

Preparing Yeast RNA Hot Acid Phenol Extraction

1. Grow yeast cells in desired medium to mid-exponential phase ($OD_{600} = 0.5-0.7$).
It is not advisable to prepare RNA from cells that have reached a density higher than $OD_{600} 1.0$ because as the stationary phase is approached, the results are less consistent and RNA yields will vary.
2. Centrifuge cells, discard supernatant, resuspend pellet in 1 ml ice-cold water. Transfer to a clean 1.5-ml microcentrifuge tube. Microcentrifuge and remove supernatant.
3. Freeze pellet by placing tube in dry ice or liquid nitrogen. Cell pellets can be stored for months at -70°C . Thaw on ice just before continuing the procedure.
4. Resuspend cell pellet in 400 μl TES solution. Add 400 μl acid phenol and vortex vigorously 10 sec. Incubate 30 to 60 min at 65°C with occasional, brief vortexing.
It is crucial to incubate for 30 min (with occasional vortexing) to obtain quantitative recovery of both large and small RNA species.
5. Place on ice 5 min. Microcentrifuge 5 min at top speed, 4°C .
6. Transfer aqueous (top) phase to a clean 1.5-ml microcentrifuge tube, add 400 μl acid phenol, and vortex vigorously. Repeat step 5.
7. Transfer aqueous phase to a clean 1.5-ml microcentrifuge tube and add 400 μl chloroform. Vortex vigorously and microcentrifuge 5 min at top speed, 4°C .
8. Transfer aqueous phase to a new tube, add 40 μl of 3 M sodium acetate, pH 5.3, and 1 ml of ice-cold 100% ethanol and precipitate for 30-60 min. (Precipitation for longer than 60 min is detrimental to RT-PCR) Microcentrifuge 5 min at top speed, 4°C . Wash RNA pellet by vortexing briefly in ice-cold 70% ethanol. Microcentrifuge as before to pellet RNA.
9. Resuspend pellet in 50 μl GIBCO RNase free H_2O . Determine the concentration spectrophotometrically by measuring the A_{260} and A_{280} . Store at -70°C , or at -20°C if it is to be used within 1 year.
Make sure that the RNA is well dissolved; if necessary, heat the resuspended pellet at 65°C for 10 to 20 min and/or dilute further with more water. Expect $\sim 0.5-2$ $\mu\text{g/ml}$ yield

Materials

- At least 3ml of culture 0.5-0.7 OD_{600}
- TES solution
 - 10 mM Tris HCl, pH 7.5
 - 10 mM EDTA
 - 0.5% SDS
- Acid phenol (citric acid buffered pH 5.3)
- Chloroform
- 3 M sodium acetate, pH 5.3
- 100% and 70% ethanol, ice-cold

DNase treatment of isolated RNA

1. Digest 5 ug of RNA with 5 ul of RQ1 DNase and 5 ul of buffer in 50 ul total reaction volume for 45 min at 37°C. (or scale to 10 ul. Typically, I store the DNase treated RNA for future experiments)
2. After 45 min add 5 ul of EDTA stop solution and bring to 65°C for 15 minutes.
3. Store at -20°C forever

Making cDNA from RNA

RT-PCR with Multiscribe (Life Technologies)

Kit contains

MultiScribe Reverse Transcriptase 5000 U, 50 U/ μ L
RNase Inhibitor 4000 U, 20 U/ μ L of RNase Inhibitor
dNTP Mixture 2.5 mM of each dATP, dCTP, dGTP, dTTP
Oligo d(T)16 100 μ L 50 μ M
Random Hexamers 100 μ L 50 μ M
10X RT Buffer 1.5 mL
MgCl₂ solution 1.5 mL 25 mM MgCl

1. Set up RT-PCR for each sample. Include a no RT control.
2. For 20 ul reaction:

2 ul of 10x buffer
0.8 ul of dNTP mixture
2 ul of Oligo d(T) or Random Hexamers
1 ul of RNase inhibitor (not necessary)
1 ul of Multiscribe
5 ul of DNase treated RNA
8.2 ul of GIBCO RNase free H₂O
3. 25°C for 10 min
37°C for 60 min
95°C for 5 min
4. Use fresh for best results or store at -20°C

Quantitative-PCR
2X SYBR Green PCR Master Mix (Biorad)

- For designing primers for qPCR aim for 80-100 nt amplicons with a Tm of 60°C. Primer3 is a useful tool for designing primers. <http://bioinfo.ut.ee/primer3/> For optimization of primers see SYBR Green PCR Master Mix and SYBR Green RT-PCR Reagents Kit User Guide. The goal is to validate specific amplification of 1 product with a melt curve or gel electrophoresis, and use the lowest primer concentrations that provide a qPCR efficiency ~100%. Use <http://www.thermoscientificbio.com/webtools/qpcr/efficiency/> to estimate efficiency. Perform a standard curve for each primer set by serially diluting your sample 1:5 6x. Use this to determine qPCR efficiency of each primer set for the calculations below. Important!! qPCR efficiencies must be the same when using ddCT method.
- For 12.5 ul reaction:
 - 1.25 ul of RT-PCR reaction ([final] 125ng)
 - 6.25 ul of 2X SYBR green
 - 4.25 ul of sterile ddH2O
 - 0.75 ul of Primer mix (5uM each [final] 300nM)
- Pipet RT-PCR into each well of 96 well plate in triplicate. The no-RT control determines your amount of gDNA contamination.
- To calculate fold changes in mRNA expression use the ddCt method. This normalizes your target to an internal ‘unchanged’ control such as ACT1 or SCR1 (dCt), and to an untreated or wildtype sample (ddCt). Set the appropriate Ct threshold that captures the linear segment of each of your samples. Using these Ct values, average the triplicate samples and exclude any outliers. PCR is logarithmic, and thus for the dCt subtract your target RNA from control RNA (dCT). Then subtract the dCt of your treatment or mutant samples from the untreated or wildtype samples (ddCt). Assuming your qPCR efficiency is 100%, each CT is a 2-fold change. Therefore fold change = 2^{ddCT} .

Ex.

	YFG#1	YFG#2	YFG#3	SCR1#1	SCR1#2	SCR1#3
WT	20.4	20.5	20.3	16	16	16
MUT	24.2	24.2	24.0	16	16	16
Avg WT	20.4			16		
Avg MUT	24.1			16		
dCT WT	20.4-16	4.4	ddCT	-3.7	Fold change	0.075
dCT MUT	24.1-16	8.1				