

## Quantifying mRNA Levels on a Budget

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[http://www.bio.davidson.edu/projects/GCAT/Pubs/QPCR\\_GCAT.pdf](http://www.bio.davidson.edu/projects/GCAT/Pubs/QPCR_GCAT.pdf)

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We chose to adapt a method of quantitative amplification of cDNA that is based on the exponential relationship between the amount of accumulated product and the initial template level. Identical PCR reactions are conducted with primers for a given gene and cDNA template derived from two RNA populations to be compared. Before the inflection point of the curve of product versus cycle number, the ratio of product amounts for the two reactions is proportional to the ratio of initial cDNA templates present. The amount of product present at several points in the exponential phase of the reactions can be measured by densitometry of the intensities of bands after electrophoresis. In this way, the relative amounts of cDNA in two samples can be measured. Preparations of cDNA can also be standardized through the measurement of genes known to be unaffected. A recommended time line for using the gel electrophoresis-based method in a series of four weekly three-hour laboratory class periods is as follows.

### List of Equipment Needed

Shaking incubator for growing yeast

Micropipettors

Microcentrifuge

UV spectrophotometer

Thermal cycler

Agarose or polyacrylamide gel electrophoresis equipment

Digital camera

### List of Materials Needed

Yeast strain – S288C

YPD medium – 10 g BactoYeast, 20 g BactoPeptone, 20 g glucose per liter

RiboPure-Yeast kit – Ambion Inc. cat. no. 1926, \$265 for 50 purifications

RNasin Plus RNase inhibitor – Promega corp.

PCR primers for reference gene *TDHI*

Forward primer 5' ATCGATGTCGCTGTTGACTCCACT 3'

Reverse primer 5' CAATGGAGCCAAACAGTTGGTGGT 3'

DNase I - Ambion Inc. cat. no. 2222, \$125 for 2000 units

Superscript II reverse transcriptase, 5X first-strand buffer, 0.1 M DTT – Invitrogen cat.

no. 18064-022, \$60 for 2000 units

oligo(dT)<sub>15</sub> primer

dNTP mix (10 mM each)

Mg-free 10X buffer (100 mM Tris pH 9.0 at 25C, 500 mM KCl, 1% Triton X-100)

25 mM MgCl<sub>2</sub>

Taq DNA Polymerase

Materials for agarose or polyacrylamide gel electrophoresis

### Day 1

The first day could begin with a discussion of the value of measuring mRNA levels for individual genes, the merits of various methods to do so, and the design of specific experiments to address hypotheses developed by the class. This discussion would probably not require the entire class time.

### Day 2

This period could be devoted to the isolation of RNA. Set up overnight yeast cultures the day before, and then grow treated and untreated cultures for RNA isolation.

Isolate RNA – 1-3 hours, depending on method chosen. Ribo-Pure takes about 2 hours.

**Growth of Yeast and RNA Isolation** – Overnight cultures of *Saccharomyces cerevisiae* strain S288C grown at 30 C in YPD with shaking at 175 rpm were used to seed two 5 ml cultures. One culture remained untreated and the other was treated with 4',6-diamidino-2-phenylindole (DAPI) at a final concentration of 10  $\mu$ M. The cultures were incubated for four hours after which both A<sub>660</sub> readings were 2.0. Total RNA was isolated using the RiboPure-Yeast kit from Ambion, Inc. The procedure uses zirconia beads for cell disruption, phenol and SDS to reduce RNase activity, and filter cartridges to bind and wash precipitated RNA. We chose to collect the RNA with two elutions of 50  $\mu$ l each, and added 1  $\mu$ l of RNasin Plus RNase inhibitor to each sample (Promega Corporation).

### Day 3

During the third class period, students could measure the quantity and quality of the isolated RNA by spectrophotometry and denaturing gel electrophoresis.

Measure RNA quality and quantity with spectrophotometry and electrophoresis – takes 1.5 hours.

Set up and complete reverse transcription reactions – takes 1.5 hours.

**Measuring RNA Quantity and Quality** - Elimination of contaminating yeast genomic DNA in RNA preparations is crucial before cDNA synthesis and PCR. We tested each RNA sample by amplifying with primers for the housekeeping gene *TDHI*. Although the RNA isolation procedure includes a DNase I digestion, about half of our samples showed a product, indicating DNA contamination. We therefore performed a second DNase I treatment with twice the enzyme recommended by the RNA isolation kit and for twice as

long, then performed the PCR check again. Spectrophotometric readings at 260 and 280 nm were then made for the RNA samples. The yield of RNA varied from 20 to 30 ug from each 5 ml yeast culture and the  $A_{260}/A_{280}$  ratios were between 1.8 and 2.1. As a further quality check, 1 ug of each RNA sample was subjected to denaturing agarose gel electrophoresis. The presence of 28S and 18S ribosomal RNA over a background smear of mRNA and the absence of both degraded RNA and high molecular weight DNA indicated good quality RNA preparations.

**Reverse Transcription** - Reverse transcription of total RNA for the preparation of cDNA to be used in PCR was also performed using Superscript II as the reverse transcriptase, but with oligo(dT)<sub>15</sub> primers. A solution of 5 ul RNA (0.2 ug/ul), 1 ul dNTP mix (10 mM each), and 5 ul dH<sub>2</sub>O was heated to 65C for 5 minutes and then chilled on ice. To this mixture was added 4 ul 5X first-strand buffer, 2 ul 0.1 M DTT, and 1 ul RNasin Plus RNase inhibitor. The contents were gently mixed, briefly centrifuged and allowed to incubate for 2 minutes at 42C. Reverse transcriptase was added (1 ul) and the reaction was incubated for 50 minutes at 42C. The reaction was stopped by heating at 70C for 15 minutes.

#### Day 4

The final lab period could be spent performing DNA amplifications with the cDNA, conducting gel electrophoresis, and analyzing the data with regard to the original hypotheses.

Run PCR reactions and take out aliquots – takes 1 hour.

Run gels with aliquots, stain, take picture – takes 1.5 hours.

**PCR** - PCR was performed in 40 ul reactions containing 84 pmol of each of two primers, 4 ul 10 mM dNTP mix, 4 ul Mg-free 10X buffer (100 mM Tris pH 9.0 at 25C, 500 mM KCl, 1% Triton X-100), 4 ul 25 mM MgCl<sub>2</sub>, and 2 units Taq DNA Polymerase. The PCR reagents were assembled as a master mix that was split into reactions that received 1 ul of either treated or untreated cDNA as template. PCR was performed with cycles of 94°C for 15 seconds, 58C to 62C for 15 seconds, and 74C for 30 seconds. Aliquots of 7 ul of the reactions were removed after a determined number of cycles and two-cycle intervals thereafter until four samples were taken from each 40 ul reaction. We designed primers for the amplification of cDNA from each of three yeast genes. The forward primer 5' ATGTCTGAATCAGTGGCCATTATAGGTGC 3' and reverse primer 5' ATCAATGCCACGAGCAGAAATAGCC 3' yielded a 180 bp product from *BNA4* using an annealing temperature of 62 C. For *OLH1*, a 124 bp product was produced by the forward primer 5' GAGCAGGTATGGTATTGCTATCGT 3' and the reverse primer 5' CCTGTAGCTTCTGATAAGGCGAAACC 3, with annealing at 58C. Forward primer 5' ATCGATGTCGCTGTTGACTCCACT 3' and reverse primer 5' CAATGGAGCCAAACAGTTGGTGGT 3' gave a 206 bp product for *TDH1* at an annealing temperature of 60 C. Although the *TDH1* reverse primer is also predicted to bind to the paralogs *TDH2* and *TDH3*, the forward primer is specific for *TDH1*.

**Gel Electrophoresis and Data Acquisition** – Aliquots taken from reactions at increasing cycles were electrophoresed on 7% polyacrylamide gels at 20 volts/cm for 30 minutes and stained with 1 ug/ml ethidium bromide for 15 minutes. Gel electrophoresis images were captured with a Kodak DC120 digital camera and the densitometry feature of Kodak Digital Science 1D version 2.0.3 was used to measure band intensities. The relative intensities of specific bands were calculated by reference to the 100 bp band from the molecular weight marker.

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