*Note: Most of the protocol below is a combination of many different protocols used by the Gresham Lab. For convenience, we have put them all in a central location for general use. Many members of the Gresham Lab have contributed to optimization of the protocols. The protocols are occasionally updated with enhancements if improvements in yield or quality of results have been observed with alteration of the protocol. All of the individual protocols are on the internal Gresham Lab Wiki.

**Cell Growth and Treatment with 4tU**

1. Make media with the desired concentration of uracil. In the original RATE-seq experiments, we used 500µM uracil in the media.
2. Inoculate an overnight culture of the strain of interest. The overnight should be 3-5 ml.
3. The next day, take a measurement of the cell density. As long as the culture density is close to saturated, proceed with the experiment.
4. Back dilute the overnight culture 1:50 into media of the same composition as the overnight. The culture should be large enough so as to obtain enough cells for the protocol. For a first experiment, it is best to do 150ml culture (3ml overnight in 147ml new media).
5. While cells are growing, determine how much 4tU to add to the culture once the cells reach the correct culture density. Take into account the volume of cells for checking cell density as well as t0. Meaning, if you remove 0.5ml for cell count and 4.5 ml for t0, then calculate based on 150-5=145ml.
6. Allow the cells to proceed through ~2-2.5 doublings. This corresponds to ~5 hrs when grown in rich conditions from a saturated culture. Take 0.5 ml for counting the cell density.
7. Take 5-10ml for your t0 time point. This is the sample BEFORE you add 4tU.
8. Add the correct amount of 4tU and take ~10 ml samples. The volume of sample depends on the number of time points you take. In our experiments in rich media, we found that 4tU is incorporated as early as 3 minutes and that sampling every 3-5 minutes for the first 25 minutes gives good resolution. Make sure to take a few samples much later, as certain transcripts approach equilibrium much slower than a majority of the transcriptome, which approaches equilibrium quite rapidly. The last time point should be around the doubling time of the culture, i.e. 100 min after 4tU addition if the culture doubles every 100 min.
9. Collect samples according to our cell collection protocol. For convenience, it is below.

**Harvesting Cells**


In most circumstances, vacuum filtration is the best way to filter cells for RNA preps. Spinning down cells may induce a stress response.

**Collection Supplies:**

- Vacuum flask, w/ vacuum source
- Rigid tube connecting liquid pump to vacuum
- Filter support, collection funnel, clamp ("glass microanalysis filter holder assembly", small)
- Filters (use appropriate size), tweezers for moving filters
- Liquid nitrogen
- 2ml Eppendorf and/or 15ml Falcon tube for each sample

*Do not harvest samples into our standard 1.5ml Eppendorf tubes* as the volume of liquid within the tube will be at capacity, some liquid will leak out, and the phenol smell will penetrate the tubes into the laboratory. Instead, use 2ml Eppendorf.

**Collection Procedure:**

1. Assemble vacuum flask with filter support and turn on vacuum source.
2. Place filter on filter support, attach funnel, and clamp. Check filter and setup using DI water, before each sample collection.
3. Pour sample into funnel and allow to drain through filter.
4. Once drained, remove the clamp and funnel, and use tweezers to remove filter.
5. Roll up filter without touching cells, and put in tube. Drop tube into liquid nitrogen.
6. Rinse funnel, and repeat for other samples.

If doing a time series, try to harvest cells as fast as possible. Switching filters takes time, but with practice, it becomes manageable. Depending on the number of samples and size of prep, it may be worth asking someone to help with the harvest.

**RNA Extraction**

Use RNase free reagents and glass-/plastic-ware throughout! If using glassware, make sure it was baked overnight.

EDTA needs to be around pH 8.5 for all of the solute to dissolve at the required concentration. Use 10M NaOH to adjust the pH, and not the standard 5M NaOH. Add EDTA in steps, first putting some EDTA and then NaOH. Each time NaOH is added, wait for most of the EDTA in the container to dissolve before adding more EDTA. Do not vortex to get SDS into solution. This leads to many bubbles and SDS becomes difficult to
Supplies/Reagents for RNA extraction:

- Lysis buffer
- Acid phenol (Acid phenol may be labeled "saturated phenol" in the 4C fridge. Make sure it has an acidic pH.)
- Heavy phase lock gel (PLG) tubes
- Pipettes and tips
- Eppendorfs
- Ice
- Chloroform
- Sodium Acetate 3M
- Ethanol 95%
- Ethanol ~70%
- RNase free water

All steps involving phenol or chloroform MUST be done in the fume hood.

Set heat block to 65°C. Bring vortex into fume hood.

- Remove samples from the -80°C. They should be in 2 ml Eppendorf tubes.
- Before they thaw, add 750 µl lysis buffer. Vortex vigorously until solution is foamy and opaque.
- Incubate 1 hour @ 65°C, vortex vigorously at 20, 40, and 60 minutes.
- Fish out the filter and discard. (Use a clean pipette tip for each sample.)
- Ice 10 min. While incubating, spin the 2 ml heavy phase lock gel (PLG) tubes for 30 sec full speed.
- Spin sample lysate 5 min, 5k RPM at RT.
- With a pipette, transfer the top aqueous layer to the PLG tube.
- Add 750 µl chloroform. Invert to mix. Do not vortex!
- Spin 5 min at 5k rpm.
- Pour aqueous layer to new 2ml tubes. If your sample is low in RNA content and the RNA concentration is low use glycogen as a carrier to help precipitate it.
- Add 75 µl (or 1/10 volume if you lost some) 3 M sodium acetate. Vortex.
- Add 1.2 ml 100% ethanol. Vortex.
- Incubate -80°C for 30 min, or if you plan to finish the prep tomorrow, incubate samples in the -20°C overnight.
- Spin 5k rpm 10 min in cold room, decant supernatant.
- Wash pellet with 400µl of 70% ethanol. Vigorous pipetting is not needed or recommended, just make sure the surface of the pellet is exposed to EtOH.
- Spin 2 min 5k rpm. Discard supernatant.
- Wash and spin one more time. Discard supernatant.
- Air dry inverted on the bench 30 min. Do not leave too long past 30 min as the RNA can degrade.
- Dissolve pellet in 100 µl water (or more or less if necessary) without stirring. If you have no idea of the expected yield, dissolve in a smaller amount and then dilute if concentration is too high.

Check the concentration of RNA on the nanodrop and measure at least 2-3 times so that you gain a clear measurement about RNA concentration. This is extremely important as the spike in which is added for normalization purposes is entirely dependent on the amount of RNA. If the measurement is incorrect, then the entire experiment will be affected.

RNA Spike-in in vitro transcription

- The sequence for spike-in comes from *B. subtilis* and is cloned into the pSP64polyA vector. The plasmid needs to be cut with EcoRI for run off transcription. We store our plasmids in DH5alpha.
- Grow the DH5alpha cells overnight.
- Miniprep plasmids and quantify.
- Set up the linearization reaction of at least two reactions per spike-in:
- Incubate 2 hours each, 37°C.
- Run 1µl on ~1% gel to check. Make sure to run some non-linearized plasmid to compare for supercoiled and relaxed plasmid.
- Assuming complete digestion, clean up reactions using PCR clean up kit. Make sure to use one miniprep column for each spike-in. This means pool the two reactions onto a single column. Quantify using nanodrop to have an idea for concentration. Concentration should be in the range of 100-200 ng/µl, when resuspend in 30µl.

For n reactions, add n+1 µl of rATP, rCTP, rGTP, and rUTP (10mM stocks) into clean tube to create “rNTP” (2.5mM each) as referenced below.

- Add 6.25µl of linearized plasmid DNA to a new Eppendorf. For a kit positive control, use 1µl of the provided standard with 5.25µl of water.
- Set up master mix:
- Add 13.75µl of master mix to each tube of linearized DNA. Incubate 1hr in 30/37°C water bath or heat block. Use 30°C if you get multiple txn products, 37°C if you need more product. NOTE: Always remember to include a positive control.
- Remove 2µl into PCR tube for later gel.
- Add 1µl of RQ1 RNase-Free DNase.
- Incubate 15min at 37°C.
- Add equal volume (19µl) saturated phenol OR phenol:chloroform 5:1.
- Vortex vigorously and centrifuge at 12k RCF for 2min.
- Transfer top aqueous layer to new Eppendorf.
- Add equal volume of chloroform. Vortex vigorously and centrifuge at 12k RCF for 2min. Transfer top aqueous layer to new Eppendorf.
- Add 1/10 volume (~1.8µl) 3M NaAcetate and 1µl of glycogen or Glycoblu for a carrier.
- Add 2.5 volumes (45µl) of absolute EtOH.
- Chill 30min in -80°C, or -20°C overnight.
- Spin down at full speed in cold room for 30 min. Remove supernatant.
Wash with 500µl 70% EtOH. Spin down 5min. Remove supernatant.
Resuspend in 10µl PCR-grade H2O. Quantify using Qubit fluorometer.

Denaturing Gel for observing in-vitro transcription products
- Add 0.6g agarose to 50.8ml DEPC water.
- Microwave till dissolved, cool to ~65C.
- In the hood,
- Add 6ml 10x MOPS.
- Add 3.24ml formaldehyde.
- Pour gel and let sit 1hr.
- Remove comb and wrap in saran wrap.
- After gel has been poured, prepare the samples as follows:
  - The EtBr is based on a bottle that is 10µg/µl. Take 2µl in 18µl for the correct diluted concentration.
  - Distribute 17µl of master mix to Eppendorfs and add 2-4µl of sample. Do not forget to make a sample for a ssRNA ladder.
  - Incubate 60min @ 55C.
  - Chill 10min on ice.
  - Centrifuge briefly and add 2µl of 10x formaldehyde gel loading buffer, mix.
  - Put on ice until ready to load.

Running gel
- After gel has sat for at least 1hr, put gel to run in 1x MOPS buffer. It will take about 400-450ml of 1x MOPS to run a gel.
- Put the gel in the box and run for 5 min at the usual 100V setting.
- Load the samples and run ~30-40 minutes. Bromophenol blue dye runs about ~500 bases fast, so adjust time as appropriate.
- Visualize in GelDoc and take picture for EtBr staining.

Biotinylation of 4tU labeled RNA
Reagents:
- RNAse free water
- Tris-HCl (1M, pH 7.4)
- EDTA (0.5M)
- 4tU RNA
- Biotin HPDP (1mg/ml)
- 75% EIOH

All reagents should be made in RNAse free water.
The pH of Tris-HCl changes with time.
Biotin HPDP should be made 10mg/10ml in DMF. It should be aliquoted into 200-300 µl samples and stored in the -20C freezer.
The amount of reagents to use is dependent on the amount of RNA being labeled. In general, it is best to label at least 25µg, but 50µg is preferred.
A reaction for 25µg of RNA that is suspended in RNAse free water at a concentration of 500ng/µl is as follows:

- Set up reaction. First put in the correct amount of water into each tube. This is equal to the reaction volume minus the volume of the other components. The reaction volume is 10x higher than the amount (in µg) of RNA in the reaction.
- Pool the three (or four) different spike ins together so that they are a single mix. Ensure that there is enough of this pool for all experiments of a timeseries and biological replicate.
- Add Tris-HCl, EDTA and spike-in as a master mix. We have found that adding approximately 4ng of each spike in to 100µg of total RNA is sufficient. Add correct amount of RNA for each sample. Add Biotin-HPDP last. Vortex briefly.
- Incubate the reaction for three hours in the dark by placing tin foil over tubes and placing in a drawer.
- Spin down the reaction after three hours (~3min full speed) and pipet the aqueous part into a new tube. The samples should be labeled in blue pen as this has become the convention in our lab for discriminating between biotinylated RNA and RNA from the original sample. This step is necessary to separate out the unbound Biotin-HPDP. We had previously done this step with a chloroform step and PLG tubes, but spinning it down seems to suffice.
- Precipitate the RNA by adding 1/10 volume of 5M NaCl (here 25µl) and 1.1 volume of isopropanol (here 275µl).
- Incubate for 10 minutes at room temperature.
- Spin for 20 minutes, at 4C, full speed.
- After spin down, discard supernatant.

*****Note: the pellet does not stick well to the tube and if not careful, can be discarded with the supernatant. *****
- Add 400µl of 75% ethanol and spin 5 minutes, at 4C, full speed.
- Remove supernatant carefully.
- Dissolve RNA in RNAse free water so sample concentration is ~500ng/µl (Here, ~50µl). Check the concentration on the nanodrop. The concentration for all samples should be slightly lower than ~500ng/µl.

Streptavidin Pulldown of biotinylated RNA
Materials:
- Streptavidin magnetic beads
- Magnetic Stand
- 5% Beta-merchaptoethanol
- Bead buffer (1M NaCl, 10mM EDTA, 100mM Tris-HCl @ 7.4)
- 1.5 ml Eppendorfs
- Take 100µl of bead buffer per sample and warm to 65C.
- Label Eppendorf and add 200µl beads.
- Place tubes in magnetic rack and let collect for ~1.5min. Discard supernatant.
• Wash the beads in 200µl of bead buffer. Mix with pipet. Place samples in magnetic stand and discard supernatant.
• Resuspend in ~100µl of bead buffer. Make sure that total volume of RNA sample and bead buffer combined will equal ~200µl. If adding more than 50µg, make sure the volume of bead buffer to sample is at least 1:1. That means if you have 100µg in 200µl, add 200µl of the buffer to the 200µl of sample.
• Add RNA sample.
• Incubate RT for 20 min on bench.
• Place samples in magnetic stand for ~2 min. Discard supernatant (unless interested in unbound mRNA).
• Resuspend in 100µl bead buffer by pipetting up and down. Incubate RT for 5min. Collect beads in rack. Discard supernatant.
• Resuspend in 100µl bead buffer by pipetting up and down. Incubate RT for 1 min. Collect beads in rack. Discard supernatant.
• Resuspend in 100µl bead buffer (65°C from earlier) by pipetting up and down. Let sit for 1 min and then collect beads in magnetic rack.
• Resuspend in 100µl bead buffer. Incubate RT for 1 min. Collect beads in rack. Discard supernatant.
• Resuspend beads in 20µl of 5% Beta-mercaptoethanol. Incubate for 10min at room temperature.
• Collect beads in magnetic rack. Transfer supernatant to new Eppendorf. The liquid should be pink/light purple color. Be careful not to take beads into new tube.
• Repeat Beta-mercaptoethanol incubation at 65°C with the beads from previous step, then pool that with the supernatant from previous step.
• Precipitate RNA with
  1. 1/10 volume 5M NaCl
  2. At least 4µg glycogen (glycoblu) but 1µl undiluted is best.
  3. 1.1 volume isopropanol
• Incubate 10 min RT, spin down at max for 25min at 4C.
• Discard supernatant and wash with 75% EtOH. Spin down for 10min.
• Resuspend pellet in 10µl RNAse free water.
• Put in rack, transfer supernatant to new tube, quantify using Qubit Fluorometer. If experimental steps were done very carefully you should see an increase in concentration of sample with time.

Ribominus depletion of ribosomal transcripts

Reagents required:

Ribominus Transcriptome isolation kit Yeast, Invitrogen, K1550-03

- Glycogen (Invitrogen 10814-010)
- NaOAC 3M, pH 5.2
- 100% EtOH
- 70% EtOH
- nuclease-free water
- Magnetic rack
- 37°C heating block
- Centrifuge
- -80°C (-20°C for 1h to o/n)

This protocol is a scaled-down version of the original Invitrogen protocol.

- Make sure your RNA sample is in 10µl.
- Mix RNA with 0.4µl of the LNA probes and 10µl of Hybridization buffer.
- Incubate at 37°C in heat block for 5min and place immediately in ice.
- In the meantime, aliquot to a new tube 25µl of Streptavidin-coated magnetic Beads (supplied with the kit) for each sample. Place in magnetic stand for 1-2min until the liquid becomes clear and all magnetic particles are visibly on the side of the tube.
- Remove liquid carefully and resuspend beads in 25µl Water (supplied with the kit). Place in magnetic stand for 1-2min until the liquid becomes clear once more, remove liquid, and repeat this step one more time.
- Resuspend the beads in 10µl Hybridization buffer and keep them at the 37°C heat block until ready to use.
- Add the 25µl of RNA waiting in ice to the beads, mix by pipeting up and down and leave at 37°C for 15 min
- Place in magnetic stand for 1-2min until the liquid becomes clear and all magnetic particles are visibly on the side of the tube.
- Transfer the liquid phase to a new tube and add 0.4µl of the LNA probes
- Place at 37°C for 5min and put immediately in ice
- In the meantime, aliquot to a new tube 25µl of Streptavidin-coated magnetic Beads and follow steps 4-5 as before.
- At the end of the bead washes do not add hybridization buffer but add the RNA sample waiting on ice.
- Mix the RNA/magnetic beads by pipeting and incubate at 37°C for 20min
- Place the Eppendorf in a magnetic stand as before and transfer the liquid to a new Eppendorf containing ~1µl glycogen and 1/10th volume 3M NaOAC, pH5.2 followed by two volumes of 100% Ethanol
- Place at -80°C for 20min
- Spin at maximum speed for 30min at 4C and discard liquid
- Add 400µl of 70% EtOH and spin for 5min at 4C
- Remove all traces of liquid carefully and let the pellet dry for 2-5min
- Resuspend carefully with 6µl Nuclease-free water

First strand synthesis

Reagents required:

- Superscript III kit (Invitrogen, 18080-051)
- Actinomycin D (Sigma, A1410-2MG) Prepare a 40x solution in ethanol. 1x working solution (125ng/µl) is prepared with ethanol and is stable for at least a year. Always keep protected from light
- Glycogen (Invitrogen 10814-010)
- NaOAC 3M, pH 5.2
- 100% EtOH
- 70% EtOH
- nuclease-free water
- Centrifuge
- -80C (-20C for 1h to o/n)
- Mix the following:
- Run the following in the PCR machine:
- When the program reaches step 3: 15C HOLD, add the following to each tube without removing from the block
- If you are not doing an enzyme mastermix, add the enzymes at the order shown above. It is best to make a master mix of the RNase OUT and Superscript and to add the actinomycin separately. Add the actinomycin first and then the master mix.
- Proceed to “next step” on the thermocycler.
- When the program is finished, to each tube add 85µl water, 1µl glycogen and 1/10th volume 3M NaOAC pH5.2
- Add two volumes of 100% EtOH and put at -80C for 20min
- Spin at maximum speed for 30min at 4C
- Remove liquid carefully and add 500µl 70% EtOH
- Spin at maximum speed for 10min at 4C
- Remove ALL liquid carefully and let the pellet dry at the bench for 1-2 minutes.
- Resuspend the pellet at:

Second strand synthesis Reagents required:
- 5x second-strand buffer (Invitrogen, 10812-014)
- Set of dATP, dCTP, dGTP, dUTP (Promega, U1335) Prepare a 10mM solution of all four deoxynucleotides in water.
- Ecoli DNA ligase (invitrogen, 18052-019)
- Ecoli DNA polymerase I (invitrogen, 18010-017)
- MinElute PCR purification kit (Qiagen, 28004)
- Centrifuge
- 16C block

Make the following reaction mix and make sure to add the enzymes at the indicated order:
- Incubate at 16C for 3 hours.
- Mini-elute (see kit for instructions) at 15µl.

From dsDNA we typically quantify with HS-DNA Qubit assay. End Repair Reagents required:
- T4 DNA ligase buffer with ATP (NEB, B0202S)
- dNTPs (from Superscript III kit (Invitrogen, 18080-051))
- T4 DNA polymerase (NEB, M0203S)
- T4 PNK (NEB, M0201S)
- MinElute PCR purification kit (Qiagen, 28004)
- Centrifuge
- 20o C block
- Nuclease-free water
- Set up End repair treatment in a PCR tube as follows:
- The amount of dsDNA to use depends on the Qubit reading after second strand synthesis. If the reading is low, use the entire 13-15µl and decrease the water.
- Incubate the tube at 20C for 30 minutes (RT is okay).
- Purify the sample using MinElute kit and elute with 16 µl of RNase free EB Buffer.

A-tailing Reagents required:
- dATP (from Set of dATP, dCTP, dGTP, dUTP (Promega, U1335))
- Klenow (3’--->5’ exo-) (NEB, M0212S)
- MinElute PCR purification kit (Qiagen, 28004)
- Centrifuge
- 37C block
- Set up the reaction in a PCR tube as follows:
- Incubate the tube at 37°C for 30 minutes.
- Purify the sample using MinElute kit and elute with 10.5 µl of RNase free EB Buffer.

Ligation Reagents required:
- Quick Ligation kit (NEB, M2200S)
- NEXTflex DNA barcodes-6 (BIOO, 514101)
- 22C block (thermocycler)
- ice
- Set up the adapter ligation in a PCR tube as follows (you can use indexed adapters):
- Incubate the tube at 22C (or RT) for exactly 15 minutes and place in ice immediately.
- NOTE: The amount of Adapters to use depends on the amount of RNA being ligated. Too much adapter will result in adapter dimers. Too little may result in biased representation of transcripts. This is the amount we have found to work for this particular protocol.

Adapter-dimer removal Reagents required:
- 2x quick ligase buffer (comes with Quick Ligation Kit (NEB, M2200S))
- Agencourt AMPure XP (Beckman Coulter, A63880)
- Magnetic rack
- Nuclease-free water
- 100% Ethanol
- Fresh 80% Ethanol
- Dilute the ligation reaction to 100µl with water (0.25x quick ligase buffer)
- Add equal volume of pre-warmed (RT) AMPure XP beads and pipet up and down 10 times
- Let stand at the bench for 15min
- Place at the magnetic rack for 1-2 minutes until the liquid is clear again and the beads are visibly at the side of the tube
- Aspirate liquid and add 500µl FRESHLY made 80% EtOH without removing from the magnetic rack
- Repeat step 5
- Aspirate ALL liquid and let the beads dry on the bench for 1-3min
- Elute the DNA with 17.5µl water; pipet up and down and let stand at the bench for 5min to ensure complete elution.
- Place tube at the magnetic rack for 1-2 minutes until the liquid is clear again and the beads are visibly at the side of the tube
- Transfer the 17.5 µl to a new tube containing 2.5 µl 2x Quick ligase buffer and repeat steps 2-7
- Elute with 20µl water as above.
- Keep half the sample and proceed to the next step with the other half. However, if the sample is very low in concentration, it may be worth proceeding with the entire sample.

**UNG treatment and PCR amplification**

Reagents required:
- dNTPs (from Superscript III kit (Invitrogen, 18080-051))
- Primer mix (from NetFlex DNA barcodes-6 (BIOO, 514101))
- Phusion high fidelity DNA polymerase (NEB, M0530S)
- UNG (Thermo, EN0361)
- MinElute PCR purification kit (Qiagen, 28004)
- Thermocycler
- Nuclease-free water
- Centrifuge
- Set up PCR reaction as follows:
  - Incubate at 37°C for 15 min and heat inactivate at 98°C for 10 min.
  - Add 0.5 µl of Phusion polymerase and cycle as follows:
  - Clean up with MinElute Columns and elute at 10µl.

References: