B) MATERIALS

▲ **CRITICAL** All reagents and equipment must be maintained free of RNase contamination. We strongly recommend that all reagents and kits are purchased at the highest molecular and manufacturing quality and to be reserved for RNA use only. Similarly, we highly recommend that equipment used for RNA-related work be reserved for RNA use only.

B-1) Reagents

- Transgenic *UAS-HA-CD:UPRT* fly lines: *pUAS-HA-CD:UPRT-attB* constructs (containing either a N-terminal HA-tagged *Saccharomyces cerevisiae S.c. CD:UPRT* fusion gene or a *Drosophila* codon-optimized *CD:UPRT* fusion gene) were used to generate second and third chromosome *UAS-CD:UPRT* lines for both the *S.c.CD:UPRT* and optimized *CD:UPRT*. All four fly lines have been deposited with the Bloomington Drosophila Stock Center (https://bdsc.indiana.edu): N-terminal HA-tagged S. cerevisiae *UAS-HA-CD:UPRT* (2^{nd} chromosome) (#77119); N-terminal HA-tagged S. cerevisiae *UAS-HA-CD:UPRT* (3^{rd} chromosome) (#77120); N-terminal HA-tagged Drosophila codon-optimized *UAS-HA-CD:UPRT* (2^{nd} chromosome) (#77121); N-terminal HA-tagged Drosophila codon-optimized *UAS-HA-CD:UPRT* (3rd chromosome) (#77122).
- Transgenic tissue-specific *Gal4* fly lines: the following Gal4 line was obtained from the Bloomington Drosophila Stock Center: *109(2)80-Gal4* (#8769) to drive the expression of *UAS-HA-CD:*UPRT in the Drosophila multi-dendritic neurons (52)
- Schneider's Drosophila media
- TRIzol reagent (Invitrogen, cat. no. 15596026) **! CAUTION** TRIzol is toxic and should be used in a fume hood; wear gloves and a lab coat.
- Chloroform (MP Biomedicals, cat. no. 194002) **! CAUTION** Chloroform is toxic and should be used in a fume hood; wear gloves and a lab coat.
- Isopropanol (Fisher Scientific, cat. no. BP2618)
- Ethanol (Fisher Scientific, cat. no. BP2818)
- Sodium Acetate
- Sodium Chloride (Invitrogen, cat. no. AM9759)
- Nuclease-free water
- RNeasy Mini Kit (Qiagen, cat. no. 74104)
- RNase-free DNase I (Qiagen, cat. no. 79254)
- 5-EC (5-ethynyl cytosine)
- 5-EUd (5-ethynyl uridine)
- Click-iT® Nascent RNA Capture Kit (Invitrogen, cat. no. C10365)
- Biotin Azide
- THPTA (tris-hydroxypropyltriazolylmethylamine) (Click Chemistry Tools, cat. no. 1010)
- Copper (II) sulfate (Sigma Aldrich, cat. no. 451657)
- (+) - Sodium L-Ascorbate (Sigma Aldrich, cat. no. A7631)
- HEPES
- Dynabeads™ MyOne™ Streptavidin T1 (Invitrogen, cat. no. 65601)
- Poly(deoxyinosinic-deoxycytidylic) acid, sodium salt (Poly(dI-dC) • Poly(dI-dC) sodium salt) (Sigma Aldrich, cat. no. P4929)
- Tris-HCl pH 7.5 (Invitrogen, cat. no. 15567-027)
- Ultrapure EDTA, pH 8.0 (Invitrogen, cat. no. 15575-038)
- TWEEN® 20 (Sigma Aldrich, cat. no. P9416)
- RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen, cat. no. 10777019)
- NuGEN Ovation® Universal RNA-Seq System 1-16 (NuGEN, cat. no. 0343-32)

- 10X PBS Buffer, pH 7.4 (Ambion, cat. no. AM9624)
- RNaseH buffer, 10X
- D-biotin, 50 mM
- RNase Cocktail™ Enzyme Mix (Invitrogen, cat. no. AM2286)
- Ribonuclease H (RNase H) (Invitrogen, cat. no. 18021014)
- Dimethyl sulfoxide (DMSO) (Sigma Aldrich, cat. no. D8418)
- Zymo DNA Clean & Concentrator-5 kit (Zymo Research, cat. no. D4013)
- Qubit™ RNA HS Assay Kit (Invitrogen, cat. no. Q32852)
- EvaGreen® Dye, 20X in Water (Biotium, cat. no. 31000)

B-2) Equipment

- RNase-free microcentrifuge tubes, 1.5 mL
- Phase-lock tubes, RNase-free
- RNase-free microcentrifuge tubes, 5 mL
- RNase-free Centrifuge tubes, 15 mL
- RNase-free Centrifuge tubes, 50 mL
- Qubit assay tubes
- Thin-walled PCR tubes
- RNase-free tubes, 0.6 mL
- Sterile syringe, 1 mL, 3 mL and 5 mL
- 26 G × 3/8 inch detachable needle
- Refrigerated centrifuge (Eppendorf)
- RNase-free pellet pestle grinder
- Pestle, RNase-free
- SpeedVac
- Thermomixer
- Thermomixer heated cover
- Thermocycler
- Vortex
- Rotator
- Covaris M-series
- Qubit 2.0 fluorometer
- Nanodrop
- Agilent bioanalyzer
- Agilent Bioanalyzer DNA high-sensitivity kit
- Agilent Bioanalyzer's RNA Pico Kit
- Illumina HiSeq ---- OR NextSeq ----

B-3) Software

- RNA-seq software
- Bioanalyzer software
- Analysis software
    - R
    - R studio
    - Excel

B-4) Reagent setup

- 5-EC (5-ethynyl cytosine)
- 5-EUd (5-ethynyl uridine)
- CuSO4 (for home-made biotinylation reaction)
- NA Ascorbate (for home-made biotinylation reaction)
- Biotin Azide
- Precipitation Solution Na Acetate, 3M, pH 5.2
- RNase-free DNase I stock
- Preparing stock of Component E from Click-iT kit
- HEPES Buffer, 10X
- Block & Washing (B&W) Buffer, 2X
- TNE bead-blocking buffer, 10X: 100 mM Tris.Cl, pH 7.5; 10 mM EDTA, pH 8.0; 2 M NaCl; 20 mg/ml nuclease-free BSA; and 10 µg/ml poly(deoxyinosinic-deoxycytidylic) acid, sodium salt. Aliquot and store up to a year at −20°C.
- Wash 65-T Buffer: 100 mM Tris.Cl, pH 7.4; 10 mM EDTA, pH 8.0; 1 M NaCl; and 0.1% Tween 20. Mix first three items. Store buffer up to a year at room temperature. Add 0.1% Tween 20 fresh each time just before use.
- TNE 2.0-T Buffer: 10 mM Tris.Cl, pH 7.4; 1 mM EDTA, pH 8.0; 2 M NaCl; and 0.1% Tween 20. Mix first three items. Store buffer up to a year at room temperature. Add 0.1% Tween 20 fresh each time just before use.
- TNE 0.2-T Buffer: 10 mM Tris.Cl, pH 7.4; 1 mM EDTA, pH 8.0; 200 mM NaCl; 0.1% Tween 20. Mix first three items. Store buffer up to a year at room temperature. Add 0.1% Tween 20 fresh each time just before use.
- TNE 0.2 Buffer: 10 mM Tris.Cl, pH 7.4; 1 mM EDTA, pH 8.0; and 200 mM NaCl in nuclease-free water. Store up to a year at room temperature.
- Adult tagging solution (Sucrose tagging solution)
- Larval tagging media (Mocha cap)
- Embryo tagging media

C) PROCEDURE

5-EC and/or 5-EUd delivery and total RNA extraction

1| Thaw 5-EC (or 5-EUd) stock solution at room temperature.

▲ **CRITICAL** 5-EC tends to precipitate out of solution, especially at higher concentration. Hence, we highly recommend vortexing the stock solution vigorously before diluting it to the required tagging concentration using the appropriate tagging media.

2| Delivery of 5-EC (or 5-EUd) into Drosophila embryos, larvae or adult stages. Here – see fly delivery protocols or replace with cell culture-specific protocol

Total RNA purification

▲ **CRITICAL** From this point on, special care should be taken to maintain RNase-free conditions. Benchtops and pipettes should be thoroughly cleaned with RNaseAWAY. RNase-free tubes and filtered pipette tips should be always used. Gloves should be worn at all times.

3| Precool microcentrifuge to 4°C.

4| Thaw TRIzol samples (stored from at -80°C from TRIzol-based total RNA extraction step). Add additional TRIzol or combine similar samples to reach a final volume of 1 mL of TRIzol.

Incubate the 1.5 mL microcentrifuge tube for 5 minutes at room temperature (~22°C). Transfer the contents of the microcentrifuge tube to a 2 mL phase lock gel tube.
! CAUTION TRIzol is toxic and should be used in a fume hood. Wear gloves and a lab coat.
▲ CRITICAL Make sure to spin down the heavy gel to the bottom of the phase lock gel tube(s) before transferring the TRIzol content to it(them).

5| Add 200 μL of chloroform. Mix well either by vigorous shaking or vortexing for 15 s. Incubate the phase lock gel tube for 3 minutes at room temperature.
! CAUTION Wear gloves and use a fume hood when working with chloroform
▲ CRITICAL The vigorous extended shaking after adding chloroform is important for high RNA yield and quality.

6| Centrifuge the phase lock gel tube at 12,000*g* for 15 minutes at 4°C.

7| Transfer the upper aqueous phase to a new RNase-free 1.5 mL microcentrifuge tube. Add 250 μL of Isopropanol and 250 μL of 3M Sodium Acetate (pH 5.2). Mix contents well by pipetting. Incubate the microcentrifuge tube for 10 minutes at room temperature.

8| Centrifuge the microcentrifuge tube at 16,000*g* for 15 minutes at 4°C.
▲ CRITICAL A pellet of precipitated RNA should be visible after this centrifugation step.

9| Decant the liquid. Wash with 500 μL of 75% (vol/vol) ethanol.

10| Centrifuge the microcentrifuge tube at 7,500*g* for 5 minutes at 4°C. Decant the liquid.

11| Fast spin the microcentrifuge tube to collect any excess ethanol to the bottom of the tube. Carefully remove the remaining liquid by pipetting without disturbing the pellet. Briefly air dry the pellet.
▲ CRITICAL Avoid over drying the RNA pellet since this could make resolubilizing it difficult.

12| Resuspend the RNA pellet in 50-100 μL of RNase-free water. Aim at having a final RNA concentration of ~ 1 μg μL^{-1}. The RNA concentration should be determined by spectrophotometry (e.g. a Nanodrop) or by using a Qubit fluorometer, according to the manufacturer's directions.

13| Treat the RNA with DNase to remove any residual genomic DNA contamination. We recommend using Qiagen's RNase-free DNase I in association with Qiagen's RNeasy Mini Kit, according to the manufacturer's direction.
▲ CRITICAL Do not exceed 100 μg of total RNA per RNeasy Mini Kit's spin column since this is the maximum binding capacity of the column. As per the manufacturer's protocol, if a yield of > 30 μg of DNase-treated RNA is expected, two rounds of elution with RNase-free water should be done. We recommend incubating the column for 1 minute at room temperature after applying RNase-free water for elution before centrifuging the tube according the manufacturer's protocol. To achieve a higher concentration of eluted DNase-treated RNA, the manufacturer's protocol suggests using the elute from the first round of elution and re-applying it to the column for the second round of elution. We recommend adding a new volume of RNase-free water to the column for the second elution step rather than re-using the elute from the first elution step since we have found that this approach yields a higher amount of RNA.

14| Determine the concentration of DNase-treated RNA by spectrophotometry (e.g. a Nanodrop) or by using a Qubit fluorometer, according to the manufacturer's directions.
▲ **CRITICAL** For the upcoming biotinylation step, the concentration of DNase-treated RNA should be ≥ 2.0 μg μL^{-1}. If the Nanodrop and/or Qubit fluorometer's measurements show a concentration of a lower value, we recommend concentration the RNA by using Qiagen's RNeasy MinElute Cleanup kit, Qiagen's RNeasy Mini kit, or ThermoFisher's SpeedVac Concentrator, as per the manufacturer's directions.

15| Determine the quality and integrity of RNA by using an Agilent Bioanalyzer's RNA Pico Kit..Label sample and store RNA at -80°C.
▲ **CRITICAL** Avoid repeated freezing and thawing of RNA samples.
■ **PAUSE POINT (end of day 1)** RNA can be frozen and stored at -80°C overnight or until ready to proceed.

RNA biotinylation
▲ **CRITICAL** For the biotinylation step, there are two alternative approaches that we non-preferentially use in our laboratory: the first is to use Invitrogen's Click-iT™ Nascent RNA capture kit, as per the manufacturer's recommendations; and the second (explained below) is dependent on reagents prepared in our laboratory. In our experience, both approaches show very comparable results in terms of yield and quality of biotinylated RNA. For both approaches, we recommend the following: (1) adding 30 μg of DNase-treated total RNA per a biotinylation reaction for which the final reaction volume should be 50 μL; (2) adding a spike-in control in the form of 0.5 pg of 5-EU-tagged Fluc in each biotinylation reaction (i.e. 0.5 pg 5-EU-tagged Fluc / 30 μg DNase-treated total RNA); and (3) incubating the biotinylation reaction in the dark at 25°C for 30-45 minutes with gentle mixing at 600-700 rpm by using Eppendorf's Thermomixer and ThermoTop.

16| In a clean RNase-free 1.5 mL microcentrifuge tube, combine 5 μL of of 20 mg/mL THPTA, 1 μL of 100 mM $CuSO_4$, 5 μL of 200 mM Sodium Ascorbate, 5 μL of 10 mM of Biotin Azide, 30 μg of DNase-treated RNA, and 0.5 pg of 5-EU-tagged Fluc. Add RNase-free water to achieve a final volume of 50 μL. Mix well by pipetting without introducing bubbles. Spin down the content using a benchtop mini spin microcentrifuge. Incubate the biotinylation reaction in the dark at 25°C for 30-45 minutes with gentle mixing at 600-700 rpm by using Eppendorf's Thermomixer and ThermoTop.

17| Terminate biotinylation reaction by adding 450 μL of 1X HEPES buffer to the microcentrifuge tube for a final volume of 500 μL. Transfer the contents of the microcentrifuge tube to a 2 mL phase lock gel tube. Add an equal volume (500 μL) of chloroform. Mix well either by vigorous shaking or vortexing for 15 s. Incubate the phase lock gel tube for 3 minutes at room temperature.
! CAUTION Wear gloves and use a fume hood when working with chloroform
▲ **CRITICAL** Make sure to spin down the heavy gel to the bottom of the phase lock gel tube(s) before transferring the sample to it.
▲ **CRITICAL** The vigorous extended shaking after adding chloroform is important for high RNA yield and quality.

18| Centrifuge the phase lock gel tube at 16,000*g* for 15 minutes at 4°C.

19| Transfer the upper aqueous phase to a new phase lock gel tube. Repeat steps 18 & 19.

20| Transfer the upper aqueous phase to a new RNase-free 1.5 mL microcentrifuge tube. Add 450 μL of Isopropanol and 50 μL of 5M NaCl. Mix contents well by pipetting. Incubate the microcentrifuge tube for 10 minutes at room temperature.

21| Centrifuge the microcentrifuge tube at 16,000*g* for 15 minutes at 4°C.
▲ **CRITICAL** A pellet of precipitated biotinylated RNA should be visible after this centrifugation step.

22| Decant the liquid. Wash with 500 μL of 75% (vol/vol) ethanol.

23| Centrifuge the microcentrifuge tube at 7,500*g* for 5 minutes at 4°C. Decant the liquid.

24| Repeat 75% ethanol wash by repeated steps 23 & 24.

25| Fast spin the microcentrifuge tube to collect any excess ethanol to the bottom of the tube. Carefully remove the remaining liquid by pipetting without disturbing the pellet. Briefly air dry the pellet
▲ **CRITICAL** Avoid over drying the RNA pellet since this could make resolubilizing it difficult.

26| Resuspend the RNA pellet in 10-15 μL of RNase-free water. Aim at having a final RNA concentration of ~ 2 μg μL^{-1}. The RNA concentration should be determined by spectrophotometry (e.g. a Nanodrop) or by using a Qubit fluorometer, according to the manufacturer's directions

27| *(optional)* Determine the quality and integrity of RNA by using an Agilent Bioanalyzer's RNA Pico Kit.. Label sample and store biotinylated RNA at -80°C.
▲ **CRITICAL** Avoid repeated freezing and thawing of biotinylated RNA samples.
■ **PAUSE POINT** Biotinylated RNA can be frozen and stored at -80°C overnight or until ready to proceed.

Isolation of biotinylated EU-tagged RNA (EU-RNA)
▲ **CRITICAL** For the biotinylated RNA isolation step, we recommend using Dynabeads MyOne Streptavidin T1. We recommend incubating the Dynabeads in a blocking solution (as described below) for ~24 hours before starting the isolation step since we have found that adding this step significantly decreases the likelihood of non-specific RNA binding to the Dynabeads during the isolation step. We recommend adding ~ 20 μg of biotinylated RNA to 50 μL of blocked Dynabeads per one isolation reaction. Accordingly, since biotinylated EU-RNA represents a tiny fraction of the total RNA going into the isolation reaction, we recommend performing at least two isolation reactions per each condition replicate.

28| Block Dynabeads MyOne Streptavidin T1 for 24 hours before isolation step
▲ **CRITICAL** Before each washing incubation, vortex the Dynabeads after adding the respective washing buffer. For washing incubations, we recommend using either a sample mixer (e.g. HulaMixer® Sample Mixer) or a thermomixer (e.g. Eppendorf's Thermomixer) at maximum speed with a heated top (e.g. Eppendorf's ThermoTop) for high temperature washes. We strongly recommend applying a cap lock to each microcentrifuge tube during washing incubations, especially for high-temperature, maximum-speed Thermomixer-based incubations, to avoid accidental spilling. After each washing incubation, fast spin the microcentrifuge tube using a benchtop mini spin microcentrifuge, apply the tube in a magnet (e.g. Dynamag-2 Magnet from Invitrogen) for 2-3 minutes to immobilize the beads, and discard the supernatant.

A. Resuspend the Dynabeads in the provided vial by vortexing for > 30 s.

B. Aliquot 50 μL of Dynabeads (for each isolation reaction) in a new RNase-free 1.5 mL microcentrifuge tube.

C. Wash with 1 mL of 1X Binding & Washing-Tween (B&W-T) buffer. Incubate for 2 minutes using a sample mixer or a thermomixer at maximum speed, at room temperature.

D. Repeat step “C” twice, for a total of 3 washes with 1X Binding & Washing-Tween (B&W-T) buffer.

E. Wash with 500 μL of Solution “A”. Incubate for 2 minutes using a sample mixer or a thermomixer at maximum speed, at room temperature.

F. Repeat step “E” once, for a total of 2 washes with Solution “A”.

G. Wash with 500 μL of Solution “B”. Incubate for 2 minutes using a sample mixer or a thermomixer at maximum speed, at room temperature.

H. Wash with 1 mL of TNE2.0-T buffer. Incubate for 2 minutes using a sample mixer or a thermomixer at maximum speed, at room temperature.

I. Wash with 1 mL of WASH65-T buffer. Incubate for 10 minutes using a thermomixer at maximum speed, at 65°C.

J. Wash with 1 mL of TNE0.2-T buffer. Incubate for 2 minutes using a sample mixer or a thermomixer at maximum speed, at room temperature.

K. After discarding the supernatant from the last washing step, add 500 μL of 1X TNE Blocking buffer. Mix the content by pipetting without introducing bubbles. Parafilm the microcentrifuge tube. Incubate for 24 hours with mixing using a sample mixer, at 4°C.

L. After 24 hours of incubation with blocking buffer, fast spin the microcentrifuge tube using a benchtop mini spin microcentrifuge. Put the tube in a magnet for 2-3 minutes to immobilize the beads and discard the supernatant.

M. Wash with 1 mL of 1X Binding & Washing-Tween (1X B&W-T) buffer. Incubate for 2 minutes using a sample mixer or a thermomixer at maximum speed, at room temperature.

N. Repeat step “M” once, for a total of 2 washes with 1X Binding & Washing-Tween (B&W-T) buffer.

O. In a new RNase-free 5 mL centrifuge tube, resuspend the blocked Dynabeads in 2.5 mL of **2X** Binding & Washing-Tween (**2X** B&W-T) buffer.

29| Bind biotinylated RNA to blocked Dynabeads to isolate EU-RNA

A. In a new RNase-free 1.5 mL microcentrifuge tube, add 21 μg of biotinylated RNA to RNase-free water for a final volume of 500 μL. Mix well by pipetting. Denature biotinylated RNA by

incubating the microcentrifuge tube on a heat block for 10 minutes at 65-68°C. Immediately place the microcentrifuge tube on ice for 5 minutes.

B. Add 2 μL of RNaseOUT. Mix well by pipetting.

C. *(optional)* Transfer 24 μL of the mixture to a new RNase-free 5 mL centrifuge tube. This represents ~ 1 μg of the "input" biotinylated total RNA. Label as "INPUT" sample for the condition replicate and store at -80°C.
▲ **CRITICAL** This step is dependent on the experimental design (as discussed in the "Experimental design" section). If a comparison to an "input" sample is not needed, then we recommend starting with 20 μg of biotinylated RNA in step "30-A" instead of 20 μg.

D. Add denatured biotinylated RNA mixture to blocked Dynabeads mixture (from step 29-O). Add nuclease-free water to bring the final volume to 5 mL. Mix well by pipetting.
▲ **CRITICAL** Avoid introducing any bubbles during pipetting.

E. Incubate the blocked Dynabeads-biotinylated RNA mixture in the dark for 30 minutes with rotation using a sample mixer (e.g. HulaMixer® Sample Mixer), at room temperature.

30| After incubation period in step "30-E", Split the Dynabeads-biotinylated RNA mixture into 3 or 4 new RNase-free 1.5 mL microcentrifuge tubes. Fast spin the microcentrifuge tubes using a benchtop mini spin microcentrifuge. Apply the tubes to a magnet for 2-3 minutes to immobilize the beads.

31| *(optional)* Transfer the supernatant to new RNase-free 1.5 mL microcentrifuge tube(s). Label as "Flow-through fraction 1 (FT1)" for the condition replicate and store at -80°C.
▲ **CRITICAL** Following steps 18-28, RNA will need to be purified from the Flow-through fraction 1 (FT1) before it can be used for any downstream analyses.

32| Add 500 μL of TNE2.0-T buffer to each of the three microcentrifuge tubes. Mix well by pipetting. Combine the total volume of 1.5 mL into a new RNase-free 1.5 mL microcentrifuge tube. Fast spin the microcentrifuge tube using a benchtop mini spin microcentrifuge. Apply the tube to a magnet for 2-3 minutes to immobilize the beads.

33| *(optional)* Transfer the supernatant to a new RNase-free 1.5 mL microcentrifuge tube. Label as "Flow-through fraction 2 (FT2)" for the condition replicate and store at -80°C.
▲ **CRITICAL** Following steps 18-28, RNA will need to be purified from the Flow-through fraction 2 (FT2) before it can be used for any downstream analyses.

34| Perform stringent washes of Dynabeads after biotinylated RNA binding step to get rid of any non-specific RNA binding to Dynabeads
▲ **CRITICAL** Before each washing incubation, vortex the Dynabeads after adding the respective washing buffer. For washing incubations, we recommend using either a sample mixer (e.g. HulaMixer® Sample Mixer) or a thermomixer (e.g. Eppendorf's Thermomixer) at maximum speed with a heated top (e.g. Eppendorf's ThermoTop) for high temperature washes. We strongly recommend applying a cap lock to each microcentrifuge tube during washing incubations, especially for high-temperature, maximum-speed Thermomixer-based incubations, to avoid accidental spilling. After each washing incubation, fast spin the microcentrifuge tube using a benchtop mini spin microcentrifuge, apply the tube in a magnet (e.g. Dynamag-2

Magnet from Invitrogen) for 2-3 minutes to immobilize the beads, and discard the supernatant unless otherwise specified.

A. Wash with 1 mL of TNE2.0-T buffer. Incubate for 2 minutes using a sample mixer or a thermomixer at maximum speed, at room temperature.

B. Repeat step "A" three times, for a total of 4 washes with TNE2.0-T buffer.

C. Wash with 1 mL of 1X Binding & Washing-Tween (1X B&W-T) buffer. Incubate for 2 minutes using a sample mixer or a thermomixer at maximum speed, at room temperature.

D. Repeat step "C" three times, for a total of 4 washes with 1X B&W-T buffer.

E. Wash with 1 mL of WASH65-T buffer. Incubate for 2 minutes using a thermomixer at maximum speed, at 65°C.
▲ **CRITICAL** We recommend prewarming WASH65-T buffer to 65°C before using it in washing steps.

F. Repeat step "E" three times, for a total of 4 washes with WASH65-T buffer.

G. Wash with 1 mL of TNE0.2-T buffer. Incubate for 2 minutes using a thermomixer at maximum speed, at 65°C.
▲ **CRITICAL** We recommend prewarming TNE0.2-T buffer to 65°C before using it in washing steps.

H. Repeat step "G" three times, for a total of 4 washes with TNE0.2-T buffer.

I. Wash with 1 mL of TNE0.2 buffer. Incubate for 2 minutes using a thermomixer at maximum speed, at 65°C.
▲ **CRITICAL** In this step, no Tween-20 is added to TNE0.2 buffer. We recommend prewarming TNE0.2 buffer to 65°C before using it in this washing step.

J. Wash with 1 mL of RNase-free water. Incubate for 2 minutes using a sample mixer or a thermomixer at maximum speed, at room temperature.

K. Repeat step "J" once, for a total of 2 washes with RNase-free water.
▲ **CRITICAL** *(optional)* Instead of discarding the supernatant of this last wash, it can be transferred to a new RNase-free 1.5 mL microcentrifuge tube and stored at -80°C as "Flow-through fraction 3 (FT3)". Following steps 18-28, RNA will need to be purified from the Flow-through fraction 3 (FT3) before it can be used for any downstream analyses.
▲ **CRITICAL STEP** At this point, we highly recommend that you ***immediately*** use the RNA captured on the Dynabeads as a template for cDNA synthesis reaction or any other desired downstream analyses. Do ***not*** store the RNA bound to the Dynabeads for future use.

Illumina RNA-Seq library preparation
▲ **CRITICAL** While there are many workflows available to prepare RNA-Seq libraries compatible with the Illumina platforms, we opted to use NuGen's Ovation® Universal RNA-Seq System since it provides a workflow suitable for whole transcriptome profiling with integrated steps for efficient depletion of uninformative transcripts like rRNAs. The system is compatible with input amounts of 10 – 100 ng of total or poly(A) selected RNA. For lower input amounts,

NuGen provides an alternative system named NuGen's Ovation® SoLo RNA-Seq System for which the input amount needs to be in the range of 10 pg – 10 ng. Both systems provide available workflows with rRNA depletion from a number of model organisms (e.g. human, mouse, rat, Drosophila, and Arabidopsis).

35| Prepare first-strand cDNA using all the Dynabeads (with all the attached RNA) that are recovered from step (35-K). Follow the manufacturer's protocol (NuGen Ovation® Universal RNA-Seq System User Guide Doc No M01373v2, 2014) beginning with section V.A of the protocol section (First Strand Primer Pre-mix Preparation), bypassing section V.B of the protocol section (First Strand Synthesis with Integrated DNase Treatment) and finishing with section V.C of the protocol section (First Strand cDNA synthesis Using DNase-treated RNA).

▲ **CRITICAL** We highly recommend running the incubations for primer annealing and first-strand cDNA synthesis with gentle mixing to prevent the beads from settling. This can be achieved by using a thermomixer (e.g. Eppendorf's Thermomixer) with a heated top (e.g. Eppendorf's ThermoTop) at the desired temperatures. Since we have recommended performing at least two EU-RNA isolation reactions per each condition replicate, it is important to remember that there are two first-strand cDNA synthesis reactions per each condition replicate.

36| Add 500 μL of 1X PBS to each reaction. Mix well by pipetting. Transfer the mixture into a new RNase-free 1.5 mL microcentrifuge tube. Fast spin the microcentrifuge tube using a benchtop mini spin microcentrifuge, apply the tube in a magnet for 2-3 minutes to immobilize the beads, and discard the supernatant.

37| Repeat step 37 for a total of two washes with 1X PBS.

38| Resuspend the beads in 50 μL of RNaseA/T1/H elution mix (tabulated below, based on a previous protocol) (52). Incubate the mixture at 37 °C for 30 min at 1,000 rpm in a Thermomixer with a ThermoTop.

Component	Amount (μL)	Final Concentration
RNaseH buffer, 10X	5	1X
D-biotin, 50mM	12.5	12.5 mM
RNase cocktail enzyme	1	0.1 U/μL
RNaseH enzyme	1	0.1 U/μL
RNase-free water	30.5	
Total	50 (per reaction)	

39| Add 1 μL of 100% DMSO to each sample. Heat to 95 °C for 4 minutes with shaking using a Thermomixer with a ThermoTop. Pulse-centrifuge the samples. Apply them to DynaMag2 magnet for 1-2 minutes. Transfer the supernatant with the eluted first-strand cDNA to a new nuclease-free 1.5 mL microcentrifuge tube.

40| Purify the eluted first-strand cDNA using a Zymogen DNA Clean & Concentrator-5 column. Add a 7X (350 μL) of DNA binding buffer. Mix by vortexing. Pulse-centrifuge the tubes. Add 1 volume (350 μL) of 96-100% ethanol and mix by vortexing. Wash the columns according to the manufacturer's protocol. Elute the cDNA twice with 10 μL of nuclease-free water (for a final volume of 20 μL).

▲ **CRITICAL** Using of the Zymogen DNA Clean & Concentrator-5 columns under the conditions detailed in step 40 will recover single-stranded cDNA as small as 20 nt. Standard binding

conditions, as detailed in the manufacturer's protocol, recover cDNA of 50 bp or larger, which would result in a loss of short single-stranded cDNA molecules.
■ **PAUSE POINT** cDNA samples can be stored at -80 °C for > 6 months.

41| Before proceeding to the second-strand cDNA synthesis step using NuGen's Ovation® Universal RNA-Seq System, combine the purified eluted first-strand cDNA from each two (or more) reactions of each condition replicate into one nuclease-free 1.5 mL microcentrifuge tube. Concentrate the combined first-strand cDNA using a SpeedVac Concentrator until you reach a final volume of 10 μL, which is the recommended volume for the second-strand cDNA synthesis step as per the Ovation® Universal RNA-Seq System User Guide.
▲ **CRITICAL** To avoid overdrying, we recommend setting the drying rate of the SpeedVac Concentrator to "low" or "medium". If the sample gets overconcentrated to a volume less than 10 μL, bring up the volume to 10 μL using nuclease-free water. By the end of this step, each condition replicate should have one single reaction since first-strand cDNA from all separate isolation reactions should be pooled and concentrated into a final concentration of 10 μL.

42| Prepare second-strand cDNA using all the pooled, concentrated first-strand cDNA recovered from step 42. Follow the directions of section V.D of the protocol section (Second Strand cDNA Synthesis) in the manufacturer's protocol (NuGen Ovation® Universal RNA-Seq System User Guide Doc No M01373v2, 2014).
■ **PAUSE POINT** The double-stranded (ds)-cDNA produced at the end of this step can be stored at -20 °C overnight or until you ready to proceed.

43| Treat ds-cDNA sample with Covaris M-series System according to the manufacturer's directions to produce fragmented cDNA with a fragment size in the desired range. After fragmentation, collect 100 μL from the Covaris tube. If the collected volume is lower than 100 μL, add nuclease-free water to bring the total volume to 100 μL.
▲ **CRITICAL** We recommend using the entire sample volume retrieved from step 43 (120 μL) for the cDNA fragmentation step. Using the Covaris M220 System, we use the "snap-cap, 130 μL, 200 bp" DNA fragmentation protocol. Alternative fragmentation platforms and protocols may be suitable for use with this RNA-Seq library preparation workflow; however, they have not been validated by NuGEN.

44| Purify and concentrate the fragmented ds-cDNA using Agencourst RNAClean XP beads following the directions in section V.F of the protocol section (cDNA Concentration After Fragmentation) in the manufacturer's protocol (NuGen Ovation® Universal RNA-Seq System User Guide Doc No M01373v2, 2014).
■ **PAUSE POINT** The purified, concentrated double-stranded (ds)-cDNA produced at the end of this step can be stored at -20 °C overnight or until you are ready to proceed.

45| Prepare the fragmented ds-cDNA sample for adaptor/barcode ligation by following the manufacturer's directions in section V.G. (End Repair) of the protocol section.
46| Ligate the appropriate adaptor/barcode to end-repaired ds-cDNA from step 46. Following the manufacturer's directions in section V.H. (Ligation) of the protocol section.
▲ **CRITICAL** The ligation buffer mix in the NuGEN Ovation® Universal RNA-Seq System is very viscous. Be sure to pipet this reagent slowly to achieve an accurate reaction volume. If you are performing a 2 (or more)-plex multiplex sequencing, it is important to follow the manufacturer's directions regarding their strict barcode pairing requirements.

47| Deplete rRNA content of the adapter-ligated ds-cDNA sample by following the manufacturer's directions in sections V.I. (Strand Selection I), V.J. (Strand Selection I Purification), and V.K. (Strand Selection II) of the protocol section.
▲ **CRITICAL** Be sure to use the appropriate AnyDeplete rRNA primer mixture for your model organism.

48| Prepare your library for amplification by cleaving the ligated adapter. Follow the manufacturer's directions in section V.L. (Adaptor Cleavage) of the protocol section.

49| Amplify the ds-cDNA library sample by following the manufacturer's directions in section V.M. (Library Amplification) of the protocol section.
▲ **CRITICAL** We ***highly*** recommend determining the appropriate number of PCR cycles used for library amplification via real-time qPCR by following the manufacturer's guidelines in Appendix section VII.B. (Using qPCR to Determine the Number of PCR Cycles). To avoid excess library amplification which could yield biases, the number of PCR cycles used for library amplification should be within the exponential phase of the real-time qPCR amplification plot.

50| Purify the RNA-Seq library with Agencourt RNAClean XP beads following the directions in section V.N of the protocol section (Bead Purification of the Amplified Material) in the manufacturer's protocol.

51| Determine the quality of the RNA-Seq amplified library by using an Agilent Bioanalyzer DNA high-sensitivity chip, according to the manufacturer's directions.

52| Quantify the concentration of the RNA-Seq library using a Qubit fluorometer, according to the manufacturer's directions.
■ **PAUSE POINT (end of day 2)** RNA-Seq libraries can be stored at -20°C until sequencing run.

Pool equimolar amounts of each RNA-Seq library, for multiplex sequencing, into the final concentration and volume recommended by the Illumina platform to be used. Sequence pooled libraries on an Illumina-based machine using standard conditions.