Nascent RNA Labeling and Purification

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Labeling of newly synthesized RNAs by addition of 4-thiouracil in growth media, followed by biotinylation of s^4U-RNA via MTS-biotin and enrichment on streptavidin beads. Purified RNAs can be analyzed by RT-qPCR or NextGen Sequencing.

References

4-thiouracil labeling, total RNA extraction, cDNA synthesis and qPCR: Bonnet J et al., 2014, *Genes Dev* **28**:1999-2012
s^4U-RNA biotinylation and enrichment: Duffy et al., 2015, *Molecular Cell* **59**:858-866 + detailed protocol manuscript in progress

Yeast Growth and Labeling

Grow 10 ml *S. cerevisiae* cells in YPD to OD 1.0 (grow to OD 1 in bigger volume, then split before 4-thiouracil addition). This corresponds to ~1 x 10^8 cells and should yield ~80-100 ug total RNA using the Ambion RiboPure Yeast Kit. Adjust volume of cells harvested if OD is slightly more or less so that total number of cells is equal for each sample (adjust volumes at the last OD measurement (before 4-thio addition), than transfer cells for spinning by dumping). To use as a spike in control for normalization, also grow and label enough *S. pombe* (label all with 4-TU) to mix with each *S. cerevisiae* sample in a 8:1 ratio (*cerevisiae*:*pombe*) – mass ratio based on OD. (*S. pombe* is grown in YE media (0.5% yeast extract, 3% glucose) at 30 deg, doubling time is ~2.5 hr).

Comments (02/18) – example of an experiment with IAA:

- Grow 40 ml of culture, divide into 3 x 10 ml (in 50 ml tubes) for each replicate when OD ~ 1. Calculate the exact volume based on OD (for OD = 1, take 10 ml).
    1. xxx –IAA, **not treated with 4-TU**
    2. xxx –IAA, +4-TU
    3. xxx +IAA, +4-TU

- For that ~4 ml o labeled Pombe culture is needed (1:8 ratio) – pombe calculation below
- After splitting cultures into individual 50 ml tubes add IAA (50 µl 100x IAA (0.2 M) to 10 ml; 0.11 g IAA in 6 ml DMSO), keep for 15' (place tubes in the beaker – four tubes in one 1l beaker, ok for short incubation)
- During IAA treatment prepare 4-thiouracil (4-TU) – 2 M stock in DMSO (260 mg/ml); keep 4-TU in foil covered tube
- Add 25 µl per 10 ml of culture, 4-TU will precipitate when added to the culture, mix the tube vigorously before placing back in the beaker
- After 4' take the tubes from the shaker and transport to the lab
- Spin for 3', 3000 rpm.
- Dump supernatant, freeze pellets at liquid nitrogen, store at -80°C

- Controls:
    1. -4-TU / +4-TU - it will show if the RNA labeling with 4-TU was successful
    2. RNA eluted from beads / total RNA (after RiboPure kit) - it will show how much of total RNA is recovered, for unlabeled sample (1 below) this should be considerably lower than for the labeled sample (2 below)

- qPCR – use 5 samples:
    1. -IAA, -4-TU
    2. -IAA, +4-TU
    3. +IAA, +4-TU
    4. -IAA, -4-TU total RNA
    5. -IAA, +4-TU total RNA

Pombe comments
For RNA-seq 8:1 cerevisiae to pombe ration is needed. Grow 20 ml of pombe to OD = 1, label with 4-TU, spin and freeze. This amount of cells will provide enough material for 16 cerevisiae samples. Resuspend pombe pellet in 850 μl of lysis buffer (no SDS and phenol) and add 50 μl to 958 μl of cerevisiae sample.

4-thiouracil is light sensitive – do all labeling and subsequent RNA purifications, biotinylation and enrichment in low lighting.

Day 1 (6-7 h for 24 samples)

Total RNA Isolation
Follow Ambion's RiboPure Yeast Kit instructions, using the following volumes. Combine *S. cerevisiae* with *S. pombe* in 8:1 ratio immediately before lysis.

Add following components from kit to each cell pellet, in order listed (from 10 ml OD 1.0 cells):

480 ul lysis buffer
48 ul 10% SDS
480 ul phenol:$CHCl_3$:IAA
1008 ul total volume per cell pellet

- Resuspend pombe cells in lysis buffer only, calculate the volume based on example above

Vortex 10-15 sec. Transfer and combine the following volumes to 2 ml screwcap tube (lower drawer in the gel room) containing 1.25 ml cold zirconia beads (stored at -20°C freezer next to the lab phone, any zirconia beads are good, the kit does not provide enough beads for our protocol):

958 ul S. cerevisiae
50 ul S. pombe
1008 ul total volume

Lyse cells in Toshi's mini bead beater ~10 min total (3 x 3 min with 1 min rests on ice). Spin 5 min at 16K x g at rm temp. Combine in 5 ml tube (5 ml Falcon 12 x 75 mm):

~400 ul supernatant
1400 ul binding buffer (350 ul buffer per 100 ul RNA)
940 ul 100% ethanol (235 ul ethanol per 100 ul RNA)

~3.1 ml

Process through filter cartridges per kit instructions – bind 700 ul mixture through column at a time followed by 1 min spin (12k x g). Repeat until all sample is loaded. Wash filter with 700 ul Wash Solution 1 (kit), spin 1 min. Wash filter with 500 ul Wash Solution 2/3 (kit). Repeat with additional 500 ul Wash Solution 2/3. Do a final 1 minute spin to remove residual ethanol. **Note – Duffy protocol suggests addition of either 0.1 mM DTT or 10 mM BME to wash steps during RNA purification in order to reduce any disulfides that have formed, although I did not include a reducing agent at this step upon the first run through of this protocol.

Elute RNA with 25 ul 95 deg preheated Elution Solution (kit), centrifuge 1 min. Repeat elution. Combine eluates. DNaseI-treat samples according to kit – 1/10 vol DNaseI buffer + 4 ul DNaseI, incubate 30 min 37 deg. Inactivate DNaseI with 5 ul Inactivation Reagent (kit) for 5 min at rm temp (pipette Inactivation reagent with a cut tip). Spin, transfer sup to new tube. Nanodrop for concentrations (obtained ~1 ug/ul) (reference sample – elution buffer + DNaseI buffer + inactivation reagent). Store RNA samples at -20 deg for 1-2 month or -80 deg for longer.

Day 2 (6-7 h for 24 samples)

Labeled RNA Biotinylation and Purification

remember to keep reactions in dark

Dilute 1 mg/ml MTSEA biotin-XX to 50 ug/ml in DMF (=82 uM) – dilute 20x. 80 ul (4 ug) in 400 ul final reaction volume = [16.4 uM] final in 20% DMF.

Combine in eppy tube:

40 ug labeled RNA	___ul	
20 mM HEPES	8 ul	1M stock
1 mM EDTA	1.6 ul	0.25 M stock
4 ug MTSEA biotin-XX	80 ul	50 ug/ml diluted stock
H_2O (nuclease free)	___ul	
Total volume	400 ul	

- Use less RNA if not enough in all samples, e.g. 35 μg

Cover with foil, incubate with rotation on nutator at room temp for 30 min. Start beads preparation – below.

Remove unreacted MTS-biotin from samples by phenol extraction. Add 400 ul phenol:$CHCl_3$:IAA (normal phenol) (25:24:1), vortex 15 sec, incubate 2 min at room temp, spin 5 min 12K x g at room temp, remove ~340 ul sup. Ppt with 1/10 vol 5M NaCl (34 ul) + equal volume isopropanol (340 ul). Vortex, spin 20 min at max speed. Wash pellet with 70% ethanol (careful – pellet does not stick very well to the bottom). Resuspend in 100 ul nuclease-free H_2O.

<u>Prepare Streptavidin beads - for 15- 20 reactions ~60 ml high salt wash buffer (remember to add Tween 20 (12.5 μl per 25 ml)!)</u>

Use 80 ul of beads suspension per reaction. Wash batch of beads (80 ul X # reactions +10%) 3X 3 ml nuclease-free H_2O. Wash beads in smaller batches – max 500 ul beads per 1.5 ml tube). Wash 3 X 0.75 ml High Salt Wash Buffer per 1.5 ml tube. Block in 1.25 ml Blocking Buffer (per tube) **<u>1 hour</u>** at room temp (nutator). Wash 2 X

0.75 ml High Salt Wash Buffer. Resuspend in original volume High Salt Wash Buffer (1.76 ml for 20 reactions +10%).

Isolate Biotinylated RNA

Aliquot 80 ul beads to new eppy tubes. Add 100 ul biotinylated RNA from previous step. Cover with foil, incubate with rotation on nutator for 15 min.

Remove sup – save as FT. Wash beads 3X 0.8 ml High Salt Wash Buffer. Elute with 25 ul Streptavidin Elution Buffer in Thermomixer at 23 deg with shaking for 15 min. Repeat 25 ul elution with fresh buffer and combine elutions = 50 ul final volume. Store at -20 deg (possible to stop at this point).

Day 3 (3-4 h for 24 samples)

Dilute 10% Input samples (total RNA samples after RiboPure Kit used for biotinylation – for each strain replicate take one unlabeled sample and a corresponding labeled sample) into Streptavidin Elution Buffer (~4 ug RNA into 50 ul final volume) and process with Eluates from this point on. Include Inputs from both labeled and unlabeled samples so that the extent of labeled and purified RNA can be determined. For normal experiment it is enough to include input only for wt (e.g. no IAA sample) both labeled and unlabeled by 4-TU.

Add 50 ul nuclease-free H_2O to each 50 ul Eluate or Input volume from above so each sample is 100 ul. Purify and concentrate on Qiagen RNeasy columns using modified protocol (RNeasy columns have a cutoff of 200 nt, smaller RNAs will be excluded using this protocol):

1. To each 100 ul sample, add 350 ul buffer RLT (10 ul 1% β-ME added per 1 ml RLT before use) and 250 ul 100% EtOH, mix well by pipetting (do not centrifuge). Apply samples to columns.
2. Centrifuge columns 15 sec at 12K xg, 4 deg. Discard FT. Repeat until all sample loaded – 700 ul total.
3. To each column, add 500 ul RPE buffer supplemented with 35 ul 1% β-ME ([10mM] final β-ME).
4. Centrifuge samples 15 sec at 12K xg, 4 deg. Discard FT.
5. Move columns to new 2 ml collection tubes. Centrifuge 5 min at max speed, 4 deg.
6. Transfer columns to microfuge tube, add 14 ul RNase-free water. Centrifuge 1 min at >12K xg, 4 deg to elute. Store at -20°C if needed or proceed to cDNA synthesis.

Measure RNA concentration with Qubit HS RNA assay (1 ul for measurment).

cDNA Synthesis

Use Roche's Transcriptor cDNA Synthesis kit

1. Assemble the following reactions in a 0.2 ml PCR tube:

RNA (from step 6)	*2 ul (ok for total RNA input 15 – 50 μg)*
random hexamer	*2 ul*
H_2O	*9 ul*
Total volume	*13 ul*

2. Incubate at 65 deg for 10 min, then hold at 4 deg.

3. Keep samples on the block. To each add 7 ul of the following and mix by pipetting up and down 3 times:

5X reverse transcriptase buffer	*4 ul*
Protector RNase Inhibitor	*0.5 ul*
10 mM dNTPs	*2 ul*
Transcriptor Reverse Transcriptase	*0.5 ul*
Total volume	*7 ul*

4. Incubate as follows: 25 deg for 10 min → 55 deg for 30 min → 85 deg for 5 min.
5. Store cDNA at -20 deg.
6. Analyze cDNAs by RT-qPCR. Depending on the gene analyzed, cDNAs may need to be diluted in water prior to PCR up to 1/100.

Primers for RT-qPCR

Use *S. pombe* tubulin (beta chain, NDA3) gene for normalization:

Tubulin_F 5' – CCGCTGGTGGAAAAGTATGTT – 3'
Tubulin_R 5' – GCCAATTCAGCACCTTCAGT – 3'

When designing primers, choose primers near the 5' end of the gene to ensure analysis of all nascent transcripts.

cDNA dilutions (tested so far) for the amount of cDNA obtained from 2 μl of final RNA prep:

EFB1 – 1x
HSP10 – 2x
HIS4 – 2x
Sp tubulin – 5x

Reagents

4-thiouracil – *light sensitive!*
Sigma item 440736-1G. Stored at room temp. 2M stock (260 mg/ml = 400X) made up in DMSO. Use 25 ul for 10 ml of culture. Store stock at -80 deg although usually this is prepared fresh.

RiboPure Yeast kit
Ambion item AM1926

Biotinylation Buffer Stocks
1 M HEPES, 7.4
0.25 M EDTA

MTSEA biotin-XX
Biotium item #90066
1 mg/ml in DMF (=1.64 mM stock), stored in 0.5 ml aliquots at -20 deg (freezer next to -80 freezers)

Streptavidin beads
Dynabeads MyOne Streptavidin C1
Invitrogen item #65001 (ThermoFisher)

High Salt Wash Buffer

100 mM Tris, 7.4	25 ml 1 M stock
10 mM EDTA	10 ml 0.25 M stock
1 M NaCl	14.61 gm
	250 ml total volume

pH to 7.4, filter sterilize.
Add 0.05% Tween-20 upon use (12.5 ul / 25 ml)

Blocking Buffer
High Salt Wash Buffer + 40 ng/ul glycogen (20 ul 20 ug/ul stock in 10 ml)

Streptavidin Elution Buffer

100 mM DTT	1 ml 1M stock
20 mM HEPES, 7.4	200 ul 1 M stock
1 mM EDTA	40 ul 0.25 M stock
100 mM NaCl	200 ul 5 M stock
0.05% Tween-20	5 ul 100%
	8.56 ml H_2O
	10 ml total volume

RNeasy Columns
Qiagen RNeasy MinElute Cleanup Kit, catalog #74204.