Yeast mRNA quantitation using Real Time PCR

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I. Yeast RNA Purification

1. Harvest ~ 10-20 mL cells at OD 0.5-1.0.

   NOTE: This amount of cells should yield ~100 ug RNA but only 15 ug is needed for subsequent steps.
2. Wash cells in cold-RNase free dH_2O and transfer to a 1.5 mL microfuge tube.
3. Pellet cells and remove supernatant.

   NOTE: Cells can be stored at -80°C.
4. Resuspend pellet in 400 uL TES and add 400 uL Acid Phenol:CHCl3.
5. Vortex for 10 sec and incubate for 60 min at 65°C in shaking thermomixer.
6. Centrifuge at max speed for 10 min at 4°C.
7. Transfer upper aqueous layer to new 1.5 mL tube and add another 400 uL Acid Phenol:CHCl3 to extracted aqueous layer.

   NOTE: It is not necessary to remove the entire upper layer.
8. Repeat steps 6 and 7 until aqueous layer is free of debris.

   NOTE: Usually two Acid Phenol extractions is sufficient, but remember that debris will pellet with RNA in downstream steps if not properly removed.
9. Add 200 uL of TE and 400 uL Chloroform to sample.
10. Vortex for 10 sec, spin for 10 min at 4°C, and transfer upper aqueous layer to new 1.5 mL tube.
11. Precipitate RNA with 10% volume of 3M NaOAc and 2-2.5 volumes 100% EtOH.
12. Incubate tubes for ~ 1hr or overnight at -80°C to precipitate RNA.

13. Pellet RNA at max speed for 15 min at 4°C and wash pellet with 500 uL 80% EtOH.
14. Dry pellet at room temperature or in speed vac.
15. Resuspend pellet in 50-100 uL RNase-free dH_2O and store at -80°C.
16. Determine concentration and purity of RNA samples using nanodrop spectrophotometer.

    NOTE: Samples should have following ratios for protein and organic solvent contamination respectively: 260/280 > 1.7 and 260/230 > 2.0.
17. Run ~ 1 ug RNA on a 1% agarose gel to ensure equivalent RNA quantities.

    NOTE: Above gel is only qualitative, need to run a 1% denaturing RNA for quantitative.

TES: 10 mM Tris pH 7.5, 10 mM EDTA, 0.5% SDS

II. Removal of Genomic DNA by Turbo DNase

1. Assemble the following reactions in 1.5 mL tubes for each sample:

x uL	**15 ug RNA**
5.0 uL	**10X Turbo DNase Buffer**
1.0 uL	**Turbo rDNase**
44-x uL	**dH_2O**
50 uL	**Total**

2. Incubate at 37°C for 30 min.
3. Spin down tubes for 1 min at max speed and add 5 uL of resuspended DNase inactivation bead slurry to tubes.

   NOTE: Ensure bead slurry is resuspended before adding to each tube.
4. Vortex tubes vigorous and incubate tubes for at least 2 min at room temperature.
5. Pellet beads at 10,000 rpm for 1 min, transfer supernatant to new 1.5 tube and store at RNA at -80°C.

   NOTE: Beads will inhibit cDNA synthesis so be sure not to carryover during transfer.

III. cDNA synthesis

1. Assemble the following reactions in 0.2 PCR strip tubes:

3.7 uL	**DNase-free RNA (1ug)**
1.0 uL	**0.5 ug/uL OligodT**
1.0 uL	**10 mM dNTPs**
8.3 uL	**RNase-free dH_2O**
14.0 uL	**Total**

NOTE: Make master mix of OligodT, dNTPs and dH_2O.

2. Incubate at 65°C for 5 min and hold at 4°C.
3. To each sample, add 6.0 uL of RT master mix.

4.0 uL	**5X RT**
1.0 uL	**100 mM DTT**
0.5 uL	**RNase-OUT**
0.5 uL	**Superscript III**
6.0 uL	**Total**

NOTE: Assemble a Superscript III minus sample separately as a negative control.

4. Incubate tubes for 50 min at 50°C, 15 min at 70°C, and hold at 4°C.
5. Store tubes at -20°C until further use.

IV. cDNA Template Preparation

1. For each cDNA reaction, make a 1:100 dilution of cDNA into RNase-free dH_2O.

   NOTE: Working cDNA dilution depends on abundance of transcript so it may be necessary to make a dilution series (e.g. 1:10, 1:100, 1:1000, 1:10,000) to determine optimum cDNA input dilution.

2. Use qPCR excel worksheet to determine reaction conditions for standards and unknowns.
3. Standard can be the following:
   i. Yeast genomic DNA purified by Promega Wizard kit diluted 10ng/uL, 1 ng/uL, 0.1 ng/uL, 0.01 ng/uL and 0.001 ng/uL.
   ii. Undiluted Wildtype sample (or WT induced sample) and diluted 1:10, 1:100, 1:1000, 1:10,000).

V. qPCR Reaction Preparation Using 96/384-well Plate Worksheet

1. Enter data “only” into blue colored boxes.
2. Use the plate configuration box to determine sample number, row A1-12 is used for duplicate samples of standards and a no template control (NTC).

   NOTE: Unknowns should either be run as duplicates or triplicates.
3. Enter the number of total samples in blue box B3.
4. Enter the name(s) of primer set in blue boxes E9-E14.
5. Enter the number reactions of corresponding primer set in blue boxes B9-B14.
6. Check the following boxes to ensure calculations have been entered correctly.
   i. Yellow box D15 should equal 1.
   ii. Red boxes C7 and C24 should be the same.
   iii. Orange boxes F25 and H14 should be the same.
7. To assemble reactions, add the indicated uL volumes of Power SYBR master mix, RNase-free dH_2O, 5 uM forward and reverse primer to separate 1.5 mL tubes for each primer set *(e.g. For Primer set 1, use row 17 boxes B thourgh E).*
8. Set out appropriate number of 1.5 tubes for each template DNA (cDNA, standards, and controls).

   NOTE: A 96 well plate can also be used instead of 1.5 tubes.
9. Dispense appropriate amount of master mix assembled in step 7 (Boxes EF3-7) to each tube.
10. Add indicated amount of DNA (Boxes GH3-7) to each tube, finger vortex tube, and briefly spin down for a few seconds.
11. To a new 96 well Optical Reaction Plate, add 15 uL of reaction to appropriate well indicated on the plate configuration box.

    NOTE: For a 384 well plate, 5 uL reaction volumes are used.
12. Seal plate with optical adhesive cover using manufacturers directions.

    NOTE: Assembled qPCR plates for cDNA samples can be assembled up to 24 hours before they are run but ChIP samples should be run within a few hours. Plates should be stored in a dark place at 4°C or room temperature.
13. Briefly spin down plate at 1,000 rpm and now plate is ready to run on the ABI 7900HT.

    NOTE: For new primer sets, add a disassociation step to cycle program to monitor primer dimer formation.

14. At end of run, analyze PCR products by electrophoresis to confirm the absence of non-specific products.

VI. Designing qPCR Primers by ABI Primer Express 3.0

1. Open new file and select: Type = Taqman Quantification, Parameters = Default.
   NOTE: Primer Express 3.0 can be accessed on most PC computers in the shared resources computing lab located in room J2-225 and the Hahn lab PC next to the -80°C freezer in A1-190.
2. Cut and paste desired DNA sequence.
3. Click on Tools and select find primers and probes.
   NOTE: If no primers suitable primers found, tweak parameters such as primer size and Tm. Also view interim results to find out where the problem lies and relax the parameter.
4. Click on Order tab to view primer sequences.

Design Guidelines

- Amplicon length = 50 to 150 bases.
- Primer Length = 20 bases.
- Tm = 58-60°C.
- GC Content = 30-80%.
- No Repeat bases - fewer than four consecutive G's.
- Select primers with the lowest amplicon length and lowest penalty score
- qPCR primers for RNA expression analysis should be designed near the 3' of the coding sequence.
- ChIP primers are sometimes harder to design since there is less sequence to search for candidate primers so it may be necessary to use alternate primer design software.

VII. Designing qPCR Primers by IDT PrimerQuest

1. PrimerQuest can be found at the following web address: http://www.idtdna.com/Scitools/Applications/Primerquest.

NOTE: PrimerQuest is particularly useful for designing primers when Primer Express 3.0 cannot find suitable primers because PrimerQuest parameters can be relaxed much easier.

2. Paste DNA sequence, select Real-Time PCR in parameters box, and click on calculate.

NOTE: If no primers can be found, relax specifications by selecting one or more of the following: Oligo size, GC content and/or TM range.