

Figure 1: *Saccharomyces cerevisiae* (Wikipedia, 2005).

RNA Analysis Prior to Use in Microarrays

Zakiat N. Darajat (Dr. Emily Schmitt, Advisor)

Math, Science, and Technology Department

Farquhar College of Arts and Sciences



INTRODUCTION

Total RNA must be analyzed prior to utilization in a microarray or other gene expression study. The early detection of mRNA (or its absence) saves time, energy and resources, allowing better error detection in an experiment (Brewster et al., 2004). *Saccharomyces cerevisiae* (Fig. 1) was the organism chosen because it is a simple eukaryote, grows rapidly, is inexpensive to grow and its genome has been well studied (Sherman, 1998). Protocols establishing mRNA presence from total RNA, including its quality and quantity, were examined to determine the most precise and least labor intensive procedure. Results of this study allowed the determination of which samples to use in a microarray-based study of gene expression of yeast when environments were not changed (Farrell, 2005).

METHODS

RNA Isolation - *S. cerevisiae* (S288C) was grown according to protocol provided by the Genome Consortium for Active Teaching (GCAT, 2005). A 1ml aliquot of yeast culture (a loopful of yeast placed in a 40 mL suspension and allowed to grow for 20 hours) was placed in 40 mL of liquid media and separated into six volumes: 11mL, 11mL, 5mL, 2mL 1.5 mL, 1.5 mL, labeled Samples 1 – 6, respectively. The RiboPure Yeast Kit (Ambion, Austin, TX, USA) was used to isolate RNA (Fig. 2).

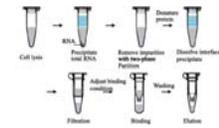


Figure 2: RNA extraction (UBI, 2005).

Spectrophotometry - Absorbances at 260nm and 280nm were taken shortly after extraction and four months later, using crystal cuvettes. A Hewlett Packard UV Visible Spectrophotometer was used for the first set of readings; while a SmartSpec, small volume (100 μ L), spectrophotometer was used for the second set. RNA quantity (μ g/mL) was calculated with the formula: $A_{260} \times \text{dilution factor} \times (40 \mu\text{g/mL}/1000)$. Samples with absorbances of 1.8 to 2.1 indicate the presence of quality RNA (RiboPure, 2004).

mRNA Electrophoresis - Electrophoresis was run for 1 hour at 130 mA using the HS Reliant Gel System (Cambrex BioScience, Baltimore, MD, USA), loaded with 5 μ L RNA and 1 μ L of loading dye. DNA ladders (1 kB and 50 bp) were used to determine molecular weight.

cDNA Electrophoresis - Protocol was followed as indicated by the Protoscript First Strand cDNA Synthesis Kit (New England Biolabs, Beverly MA, USA). Oligo-dT primers, reverse transcriptase, and dNTPs were used to target PolyA tails of mRNA. TDH1 (a "housekeeping gene," which is always expressed in yeast) was amplified by PCR using the following: 25 μ L deionized water, 1 μ L 25 mM MgCl₂, 1 μ L of forward and reverse primer each at 5mM concentration, 13 μ L Quiagen Master Mix (Quiagen, Valencia, CA, USA) and 5 μ L cDNA sample; with the thermocycler set to 20 cycles of 94°C, 58°C and 74°C for 15, 15 and 30 seconds, respectively. 1 μ L loading dye, 1 μ L (1:1000) Sybr dye and 5 μ L of amplified TDH1 gene was loaded into a 1.0% TBE (native) agarose gel for approximately 1 hour at 100V and 130 mA.

Denaturing Buffer - 2 μ L of extracted RNA, 1 μ L buffer A, 3 μ L buffer B, 1 μ L Sybr Gold (1:1000) and 1 μ L loading dye was run on a 1.5% TBE agarose gel, run at 100 volts for one hour and 30 minutes (Gregg et al., 2004).

RESULTS

Spectrophotometric Analysis - The first set of absorbance demonstrated quality RNA in Samples 2 and 3, with corresponding quantities of 2.3 μ g/ μ L and 1.7 μ g/ μ L (Tables 1 and 2). When RNA was quantified four months later, Sample 2 still showed high quality and quantity.

Table 1: Observed Absorbance of total RNA samples after extraction, on September 20, 2004 using SP-110 Visible Spectrophotometer. Samples were diluted in deionized water.

RNA Sample	Yeast culture originally taken (mL)	Dilution Factor (2xL deionized water)	Absorbance (260nm)	Absorbance (280nm)	RNA Quantity (μ g/ μ L)**
1	11	100	0.93	0.82	1.3
2	11	1000	2.32	2.24	2.3
3	11	1000	1.74	1.64	1.7
4	2	1000	1.74	1.64	1.7
5	1.5	1000	2.64	2.54	2.3
6	1.5	1000	2.64	2.54	2.3

Table 2: Absorbance on remains of total RNA samples, performed on January 20, 2005 using the same SP-110 Visible Spectrophotometer. Samples were diluted with TBE buffer.

RNA Sample	Amount of yeast culture originally taken (mL)	Dilution Factor (2xL TBE buffer)	A-260nm	A-280nm	RNA Quantity (μ g/ μ L)**
1	11	100	0.666	0.33	1.90
2	11	1000	1.54	0.74	2.02
3	11	1000	1.54	0.74	2.02
4	2	1000	0.369	0.178	0.07
5	1.5	1000	0.06	0.03	0.04
6	1.5	1000	0.104	0.066	0.07

mRNA Electrophoresis - Smears were visualized indicating the presence of molecules smaller than 100bp (Fig. 3) When mRNA was treated with Superload Denaturing Buffer, a light smear was present in between bands at approximately 2500bp and 1500bp, with the 2500bp band approximately one and a half times brighter than the 1500 bp band; samples 4, 5 and 6 had faint bands (of the same molecular weight) that were not visualized by the camera (Fig. 4).

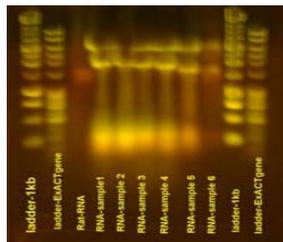


Figure 3: mRNA electrophoresed in a native agarose gel.

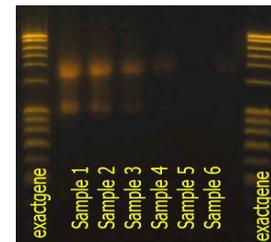


Figure 4: Denatured mRNA electrophoresed on a native agarose gel.

TDH 1 Visualization - 200 bp bands were visualized for Samples 2 and 5, which were chosen for hybridization onto a microarray (Fig. 5).

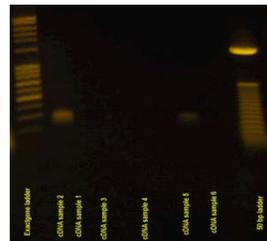


Figure 5: TDH1 is visualized in cDNA samples 2 and 5.

Microarray Hybridization - Visualization of yellow spots indicates successful hybridization of Sample 2 (Fig. 6).

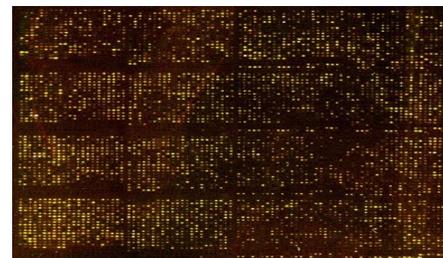


Figure 6: Sample 2 hybridized onto a microarray.

DISCUSSION

mRNA presence is necessary for cDNA synthesis (Fig. 7) prior to microarray production. First, absorbances were used for baseline quantity and quality analysis. Then, other experiments were performed to confirm physical mRNA presence in the samples by electrophoresing RNA, and electrophoresing cDNA after TDH1 amplification.

Extracted RNA electrophoresis displayed 1800bp and 3000bp bands; however, smears of a low molecular weight (<100bp) were also visualized (Fig. 3). These were likely degraded DNA, which is not useful for microarray labeling (Brewster et al, 2004). Other protocols recommend the use of a denaturing gel to eliminate the visualization of secondary RNA structure (RiboPure Yeast Kit, 2004) but denaturing gels require carcinogenic chemicals and are complicated to perform.

To eliminate doubt, the researchers synthesized cDNA from the extracted RNA on the premise that cDNA can only be produced when mRNA is present.

Amplification of the TDH1 gene (glyceraldehyde 3 phosphate dehydrogenase), needed for metabolism, followed and the product was electrophoresed. 200 bp bands were visualized, indicating mRNA presence in Samples 2 and 5 (Fig. 5). These samples were chosen for microarray hybridization, successfully yielding a microarray from Sample 2 (Fig. 6).

Four months after the production of a microarray, another protocol was tested, attempting to provide a quicker, less labor intensive method of assessing RNA. Instead of producing cDNA and amplifying TDH1, the extracted RNA was exposed to denaturing buffers and then electrophoresed (Fig. 4). 1500bp and 3000bp bands were seen, equivalent to the 18s and 28s ribosomal RNA subunits, with a light smear in between the bands, indicative of mRNA.

Besides allowing experimental progression, the Superload Denaturing Gel (ViaGen) protocol requires a small volume of mRNA (2 μ L). The visualization of bands at a slightly lower molecular size, at 3000bp and 1500bp (Fig. 4), and the lower absorbances (Table 2) are most likely due to RNA degradation that may have taken place since extraction. It is believed that if this protocol was used directly after RNA isolation, adequate evidence supporting mRNA presence would have been provided, allowing faster progression of the experiment, decreasing the amount of energy and resources required.

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