Pombe total RNA extraction and RT-qPCR protocol

Requirements

RNA extraction:
- 0.55 mm zirconia/silica beads
- TRIzol
- Chloroform
- RNAse-free water (e.g. Gibco #10977)
- 75% EtOH (prepared w/ RNAse-free H_2O)
- Ambion TURBO DNA-free Treatment and Removal kit (#1907)

cDNA synthesis:
- Superscript III RT (Invitrogen/Life technologies, #18080-044 or -085)
- 10 mM dNTP mix (RNase-free)
- 50 uM oligo (dT)20

Growth & Harvest

Day 1: Set up pre-cultures. Growth for one day at 30ºC.

Day 2: Set up main cultures: depending on individual strain growth use a dilution of 1:100 – 1:500 of post-log phase pre-culture ($3 < OD_{pre} < 5$; OD_{start} of main culture should be ~ 0.01-0.05) and inoculate into 25-50 ml YES main culture. Grow 14-16 hrs at 30ºC.
optional: skip pre-culture and i/o main culture directly from plate
(WT = 0.02-0.03 OD_{start}; mutant = 0.04-0.08 OD_{start}; 12-16 hour growth)

Day 3:
- Harvest cultures between OD = 0.4 - 0.8.
- Transfer into 50 mL conical tubes, spin at 3000 rpm for 5 min.
- Wash 1x cell pellet with ice-cold H_2O and transfer to 1.5ml **screw cap** tubes.
- Freeze pellet in liquid N_2 and store at -80ºC.

Day 4 (or directly after harvest/flash freezing): estimated time 6 hr (per ~12 samples)

RNA extraction – important: use only filter tips!
- Thaw cell pellets on ice (almost completely) and resuspend in 1 mL TRIzol
- Add 2 scoops (i.e. a small PCR tube ~ 250 µl vol equivalent) of zirconia beads
- Break up cells in *Precyllis 24* (Peqlab) for 3 x 30 s (program 1: 6800) with 5 min rest on ice. **Important**: make sure that **only screw cap tubes** are used and **tightly sealed**!
- Spin the tubes at 12,000 x g at 4ºC for 10 min
- Transfer the cleared lysate to 1.5 mL Eppendorf tube and add 200 µl chloroform (note: chloroform may be added to empty tubes before)
  important: mix thoroughly (vortex) immediately upon addition of chloroform! (delays will strongly affect RNA yield!)
- Vortex for 15 s, let sit at RT for 10 min, and spin at 12,000 x g at 4ºC for 10 min
- [**optional**: Extract the aqueous phase and add 500 µl chloroform. Briefly vortex and spin at 12,000 x g at 4º for 10 min; note: this step is not required but recommended]
- Extract the aqueous phase and add 500 µl isopropanol. Briefly vortex and allow it to sit at 4ºC for 15 min. Spin at 12,000 x g at 4º for 10 min
  (note: isopropanol may be added to empty tubes before)

- Remove supernatant (SN) and wash pellets 2x w/ 1 mL 75% ethanol (RNase-free), vortex to mix and spin at 9600 rpm for 5 min.
- Remove SN and dry the pellets in speed vac **briefly** and without heat for ~ 5-10 min.
  (it's important to prevent over-drying of the RNA pellet!)
- Resuspend pellets in 60 µl RNase-free H_2O and incubate at 55ºC over 30-45 min with occasionally flicking or pipetting (make sure that the RNA is totally dissolved).
  optional: RNA can be stored after this step at -80ºC (label "**RNA**" + smpl-ID + date)

DNase treatment – important: use only filter tips!

- Determine the RNA concentration and yield using the Nano-drop
  (check also 260:230 ratio — values < 1.6 indicate protein/phenol contaminations).
- Dilute 20 µg RNA in 36 µl RNase-free H_2O
  (final RNA concentration should be in the range of 0.2-0.5 ug/µl).
- Add 4 µl of TURBO DNA-free 10x buffer and 0.5 µl TURBO DNA-free DNase I (Ambion, M1907) to the sample and incubate at 37ºC for 30 min.
- After 30 min, add again 0.5 µl DNase I to the sample and incubate for 30 min.
- Add 6 µl of TURBO DNase inactivation reagent (important: resuspend thoroughly before adding) and mix well; incubate at RT w/ occasional mixing for 2 min.
- Spin at 10,000x g for 1.5 min and transfer 35 µl supernatant to a fresh tube
- Samples (~10 ug RNA) can be immediately used for the RT reaction w/o any further clean up.
  optional: If samples are stored (-80ºC), label "**RNA DNAse**" + smpl-ID + date

cDNA synthesis (estimated time: set up 1 hr; reaction 1.5 - 2 hours)

Note: when using –RT samples as negative controls, each reaction sample has to be set up 2x (only recommended for a few samples, e.g. WT and clr4 or any other HC-deficient mutant

- Use ~ 5 ug total RNA for RT reaction (i.e. 10 µl of the TURBO DNase treatment rxn)
- Denature RNA:
  add the following components to nuclease-free Eppi tube (or PCR strip)
  - 11 µl RNA sample (5 µg)
  - 1 µl of oligo-$(dT)_{20}$ primers (50 µM)
    or 50-250 ng random primers or 2 pmol of gene specific primers
  - 1 µl of 10 mM dNTP mix
- Incubate at 70ºC for 10 min and then let sit on ice for 10 min
  (or use PCR program with a hold step at 4ºC for up 10 min)
- Add enzyme mix (total 7 µl each per sample); all components are provided w/ the superscript III kit
  - 4 µl 5x First-Strand Buffer
  - 1 µl 0.1 M DTT
  - 0.25 µl SuperScript III (can be varied in a range of 0.1-0.5 µl)
    or equivalent amount of H_2O for –RT control
  - 1.75 µl RNAse-free H2O to 7 µl
- Incubate a t 50ºC (55ºC for gene-specific primers) for 30-60 min
- Heat-inactivate at 70ºC for 15 min
- **optional:** Add 0.5 µl per sample RNaseH for degrading any RNA:DNA hybrids, incubate at 37ºC for 30 min
- store samples at -20ºC (optionally transfer into 1.5 mL tubes and label "**cDNA**" + smpl-ID + date) and/or continue immediately

quantitative PCR (qPCR, real-time PCR)

for heterochromatic single-copy genes (e.g. ura4 reporter) use 1:25 dilutions
for heterochromatic repeats (e.g. dg or dh repeats) use 1: 50 dilutions
for euchromatic genes (e.g. act1) use 1:1000 or 1:2000 dilutions

in general: for –RT control samples use 1:25 dilutions (irrespective of transcripts)

- reaction mixture:
  5 µl of cDNA + 2.5 µl of FOR/REV primer mix (1.5 µM) + 7.5 µl 2x SYBR mix (PQ),
- or for INTEGRA 3.35 µl of cDNA + 1.65 µl of FOR/REV primer mix (1.5 µM) + 5 µl 2x SYBR
- Use 96-well MicroAmp® Optical 96-Well Reaction Plate or 384-well MicroAmp® and qPCR Seal (e.g. Peqlab)
- qPCR program stored on Thermo Fisher Cloud
- program: 95ºC: 2 min,
  [92ºC: 6 s, 60ºC: 20 s, 72ºC: 20 s, read] X40 cycles
  record melting curve 95ºC: 15 s, 60ºC: 1 min, 95ºC: 15 s; cool down to 10º.

Appendix:

Information on Trizol

TRIzol is acidified phenol with guanidine isothiocyanate. Low pH phenol keeps DNA in the organic phase while RNA is extracted from the aqueous phase. Guanidine isothiocyanate is a chaotropic agent used to rapidly eliminate enzyme activity.
Components:
Phenol in saturated buffer (38 %) -- 380 ml/liter
Guanidine thiocyanate (0.8 M) -- 118.16 g
Ammonium thiocyanate (0.4 M) -- 76.12 g
Sodium acetate, pH5 (0.1 M) -- 33.4 ml of 3M stock
Glycerol -- 50 ml
H_2O to 1.0 liter

Information on Ambion TURBO DNase I treatment and removal kit (M1907)

Ambion uses a recombinant, engineered DNase I having a higher activity than the WT enzyme. In addition, it can be efficiently removed from the reaction sample by adding the removal resin reagent, followed by a quick spin and the transfer of the RNA sample to a fresh tube. By this, any further purification that might be either expensive or tedious (e.g. columns, phenol-chloroform extraction) becomes unnecessary. In addition, divalent cations from the reaction buffer will also be bound and removed by the removal reagent. The ideal concentration of nucleic acids for the DNase I digestion is 0.2 ug/µl, however, concentrations up to 0.5 ug/µl usually give good results, too. Optionally, the amount of enzyme can be increased if lower concentrations are not suitable (e.g. for the subsequent RT reaction). However, the volume of the removal reagent should then be equally increased (> 5 µl removal reagent per µl DNase I).

Primers used for qPCR (FOR and REV mixes, each 1.5 uM):

locus	oligo ID	oligo name	FOR oligo	REV oligo	reference
act1+	Sg1028/29	act1_mid(4) (act1-4)	GATTCTCATGGAGCGTGGTT	CGCTCGTTTCCGATAGTGAT	Braun 2011
ura4+	Sg0959/60	ura4_qPCR	CAGCAATATCGTACTCCTGAA	ATGCTGAGAAAGTCTTTGCTG	Braun 2011
cen-dh	Sg1022/23	cen-dh	TGAATCGTGTCACTCAACCC	TGAATCGTGTCACTCAACCC	Buehler et al., 2007
cen-dg	Sg1020/21	cen-dg (dg-1)	TGCTCTGACTTGGCTTGTCTT	CCCTAACTTGGAAAGGCACA	Braun 2011
mat3M	Sg1573/74	mat3-Mc-3'	CGAGACCCCCTAATGCTTTT	CCAGGGTACATTTTCTGATGTTG	-
tlh1+/ thl2+	Sg1024/25	tlh1- mb274/276	ATGGTCGTCGCTTCAGAAATTGC	CTCCTTGGAAGAATTGCAAGCCTC	Buehler et al., 2007

Quick Summary: Total RNA extraction and RT-qPCR protocol RNA_1v5

Harvest

- Harvest at OD = 0.4 - 0.8.
- Wash 1x w/ ice-cold H_2O + transfer to **screw cap** tubes + flash-freeze -> -80ºC

RNA extraction

- Resuspend in 1 mL TRIzol + zirconia beads
- Lyse (*Precyllis,* program 1: 6800) 3x 30 s with 5 min rest on ice.
- Spin at max speed at 4ºC for minimum 5 min – max 10 min
- Transfer + 200 µl chloroform + vortex 15 s, incubate 10 min at RT
- Spin at max speed at 4ºC for minimum 5 min – max 10 min
- Transfer + 500 µl chloroform + briefly vortex
- Spin at max speed at 4ºC for minimum 5 min – max 10 min
- Transfer + 500 isopropanol + briefly vortex, incubate 15 min on ice
- Spin at max speed at 4ºC for 10 min
- Remove SN + wash pellets 2x w/ 1 mL 75% ethanol + briefly
- Spin at max speed at 4ºC for 5 min
- Remove SN + dry pellets in speed vac **briefly** without heat for ~ 5-10 min
- Resuspend in 60 µl RNase-free H_2O + incubate at 55ºC over 30-45 min
- Label "**RNA**" + smpl-ID + date (store at -80ºC)

DNase treatment (TURBO DNA-free, Ambion)

- Dilute 20 ug RNA in 36 µl RNase-free H2O
- Add 4 µl of 10x buffer + 0.5 µl DNase I + incubate at 37ºC for 30 min.
- Add again 0.5 µl DNase I + incubate for 30 min.
- Add 6 µl inactivation reagent + incubate at RT w/ occasional mixing for 2 min.
- Spin at 10,000x g for 1.5 min and transfer 35 µl supernatant to a fresh tube
- Label "**RNA DNAse**" + smpl-ID + date (store at -80ºC)

cDNA synthesis (Superscript III, Invitrogen)

- Denature RNA: **11 µl** RNA sample (5 ug)
  1 µl of **oligo-(dT)$_{20}$** primers (50 uM)
  1 µl of 10 mM dNTP mix
- Incubate at 70ºC for 10 min -> 4º (ice) for 10 min (PCR machine)
- Add enzyme mix (total 7 µl each per sample)
  4 µl 5x First-Strand Buffer
  1 µl 0.1 M DTT
  0.25 µl SuperScript III (H_2O for negative control)
  1.75 µl RNAse-free H2O to 7 µl
- Incubate at 50º for 60 min
- Heat-inactivate at 70ºC for 15 min
- Label "**cDNA**" + smpl-ID + date (store at -20ºC)

quantitative PCR (qPCR, real-time PCR)

for heterochromatic single-copy genes and repeats: 1:25 – 1:50 dilutions
for euchromatic genes (e.g. act1) use 1:100 dilutions

- reaction mix: 5µl cDNA+2.5µl of primer mix (1.5 uM)+7.5µl 2x SYBRmix (PQ)
- or for INTEGRA 3.35µl of cDNA+1.65µl of primer mix (1.5 µM) + 5 µl 2x SYBR
- program: 95ºC: 2 min, [92ºC: 6 s, 60ºC: 20 s, 72ºC: 20s, read] X40 cycles
  record melting curve 95ºC: 15 s, 60ºC: 1 min, 95ºC: 15 s; cool down to 10º.