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Extremely Rapid and Specific Metabolic Labelling of RNA In Vivo with 4-Thiouracil (Ers4tU)

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JoVE,
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Dear Dr Upponi,

I am submitting this manuscript as an original research article entitled "Extremely rapid and specific metabolic labelling of RNA in vivo with 4-thiouracil" for consideration by JoVE.

This protocol has been extensively optimised to specifically isolate thio-labelled RNA within seconds of its synthesis, by using extremely short labelling times and pulse/chase experiments with fine temporal resolution. Every step of the protocol has been optimised, and many parameters were adjusted to improve robustness. We also provide an exhaustive troubleshooting guidance. This is a thoroughly tested protocol and provides comprehensive tips and advice for the user throughout.

Although the protocol is optimised for extraction of RNA from yeast cells, it should be usable with RNA from any source. The purified RNA can be used in many downstream applications such as RT-qPCR and SLAM-seq, accommodating the user's needs and resources.

I can confirm that this work is original and has not been published elsewhere, nor is it currently under consideration for publication elsewhere. We also declare that we have no conflicts of interest and that there are no ethical considerations associated with this work.

Thank you so much for your time

Yours sincerely

David Barrass
Corresponding author

TITLE:

Extremely Rapid and Specific Metabolic Labelling of RNA In Vivo with 4-Thiouracil (Ers4tU)

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KEYWORDS:

Saccharomyces cerevisiae, nascent RNA, newly synthesized, transcription, splicing, RNA processing, RNA degradation, RNA metabolism, pulse chase

SUMMARY:

Here, we describe the use of thiolated uracil to sensitively and specifically purify newly transcribed RNA from the yeast *Saccharomyces cerevisiae*.

ABSTRACT:

The nucleotide analogue, 4-thiouracil (4tU), is readily taken up by cells and incorporated into RNA as it is transcribed in vivo, allowing isolation of the RNA produced during a brief period of labelling. This is done by attaching a biotin moiety to the incorporated thio group and affinity purifying, using streptavidin coated beads. Achieving a good yield of pure, newly synthesized RNA that is free of pre-existing RNA makes shorter labelling times possible and permits increased temporal resolution in kinetic studies. Here we present a protocol for very specific, high yield purification of newly synthesized RNA. The protocol presented here describes how RNA is extracted from the yeast *Saccharomyces cerevisiae.* However, the protocol for purification of thiolated RNA from total RNA should be effective using RNA from any organism once it has been extracted from the cells. The purified RNA is suitable for analysis by many widely used techniques, such as reverse transcriptase-qPCR, RNA-seq and SLAM-seq. The specificity, sensitivity and flexibility of this technique allow unparalleled insights into RNA metabolism.

INTRODUCTION:

RNA has a dynamic nature; soon after it is produced much RNA is rapidly processed and degraded. Currently, most studies of RNA metabolism analyze the total cellular RNA, which is mostly fully processed and at steady state level. This level depends on the balance between the rates of transcription, post-transcriptional maturation and degradation. Analysis of the processes that lead to the steady state equilibrium requires specialized techniques to capture very short-lived RNA species.

Metabolic labelling of RNA with nucleotide analogues such as 4-thiouracil (4tU) or 4-thiouridine (4sU) (see Duffy et al.[1] for an excellent review), offers the ability to isolate thio-labelled nascent RNAs and their processing intermediates. However, published protocols involve labelling times of several minutes[2,3], which is slow relative to the rate of production of many transcripts. It takes in the order of one minute to transcribe the average yeast gene, so labelling yeast RNA for less than one minute can be considered extremely short. The extremely rapid and specific 4 thiouracil protocol (ers4tU) maximizes the signal to noise ratio by maximizing 4tU incorporation and minimizing the recovery of unlabeled, pre-existing RNA making very short labelling times possible[4].

The thio-modified base must be imported into the cells rapidly and in sufficient quantity to efficiently label the newly synthesized RNA (nsRNA). To promote this, cells are grown in uracil-free medium, and expression of an appropriate permease helps to boost 4tU or 4sU uptake (see **Table 1** for a list of plasmids that carry suitable permease genes and **Supplementary Figure 1**). 4tU's solubility in sodium hydroxide avoids the need for toxic organic solvents required by other nucleotide analogues. Unfortunately, growing cultures for long periods with thio-modified nucleosides at concentrations greater than 50 µM has been observed to disrupt ribosomes[5]. However, the concentration (10 µM) used here, and the extremely short labelling times, minimize deleterious effects[5] (**Figure 1a**), while still yielding sufficient RNA for analysis.

This technique can be combined with rapid and specific auxin-mediated depletion of a target protein[6,7] (**Figure 2**), referred to as the "β-est AID 4U" protocol, in which β-estradiol regulated expression of the auxin inducible degron (AID) system is combined with 4tU labelling. With the β-est AID 4U approach, a target protein can be depleted and the effect on RNA metabolism closely monitored (**Figure 2**). The timing is critical; it is advisable to view the accompanying video and pay close attention to **Figure 2** and its animated form (see **Supplementary Figure 2**).

Processing and degradation of RNA must be stopped extremely rapidly for accurate time resolution. This is achieved using methanol at low temperature, which fixes the cell contents very rapidly and degrades the cell membrane while preserving the nucleic acid content[8]. The RNA extraction should be efficient and not damage the RNA. Mechanical lysis is effective in the absence of chaotropic agents (often these contain thio groups, so should be avoided). Lithium chloride precipitation of RNA is preferred, as tRNAs are less efficiently precipitated. tRNAs are rapidly transcribed and naturally thiolated[9], so removing tRNAs reduces competition for the biotinylation reagent. If small, highly structured RNAs are of interest, alcohol-based RNA precipitation methods are recommended.

To recover the thiolated RNA, biotin is covalently attached via the thio groups incorporated into the RNA with 4tU. The use of modified biotin, which attaches via a cleavable disulfide bond (e.g., N-[6-(biotinamido)hexyl]-3´-(2´-pyridyldithio)propionamide [HPDP-biotin] or methane thiosulfonate [MTS-biotin]) is recommended as it permits release of the RNA by addition of a reducing agent. The biotinylated RNA is affinity purified on streptavidin coupled to magnetic

beads. This protocol is similar to others listed previously[10] but has been intensively optimized to reduce background.

There are two types of thio-labelling experiment that can be performed, continuous and discontinuous labelling. Each has its own advantages. In continuous labelling the 4tU is added to the culture and samples taken at regular intervals. This type of experiment shows how the RNA is processed and how levels change over time. Examples include comparison of mutant with wild-type experiments and a pulse-chase experiment. The experiments shown in **Figure 3b,c** are of this type. For discontinuous labelling a change is induced into the system and the RNA monitored. Once the change has been induced the culture must be split into several sub-cultures, and at specific times, each one is then thio-labelled for a brief period. One example is β-est AID 4U shown in **Figure 2**[7]. This type of experiment is particularly useful for monitoring the effect of a metabolic change on RNA processing (see **Figure 3d**).

A graphical representation of a thio-labelling experiment is presented in **Figure 4** and **Figure 5**, and a spreadsheet that greatly simplifies the performance of the protocol is available (see **4tU experiment template.xlsx**). As well as this the Supplementary Information contains an extensive troubleshooting guide. For the β-est AID 4U protocol that integrates 4tU labelling with the auxin depletion protocol, see **Figure 2** and **Supplementary Figure 2**. See Barrass et al.[7] for the detailed AID depletion protocol.

PROTOCOL:

1. Growth and thio-labelling

NOTE: Time for completion of this section of the protocol is highly variable, depending on cell growth rate. Allow 1 h to prepare the solutions and equipment prior to thio-labelling and 30 min post-labelling to process samples.

1.1. Ensure the *S. cerevisiae* strain contains a plasmid encoding a permease (**Table 1**) to boost 4tU import into the cell.

NOTE: Without an importer, labelling for less than 2 min is unlikely to be successful[11] (see **Supplementary Figure 1**). 4tU incorporation is more efficient if growth is in medium without uracil, so the strain must be *URA3+;* several of the plasmids in **Table 1** carry *URA3* as marker. If this protocol is to be combined with β-est AID depletion[7], additional strain modifications are required.

1.2. Prepare YMM uracil-free medium by adding 6.9 g of yeast nitrogen base without amino acids, 20 g of glucose, and 1.92 g of SCSM single drop-out−ura (**Table of Materials**) to 1 L of water. Autoclave or filter sterilize the growth medium before use.

NOTE: Filter sterilization is preferred as peptide/sugar complexes produced by autoclaving co-precipitate with the cells in the methanol used in sample collection.

1.3. Grow yeast in YMM uracil-free medium to an optical density at 600 nm (OD_{600}) of 0.6–0.8. Ensure the culture is in log phase growth and has been for at least two doublings. Growth at 30 °C is normally recommended, but other temperatures may be used, for example, for temperature-sensitive strains.

NOTE: Depending on the strain, growth conditions and RNA yield, approximately 30 mL sample volume will be needed. This amount will be assumed throughout the protocol. 30 mL of culture is the most that will fit into a 50 mL centrifuge tube with 20 mL of methanol, so is a convenient volume to start optimization. Consider using more sample volume for early time points to increase RNA recovery, up to 2000 mL has been used for slower growing cells at really short labelling times (<1 min).

1.4. Chill about 50 mL of H_2O on ice. For each sample, add 200 µL of zirconia beads to a 2 mL screw-cap tube and chill on ice. Also put 20 mL of methanol (CAUTION), into 50 mL centrifuge tubes, and place on dry ice (CAUTION). The methanol should be $^1/_3$ to $^2/_3$ the volume of the sample.

CAUTION: Methanol is toxic by inhalation, contact and consumption. Dispense large volumes in a fume hood, and wear two pairs of gloves, as methanol can penetrate nitrile laboratory gloves. Methanol is highly flammable, keep away from all sources of ignition. As dry ice can cause cold burns on contact and produces asphixiant gas, use gloves when handling and use in a well-ventilated space.

NOTE: Adding the beads at this point is easier than after the sample has been added as the tube is dry and when spinning down the cell pellet the beads are also spun clear of the tube thread saving some time. Additionally, this allows the beads to cool before the sample is added.

1.5. If an *S. pombe* spike is to be added to the culture (rather than later), thaw an aliquot of thiolated *S. pombe* cells on ice and vortex thoroughly, at least 30 s, then add to the culture. If prepared according to the instructions below, one *S. pombe* aliquot is sufficient for 400 mL of culture (enough for twelve 30 mL samples plus a little to allow for errors in handling). If more or less culture is used, adjust the volume of *S. pombe* added to the culture.

1.5.1. Grow 1 L of *S. pombe* culture to OD_{600} to 0.8 exactly as described in the protocol for *S. cerevisiae*.

1.5.2. Thio-label as step 1.7, but for 10 min.

1.5.3. Fix all of the culture using 400 mL of methanol on dry ice, essentially as described in step 1.9

1.5.4. Pellet the cells by centrifugation at 3000 x *g* for 3 min.

1.5.5. Discard the supernatant and resuspend the cell pellet in 3.3 mL of H_2O.

1.5.6. Split into aliquots of 80 µL each. Store at -80 °C.

1.5.7. Use all of one aliquot for 400 mL culture or 10 µL per 30 mL sample.

NOTE: Reduce the volume of spike to $^1/_{10}$ if performing RNAseq. Do not reuse aliquots; discard any unused spike. This spike is useful to normalize and compare results across time points and experiments.

1.6. For discontinuous labelling, induce the required metabolic perturbation (e.g., growth conditions, gene induction or depletion such as β-est AID[7] (**Figure 2** and **Supplementary Figure 2**), then split the culture. Ensure all flasks and media are at the required temperature and, if possible, aerate the medium before adding the culture.

1.7. Add 4tU to the culture to a concentration of 10 µM and mix vigorously ($^1/_{10,000}$ of the culture volume of 100 µM 4tU dissolved in 1 M NaOH). Thiol-label for 15 s to 5 min.

NOTE: Thirty seconds is a good starting point. Thio-labelling for less than 20 s gives more variable results due to difficulties manipulating the culture under time pressure. However, labelling for more than 1 min reduces the temporal resolution of the technique.

1.8. If a chase experiment is to be performed; allow thio-labelling for 20–30 s then chase by adding $^1/_{200}$ culture volume of 1 M uridine (not thiolated), to a final concentration of 5 mM.

NOTE: Uridine is preferable to uracil for the chase, as uridine is more water soluble allowing a smaller volume to be added to the culture and so there is less disturbance to the growth of the cells.

1.9. Take samples of culture at regular intervals (at least 15 s), to the end of the time course. Sampling intervals shorter than this are difficult to perform reliably. Add the sample to the methanol on dry ice prepared in step 1.4. For convenience, add 30 mL of culture to a 50 mL tube containing 20 mL of methanol.

NOTE: Carbon dioxide dissolves in the methanol when cold; this comes out of solution on addition of the sample and foams vigorously upon mixing—resulting in sample loss. To avoid this, chill the methanol to <-70 °C in a tightly sealed tube until close to the time it is needed, then transfer to dry ice.

1.10. Seal the tube and mix thoroughly by shaking. Place the samples on ice. Check that none of the samples have frozen; if so, gently warm in the hand, inverting constantly. This is best done in the hand as the sample's temperature can be assessed, it should always feel cold. Place on ice. This is not a pause point; once all the sample is fluid proceed to the next step.

1.11. Spin at 3000 x *g* for 2 min (at 4 °C if possible) to pellet the cells. Pour off the liquid and resuspend the pellet in at least 1 mL of ice-cold water by gently pipetting up and down.

NOTE: The residual methanol in the sample pellet aids resuspension.

1.12. Transfer to 2 mL screw cap tubes as prepared in step 1.4. Spin briefly (e.g., 10 s total time) at >13,000 x *g* to re-pellet the cells, place back on ice and remove liquid.

NOTE: The cell pellet can be stored at -70 to -80 °C for several months.

2. Preparation of total RNA

NOTE: The time for completion is 90 min.

2.1. Use diethyl pyrocarbonate (DEPC)-treated solutions to protect the RNA from degradation. Aliquot the solutions using filter pipette tips and wear gloves at all times.

2.1.1. To a solution add $^{1}/_{1000}$ volume of DEPC and mix by vigorous shaking.

2.1.2. Leave at room temperature (RT) for 24 h, then autoclave.

2.1.3. Solutions with amine groups (such as tris) cannot be DEPC treated. Aliquot the powder and store specially for RNA work. Use previously DEPC treated H_2O to make the solution.

NOTE: As the thio-group on the RNA is photoactivatable, minimize exposure to UV light from this point on. Storage should be in the dark and incubation is best done in a PCR machine with a lid.

2.2. If an *S. pombe* spike is to be added to the cell pellet rather than the culture (do not do both), add it now. Thaw an aliquot of thiolated *S. pombe* cells on ice and vortex thoroughly, at least 30 s, before adding to the pellet.

NOTE: If prepared according to steps 1.5.1–1.5.7, 10 µL of *S. pombe* aliquot is required for one pellet derived from 30 mL of culture.

2.3. Before putting on the cap, spin very briefly for 1–2 s to ensure that no zirconia beads are trapped between the cap and the tube, which can cause sample and phenol to leak from the tube.

2.4. Resuspend the cells in 400 µL of acetate EDTA (AE) buffer (50 mM sodium acetate pH 5.3, 10 mM EDTA pH 8.0), by vortexing vigorously. Add 40 µL of 10% (w/v) sodium dodecyl sulfate (SDS). Do not vortex, as SDS will foam.

2.5. If the β-est AID 4U protocol is to be used, take 40 µL of the cell suspension for protein analysis[7]. Add 40 µL of AE to make the volume back up to 400 µL.

2.6. Add 800 µL of phenol (CAUTION) at low pH and vortex for 10 s.

CAUTION: Phenol is toxic and corrosive by inhalation and contact. Always perform procedures involving phenol in a fume hood and wear two pairs of gloves.

2.7. Lyse the cells in a homogenizer (e.g., **Table of Materials**) for three 2-min bursts at the lowest power setting. Leave the samples on ice for 2 min between pulses of homogenization.

NOTE: Optimize the conditions if using other homogenizers. Insufficient shaking will result in poor yields, whereas excessive shaking results in apparent higher yield, as determined by absorbance at 260 nm (A_{260}), but the RNA may be degraded. A homogenizer is preferred, but hot phenol RNA purification[12] can be used.

2.8. Place the lysed sample on dry ice for 5 min, until it solidifies, this reduces genomic DNA carry over into the RNA. Do not freeze for too long as the sample will not thaw. Spin 5 min in microfuge at >13,000 x *g* at RT; do not be tempted to do this at 4 °C, as the sample/phenol mix will remain solid throughout the spin if performed at low temperature.

NOTE: If the sample is still frozen at the end of the spin, re-spin for another 5 min until the sample has completely thawed.

2.9. Phenol/chloroform extract then chloroform extract with an equal volume (approximately 600 µL) of phenol:chloroform 5:1 then chloroform (CAUTION). Transfer the top phase to another tube containing phenol:chloroform 5:1 or chloroform. Vortex, then spin for 5 min in a microfuge at RT. Then transfer the top phase to a new 1.5 mL tube.

CAUTION: Chloroform is toxic by inhalation and contact. Always perform procedures involving chloroform in a fume hood and wear two pairs of gloves.

2.10. Add a third to half volume (approximately 300 µL) of 10 M LiCl, and mix to precipitate the RNA. The sample should go cloudy immediately but leave for at least 10 min on ice or at 4 °C (do not store below -20 °C as it will freeze), or until the precipitate flocculates.

2.11. Spin for 5 min at >13,000 x *g* in a microfuge. Remove the fluid, briefly re-spin and remove the dregs. Wash pellet with 300–500 µL of 70% ethanol, spin briefly and remove remaining ethanol.

NOTE: During these washes keep the pellet on the same side of the tube as the first spin, this way the pellet will not move and break; if it breaks some of the RNA could be lost accidently. Do not dry the pellet; as long as most of the fluid has been removed it will not interfere with subsequent steps. The RNA can also be stored at this stage at -20 °C for a few months or -70 to -80 °C for long-term storage.

2.12. Re-dissolve the RNA pellet in 90 µL of TE pH 7.0 (10 mM Tris HCl pH 7.0, 1 mM EDTA pH 8.0) by heating at 65 °C with shaking as the RNA pellet can be difficult to re-dissolve. This must be for **no more than 5 min** as RNA degrades at higher temperatures. Check for full RNA solubilization and then transfer to a 0.2 mL tube. Pipette the sample up and down; there should be no "lumps", and the fluid should rise and fall smoothly in the tip. This solution is viscous so the final pipetting motion should be slow.

NOTE: The RNA can be stored at -20 °C in the dark at this stage; this can also be beneficial to RNA solubility.

3. Biotinylation

NOTE: The time for completion is 60 min. The following steps are conveniently done in a strip of tubes with integral caps as they have less tendency to open on vortexing than strips with separate caps.

3.1. Biotinylate by adding 10 µL ($^{1}/_{10}$ final volume) of HPDP-biotin solution (MTS-biotin can be used in exactly the same way as HPDP-biotin), to the RNA and mix thoroughly. Preheat the RNA for no more than a few seconds at 65 °C before adding the biotin. Incubate at 65 °C for 15 min to a **maximum of** 30 min in the dark.

NOTE: This heating is required as some HPDP batches precipitate at RT in the RNA sample. A PCR block with a heated lid is ideal for this.

3.2. Prepare a small resin volume, size exclusion column (**Table of Materials**) to exclude the unincorporated biotin. Remove the bottom tag of the column and loosen the cap, place in a 2 mL centrifuge tube. Spin at 1500 x *g* for 1 min to flush out the buffer and discard the flow through. Add 0.3 mL of TE gently to the top of the column and spin again.

3.3. Repeat the wash and spin twice more for a total of 3 washes. Finally transfer the washed column to a fresh 1.5 mL tube.

3.4. Once the sample incubation (step 3.1) is complete, add the sample to the top of the column. Spin at 1500 x *g* for **2 min.** The biotinylated RNA sample is now in the bottom of the tube.

NOTE: A 1 min spin is not sufficient to elute the entire sample.

3.5. Add a third to half volume (approximately 40 µL) of 10 M LiCl, mix to re-precipitate the RNA as step 2.10. The sample should go cloudy immediately but leave for at least 5 min on ice or at 4 °C or until the precipitate flocculates; do not store below -20 °C as it will freeze. Centrifuge the sample for 5 min at >13,000 x *g* in a microfuge.

3.6. Wash with 80% ethanol, ≤1 h rotating. Follow the procedure in step 2.11 to remove as much of the fluid as possible.

NOTE: As HPDP-biotin is very soluble in 80% ethanol, this is an additional purification step.

3.7. Repeat the 80% ethanol wash to remove as much un-incorporated biotin as possible.

NOTE: The RNA can also be stored at this stage at -20 °C in the dark.

4. Purification of the newly synthesized RNA

NOTE: The time for completion is 2 h.

4.1. Re-dissolve the RNA in 200 µL of DEPC-treated H_2O (65 °C incubation can be used, similar to the procedure in step 2.12).

4.2. Measure the RNA concentration at A_{260} using a spectrophotometer; the sample may have to be diluted $^1/_{10}$ to get it within the linear range of the spectrophotometer. Vortex this dilution for at least 10 s to ensure the viscous RNA is evenly dissolved.

NOTE: The efficiency of biotinylation can be assessed by dot blot[13] if required.

4.3. Add equal amounts of RNA to a fresh tube and make up to 200 µL in DEPC-treated H_2O. Use all of the sample with the lowest RNA concentration and use an appropriate volume of the other samples to have a similar amount of RNA for each.

NOTE: The spreadsheet **4tU experiment template.xlsx** has a form to aid this calculation.

4.4. When the sample is at RT, add 25 µL of NaTM buffer (0.1 M Tris HCl pH 7.0, 2 M NaCl, 250 mM $MgCl_2$), 25 µL of 1 M NaPi pH 6.8 (0.5 M NaH_2PO_4 0.5 M Na_2HPO_4), and 2.5 µL of 10% SDS. Mix thoroughly and spin gently (<30 s; approximately 100 x *g*).

NOTE: To avoid precipitation of the SDS and salts, the samples must be kept at RT throughout the following procedures up to step 4.13.

4.5. Make the bead buffer containing 1x NaTM buffer, 0.1 M NaPi, and 0.1% SDS, 2 mL per sample. Add the required amount of H_2O first and the SDS last. This must be made fresh each time as a precipitate forms after 24 h.

NOTE: To avoid the formation of precipitates, the bead buffer must be kept at RT throughout the following procedures. Do not DEPC treat or autoclave.

4.6. Add 50 µL of streptavidin beads to a low retention 1.5 mL tube. Place the tube on the magnetic rack, wait for the beads to settle and then remove the fluid by aspiration.

4.7. Wash the streptavidin beads.

4.7.1. Add 200 µL of bead buffer and vortex until the bead pellet is fully resuspended. Usually 3−5 s is all that is required. For washes before the RNA sample is added it is sufficient to turn the tubes round so the beads travel across the tube to the other side. Then turn the tubes back to the original side so the beads travel across the tube once again.

4.7.2. Spin the tube at low speed (approximately 100 x *g*) for a maximum of 5 s to spin down the fluid, but not the beads.

4.7.3. Place in the magnetic rack to allow the beads to be captured by the magnet.

4.7.4. Remove the fluid by aspiration for a small number of samples; pour off the liquid if many samples.

NOTE: With a large number of samples, removing all the fluid purely by aspiration can be problematic, as the beads in the first sample may be dried out before the last sample is finished, this increases background. Washing can be expedited by pouring off the fluid from all samples at once whilst on the magnet. They should be left a little longer on the magnet before pouring and the small amount of fluid that remains has to be aspirated away but, overall, it means less time on the magnet and without fluid. In this way, it is possible to do 24 or more extractions quickly.

4.8. Block with 200 µL bead buffer, 10 µL 20 mg/mL glycogen, and 2.5 µL 5 mg/mL tRNA, 20 min rotating end over end at moderate speed at RT. The rotation is to keep the beads in suspension. Once the blocking is complete remove the fluid as steps 4.7.2−4.7.4 and wash again, as the steps in section 4.7.

4.9. Resuspend the beads in the sample. Incubate at RT with rotation for 30 min.

4.10. During the incubation, prepare a fresh 1.5 mL tube for each sample. Add $^{1}/_{10}$ volume (approximately 10 µL) of 3 M sodium acetate pH 5.3 and 20 µg of glycogen, and spin at approximately 100 x *g* for 3 s. Store in a rack until needed.

4.11. Remove the unbound RNA from the beads, as steps 4.7.2−4.7.4. The unbound RNA can be persevered in a fresh tube, but the salts and SDS make it very difficult to purify. Then wash the beads, as section 4.7 with vortexing, for a minimum of 3 to a maximum of 5 times.

4.12. After the final wash take special care to aspirate all the liquid; return to each tube and aspirate the dregs of the buffer once more.

4.13. To elute the RNA, add 50 µL of freshly prepared 0.7 M β-mercaptoethanol (βME) to the beads ($^{1}/_{20}$ dilution of the commercially supplied stock solution). Vortex and spin briefly, as steps 4.7.1 and 4.7.2. Place the slurry in the magnetic rack and pipette the RNA containing solution into the 1.5 mL centrifuge tube prepared in step 4.10.

4.14. Elute once more as step 4.13 to recover residual RNA from the beads and add the eluted sample to the tube containing the first elution from these beads.

4.15. Remove residual beads from the eluted RNA by placing the sample back in the magnetic rack and transferring the fluid to a fresh, low binding 0.5 mL centrifuge tube.

4.16. Mix the sample and then precipitate the nsRNA by adding 2.5x volumes (280 µL) of ethanol and mix once more. Leave for 1 h to overnight at -20 °C. Spin in a pre-chilled centrifuge (4 °C) for 20 min at the maximum speed (at least 13,000 x *g*).

4.17. Wash thoroughly with 200 µL of 70% ethanol at -20 °C. As residual βME will inhibit downstream applications, spin at every step to remove as much of the dregs as possible; at the end the sample should not smell of βME.

4.18. Re-dissolve in 10−20 µL of DEPC-treated 1x TE with the equivalent of 0.005 µL RNase inhibitor.

NOTE: All subsequent stages should be performed on ice.

4.19. Measure the RNA concentration and purity.

4.19.1. Measure the A_{260} and A_{225} in a low sample volume spectrophotometer.

NOTE: An absorbance maximum near λ = 225 nm is from an unavoidable contaminant from the beads. In the absence of RNA the signal from the contaminant declines to 35% at λ = 260 nm. Therefore, the actual amount of RNA is approximated by the formula: $(A_{260}-(A_{225}*0.35))*40$ ng/µL.

4.19.2. Alternatively, analyze the sample on a micro-fluidics electrophoresis system such as a bioanalyzer.

NOTE: This analysis is preferable to using a spectrophotometer as RNA integrity can be assessed, the contaminant does not interfere with the quantitation and less sample is required.

4.20. Analyze the nsRNA.

NOTE: For example, specific RNAs can be quantified by standard reverse transcriptase qPCR techniques. RNA prepared this way is compatible with library preparation for RNA-seq. Removal of rRNA is not necessary for labelling times of less than 5 min. Recoding SLAMseq[14] can also be performed on this RNA.

REPRESENTATIVE RESULTS:

Typical yields for nsRNA recovered using this ers4tU protocol are displayed in **Figure 1b**, this has been produced by a bioanalyzer and the trace shows yield of RNA versus size (nucleotides [nt]).

Note, in both the bioanalyzer trace and the inset graph, that RNA recovery from time point 0 is a very small portion of that recovered from longer time points — approximately 0.3 µg of RNA recovered from approximately 10^9 cells compared with over twice as much after just 30 s of labelling (0.8 µg of nsRNA) from the same number of cells. RNA recovery at 15 s is more variable as small differences in performing the sampling have a proportionately larger effect on RNA recovery. In the bioanalyzer trace, rRNA precursors can be seen as a peak near 1000 nt and a doublet of peaks at 1700−1800 nt. The abundance of these intermediates increases as thiolation continues.

Thio-labelling was used to quantify splicing of the *ACT1* transcript (**Figure 3**). Thiolation was performed and samples taken at 15 s intervals from the start of thio-labelling and the processing of *ACT1* RNA monitored (**Figure 3a,b**). As can be seen, pre-mRNA is generated (by transcription), and lariats (by the first step of splicing from pre-mRNA), even after just 15 s of labelling. After about 45 s to 1 min, the amounts of lariats and pre-mRNA reach equilibrium with as much of these RNA species being created by transcription as are processed away by splicing.

To produce the data shown in **Figure 3c** the strain was pulsed with 4tU for 25 s and then chased with uridine. The generation of pre-mRNA and lariats reaches a maximum at 1 minute. This compares well with **Figure 3b**; the maximum being achieved after 45 s to reach equilibrium plus the 25 s of the labelling. After the peak, the levels decline as the thio-labelled RNAs are chased through the splicing process.

Figure 3d shows depletion of a protein splicing factor and its effect on RNA metabolism, using the β-est AID 4U system[6,7]. Here, Prp16p is reduced from near physiological levels to 5% of this level after 25 min of depletion. Prp16p is an essential splicing factor for the second step of splicing[15]. Lariats are removed during the second step of splicing (**Figure 3a**), but here they increase above the level of pre-mRNA as Prp16 becomes limiting. At later depletion times, other factors become limiting due to secondary effects, so that levels of lariat decrease, and pre-mRNA levels rise. The level of spliced mRNA declines.

FIGURE AND TABLE LEGENDS:

Figure 1: Growth in 4tU and RNA recovery. (**a**) 4-thiouracil affects growth. Increasing the concentration of 4tU in YMM drop-out growth medium without uracil increases the doubling time of *S. cerevisiae* (BY4741) carrying the p4FuiΔPEST plasmid. Growth of four replicate cultures was monitored at 30 °C in a Tecan Infinite Pro 200. All cultures were in log phase throughout, with OD_{600} between 0.1 and 0.6. Mock is a control culture with an equivalent amount of NaOH added, which does not by itself change the growth rate. This graph demonstrates that thio-labelling is compromise between rapid labelling and damage to the cell. Error bars are standard error of 4 replicates. (**b**) nsRNA yield increases linearly from about 15 s of labelling. The main figure shows the bioanalyzer traces of purified, nsRNA from 0 (not thiolated) to 2 min after addition of 4tU at 15 s intervals. Note that the 15 s sample is not shown, as it was indistinguishable from the unlabelled sample. The two large peaks correspond to ribosomal RNAs (rRNAs). The rRNA precursors and intermediates are visible as several peaks at greater molecular

weight than mature rRNAs. The recovery of these precursors and intermediates increases with time. Results from one representative experiment are shown. The inset graph shows the recovery of nsRNA with increasing incubation with 4tU. The yield of nsRNA increases with increasing time of growth with 4tU. The recovery is remarkably linear (R^2 = 0.934) throughout the timescale of this experiment and shows a slight increase over background even at 15 s labelling with 4tU even though not distinguishable from the unlabelled sample by eye from the bioanalyzer trace. Error bars show standard error for three biological replicates.

Figure 2: β-est AID 4U β-est AID 4U graphical protocol. A graphical summary of the protocol of the β-est AID 4U protocol. β-estradiol (β-est) promotes the expression of the auxin inducible degron (AID) system which in turn depletes an AID* tagged target protein, refer to Barrass et al.[7] for a detailed protocol. In this case, degron system expression is initiated 25 min before protein degradation commences and thiolation at each time point is for 1 min. Samples are taken before induction and every 2 minutes during depletion. An animated version appears in the **Supplementary Figure 2**.

Figure 3: Precursors and intermediates of *ACT1* RNA splicing. Splicing of *ACT1* pre-mRNA transcripts was monitored by quantitative reverse transcription PCR[16]. The levels of *ACT1* precursor (pre-mRNA), intermediate lariat-exon2 (Lariat) and spliced product (mRNA) are shown normalized against the level of *ACT1* Exon2 and steady state levels of these RNAs. (**a**) Location of qPCR products on the ACT1 transcript. Schematic of the locations of the qPCR products used to assay the levels of precursors, intermediates and products of the splicing reaction of the *ACT1* transcripts[16]. Exons are represented by boxes, intron as a line and the qPCR products as lines with diamonds at either end, the color matches those used in the graphs. The pre-mRNA PCR is specific for pre-mRNA and not any intermediates of splicing as this product crosses the branch point which is disrupted after the first step of splicing. Lariat PCR will detect the product of the first step of splicing and the excised lariat produced after the second. The mRNA PCR is specific for the product of splicing, mRNA. Results from the exon PCR (present in all precursors, intermediates and products, except the excised lariat) is not shown in the graphs as this was used to normalize the data and is therefore always equal to 1. (**b**) Continuous thiolabelling. The amount of pre-mRNA increases with time as 4tU is incorporated by transcription and, after a short delay, splicing converts it to lariat-exon2 intermediate and spliced products. The levels of these pre-mRNA and lariat species are detectable above background after as little as 15 s of growth with 4tU and reach a maximum after approximately 45 s of continuous labelling with 4tU, at which point their production is balanced by conversion to spliced mRNA and/or degradation. Values are normalized to their steady state (left-most point of the graph), and exon 2 levels to show their appearance and processing in comparison to transcription of exon 2. As RNA splicing of *ACT1* is largely co-transcriptional[4,17] spliced mRNA rapidly becomes the most abundant species, its level is similar to that of exon 2. Standard error of three biological replicates, each assayed in triplicate. (**c**) Pulse/chase. Thiolation pulse of 25 seconds followed by chase with uridine. Compared to the steady state levels of these RNAs (left-most point), they are initially very abundant in the newly synthesized pool. The levels gradually decline as they are processed into mRNA (or degraded), approaching levels very similar to steady state levels by 5 min. Standard error of three biological replicates, each assayed in triplicate. (**d**) nsRNA and protein depletion.

Splicing of *ACT1* pre-mRNA transcripts monitored by quantitative reverse transcription PCR as in panel (**a**) upon depletion of the Prp16 protein using the auxin degron system as described in **Figure 2**. The Prp16 protein levels are also displayed in the graph plotted against the second Y-axis as percentage of levels prior to auxin depletion. Prp16 is a vital component of the spliceosome, particularly important for the second step of splicing shown in panel (**a**), after which lariats are degraded. When this step becomes limiting lariats accumulate initially. At later time points splicing fails completely, lariats are no longer produced and pre-mRNA levels rise. Error bars are standard error of three biological replicates, each assayed in triplicate.

Figure 4: Graphical summary of the protocol sections 1 to 3. The cells are thiolated with 4tU and allowed to grow to incorporate the modified nucleotide into the RNA. A thiolated *S. pombe* spike can be added to allow normalization across time points and experiments. The pulse of 4tU can be chased using un-thiolated uridine. Labelling can either be performed continuously from 4tU addition or from a change to growth conditions, the culture split and 4tU added to cultures at increasing times from the growth condition change, but each labelling only for a brief time. The cells are collected, and RNA prepared from the cells, preferably using a homogenizer and phenol-based methods. The RNA is biotinylated and then the biotinylated RNA purified from unincorporated biotin using a size exclusion column. The nsRNA is now ready for purification with streptavidin beads (section 4, **Figure 5**). Numbers in red correspond to the step numbers in the protocol.

Figure 5: Graphical summary of the protocol section 4. Following on from sections 1 to 3 (**Figure 4**), the streptavidin beads are blocked and the biotinylated RNA sample added to the prepared beads. The biotinylated RNA binds to the streptavidin beads and the un-biotinylated RNA removed and washed. The biotinylated RNA is eluted from the beads using βME and precipitated ready for further research. Numbers in red correspond to the step numbers in the protocol.

Supplementary Figure 1: Improvement of nsRNA recovery from yeast cells with and without additional copies of the importer at 1 and 3 minutes of thio-labelling. Note that *Fui1* is the yeast's own promoter expressed from a 2 μm plasmid. The genomic copy of this gene is present in both of these strains.

Supplementary Figure 2: Animated version of the β-est AID 4U β-est AID 4U graphical protocol.

Table 1: Plasmids used with this protocol.

DISCUSSION:

This article presents a protocol for extremely rapid and specific 4tU labelling, for recovery of nascent, newly synthesized RNA from *S. cerevisiae* after as little as 15 s of labelling, with very low contamination by unlabeled RNA.

The user should always take care to maintain the integrity of the RNA by use of cold temperatures and DEPC-treated reagents. Streptavidin bead purification is generally reliable; however, the bead buffer is difficult to handle; it must be made freshly, with its components added in the right

order, and not chilled or autoclaved. Common failings include the RNA being incompletely dissolved after the precipitation steps, and so being either not biotinylated or otherwise lost during the processing steps. There is extensive troubleshooting help in the supplementary material.

There are some limitations to be aware of in ers4tU. One already mentioned is that 4tU slows growth of the yeast (**Figure 1a**). Apart from endogenously thiolated RNAs[9], only RNAs that have been transcribed during the labelling period can be purified by this method. Polymerases paused on genes throughout the thiolation time will not produce thiolated transcripts that can be purified, although transcripts that are partially labelled due to polymerases entering or leaving a paused state during thiolation can be recovered. Strains that transcribe poorly, either because of mutation or growth conditions, produce little nsRNA, although the techniques used here will nevertheless improve recovery of nsRNA compared to other methods. Longer times and increased culture volumes may be necessary in these strains and conditions. Note that uracil is a good source of nitrogen and so this method should be trialed before being used for studies involving nitrogen starvation.

The ers4tU protocol is particularly useful for analysis of short-lived RNAs, many of which are so rapidly degraded that they cannot be identified without crippling the degradation machinery. Examples include cryptic unstable transcripts (CUTs)[4], and short transcripts produced by premature termination or promoter proximal pausing[18] and antisense transcription "upstream" from a promoter (PROMPTs)[19]. The intermediates produced during processing of stable RNA species are also transient but can be enriched using ers4tU transcription[4]. The ers4tU protocol is therefore exceptional in permitting highly transient RNA species to be analyzed and captured under near physiological conditions, which is a huge advantage over other methods. This technique has been used to study transcription and downstream RNA processing kinetics in RNA polymerase mutants that elongate faster or slower than normal[20].

Thiolation is also compatible with RNA-seq and SLAM-seq[21], allowing all RNA produced within a very short time window to be characterized in exquisite detail.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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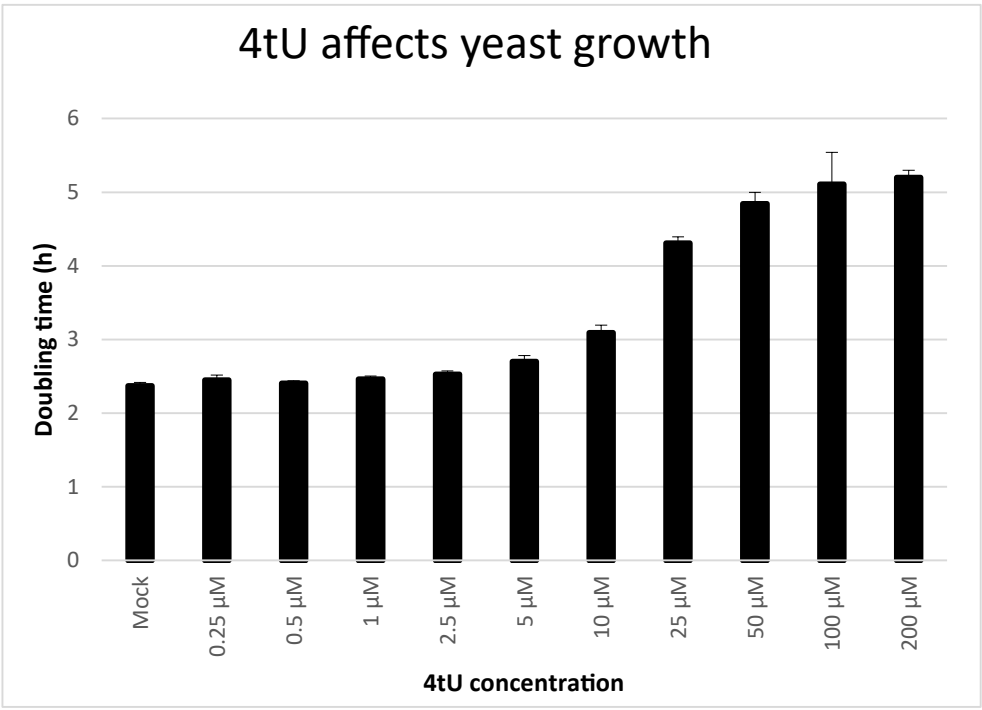
a



b

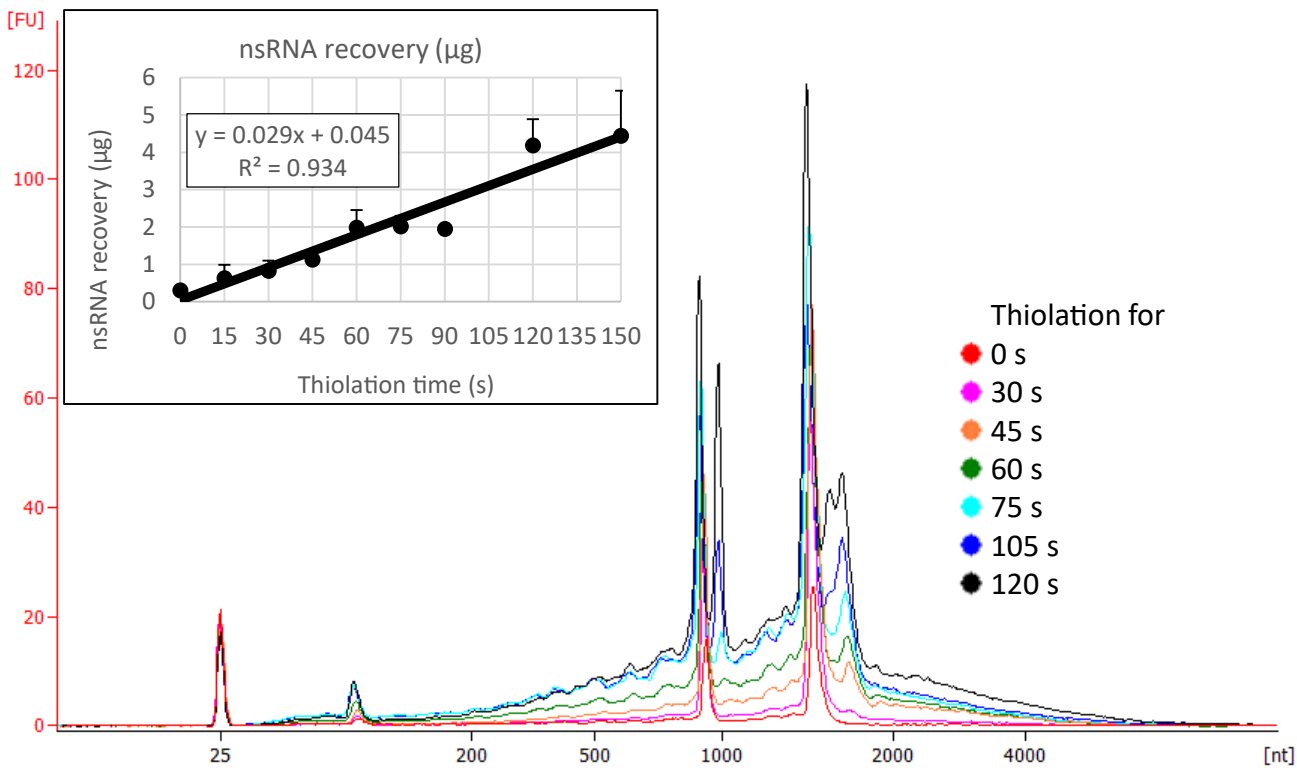


Figure 2

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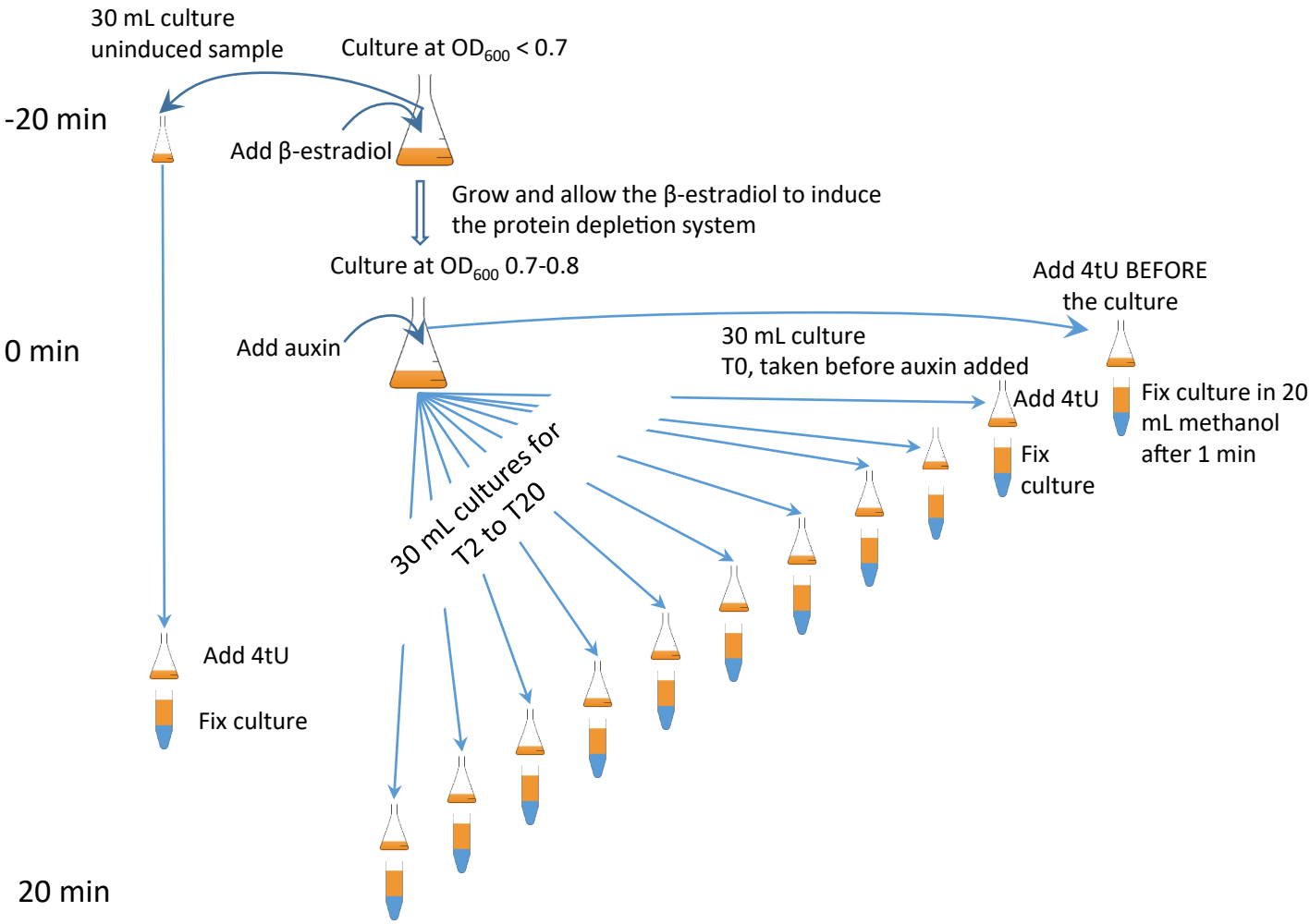


Figure 3

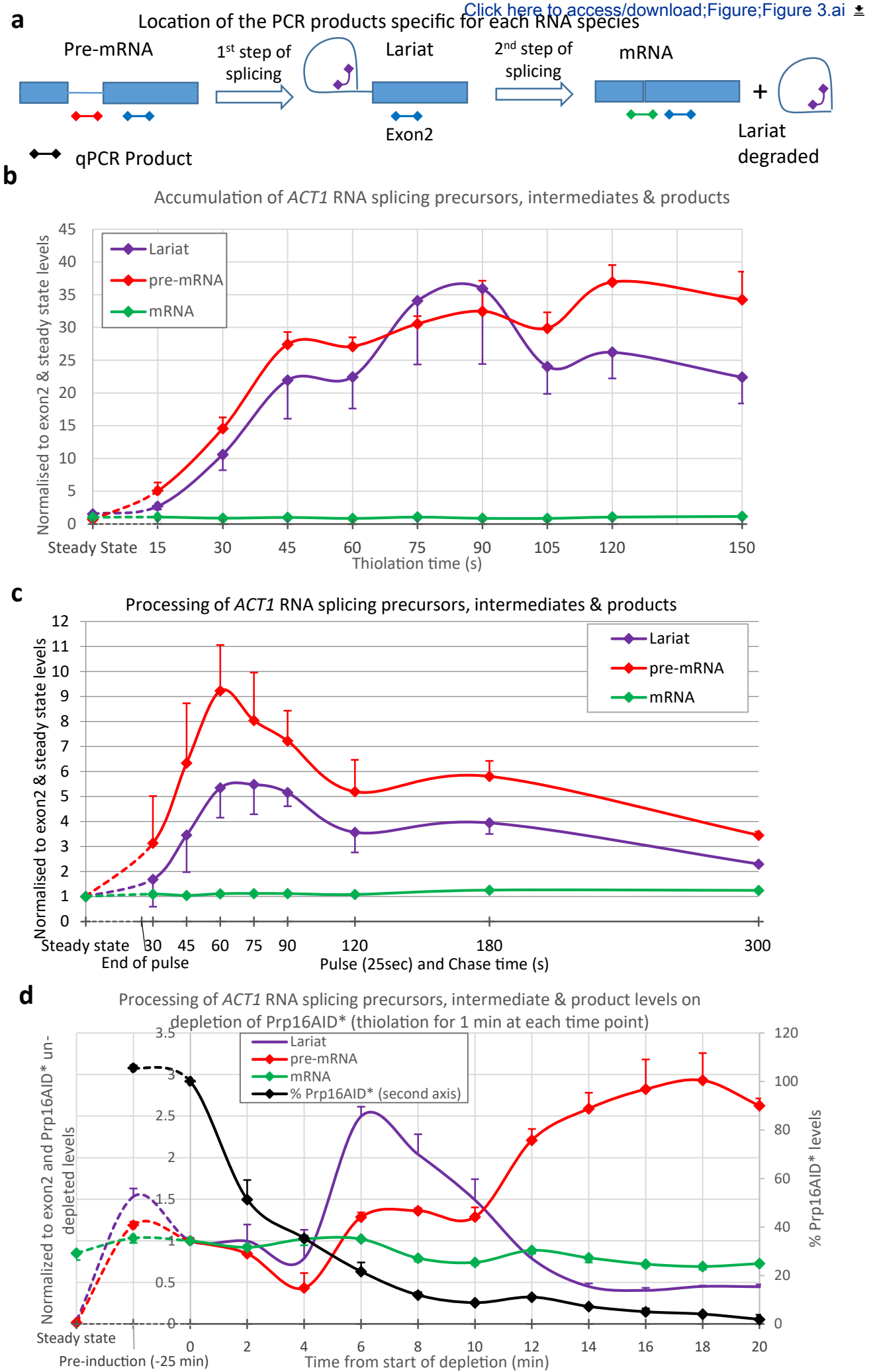
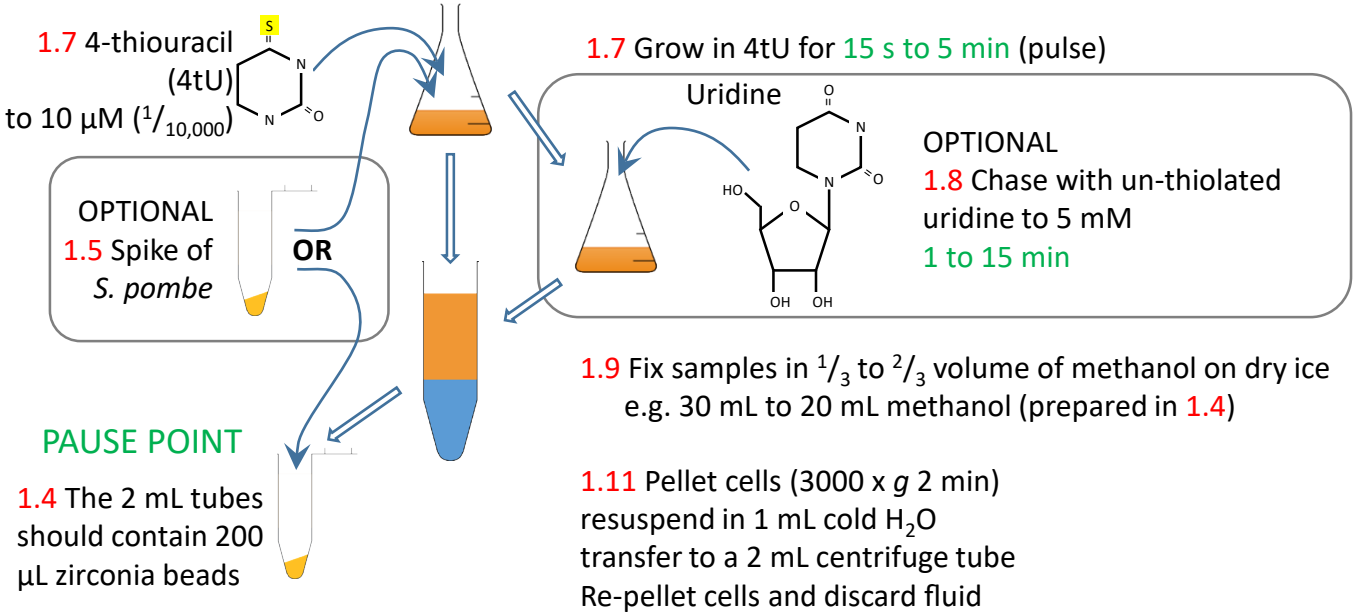


Figure 4

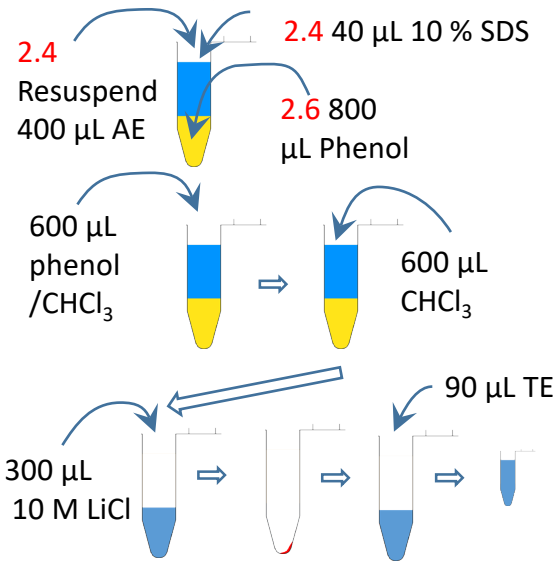
Section 1 Growth and thio-labelling



1.9 Fix samples in $^{1}/_{3}$ to $^{2}/_{3}$ volume of methanol on dry ice
e.g. 30 mL to 20 mL methanol (prepared in 1.4)

1.11 Pellet cells (3000 x *g* 2 min)
resuspend in 1 mL cold H_2O
transfer to a 2 mL centrifuge tube
Re-pellet cells and discard fluid
Store -80 °C

Section 2 Preparation of total RNA



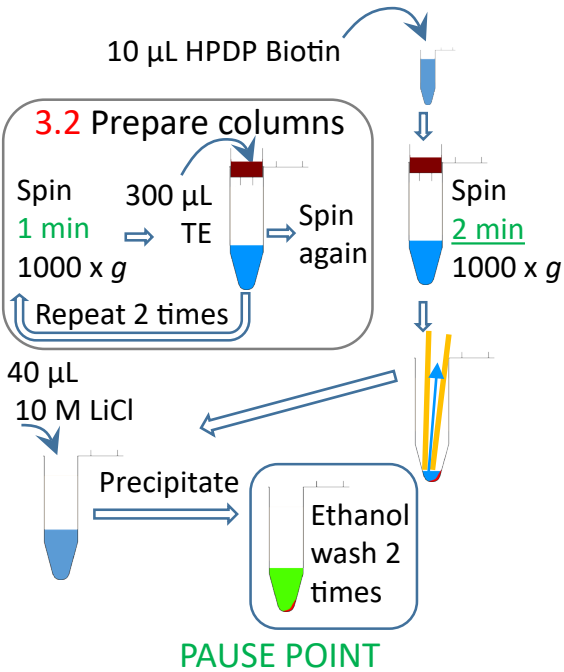
2.7 Lyse cells either by mechanical means, beat for 3 x 2 min (or hot phenol)
2.8 Put on dry ice until frozen

2.8 Spin 13,000 x *g* 5 min room temperature

2.9 Phenol/chloroform (5:1) and then chloroform extract. All spins at >13,000 x *g* for 5 min at room temperature

2.10 Add ½ volume of 10 M LiCl on ice for at least 5 min
2.11 Centrifuge 5 min at >13,000 x *g* to pellet the RNA
2.12 Resuspend in 90 µL TE, check for complete re-dissolving, and transfer to a 0.2 mL tube

Section 3 Biotinylation



3.1 Add 10 µL of biotinylation reagent
65 °C 15 min

3.3 Use a desalting column to purify away the un-incorporated HPDP biotin

3.4 Pellet the RNA once more with ½ volume of 10 M LiCl. Spin down >13,000 x *g* for 5 min and remove as much of the fluid as possible

3.5-3.6 Wash the pellet twice with 80% ethanol, each one for at least 1 h preferably with gentle mixing.
Each time remove as much of the fluid as possible

Figure 5

Section 4 Purification of newly synthesised RNA

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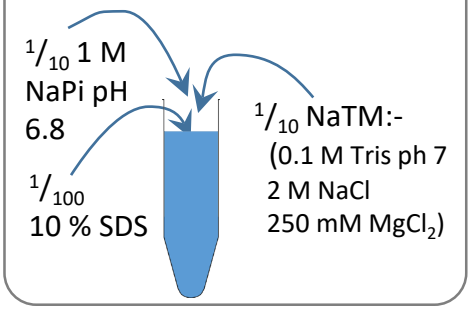
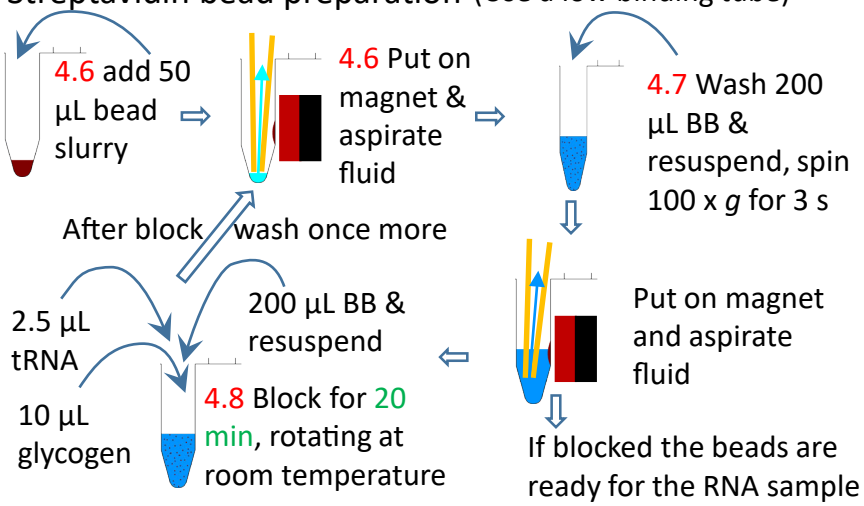
Bead Buffer (BB) preparation

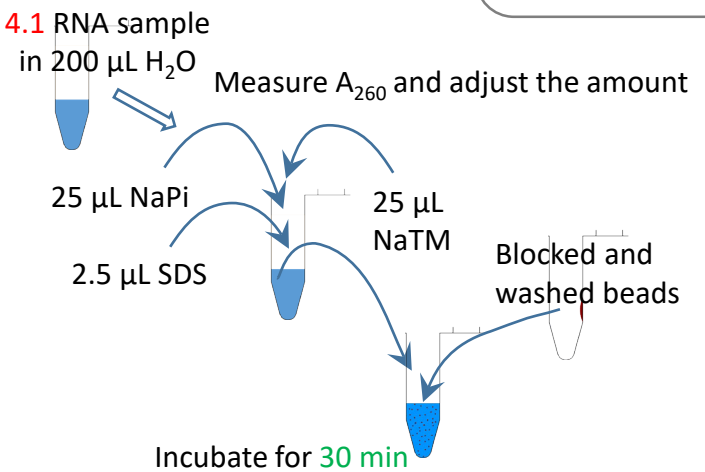
4.5 Prepare Bead Buffer fresh from the stock solutions; at least 2 mL per sample

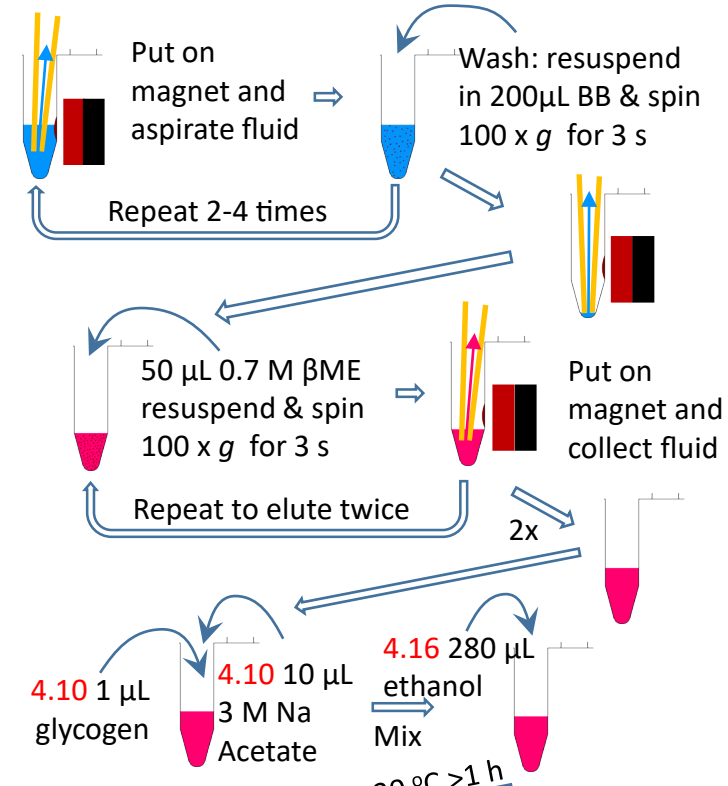
Add H_2O first & SDS last



Streptavidin bead preparation (Use a low binding tube)







4.1 Resuspend in 200 µL DEPC treated H_2O
Check all the RNA has re-dissolved

4.2 & 4.3 Measure the A_{260} and equalise the RNA content of the samples, make back up to 200 µL

4.4 Add the salts & detergent separately to the RNA. Mix gently. Spin 100 x *g* for 10 s

4.9 Add the RNA sample to the prepared beads and resuspend. Incubate for 30 min, gently rotating at room temperature end over end to keep in suspension

4.10 During incubation add 10 µL 3 M Na acetate and 1 µg glycogen to a 0.5 mL low-binding tube for each sample

4.11 After the incubation discard the sample and thoroughly wash the beads with Bead Buffer

4.12 At the final wash remove as much of the Bead Buffer as possible

4.13 & 4.14 Elute twice with βME, combine both eluates in the same low binding tube
This tube should already contain 10 µL 3 M sodium acetate and 1 µg glycogen (4.10), but if forgotten can be added before 4.16.

4.16 Ethanol precipitate
-20 °C at least 1 hour
Centrifuge >13,000 x *g* at 4 °C for 20 min

4.17 Wash the pellet with 200 µL 70 % ethanol, Remove as much of the fluid as possible

4.18 Resuspend the RNA in TE & RNase inhibitor

The sample is now ready for QC and downstream applications

Plasmid Name	Importer/permease	Marker	Comment
p4Fui	*S. cerevisiae* Fui1	*URA3*	Fui1 imports Uracil and Uridine, making it ideal for pulse/chase experiments.
pAT2	*S. cerevisiae* Fui1	*LEU2*	
p4Fui-ΔPEST	*S. cerevisiae* Fui1	*URA3*	The PEST motif of Fui1 has been deactivated, so the permease is not degraded when there is sufficient intracellular uracil for the cell's needs. Works well in labelling experiments and improves pulse/chase performance.
p4Fur	*S. cerevisiae* Fur4	*URA3*	Uracil permease
YEpEBI311	*H. Sapiens ENT1* (equilibrative nucleoside transporter)	*LEU2*	Miller et al.[11]. Also contains an HSV thymidine kinase gene.

All plasmids are 2 µm based. All p4 plasmids and pAT are based on the pRS[16] series of plasmids. *FUI1* and *FUR4* are expressed from their own, endogenous promoters.

Table of Materials

Click here to access/download;Table of Materials;Reagents JoVE v1.xlsx

β-mercaptoethanol (βME)	Sigma-Aldrich	M3148
Chloroform	Sigma-Aldrich	25668
Diethyl pyrocarbonate (DEPC)	Sigma-Aldrich	D5758
DMF (N,N-dimethylformamide)	Sigma-Aldrich	227056
EDTA	Sigma-Aldrich	3609
Ethanol	Sigma-Aldrich	29221
EZ-link HPDP Biotin	Thermo scientific	21341
Glucose	Fisher Scientific	G/0500/60
Glycogen [20 mg/mL]	Sigma-Aldrich	10901393001
Immobilised TCEP Disulfide Reducing Gel	Thermo Scientific	77712
LiCl	Sigma-Aldrich	793620
Magnesium chloride ($MgCl_2$)	Sigma-Aldrich	63033
Methanol	Fisher Scientific	M/4000/PC17
NaH_2PO_4	Sigma-Aldrich	S3139
Na_2HPO_4	Sigma-Aldrich	S3264
NaCl	Sigma-Aldrich	S9888-M
Phenol, low pH.	Sigma-Aldrich	P4682
Phenol Chloroform 5:1 (125:24:1) low pH.	Sigma-Aldrich	P1944
Pierce Spin Columns	Thermo Scientific	69702
SCSM single drop-out –ura	Formedium	DSCS101
Sodium Acetate	Sigma-Aldrich	32318-M
Sodium hydroxide	Sigma-Aldrich	795429
SDS (Sodium dodecyl sulfate)	Sigma-Aldrich	436143
Streptavidin Magnetic beads	NEB	1420S
SUPERase-In, RNase inhibitor	Life technologies	AM2696
Thiolated *Schizosaccharomyces pombe* for spike 4-thiouracil (4tU)	ACROS ORGANICS	359930010
Tris base	Sigma-Aldrich	93362
tRNA	Sigma-Aldrich	10109541001
Uridine	Sigma-Aldrich	U3750
Yeast nitrogen base without amino acids with amonium sulphate	Formedium	CYN0410
Zeba Columns 0.5ml	Thermo Scientific	89882
Zirconia beads	Thistle Scientific	110791052

CAUTION toxic. Stock solutions are aproximatly 14 M, make at $^{1}/_{20}$ dilution for use
CAUTION toxic
add $^{1}/_{1000}$ volume to a solution, leave at room temperature for 24 h, then autoclave
CAUTION toxic
Make 0.5 M and pH to 8.0 with sodium hydroxide

Store protected from light. Disolve all the vial contents in DMF. Store away from water, in the dark & at -20 °C. Check the solution before using, as some batches of HPDP precipitate in storage; heat at 42 °C to resuspend.

Store at -20 °C
Optional
10 M solution. CAUTION: this gets very hot as is dissolves and can even boil at greater than 100 ^{o}C, add the LiCl crystals to the water slowly.
1 M solution. CAUTION: this gets very hot as is dissolves and can even boil at greater than 100 ^{o}C, add the $MgCl_2$ crystals to the water slowly.
CAUTION Toxic and flammable

Make 1 M solutions of each and mix in equal amount to obtain a solution of the appropriate pH

5 M solution
Store in the dark at 4 °C. CAUTION toxic
Store in the dark at 4 °C. CAUTION toxic
Optional

Make a 3 M solution and pH to 5.3 with acetic acid
CAUTION corrosive
CAUTION irritant, do not inhale
Store at 4°C
Store at -20°C
See section 1.7 of the protocol
Store in the dark. Make 100 mM Stock in 1M NaOH, store solutions at -20°C.
1 M solutions at various pH
5mg/ml, store at -20°C
Make 1 M solution in H_2O. Split into 2 mL aliquots and store at -20 C.

Store at 4 °C

Beadbeater	Biospec	112011EUR

Bioanalyser (Agilent) or similar to assess RNA quality. If this is not important a spect
Centrifuge: capable of spinning cultures at 4 °C and at least 3000 g. Pre-chill if possi
Centrifuge: capable of spinning up to 2 mL tubes at variable speeds upto 13,000 g a
Magnetic rack for separating the beads from the sample. The one used in the paper
PCR machine with a heated lid that will allow incubation in the dark.
Rotating wheel to rotate 1.5 mL tubes end over end
Shaking heating block (such as Eppendorf Thermomixer) is recomended

Tubes, centrifuge, Low retention, RNase free 0.5mL	Eppendorf	H179467N
Tubes, centrifuge, Low retention, RNase free 1.5mL	Ambion	AM12350
Tubes, centrifuge, 50 mL	Sarstedt	62.547.004
Tubes, centrifuge, 15 mL	Sarstedt	62.554.001
Tubes, 2 mL, screw cap	Greiner	723361
Tubes 0.2 mL strip of 8 with integral lids	Brand	781332

Other homogenisers can be used; the correct conditions for each homogeniser and strain must be established.
trophotometer is useful to quantify the RNA.
ble.
ınd down to 1000 g
r is 3D printed, available from Thingiverse (thing:3562952). Comercially available racks exist

Other centrifuge tubes are not gas proof allowing CO_2 to disolve in the methanol, this comes out of solution vigorously on adding warm culture, leading to sample loss

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Author(s):	J. David Barrass and Jean D. Beggs

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CORRESPONDING AUTHOR

Name:	David Barrass
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Title:	Mr
Signature:	David Barrass
Date:	5th March 2019

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Dear Dr. Beggs,

Your manuscript, JoVE59952 "Extremely rapid and specific metabolic labelling of RNA in vivo with 4-thiouracil (ers4tU)," has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually. Please submit each figure as a vector image file to ensure high resolution throughout production: (.svg, .eps, .ai). If submitting as a .tif or .psd, please ensure that the image is 1920 x 1080 pixels or 300 dpi. Additionally, please upload tables as .xlsx files.

Your revision is due by **Apr 15, 2019**.

To submit a revision, go to the JoVE submission site and log in as an author. You will find your submission under the heading "Submission Needing Revision".

Best,

Phillip Steindel, Ph.D.
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Editorial comments:
General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Some issues found and fixed

2. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5” x 11”) page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.

Fixed

3. Please use American spelling (liter, sterilize, etc.).

I hope I have achieved this.

4. Please remove the ‘Video’ section and please highlight the steps to be filmed in the protocol section itself. Please leave any other notes about the video in Editorial manager (in the author notes section).

Removed. Steps to be part of the video highlighted in the protocol.

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For example: Beadbeater, Nanodrop, Bioanalyzer

Equipment made more generic, some left in as an example of a type that can be used

Protocol:
1. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.).

Done

2. Everything in the protocol should be in a numbered header, numbered step (in the imperative tense and of no more than 4 sentences), or 'Note'. Please move the introductory paragraphs of the protocol to the Introduction, Results, or Discussion (as appropriate) or break into steps.

Done

3. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 'Growth and thio-labelling' should be section 1, and contain steps 1.1, 1.2, 1.2.1, etc.

Done

4. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Highlighted in yellow

5. For each protocol step, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Done

Figures:
1. Please remove titles from the Figures themselves.

Done

2. Figure 1: Please combine into 1 single page. Note that we have no size limits for Figures. Please use ‘mL’ and ‘µL’ instead of ‘ml’ and ‘µl’, respectively, and please include a space between numbers and units. Lastly, please ensure the protocol shown here matches with that in the written protocol (e.g., step 11 mentions thiolabeling for 15 s to 1 min, but the figure mentions 15 s to 5 min).

Mostly done, but I feel that this figure is still better presented in a format that the user can print out easily. Figure split into two on advice from Editor

3. Figure 2; Please use ‘h’ and ‘s’ instead of ‘hrs’ and ‘sec’, respectively.

Done

4. Figure 3: ‘plulse’ is a typo; please also use ‘s’ here instead of ‘sec’.

Done

NOTE The numbering of the figures has been changed to correspond to the order in which they appear in the manuscript

OLD	*NEW*
fig 1a	*fig 4*
fig 1b	*fig 5*
fig 2a	*fig 1*
fig 2b	*fig 3d*
fig 3	*fig 3*
fig 4	*fig 3*

References:
1. Please do not abbreviate journal titles.

Done

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

~~*Check*~~*Done*

Reviewers' comments:

Reviewer #1:

This manuscript by David Barrass and Jean Beggs describes a step by step protocol enabling very short labelling of nascent RNA with the nucleotide analogue 4tU.

Overall the workflow is properly described, and the planned videos are expected to cover most of the critical steps.

As a side note, the authors should emphasize the fast handling requirement of the experimental set-up during the video production, but meanwhile the video should be self-explanatory, meaning that the sequence of events should be slow enough to be understandable. If required, the authors and filming crew should keep in mind to implement slow motion replay and/ or multiple angle view of the most critical steps.

Specific comments (without order of preferences):

1) Page 2 lane 33 "purification of thiolated RNA from any organism."
Whereas 4tU labeling might be working in many or most organisms (the list is currently growing), there is no strong evidence available in the literature that very short labelling maybe efficiently working in ANY organism as stated by the authors. For example, to be efficient the yeast cells are engineered to uptake more of the 4tU, this strategy might not be easily applicable in different biological system and could represent an obstacle that might be difficult to overcome in many cases. This also implies that such analysis might be restricted to genetically tractable organisms. Along with this line, the Methanol step to "fix the cells" will be also detrimental for certain type of organisms that nay not withstand this treatment. Overall, the authors should be more cautious describing the potential of the method and the reader should be properly informed about these limitations.

In compacting the abstract the statement that this was only after RNA was purified from other organisms was removed. This has now been added back, but the abstract is now over 150 words.

2) Page 2 lane 48 "The extremely rapid and specific 4 thio uridine protocol (ers4tU)" the authors mean 4-thiouracil.

Thank you for pointing this out

3) The authors use the term extremely rapid based on labelling time under the minute scale. On the other hand, the definition of short labelling may vary depending on the cellular system investigated (for example doubling time and/ or average length of mRNA are important parameters to consider....). The current extremely rapid definition should not be only founded on manual/ technical handling considerations but based on the nature of the biological system (I believe that is not the original intentions of the authors, but it reads this way).
I think the authors should better define very fast labelling in relation to the biological system used. For example, as a fraction of cell division or the time that would allow the average synthesis of a certain number of average transcriptome…For instance, very short could be define as the labelling time that is below 1% of the time required for the biological system to divide or consider the average transcript size of a biological system and the estimated time it would require to be "transcribed" (e.g. average transcript size of 1000 nt, assuming 50 nt/s as a transcription rate and 1 min labelling would represent ~ 3 full length transcriptome on average).

This is an interesting idea. Extra short labelling has now been defined as less than the time to transcribe the typical S. cerevisiae gene.

4) HDPD usage. The authors described a protocol which was originally performed/ optimized using HDPD-Biotin chemistry, however according to recent studies (e.g. Duffy et al 2015 and Knüppel et al 2017) MTS-Biotin chemistry is superior. The authors should clearly indicate

this point to the readers, as I also believe the results may be of even better quality using this improved chemistry.

In my experience MTS is a viable method of biotinylating thio groups. However, in my hands, it leads to higher background of RNA binding. The protocols using MTS are not optimised to reduce background to anything like the degree in this protocol so are not comparable.
This is not an easy effect to quantify, with such a low background, variability in the technique makes a big difference. Given my experience I cannot whole heartedly recommend MTS, but I am happy to refer to it as an alternative reagent.

5) Amount of 4tU/ toxicity. The authors should better indicate/highlight that the amount of 4tU used may vary between organisms and needs to be experimentally adjusted (trade-off between toxicity and labelling efficiency).

This is a good point and a sentence has been added to the caption of figure 2a to highlight this

6) Graphical summary of the protocol (Figure 1) is already a good start but the design is somehow confusing.
a) I would suggest the authors to do a 2 columns figure where the left side is a schematic representation with the written steps on the right side. The steps should be numbered for clarity (the numbering should somehow reflect the protocol numbering).
Ideally the figure should contain all the necessary information to perform the whole procedure without stepping back to the rest of the protocol (like quick cards protocols found in some molecular biology kits).

The design of this figure is a compromise between utility, space available to display the information and readability. The design philosophy was to provide a sheet that could be printed double sided and used at the bench away from a computer. ~~I am awaiting clarification from the editors about this~~On advice from the editor this figure was split into two.~~.~~ Equivalent step numbers from the text have been added to the figure

The supplementary excel sheet also provides a quick reference, as well as many useful calculations

b) Figure 1a indicate 45 min 65°C hot-phenol!!! It seems to me to be an excessive time.
The hot-procedure is also not described in the protocol.

*This is the time we, in our lab, found to be ~~the~~ optim*al*~~um~~ for the very large cell pellets produced by the sampling process.*
A reference for the hot phenol protocol was provided in the main text, but was awkwardly sited. This has been moved to a more logical location

7) In several instance the Chemical name is given before its concentration (xx 0.1M) instead of the conventional notation: concentration followed by the chemical name (0.1 M xx).

Thank you for pointing this out

8) General Consistency:
pH values are sometime provided as pH 7 or pH 7.0
space usage between numbers and unit is randomly used

The authors should make sure that ALL the abbreviations are properly introduced at some point in the text.

Thank you for pointing these out, I have been more consistent in the use of abbreviations and spaces.

9) "S. pombe spike": the authors should shortly explain the advantage of using such spike and how it is obtained (a detailed protocol should be eventually added).

A detailed protocol is in the Reagents section as it is something that should be prepared before a thio-labelling experiment starts but it is less than ideal. Additional text has now been added to the protocol to explain its utility, give more details and to reference the Reagents section

Reviewer #2:

Manuscript Summary:
The manuscript by Barrass and Beggs describes a metabolic labelling approach using nucleotide analogue for the analysis of newly-synthesized RNA in S. cerevisiae. This is a very nice method that has already been used to detect and analyze short-lived non-coding RNA and to measure pre-mRNA splicing kinetics in yeast (Barrass et al, Genome Biol., 2015). Existing 4-thiouracil (4tU) labelling protocols have been improved to allow the specific purification of labelled RNA following extremely short labeling times (from 15 sec to 5 min). The protocol called 'extremely rapid and specific 4tU labelling (ers4tU)', is presented in different experimental setups, including continuous labelling (or pulse-chase experiment) or discontinuous labelling in which a change is induced into the system. The manuscript provides a very clear and detailed description of the protocol which should be of great interest to many readers interested in RNA metabolism. I have no major issues with the manuscript.

Major Concerns:
none

Minor Concerns:

- In the Introduction, the authors mention two different biotinylation reagents (HPDP-biotin or MES-biotin: do the authors means MTS-biotin here?). However, only HPDP-biotin is described in the detailed protocol. As MTS-biotin was reported to dramatically increase the yields in nascent RNA recovery, I suggest a brief discussion on this question, based on the author's experience.

Thank you for spotting this I had thought I'd found all of these.
See response to Reviewer #1 point 4)

- Is it possible to provide an estimation of the efficiency in labelled RNA recovery? How much variability is observed in the proportion of newly-synthesized RNA across technical replicates?

Without knowing how much RNA is actually transcribed during the time course the efficiency is impossible to estimate. The variability is addressed in figure 2b (inset) the error bars (standard error of 3 replicates), and gives an indication of the variabillity

- A major advantage of this protocol is the very short labelling time, allowing the purification and kinetic analysis of extremely short-lived RNAs. In a more general perspective, when longer labelling times are applied (5 min or more), which steps of the proposed protocol should be applied to improve labelled RNA purification? In other words, which are the key factors of the proposed protocol that improve the efficiency and specificity of the thio-labelled RNA purification?

It is difficult to say, as all steps have been optimised. If the question is "what is really critical to get right?" I would say the Streptavidin bead preparation is the part of the protocol that most often goes wrong and the user should take particular care reading the protocol in this section. This should be made clear; the video is probably the best place to discuss this.

- Biotinylation reaction (steps 45-47) is performed at 65°C, although most protocols are incubating HPDP-biotin with RNA at 24°C. I guess that this is part of the protocol optimization. Could the authors comment on that?

This is to deal with poor batches of HPDP-biotin. Some ~~of the~~ batches have a tendency to precipitate during the biotinylation, and performing this at 65 ^{o}C eliminates this. This is mentioned in the step "This heating is required as some HPDP batches precipitate at room temperature in the RNA sample"

- page 4, line 128. Spike-in with S. pombe cells or labelled RNA is proposed at different steps (step 8 or step 23). Could the authors give more information on the spike-in procedure (ratio of S. pombe cells, expression of a permease…)?

See reviewer 1 point 9)

- From the Figure 3a, I understand that the quantification of pre-mRNA is based on the amplification of an intronic sequence which should be also detected from the lariat. If this would be right, then the levels of lariat could not be higher than those of pre-mRNA.

The pre-mRNA PCR cannot detect lariats as the PCR crosses the sequences modified by the creation of a nucleotide that is in branched (2'-5' linkage), form in the lariat (the branch point) and the branched nucleotide blocks reverse transcriptase, so these measurements are independent. This is detailed in the reference associated with the figure.

- Typos: page 1, line 25: replace '4-thouracil' by '4-thiouracil' - page 2, line 48: replace '4 thiouridine protocol…' by '4-thiouracil' - page 2, line 56: '4-thiouracil…(reference 5: Burger et al.)'. Only toxicity of 4-thiouridine (4sU) was shown in this paper - page 6, line 26: replace 'Resupended' by 'Resuspend' - page 8, line 264 (same typo page 9, line 273): replace '0.5 m NaH2PO4' by '0.5 M NaH2PO4' - page 9, line 273 : replace 'Tris Cl' by 'Tris HCl'

Thank you for point these out

Reviewer #3:

This is a useful and thorough protocol for 4 thiouracil labeling in yeast. The text would be enhanced by addressing the following points:

I find the acronyms to be quite awkward and not helpful in reading the text. Moreover, the are defined at least twice in the text which reflects their lack of utility.

Thank you for point these out. I have edited the text to be consistent in the use of abbreviations.

I am not convinced that it is necessary to overexpress a transporter gene for efficient incorporation. Can the authors point to a reference showing this is required?

Ref 19, Miller et all 2014 "This significantly enhanced 4sU incorporation" (all be it with thio-uridine). I can also provide my own experimental data to show this, added as Supplementary Information Figure S2, although these experiments were 9 years ago when the protocol was not as optimised as it is now. I only present one experiment but there are other older, more difficult to find, experiments using the permease under a different promoter that also showed increased incorporation. These experiments were analysed in a different way, so the data is not strictly comparable.

The motivation for using the auxin system is well justified. Presumably this is useful for studying essential genes and transient effects. It is introduced without any rationale.

This is discussed in the introduction section, lines 63 to 68 (in the new document)

I think there is pretty compelling evidence that MES-biotin from the Simon lab is superior. It would be useful to explain why HPDP-biotin is used in this case.

See response to Reviewer #1 point 4)

The use of an S. pombe spike is not explained. What is this for and does it need to the thiolated? (yes). And, how should that be done.

All of the reviewers have asked for more details and this is discussed in comments to reviewer 1 point 9)

Can liquid nitrogen be used instead of cold methanol?

It could but the sample would be frozen and much, much harder to process, involving grinding, and so it is not suitable for processing a large number of samples

In the results section - how many cells produce 0.8ug of thiolated RNA?

Approximately 10^9 cells, this has been added to the text.

What is the evidence that 4yU is a nitrogen source.

~~*This is my own observation, I do have a growth curve that demonstrates improved growth with 4-thiouracil in low nitrogen medium. This could be put in a supplementary section and I am awaiting clarification .*~~
~~*It is not un-expected that 4tU can act as a nitrogen source.*~~

Looking back at my experiment from over 6 years ago I realise it was actually uracil not 4-thiouracil used. This sentence has been modified. Everyone will be willing to accept that

uracil is a nitrogen source

Why are chases performed with uridine and not uracil?

Uracil is not as soluble as uridine. In order to act as a chase large amounts of uracil or uridine have to be added. If uracil were to be used an unfeasibly large volume of uracil would have to be added, this would involve a considerable change in the growth conditions, whereas a small volume of a more concentrated uridine solution can be added. I have added a sentence to the relevant step to explain this.

Trouble shooting table

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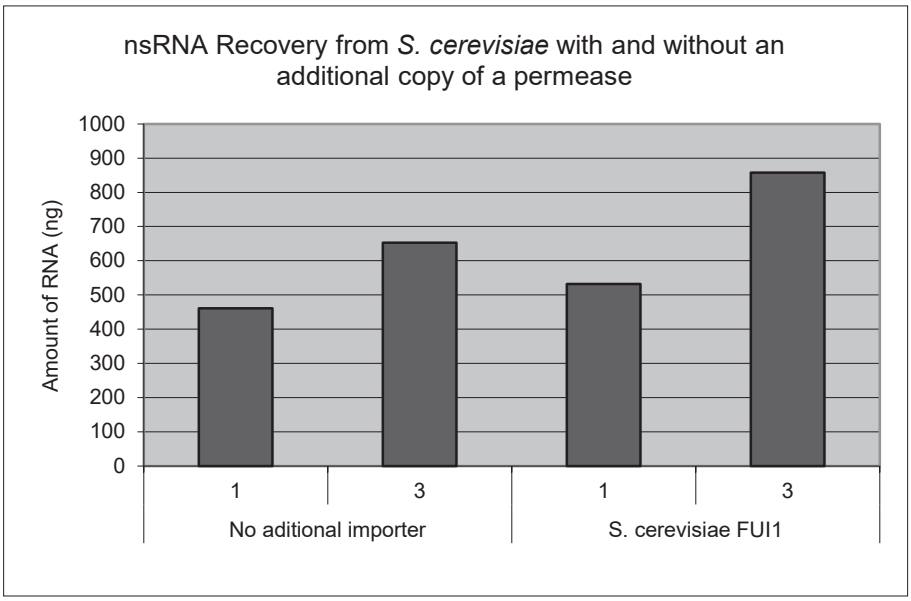
Problem	Possible reason
Cell pellet not firm or secure	Insufficiently spun
	Too long on dry ice
Discolouration in the cell pellet, often at the top	Autoclaved growth medium
Large white mass in the phenol after spinning	The sample has not adequately thawed
The sample does not flocculate	Not enough LiCl
	Not Enough RNA
The RNA will not re-dissolve	Too much RNA
	Too much genomic DNA contamination
Low RNA recovery	Not enough material
	RNA degradation
	RNA pellet not re-suspended, generally one or a few samples have much less RNA than the others
HPDP precipitation	Poor batch of HPDP
	Water in DMF
After zeba column purification the sample volume appears to be less than 90 µL	Inadequate centrifugation
Bead Buffer precipitates	Solutions added in wrong order
	Solutions too cold
Precipitation in the sample	Solutions added in wrong order
	Sample too cold
Magnetic beads will not resuspend	Sample left too long on magnet
	Bead Buffer has precipitated; the pellet will have more cohesion than in the case above

Brown discolouration of the liquid	The beads are degrading
RNA pellet is diffuse and spread up the side of the tube	Too much detergent left after washing
RNA will not resuspend	Too much SDS or glycogen
Low nsRNA yield	Poor RNA resuspension
	Poor RNA precipitation
	Not enough material
High background	Blocking not complete
RNA degraded	RNases
	There is not actually a problem

Solution
The centrifuge might take a while to reach maximum speed. Re-spin for at least 1 min more at 3000 g
Remove the liquid carefully, do not pour. In future, if there is insufficient time to fully process the samples, spin quickly, remove the liquid and put the pellet in dry ice.
The methanol will precipitate brown peptide/sugar complexes produced by autoclaving the growth medium. Proceed as normal, phenol extraction will remove this matter. To avoid this, filter sterilise media in the future.
Continue with extraction after thawing and re-spinning. However, there will be increased genomic DNA contamination, which, although undesirable, should not ruin the experiment. Next time, reduce the amount of time in dry ice in step 2.11. Do not used a chilled centrifuge.
Add more LiCl, up to the same volume as the sample. Ensure that the concentration of LiCl is 10 M, remake the solution if unsure.
Repeat the experiment with a greater sample volume taken from the culture.
Redisolve in more TE, but do not use more than 90 µL in the biotinylation reaction.
Discard and start again from the beginning, paying particular attention to the dry ice step with phenol. DNase treatment is not recommended as the DDT in most DNase buffers will deactivate the HPDP in later stages unless strenuous steps are taken to re-purify the RNA. This may not be a problem if using permanent biotin linker such as maleimide biotin.
Repeat using more sample.
Run on a gel or bioanalyser to confirm. Repeat the experiment using RNase free reagents and equipment, reduce the time the RNA spends at 65 °C. DEPC treat reagents, use fresh aliquots
Check that all of the RNA has re-dissolved by pipetting up and down and checking for smooth movement; incubate at 65 °C briefly.
Heat to 42 °C, replace batch.
Heat to 42 °C, replace the batch or take a fresh aliquot.
Do not discard the column; place back in the sample tube and spin at 1000 g for another minute.
Remake the solution, adding the water first.
The solutions and sample must be at room temperature; if the lab is cold, gently warm sample to 30 °C.
Spin down the precipitate, transfer the liquid into a new tube, add any components not yet added to the liquid, and then mix before adding the SDS last. The yield and /or background may be unfavourable.
Spin down the precipitate and collect the liquid. Ensure that the sample is at room temperature, then add any components not yet added, with the SDS added last. The yield and/or background may be unfavourable.
Vortex vigorously, pipette up and down to break up the bead mass.
Vortex vigorously, pipette up and down to break up the bead mass. The experiment may have to be repeated.

The βME is degrading the beads, which can happen, particularly with the NEB beads, whereas Dynabeads are less sensitive. This is normal and the discoloration is often removed by the second magnetic purification. It does not seem to affect downstream steps. However, if you are concerned, leave on the magnet longer during the second magnetic purefication and reduce the time the beads are in contact with the βME.
Be careful when removing the liquid and when washing. There is no reason to worry about RNA yield. Take special care to remove as much of the bead wash as possible in future.
Increase the volume and pipette to break up the pellet. Consider heating briefly to 65 °C and then leaving at minus 20 °C overnight.
Check that the RNA has resuspended and treat as above.
Make sure the RNA, sodium acetate and glycogen are mixed well before adding the ethanol.
Repeat using more sample.
Repeat the experiment or affected sample, using new batches of glycogen and tRNA, make sure the RNA sample is well mixed with the Bead Buffer before adding to the beads.
Ensure that all solutions are RNase free by DEPC-treating wherever possible. Add SUPERase-In to solutions where the RNA concentration is low, i.e. from step 4.26.
The RNA may not, in fact, be degraded; the nsRNA profile, as produced by a bioanalyzer, will not be the same as for total RNA; see Figure 1b for expected RNA profiles. Similarly the RIN number will indicate that the RNA is of poor quality, however values down to 4 are not cause for concern.



1. Growth and Thiolation				Culture 1	Culture 2	Culture 3	Culture
Date 4/29/2019			Name				
			1.3 OD_{600}				
	Stock Conc	Final Conc	Time				
Culture volume							
1.5 *S. pombe* spike enter in this row → 1 aliquot = 400 mL of culture							
1.6 β-est	10 mM	10 µM	1 in 1000	0	0	0	
Incubation time			For				
Sample taken out?			Vol				
1.6 IAA	1.5 M	0.75 mM	1 in 2000	0	0	0	
Incubation time			For				
Sample taken out?			Vol				
1.7 4tU	100 mM	10 µM	1 in 10000	0	0	0	
Incubation time			For				
Sample taken out?			Vol				
1.7 Uridine	1 M	5 mM	1 in 200	0	0	0	

Time Course

1.7 Samples taken and put into methanol on dry ice

1.11 Span 3 *g* x1000 for 2 min

1.11 Supernate removed

1.11 Resuspend in H_2O 1 mL

1.12 Transfered to screwcap tube with 200 µL zirconia

1.12 Span 10 sec >13,000 x *g*

Supernate removed

Stored: Storage at -80 °C

Sample #	Time (s or min)	Vol (mL) Culture	Vol (mL) MeOH	Time (s or min)	Vol (mL) Culture	Vol (mL) MeOH	Time (s or min)	Vol (mL) Culture	Vol (mL) MeOH	Time (s or min)	Vol (mL) Culture
1		30	20		30	20		30	20		30
2		30	20		30	20		30	20		30
3		30	20		30	20		30	20		30
4		30	20		30	20		30	20		30
5		30	20		30	20		30	20		30
6		30	20		30	20		30	20		30
7		30	20		30	20		30	20		30
8		30	20		30	20		30	20		30
9		30	20		30	20		30	20		30
10		30	20		30	20		30	20		30
11		30	20		30	20		30	20		30
12		30	20		30	20		30	20		30
13		30	20		30	20		30	20		30
14		30	20		30	20		30	20		30

4	
	mL
	µL
0	µL
	min
	mL
0	µL
	min
0	mL
0	µL
	sec
0	mL
0	mL

(mL) MeOH	Time Minutes (min) or Seconds (s) - Vol in mL
20	
20	
20	
20	
20	
20	
20	
20	
20	
20	
20	
20	
20	
20	

2. RNA Extraction		**Culture 1**	**Culture 2**	**Culture 3**	**Culture 4**
	Date	4/29/2019	4/29/2019	4/29/2019	4/29/2019
	Name				
2.2 *S. pombe* spike (Optional)	Vol	10 µL	10 µL	10 µL	10 µL
2.4 AE buffer	Vol	400 µL	400 µL	400 µL	400 µL
2.4 SDS [10%]	Vol	40 µL	40 µL	40 µL	40 µL
2.6 Phenol, low pH	Vol	800 µL	800 µL	800 µL	800 µL
2.7 Lyse in Beadbeater	For	3x times	3x times	3x times	3x times
rest in ice between	min	2 min	2 min	2 min	2 min
2.8 Dry Ice	For	5 min	5 min	5 min	5 min
2.8 Spin >13,000 x *g* (room temp)	For	5 min	5 min	5 min	5 min
Extract supernate to new tube					
2.9 Phenol:$CHCl_3$ 5:1, low pH	Vol	600 µL	600 µL	600 µL	600 µL
vortex vigourously					
Spin >13,000 x g	For	5 min	5 min	5 min	5 min
Extract supernate to new tube					
2.9 $CHCl_3$	Vol	600 µL	600 µL	600 µL	600 µL
vortex vigourously					
Spin >13,000 x g	For	5 min	5 min	5 min	5 min
Extract supernate to new tube					
2.10 LiCl [10M]	Vol	300 µL	300 µL	300 µL	300 µL
Mix					
Precipitate	Temp	4 °C	4 °C	4 °C	4 °C
	For	10 min	10 min	10 min	10 min
2.11 Spin >13,000 x *g*	For	5 min	5 min	5 min	5 min
Wash 70% EtOH					
2.12 Resuspend 1x TE pH7.0	Vol	90 µL	90 µL	90 µL	90 µL
Ensure complete resuspension and transfer to 0.2ml tube strip					

3. Biotinylation		**Culture 1**		**Culture 2**		**Culture 3**		**Culture 4**	
	Date	4/29/2019		4/29/2019		4/29/2019		4/29/2019	
	Name								
3.1 Begin incubation	Temp	65	°C	65	°C	65	°C	65	°C
3.1 HPDP or MTS biotin	Vol	10	µL	10	µL	10	µL	10	µL
Vortex									
Incubation	Temp	65	°C	65	°C	65	°C	65	°C
In dark eg PCR block	For	15	min	15	min	15	min	15	min
3.2 Prepare Zeba columns during incubation									
Wash - spin 1500 x g, 1min	Vol	300	µL	300	µL	300	µL	300	µL
remove fluid then add 1x TE	Washes	inital	wash 1	inital	wash 1	inital	wash 1	inital	wash 1
pH7.0. Repeat 3 times		wash 2	wash 3	wash 2	wash 3	wash 2	wash 3	wash 2	wash 3
3.3 After incubation put column in a new tube, add RNA									
Spin 1500 x *g* **2 min**									
3.4 Precipitate	10M LiCl	40	µL	40	µL	40	µL	40	µL
Precipitate	Temp	4	°C	4	°C	4	°C	4	°C
	For	10	min	10	min	10	min	10	min
Spin >13,000 x *g*	For	5	min	5	min	5	min	5	min
3.5 & 3.6 Wash 80% EtOH		wash 1	wash 2	wash 1	wash 2	wash 1	wash 2	wash 1	wash 2

Beads			**Culture 1**	**Culture 2**	**Culture 3**	**Cultu**
		Date	4/29/2019	4/29/2019	4/29/2019	4/29/
		Name				
4.1 Redisolve the RNA in H_2O			200 µL	200 µL	200 µL	200
4.2 Measure the concentration of the RNA and adjust volumes to use. See form to right						
4.4 Add Beadbuffer	1st NaTM	Vol	25 µL	25 µL	25 µL	25
	2nd 1 M NaPi	Vol	25 µL	25 µL	25 µL	25
	3rd 10% SDS	Vol	2.5 µL	2.5 µL	2.5 µL	2.5
4.5 Make Bead Buffer, see form to far right						
4.6 Bead aliquot		Vol	50 µL	50 µL	50 µL	50
Type	NEB streptavidin					
4.7 Wash	Bead Buffer	Vol	200 µL	200 µL	200 µL	200
4.8 Block	Bead Buffer	Vol	200 µL	200 µL	200 µL	200
	Glycogen 20 mg/mL	Vol	10 µL	10 µL	10 µL	10
	tRNA 5 mg/mL	Vol	2.5 µL	2.5 µL	2.5 µL	2.5
	Time	For	20 min	20 min	20 min	20
Wash as 4.7	Bead Buffer	Vol	200 µL	200 µL	200 µL	200
4.9 Add Sample		Vol	250 µL	250 µL	250 µL	250
Incubate		For	30 min	30 min	30 min	30
4.10 and Wash as 4.7 with Bead Buffer	1. On magnet	Vol	200 µL	200 µL	200 µL	200
	2. pour off & aspirate		wash 1	wash 1	wash 1	wash 1
	3. add wash	repeat	wash 2	wash 2	wash 2	wash 2
	4. vortex	at least	wash 3	wash 3	wash 3	wash 3
	5. Spin 1krpm a few sec	3 times	wash 4	wash 4	wash 4	wash 4
Other wash [Opt]		Vol	0 µL	0 µL	0 µL	0
			wash 1 / wash 2	wash 1 / wash 2	wash 1 / wash 2	wash 1
			wash 3 / wash 4	wash 3 / wash 4	wash 3 / wash 4	wash 3
4.13 Elution 1	BME 0.7 M	Vol	50 µL	50 µL	50 µL	50
4.14 Elution 2	BME 0.7 M	Vol	50 µL	50 µL	50 µL	50
4.15 Final bead removal, transfer to a freash tube						
4.16 Ethanol precipitate	4.10 Na acetate 3 M pH 5.3	Vol	10 µL	10 µL	10 µL	10
	4.10 Glycogen 20 mg/mL	Vol	1 µL	1 µL	1 µL	1
	Mix					
	Ethanol	Vol	280 µL	280 µL	280 µL	280
	Mix					
Precipitate		Temp	-20 °C	-20 °C	-20 °C	-20
		For	O/N	O/N	O/N	O/N
		Date	4/30/2019	4/30/2019	4/30/2019	4/30/
Spin >13 krpm		For	20 min	20 min	20 min	20
		Temp	4 °C	4 °C	4 °C	4
4.17 Wash 70% EtOH						
4.18 Resuspend TE + RNase inhibitor		Vol	10 µL	10 µL	10 µL	10

ure 4
'2019
µL
µL
µL
µL
µL
µL
µL
µL
µL
min
µL
µL
min
µL
µL
wash 2
wash 4
µL
µL
µL
µL
µL
°C
'2019
min
°C
µL

4.2 Sample Volumes

Date 4/29/2019

Typical sample vol	198.0	µL	RNA to lable	0 mg
			Total vol	250.0 µL

Sample			Add to sample (µL) (in this order)			
Sample name	conc µg/µL	vol to use (µL)	NaTM	NaPi	SDS	H2O
		0.00	25	25	2.5	197.5
		0.00	25	25	2.5	197.5
		0.00	25	25	2.5	197.5
		0.00	25	25	2.5	197.5
		0.00	25	25	2.5	197.5
		0.00	25	25	2.5	197.5
		0.00	25	25	2.5	197.5
		0.00	25	25	2.5	197.5
		0.00	25	25	2.5	197.5
		0.00	25	25	2.5	197.5
		0.00	25	25	2.5	197.5
		0.00	25	25	2.5	197.5
		0.00	25	25	2.5	197.5
		0.00	25	25	2.5	197.5
		0.00	25	25	2.5	197.5
		0.00	25	25	2.5	197.5
		0.00	25	25	2.5	197.5
		0.00	25	25	2.5	197.5
		0.00	25	25	2.5	197.5
		0.00	25	25	2.5	197.5
		0.00	25	25	2.5	197.5
		0.00	25	25	2.5	197.5
		0.00	25	25	2.5	197.5
		0.00	25	25	2.5	197.5
		0.00	25	25	2.5	197.5
		0.00	25	25	2.5	197.5
		0.00	25	25	2.5	197.5

4.5 Ammount of Bead Buffer to make up

#Samples	0		
Minumum	0 mL	Minimum volume needed	
So make up	20 mL	← Change this to have excess	

Bead Buffer	**Stock Conc**		**Final Conc**		**Volume**		
H2O for 1x		to	20	mL	16 mL	1st	Add in this order
NaTM	10	x	1	x	2 mL	2nd	
NaPi ph 6.8	1	M	0.1	mM	2 mL	3rd	
SDS	10	%	0.1	%	0.2 mL	4th	

Supplemental File Figure S2

Click here to access/download

Supplemental Coding Files

Figure S2 Best AID 4U.mp4