspirate the M9 from each conical.
4. Add 8 mL of bleach solution to each conical. Bleach no more than 10 conicals at a time per person. If you do more, you run the chance of over-bleaching the worms.
5. Cap the conicals and start a 3 minute timer. Shake five conicals in each hand.
6. After 3 minutes, centrifuge the conicals at 1200 rpm for 30 seconds. Quickly remove the bleach solution.
7. Add 6 mL of fresh bleach solution, cap, and quickly shake for 2 minutes.
8. Centrifuge the conicals at 1200 rpm for 30 seconds. Quickly remove the bleach solution.
9. Add 10 mL of sterile M9, cap, invert, and quickly centrifuge the conicals at 1200 rpm for 30 seconds. Quickly remove the wash.
10. Repeat the wash two times.
11. After the last M9 wash step, add 2 mL of K medium (recipe below). Vortex to resuspend.
12. Pipet the embryo solution from both conicals for the same strain into a 125 mL Erlenmeyer flask containing 25 mL of K medium.
13. Shake at 180 rpm at 20ºC overnight.
14. The next day, titer the L1 concentration by pipetting five µL of solution to five different spots on a fresh unseeded 6 cm plate and counting then averaging the number of L1s in each spot. Count the number of thrashing L1s that have good-looking morphology (no blebs or malformations).
15. Ensure that you have ~100,000 L1 animals in 25 mL of K medium in a 125 mL Erlenmeyer flask. Each replicate of each genotype goes in its own 125 mL flask.
16. Add HB101 bacterial lysate to a final concentration of 10 mg/mL.
17. Go to growth and harvest.

Settle procedure:
1. Label three sterilized glass culture tubes for each of the strains.
2. Wash all of the animals from one plate to the next for five plates by pipetting 10 mL of sterile M9 from plate to plate. Then, pipette from the final plate into a labeled glass culture tube.
3. Perform a serial rinse of all ten plates with 10 mL of sterile M9.
    Note: Do not prepare more than 10 strains a time. You want to ensure no one strain sits for much longer than others.
4. Wait 10 minutes at room temperature for all non-L1 stages to pellet by gravity.
5. Remove the top 15 mL of M9 and L1s from each tube to a new labeled glass culture tube.
6. Wait 10 minutes at room temperature for all non-L1 stages to pellet by gravity.
7. Remove the top 10 mL of M9 and L1s from each tube to a new labeled glass culture tube.
8. Titer the L1 concentration by pipetting five µL of solution to five different spots on a fresh unseeded 6 cm plate and counting then averaging the number of L1s in each spot.
9. Take five independently sampled pictures using XXX to titer and look at animal stage.
10. Resuspend ~100,000 L1 animals in 25 mL of K medium with 10 mg/mL HB101 bacterial lysate in a 125 mL Erlenmeyer flask. Each replicate of each genotype goes in its own 125 mL flask.
11. Go to growth and harvest.

Growth and harvest:
1. Shake at 180 rpm at 20ºC.
2. Grow for XXX hours.
3. Decant 2 aliquots of 2.5 mL (~10,000 animals) into 2 x 15 mL conical for RNA extraction.
    *Decant the remaining 20 mL from the growth flask into a 50 mL conical and allow to settle on bench top during the following steps.
4. Pellet at 2,000 rpm in a tabletop centrifuge for one minute.
5. Wash twice with M9 (Aspirate supernatant, add 10 mL of M9, pellet, repeat).

Comment [5]: We need to add photograph step for tittering and quality.

Comment [6]: DL238: ~90K / 10 plates
Hawaii: ~200K / 10 plates
BRC20067: ~200K / 10 plates
I estimated 15-20% L2 in the final tube, although it's hard for me to discern big L1s from L2s.

Comment [7]: If we are going to include it, how are we going to do it?

Comment [8]: Consider YongUk's advice, I think we can take 30 uL of pack worms and transfer to a 96-well plate, add few uL of NaAzide to anesthetize worms and waits for them to settle down in the well. Then take pictures with 50X

After the two M9 wash in 15ml conicals, I transfer the worm packs to 1.5ml tubes, centrifuge and aspirate M9 again. 1.5 ml tubes save stock space and they can be directly used in RNA isolation.

Comment [9]: Please enter your times in this comment thread.

6. Aspirate the supernatant of the final wash and then carefully remove as much M9 as possible with a P200.
7. Freeze in liquid nitrogen and transfer to -80ºC. Proceed to the RNA isolation protocol section.
8. *50 mL conical containing the 20 mL culture (settle at least 10 m): remove the supernatant from the settled 50 mL conical to a fresh labeled 50 mL conical and freeze in LN2.
9. Wash twice with M9 - either by iterative settling or by centrifugation - and aspirate supernatant after each wash.
10. Freeze the pellet in LN2.
11. Ship both 50 mL tubes (containing growth supernatant and worm pellet) on dry ice to Schroeder lab.

Comment [10]: I remove 1mL pellet of worms to a 1.5mL tube, vortex and take pictures with 30µL in a 96-well plate, 50X magnification. Then I pellet the rest ~970µL and aspirate as much M9 as possible.

Comment [11]: Please add details above too. Thanks.

Comment [12]: Settling helps to reduce contamination from larval stages. I think centrifugation is probably acceptable in lieu of settling following bleach b/c less larval contamination following egg prep.

NGMA recipe:
Mix the following reagents in a sterile manner:

	1 L	2 L
Peptone (US Biological P3300)	2.5 g	5 g
NaCl (Fisher BP358)	3 g	6 g
Agarose (Denville GR140-500)	7 g	14 g
Agar (US Biological A0930-05)	10 g	20 g
Sterile water	975 mL	1950 mL

Autoclave the media.
After the media has cooled for at least an hour in a 65°C incubator or water bath, add the following IN THE ORDER LISTED using a plastic, disposable pipette:

K medium recipe:

Per 500mL:
51 mM NaCl (5.1 mL of 5 M NaCl)
32 mM KCl (16 mL of 1 M KCl)
3mM CaCl2 (1.5 mL of 1 M CaCl2) 3mM MgSO4 (1.5 mL of 1 M MgSO4)

1. Mix the four salts with increasing water, then fill to 500 mL of dH2O. Filter sterilize with Thermo Filter Unit (Cat #566-0020)
2. Add 1.25 µg/mL filtered cholesterol (125 µL of 5 mg/mL cholesterol) (Cat. # SLLG025SS, Millipore) after filtering
3. Mix, label, and store for up to two weeks. Be sure to check the K medium prior to use for any floating particulate, which is a sign of contamination. If K medium is contaminated, pour it down the drain and make new K medium.

Cholesterol Recipe:

1. Add 125 mg of powdered cholesterol (Sigma C8667-25G) to a 50 ml conical tube.
2. Add 25 mL of 100% ethanol to the tube.
3. Cap and shake to resuspend. Ensure that cholesterol is fully dissolved.

4. Aliquot using a 25 mL syringe fitted with a filter (Millipore Millex-LG cat #SLLG025SS)
5. Dispense 1 mL of 5 mg/mL cholesterol through the filter into autoclaved sterile 1.7 mL microfuge tubes
6. Cap and store at room temperature for up to six months
7. 125 µL of 5 mg/mL cholesterol in ethanol should be added to 500 mL of K medium.

Bleach recipe (per 200 mL):

40 mL NaOCl (from Fisher, cat #SS290-1)
Add 100 mL ddH2O
10 mL of freshly made 10 M NaOH (add 4g NaOH pellets to 10 mL of ddH2O)
Add ddH2O up to 200 mL

Mix well, store at 4ºC until needed. This bleach should be made immediately before use. If you make it earlier than immediately, you must adjust the bleaching time for the weaker bleach, respectively.

Addendum: Bleach Protocol from WormBook

Reagents

- 5 N NaOH
- Household bleach (5% solution of sodium hypochlorite)

Methods

1. Use C. elegans stock plates that have many gravid hermaphrodites. Wash the plates with sterile H2O. Pipet the H2O across the plate several times to loosen worms and eggs that are stuck in the bacteria.
2. Collect the liquid in a sterile 5 ml conical centrifuge tube with cap. Add H2O to total 3.5 ml.
3. Mix 0.5 ml 5 N NaOH with 1 ml bleach. Make this solution fresh just before use! Add to the centrifuge tube with the worms.
4. Shake well or vortex the tube for a few seconds. Repeat shaking/vortexing every 2 minutes for a total of 10 minutes.
5. Spin the tube in a table top centrifuge for 30 seconds at 1300 x g to pellet released eggs.
6. Aspirate to 0.1 ml.
7. Add sterile H2O to 5 ml. Shake well or vortex for a few seconds.
8. Repeat steps 5 and 6.

There are minor deviations in the Schroeder lab. My understanding is people typically bleach by suspending worms in 8 mL water, add 2 mL bleach (Clorox), and then 300 uL 10M NaOH. The bleaching process is ~6 m long: 4 m of shaking / vortexing, then a sturdy bang on the countertop, another vortex, then spin.

I have pretty limited experience bleaching worms, so if the worm heavyweights have opinions, preferred methods, tips+tricks, etc., please share them here.

Comment [13]: At this point, eggs would be collected and rocked in either S-Basal or M9 overnight to allow for eggs at different stages to synchronize, yielding an arrested L1 population the following day

Comment [14]: I definitely don't agree with all points in the WB protocol. Household bleach is too variable.

Hypochlorite concentration is higher in our recipe. 1/5 in both but Fisher bleach is more exact and 6% hypochlorite.

NaOH is the same in both. 10 mL in 200 mL of 10 M = 0.5 mL in 5 mL of 5 M.

I think we are good on concentrations.

The protocol as I wrote out above is more exact and will work more reliably than these differences.

Good?

RNA isolation protocol (Wear gloves and use RNAse-free reagents and plastic):

Prep: Label XX tubes, prepare sand, XXX

1. Remove worm tube from freezer.
2. Add 100 µL of prepped sand* before the worm pellet (100ul) thaws.
3. Add 1 mL TRIzol (cn 15596018, Thermo Fisher) to each frozen sample.
4. Vortex vigorously for 10 minutes at room temperature.

If >1mL TRIzol added in 15mL conical, allow the sand to settle at room temperature for 5 min.
Aliquot the supernatant in 1 ml aliquots into labeled 1.5 ml tubes.

5. Add 0.2 mL chloroform.
6. Vortex for 3 minutes.
7. Spin for 3 minutes at full speed.
8. Transfer the aqueous layer (XX) to a new labeled tube.
9. Add 0.5 mL isopropanol.
10. Mix by short vortex for 30 seconds and incubate for 8 minutes at room temperature.
11. Transfer to ice for 2 minutes.
12. Centrifuge at full speed for 10 minutes.
13. Remove supernatant and add 1 mL 75% ethanol (made with RNAse-free water).
14. Vortex vigorously and spin at full speed for 3 minutes to remove as much ethanol as possible.
15. Remove supernatant.
16. Centrifuge at full speed to spin down the residual wash. Pipette to remove the residual wash. Be careful not to disturb the RNA pellet.
17. Air dry for 3 minutes or until the pellet appears almost completely dry.
18. Resuspend in 150 µL of RNAse-free water.
19. Aliquot 2.5 µL to a separate tube for QC and store on ice. Transfer the master RNA tube to -80ºC freezer.
20. Assay 1 µL of RNA on the NanoDrop.
A260/280 = 2.0 - 2.1 (1.8 or higher is acceptable)
A260/230 = 2.0 - 2.3 (often lower without problems)
21. Assay RNA concentration using 1 µL on the Qubit with Qubit™ RNA XR Assay Kit (cat. Q33224, Invitrogen via Life Technologies). The Qubit concentration is often half that of the NanoDrop but it is more correct.
22. Transfer QC data to the master google sheet (here). Go to Illumina RNA-seq library preparation protocol.

*[Sand is from Sigma (#274739). To prep sand, wash 2x in 1 M HCl, wash ~8x in RNAse-free water (until pH is ~7.0), bake to dry. Predispense ~100 µL aliquots before starting RNA prep so they are handy for quick addition to each sample]

Comment [15]: 100uL sand per 100ul worms

Comment [16]: 1 ml Trizol for ≤ 100 µl worms.
10 volumes of Trizol for worm >100µl.

Comment [17]: about 60% of the volume of TRIZOL Reagent used for homogenization?

Comment [18]: Definitely will have to be adjusted with 20,000 animals.

Comment [19]: User guide of nanodrop says detection range of RNA is from 3 ng/ul to 1200 ng/ul. Extracted RNA conc. from x300 young adults is around 70-100 ng/ul in 50 ul RNAse-free water on OP50 diet and 90-110 ng/ul in 50 R.F.W. on Coma. diet. So is it too small volume for 20k worms to measure precisely by nanodrop and maybe Qubit (but I never experienced Qubit)?

Comment [20]: Yes, it is definitely too small a volume. We might have to go with 100-200 µL. Also, some other parts of the RNA isolation might have to be scaled like the Trizol, chloroform, and isopropanol.

Illumina RNA-seq library preparation protocol:

We should multiplex 24 libraries per HiSeq4000 lane. That will give us ~15 million reads per library assuming a 360 million read lane. From RNA isolation to library construction will be performed in our labs (or one lab). Given the scale and complexity, it might be worthwhile doing robotic RNA preps and pooling. We can do our own QC. At the number above, we will be sequencing 55 lanes on the HiSeq4000.

PCR Amplification Specs
50ng input - 15 cycles
100ng input - 14 cycles
250-400ng input - 13 cycles
500ng input - 12 cycles

NEBNext Poly(A) mRNA Magnetic Isolation Module - 24 rxns - 24 reactions, New England BioLabs, E7490S

NEBNext Multiplex Oligos for Illumina (Dual Index Primers Set 1) - 96 rxns - 96 reactions, New England BioLabs, E7600S

Notes: Keep all the buffers on ice, unless otherwise indicated.
Input RNA samples, RIN >7

Comment [21]: RNA integrity number, by Bioanalyzer
Comment [22]: explain

Which tubes to use? Which tips? Clean bench and pipettes?

Preparation of First Strand Reaction Buffer and Random Primer Mix (NEBNext Ultra II RNA Library Prep with Sample Purification Beads - 24 - 24 reactions, New England BioLabs, E7775S)

1. Prepare the First Strand Synthesis Reaction Buffer and Random Primer. Mix (2X) in a nuclease-free microcentrifuge tube as follows:

COMPONENT	VOLUME
(lilac) NEBNext First Strand Synthesis Reaction Buffer (5X)	8 µl
(lilac) NEBNext Random Primers	2 µl
Nuclease-free water	10 µl
Total Volume	20 µl

1.1.2. Mix thoroughly by pipetting up and down several times.
Note: Keep the mix on ice until mRNA is purified. It will be used in Step 1.2.36 and 1.2.39.

Comment [23]: update

1.2. mRNA Isolation, Fragmentation and Priming Starting with Total RNA (NEBNext Poly(A) mRNA Magnetic Isolation Module - 24 rxns - 24 reactions, New England BioLabs, E7490S**)**
1.2.1. Dilute the total RNA (1 µg) with nuclease-free water to a final volume of 50 µl in a nuclease-free 0.2 ml PCR tube and keep on ice.
1.2.2. To wash the Oligo dT Beads, add the following to a 1.5 ml nuclease-free tube. If preparing multiple libraries, beads for up to 10 samples can be added to a single 1.5 ml tube for subsequent washes.

COMPONENT	VOLUME PER ONE LIBRARY
Oligo dT Beads $d(T)_{25}$	20 µl
RNA Binding Buffer (2X)	100 µl
Total Volume	120 µl

1.2.3. Wash the beads by pipetting up and down several times.
1.2.4. Place the tube on the magnet and incubate at room temperature until the solution is clear (~2 minutes).
1.2.5. Remove and discard all of the supernatant from the tube. Take care not to disturb the beads.
1.2.6. Remove the tube from the magnetic rack.
1.2.7. Add 100 µl RNA Binding Buffer (2X) to the beads and wash by pipetting up and down. If preparing multiple libraries, add 100 µl RNA Binding Buffer (2X) per sample.
1.2.8. Place the tubes on the magnet and incubate at room temperature until the solution is clear (~2 minutes).
1.2.9. Remove and discard the supernatant from the tube. Take care not to disturb the beads.
1.2.10. Add 50 µl RNA Binding Buffer (2X) to the beads and mix by pipetting up and down until beads are homogenous. If preparing multiple libraries, add 50 µl RNA Binding Buffer (2X) per sample.
1.2.11. Add 50 µl beads to each RNA sample from Step 1.2.1. Mix thoroughly by pipetting up and down several times.

Comment [24]: about how much you add? what volume?

1.2.12. Place the tube in a thermal cycler and close the lid. Heat the sample at **65°C for 5 minutes and cool to 4°C with the heated lid set at ≥ 75°C** to denature the RNA and facilitate binding of the mRNA to the beads.
1.2.13. Remove the tube from the thermal cycler when the temperature reaches **4°C**.
1.2.14. Mix thoroughly by pipetting up and down several times. Place the tube on the bench and incubate at room temperature for 5 minutes to allow the mRNA to bind to the beads.
1.2.15. Place the tube on the magnetic rack at room temperature until the solution is clear (~2 minutes).
1.2.16. Remove and discard all of the supernatant. Take care not to disturb the beads.
1.2.17. Remove the tube from the magnetic rack.
1.2.18. Wash the beads by adding 200 µl of Wash Buffer to the tube to remove unbound RNA. Gently pipette the entire volume up and down 6 times to mix thoroughly.
1.2.19. Place the tube on the magnetic rack at room temperature until the solution is clear (~2 minutes).
1.2.20. Remove and discard all of the supernatant from the tube. Take care not to disturb the beads.
1.2.21. Remove the tube from the magnetic rack.
1.2.22. Repeat steps 1.2.18–1.2.21.
1.2.23. Add 50 µl of Tris Buffer (provided) to each tube. Gently pipette up and down 6 times to mix thoroughly.
1.2.24. Place the tube on the thermal cycler. Close the lid and heat the samples at **80°C for 2 minutes, then hold at 25°C with the heated lid set at ≥ 90°C** to do the first elution of the mRNA from the beads.
1.2.25. Remove the tube from the thermal cycler when the temperature reaches **25°C**.
1.2.26. Add 50 µl of RNA Binding Buffer (2X) to the sample to allow the mRNA to re-bind to the beads. Mix thoroughly by gently pipetting up and down several times.
1.2.27. Incubate the tube at **room temperature for 5 minutes**.
1.2.28. Place the tube on the magnetic rack at room temperature until the solution is clear (~2 minutes).
1.2.29. Remove and discard the supernatant from the tube. Take care not to disturb the beads.
1.2.30. Remove the tube from the magnetic rack.
1.2.31. Wash the beads by adding 200 µl of Wash Buffer. Gently pipette the entire volume up and down 6 times to mix thoroughly.

1.2.32. Spin down the tube briefly to collect the liquid from the wall and lid of the tube.
Note: It is important to spin down the tube to prevent carryover of the Wash Buffer in subsequent steps.
1.2.33 Place the tube on the magnet at room temperature until the solution is clear (~2 minutes).
1.2.34. Remove and discard all of the supernatant from the tube. Take care not to disturb the beads that contains the mRNA.
Note: It is important to remove all of the supernatant to successfully fragment the mRNA in the subsequent steps.
1.2.35. Remove the tube from the magnetic rack.
1.2.36. To elute the mRNA from the beads and fragment, add 11.5 µl of the First Strand Synthesis Reaction Buffer and Random Primer Mix (2X) prepared in Step 1.1.2, pipette up and down several times to resuspend the beads.
1.2.37 Incubate the sample in a thermal cycler with the heated lid set at 105°C as follows:

15 minutes at 94°C
Hold at 4°C*

*Immediately transfer the tube to ice for 1 minute as soon as it is cool enough to handle (~65°C)

1.2.38. Quickly spin down the tube in a microcentrifuge to collect the liquid from the sides of the tube and place on the magnet right away until the solution is clear (~1-2 minutes).
1.2.39. Collect the fragmented mRNA by transferring 10 µl of the supernatant to a nuclease-free 0.2 ml PCR tube.

Note 1: If the supernatant volume recovered is less than 10 µl for any reason, bring the volume up to 10 µl by adding the First Strand Synthesis Reaction Buffer and Random Primer Mix (2X) prepared in Step 1.1.2 and continue with the protocol.
Note 2: Avoid transferring the magnetic beads.

1.2.40. Place the tube **on ice** and proceed directly to First Strand cDNA Synthesis.

1.3 First Strand cDNA Synthesis (NEBNext Ultra II RNA Library Prep with Sample Purification Beads - 24 - 24 reactions, New England BioLabs, E7775S**)**

1.3.1. Assemble the first strand cDNA synthesis reaction **on ice** by adding the following components into fragmented and primed RNA from Step 1.2.40.

FIRST STRAND cDNA SYNTHESIS REACTION	**VOLUME**
Fragmented and primed RNA (Step 1.2.40)	10 µl
Nuclease-free Water	8 µl
(lilac) NEBNext First Strand Synthesis Enzyme Mix	2 µl
Total Volume	20 µl

1.3.2. Mix thoroughly by pipetting up and down several times.

1.3.3. Incubate the sample in a preheated thermal cycler with the heated lid set at ≥ 80°C as follows:

Step 1: 10 minutes at 25°C
Step 2: 15 minutes at 42°C
Step 3: 15 minutes at 70°C
Step 4: Hold at 4°C

1.3.4. Immediately, perform Second Strand cDNA Synthesis.

1.4 Second Strand cDNA Synthesis (NEBNext Ultra II RNA Library Prep with Sample Purification Beads - 24 - 24 reactions, New England BioLabs, E7775S**)**

1.4.1. Assemble the second strand cDNA synthesis reaction **on ice** by adding the following components into the first strand synthesis reaction product from Step 1.3.4.

SECOND STRAND SYNTHESIS REACTION	**VOLUME**
First-Strand Synthesis Product (Step 1.3.4)	20 µl
(orange) NEBNext Second Strand Synthesis Reaction Buffer (10X)	8 µl
(orange) NEBNext Second Strand Synthesis Enzyme Mix	4 µl
Nuclease-free Water	48 µl
Total Volume	80 µl

1.4.2. Keeping the tube on ice, mix thoroughly by pipetting the reaction up and down several times.
1.4.3. Incubate in a thermal cycler for **1 hour at 16°C** with the heated lid set at ≤ 40°C.

1.5 Purification of Double-stranded cDNA using NEBNext Sample Purification Beads (NEBNext Ultra II RNA Library Prep with Sample Purification Beads - 24 - 24 reactions, New England BioLabs, E7775S**)**

1.5.1. Vortex NEBNext Sample Purification Beads to resuspend.
1.5.2. Add 144 µl (1.8X) of resuspended beads to the second strand synthesis reaction (~80 µl). Mix well by pipetting up and down at least 10 times.
1.5.3. Incubate for 5 minutes at room temperature.
1.5.4. Briefly spin the tube in a microcentrifuge to collect any sample from the sides of the tube. Place the tube on a magnetic rack to separate beads from the supernatant. After the solution is clear, carefully remove and discard the supernatant. Be careful not to disturb the beads, which contain DNA.
1.5.5. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
1.5.6. Repeat Step 1.5.5 once for a total of 2 washing steps.
1.5.7. Air dry the beads for 5 minutes while the tube is on the magnetic rack with the lid open.
Caution: Do not overdry the beads. This may result in lower recovery of DNA.

1.5.8. Remove the tube from the magnet. Elute the DNA target from the beads by adding 53 µl 0.1X TE Buffer (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down several times. Briefly spin the tube and incubate for 2 minutes at room temperature. Place the tube on the magnetic rack until the solution is clear.
1.5.9. Remove 50 µl of the supernatant and transfer to a clean nuclease-free PCR tube.

SAFE STOP **Note: If you need to stop at this point in the protocol, samples can be stored at –20°C.**

1.6 End Prep of cDNA Library (NEBNext Ultra II RNA Library Prep with Sample Purification Beads - 24 - 24 reactions, New England BioLabs, E7775S**)**

1.6.1. Assemble the end prep reaction on ice by adding the following components to second strand synthesis product from Step 1.5.9.

END PREP REACTION	**VOLUME**
Second Strand cDNA Synthesis Product (Step 1.5.9)	50 µl
(green) NEBNext Ultra II End Prep Reaction Buffer (10X)	7 µl
(green) NEBNext Ultra II End Prep Enzyme Mix	3 µl
Total Volume	60 µl

1.6.2. Set a 100 µl or 200 µl pipette to 50 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.
Note: It is important to mix well. The presence of a small amount of bubbles will not interfere with performance.

1.6.3. Incubate the sample in a thermal cycler with the heated lid set at ≥ 75°C as follows:

30 minutes at 20°C
30 minutes at 65°C
Hold at 4°C

1.6.4. Proceed immediately to Adaptor Ligation.

1.7 Adaptor Ligation (NEBNext Ultra II RNA Library Prep with Sample Purification Beads - 24 - 24 reactions, New England BioLabs, E7775S; NEBNext Multiplex Oligos for Illumina (Dual Index Primers Set 1) - 96 rxns - 96 reactions, New England BioLabs, E7600S **)**

1.7.1. **Dilute the (red) NEBNext Adaptor* 5-fold dilution in Adaptor Dilution Buffer prior to setting up the ligation reaction in ice-cold Adaptor Dilution Buffer and keep the diluted adaptor on ice. X**

*The adaptor is provided in NEBNext Multiplex (NEB #E7600) Oligos for Illumina.

1.7.2. Assemble the ligation reaction on ice by adding the following components, in the order given, to the end prep reaction product from Step 1.6.4.

LIGATION REACTION	VOLUME PER ONE LIBRARY
End Prepped DNA (Step 1.6.4)	60 µl
Diluted Adaptor (Step 1.7.1)	2.5 µl
(red) NEBNext Ligation Enhancer	1 µl
(red) NEBNext Ultra II Ligation Master Mix	30 µl
Total Volume	93.5 µl

The Ligation Master Mix and Ligation Enhancer can be mixed ahead of time and is stable for at least 8 hours at 4°C. We do not recommend premixing the Ligation Master Mix, Ligation Enhancer, and adaptor prior to use in the Adaptor Ligation Step.

1.7.3. Set a 100 µl or 200 µl pipette to 80 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.
Caution: The NEBNext Ultra II Ligation Master Mix is very viscous. Care should be taken to ensure adequate mixing of the ligation reaction, as incomplete mixing will result in reduced ligation efficiency. The presence of a small amount of bubbles will not interfere with performance.

1.7.4. Incubate **15 minutes at 20°C** in a thermal cycler.

1.7.5 Add 3 µl (red) USER Enzyme to the ligation mixture from Step 1.7.4, resulting in total volume of 96.5 µl.
Note: USER enzyme can be found in the NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E6609 or #E7600) Oligos for Illumina.

1.7.6 Mix well and incubate at **37°C for 15 minutes** with the heated lid set to ≥ 45°C.
1.7.7 Proceed immediately to Purification of the Ligation Reaction.

1.8 Purification of the Ligation Reaction Using NEBNext Sample Purification Beads
1.8.1. Add 87 µl (0.9X) resuspended NEBNext Sample Purification Beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.
1.8.2. Incubate for 5 minutes at room temperature.

1.8.3. Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (~ 5 minutes), discard the supernatant that contain unwanted fragments **(Caution: do not discard the beads)**.
1.8.4. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
1.8.5. Repeat Step 1.8.4 once for a total of 2 washing steps.
1.8.6. Briefly spin the tube, and put the tube back in the magnetic rack.
1.8.7. Completely remove the residual ethanol, and air dry beads until the beads are dry for 5 minutes while the tube is on the magnetic rack with the lid open.
Caution: Do not overdry the beads. This may result in lower recovery of DNA target.
1.8.8. Remove the tube from the magnet. Elute DNA target from the beads by adding 17 µl 0.1X TE (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down. Quickly spin the tube and incubate for 2 minutes at room temperature. Put the tube in the magnetic rack until the solution is clear.
1.8.9. Without disturbing the bead pellet, transfer 15 µl of the supernatant to a clean PCR tube and proceed to PCR enrichment.

SAFE STOP **Note: If you need to stop at this point in the protocol samples can be stored at –20°C.**

1.9. PCR Enrichment of Adaptor Ligated DNA
Check and verify that the concentration of your oligos is 10 µM on the label.

NEBNext Multiplex Oligos for Illumina (Dual Index Primers, NEB #E7600)

1.9.1. Set up the PCR reaction as described below based on the type of oligos (PCR primers) used.

1.9.1A. Forward and Reverse Primers Separate	
COMPONENT	**VOLUME PER ONE LIBRARY**
Adaptor Ligated DNA (Step 1.8.9)	15 µl
(blue) NEBNext Ultra II Q5 Master Mix	25 µl
i5 Primer*	5 µl
i7 Primer*	5 µl
Total Volume	50 µl

* The primers are provided in NEBNext Multiplex (NEB #E7600) Oligos for Illumina. Look at the NEB #E7600 manual for valid barcode combinations and tips for setting up PCR reactions.

1.9.2. Mix well by gently pipetting up and down 10 times. Quickly spin the tube in a microcentrifuge.
1.9.3. Place the tube on a thermocycler with the heated lid set to 105°C and perform PCR amplification using the following PCR cycling conditions (refer to Table 1.9.3A and Table 1.9.3B):

Table 1.9.3A:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation Annealing/Extension	98°C 65°C	10 seconds 75 seconds	10
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

** It is important to limit the number of PCR cycles to avoid overamplification. If overamplification occurs, a second peak ~ 1,000 bp will appear on the Bioanalyzer trace (see page 76).

Note: PCR cycles are recommended based on high-quality Universal Human Reference Total RNA. It may require optimization based on the sample quality to prevent PCR over-amplification.

1.10. Purification of the PCR Reaction using NEBNext Sample Purification Beads
1.10.1. Vortex NEBNext Sample Purification Beads to resuspend.
1.10.2. Add 45 µl (0.9X) of resuspended beads to the PCR reaction (~ 50 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.
1.10.3. Incubate for 5 minutes at room temperature.
1.10.4. Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.
1.10.5. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
1.10.6. Repeat Step 1.10.5 once for a total of 2 washing steps.
1.10.7. Air dry the beads for 5 minutes while the tube is on the magnetic rack with the lid open.

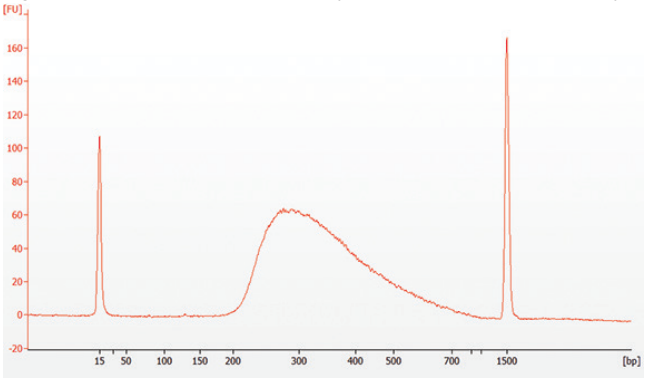
Caution: Do not overdry the beads. This may result in lower recovery of DNA.

1.10.8. Remove the tube from the magnetic rack. Elute the DNA target from the beads by adding 23 µl 0.1X TE (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down several times. Quickly spin the tube in a microcentrifuge and incubate for 2 minutes at room temperature. Place the tube in the magnetic rack until the solution is clear.
1.10.9. Transfer 20 µl of the supernatant to a clean PCR tube, and store at –20°C.

1.11 Assess Library Quality on a Bioanalyzer (Agilent DNA 1000 Chip)
1.11.1. Run 1 µl library on a DNA 1000 chip. If the library yield is too low to quantify on this chip, please run the samples on a DNA High Sensitivity chip.
1.11.3. Check that the electropherogram shows a narrow distribution with a peak size approximately 300 bp.

Note: If a peak at ~ 80 bp (primers) or 128 bp (adaptor-dimer) is visible in the Bioanalyzer traces, bring up the sample volume (from Step 1.10.9) to 50 µl with 0.1X TE buffer and repeat the SPRIselect or NEBNext Sample Purification Bead Cleanup Step (Section 1.10).

Figure 1.11.1: Example of RNA library size distribution on a Bioanalyzer.



Metabolite extraction protocol:

1.

All packages should be shipped overnight (?) on Mondays, Tuesdays, or Wednesdays to avoid packages being lost or delayed over weekends.

Shipping Addresses

Walhout lab:
University of Massachusetts Medical School
Albert Sherman Center, AS5.1008
368 Plantation Street
Worcester, MA 01605

Schroeder Lab
Cornell University
Boyce Thompson Institute, Rm 420
533 Tower Road
Ithaca, NY 14853

Andersen Lab
Department of Molecular Biosciences
Northwestern University
2205 Tech Dr, Hogan 1-500
Evanston, IL 60208-3500