Laboratory 7
Analysis of the RNA Content of Yeast Cells, Part B

I. Introduction

In the first part of this project, you examined the yeast *Saccharomyces cerevisiae* microscopically, prepared a perchloric acid (PCA) extract of an overnight culture, made a highly-purified mini-prep of the yeast RNA, and determined the total cell count, viable cell count, and turbidity of the yeast culture.

In the second part of this project, you will

- finish up viable cell counts from last week
- separate the RNA molecules in the mini-prep by horizontal gel electrophoresis
- determine the concentration of RNA in your PCA extract of yeast cells
- complete a series of calculations to determine the RNA content per cell.

Because it takes some time for the gel electrophoresis to run, you should start this part of the experiment early in the lab period.

II. Pre-Lab Preparation

Read the Introduction, Background Information, and Experimental Procedures for this experiment. Also, review all of the background information and calculations described in **Laboratory 6 (Analysis of the RNA Content of Yeast Cells, Part A).** Also, review the material in **Laboratory 3 (Determination of Protein Concentrations by Spectrophotometry**). After preparing for the lab, you should be able to answer the following questions.

A. What cellular molecules should be present in the PCA extract made last week?

B. Which cellular molecules should not be present in this extract?

C. What are the major differences between RNA and DNA?

D. What chemicals are present in the orcinol reagent?

E. How does RNA react with these chemicals to produce a colored product?

F. What is the purpose of making a RNA standard curve?

G. What wavelength will be used to read the RNA reactions?

H. How will the RNA content of the yeast cells be calculated?

I. What is the overall net charge on a RNA molecule?

J. Which groups contribute to this charge?

K. What determines the rate of movement of molecules like RNA in an electric field?

L. How will the RNA molecules in the gel be visualized after electrophoresis?

III. Background Information

A. Biochemical Assay for RNA

The total amount of RNA in a cellular extract can be determined with the orcinol reaction. In the presence of strong acids such as HCl, purine bases such as adenine and guanine are removed from ribose by hydrolysis and the RNA chain is split to release pyrimidine nucleotides and free phosphate (Figure 7.1).

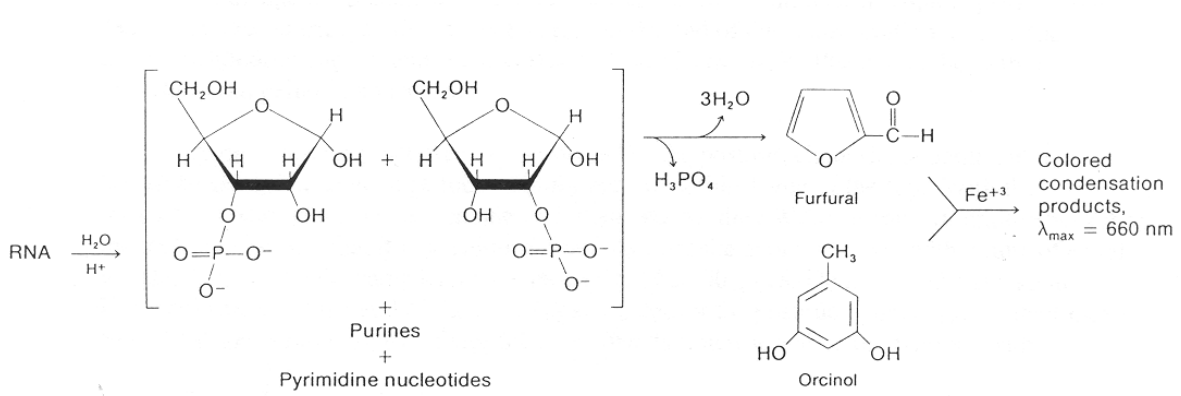


Figure 7.1. The free ribose undergoes dehydration to form furfural, which then reacts with the aromatic compound orcinol in the presence of iron to form a blue-green product. The amount of this product can be measured spectrophotometrically at 660 nm.

As with other spectrophotometric assays, the use of this reagent requires the construction of a standard curve that relates the absorbance to the amount of the compound of interest. In this experiment, yeast RNA will be used as the standard. While DNA is also hydrolyzed under these conditions, deoxyribose only gives 10% of the color yielded with ribose. Thus, the presence of DNA in a sample is not a major problem in the analysis in most cases. This is particularly true for yeast and bacterial cells, where RNA concentrations in the cytoplasm are very high compared to the amount of DNA.

B. Agarose Gel electrophoresis

Electrophoresis is a powerful preparative and analytical technique that is widely used to separate molecules such as proteins and nucleic acids. Agarose gel electrophoresis is a convenient way to separate relatively large DNA or RNA molecules that differ in size or shape. A gel of agarose (a purified component of agar) is usually cast in a flat UV-transparent plastic tray with a Teflon comb positioned near one end. Once the gel has hardened, the comb is removed to create a series of wells into which small samples of DNA or RNA can be loaded. The gel in its tray then is placed in an electrophoresis chamber between two buffer chambers, and submerged beneath about 1-2 mm of buffer. One of the buffer chambers contains a positive electrode and the other a negative electrode.

The DNA or RNA samples are then added to the wells through the layer of buffer with a micropipetter. To keep the molecules in the wells and to monitor the movement of molecules through the gel, the samples are usually made up in a sample buffer that contains glycerol, bromophenol blue, and xylene cyanole. The latter two dyes are colored blue and so they are easy to follow as electrophoresis occurs. After the samples have been added to the wells with a micropipetter, the electrophoresis chamber is connected to a power supply and a voltage applied to the system (Figure 7.2). This causes the molecules to move out of the wells and through the agarose gel towards the positive electrode. Once electrophoresis has been completed, the gel is stained with the dye ethidium bromide, which exhibits a bright orange fluorescence when it is bound to DNA or RNA and exposed to ultraviolet light (Figure 7.2). (Some scientists prefer to add the ethidium bromide to the agarose solution before the gel is cast.) The gel is then examined with the UV transilluminator and a permanent record of the gel made by creating a digitized image of the gel.

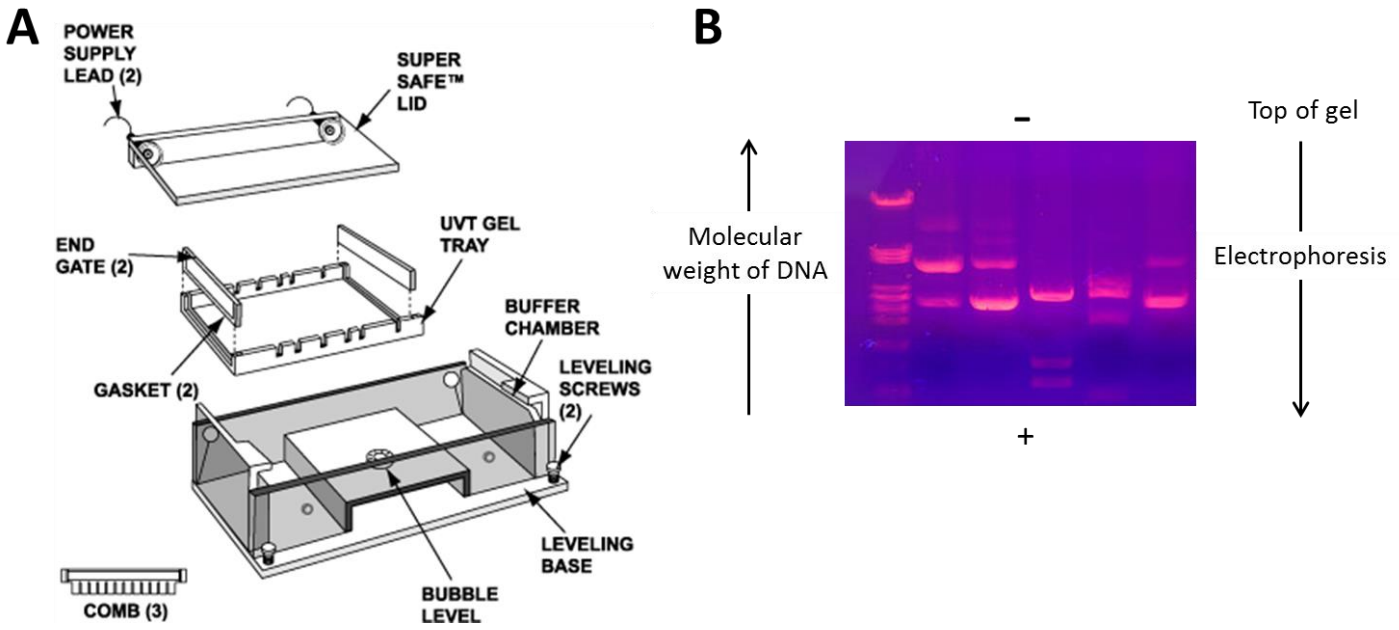


Figure 7.2 (A) Diagram of a typical Gel Box (B) Gel showing separation of DNA fragments stained with ethidium bromide

The rate of movement of DNA or RNA molecules through an agarose gel is determined by their size and shape. Smaller molecules move through the gel faster that large molecules. Plasmid DNAs that are supercoiled or twisted are more compact than non-supercoiled circular DNAs or linear pieces of DNAs and so often move through the gel faster. While most DNA molecules are analyzed under nondenaturing conditions, RNAs often are separated in the presence of formaldehyde and formamide. These molecules denature the RNA so that secondary structures resulting from intrachain hydrogen bonds are eliminated. This forces the molecules to move through the gels as more or less linear chains in order of decreasing size. Size markers are usually included during agarose gel electrophoresis so the sizes of particular DNA or RNA fragments can be estimated. In this experiment, a RNA ladder from New England BioLabs will be used, which contains RNAs of 500, 1,000, 2,000, 3,000, 5,000, 7,000, and 9,000 nucleotides in length. The RNA ladder and the RNA samples will be treated with a denaturing sample buffer, but then run under nondenaturing conditions.

C. Mobility of RNA or DNA Fragments in Agarose Gels

The mobility of molecules during agarose gel electrophoresis is usually not a direct function of the size of the molecules. Rather it is a function of the **log of the size** (Figure 7.3).

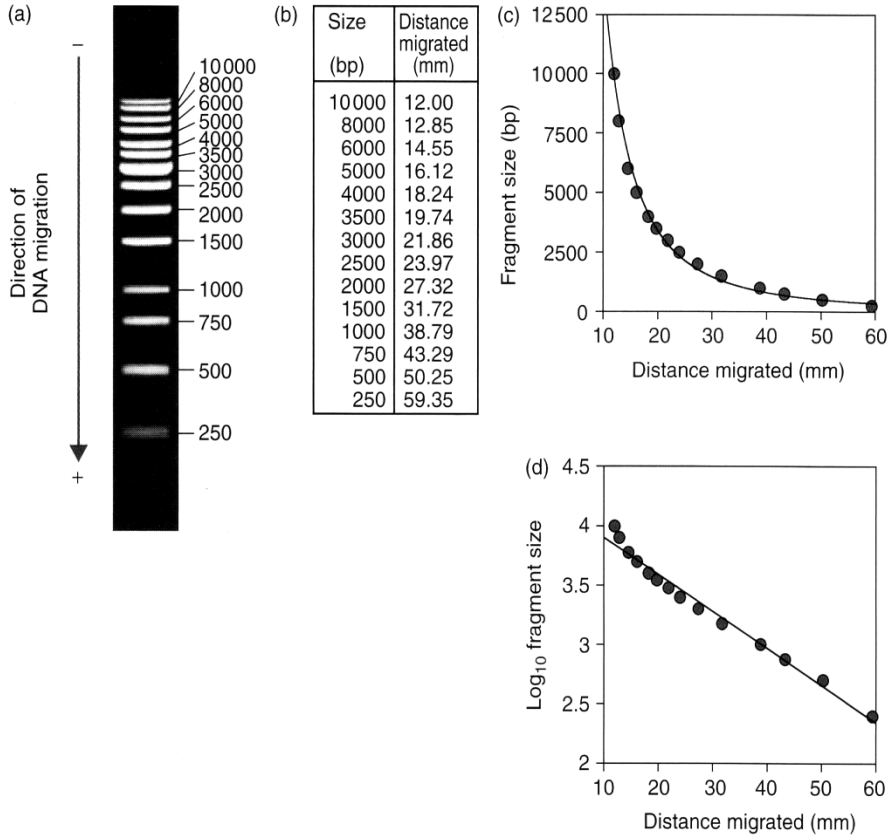


Figure 7.3. The migration of DNA fragments through an agarose gel. (a) An agarose gel showing the separation of DNA fragments of known size. (b) The size of a DNA fragment and the distance migrated through the gel. (c) A plot of fragment size against distance migrated taken from the data shown in (b). This indicates that the relationship between fragment size and distance migrated is not linear. (d) A plot of the log of fragment size against distance migrated. This indicates that there is a direct, inverse, relationship between distance migrated and the log of the size of a DNA fragment.

When fragment size in nucleotides or base pairs is plotted on the Y axis and the distance migrated is plotted on the X axis, a curved line is formed. However, when the log of the fragment size is plotted on the Y axis and the distance migrated is plotted on the X axis, a straight line is formed. Because it is much easier to interpolate between known points on the straight line, the latter method is clearly preferred.

There are two ways to generate the log size vs. distance plot. One is to actually calculate the log of the fragment size using a calculator and then to plot the data on graph paper. You can also do this using Excel. The other is to use **semi-log graph paper**. This type of paper has a linear scale on the X axis and a log scale on the Y axis. Depending on the type of graph paper, there may be 1 to 5 log cycles along the Y axis. When you plot numbers on the paper, it is as if you were plotting the log of a certain number because the intervals get smaller as you go along each cycle.

For example, suppose you collect the following set of data:

Distance (X variable):	12	12.85	14.55	16.12	18.24	19.74	21.86	23.97	27.32	31.72	38.79	43.29	50.25	59.35
Fragment Size (Y variable):	10,000	8,000	6,000	5,000	4,000	3,500	3,000	2,500	2,000	1,500	1,000	750	500	250

Since the Y variable changes over a very wide range of values, it is easier to plot log Y as a function of X rather than Y itself. Figure 7.4 shows the two ways to plot of these data.

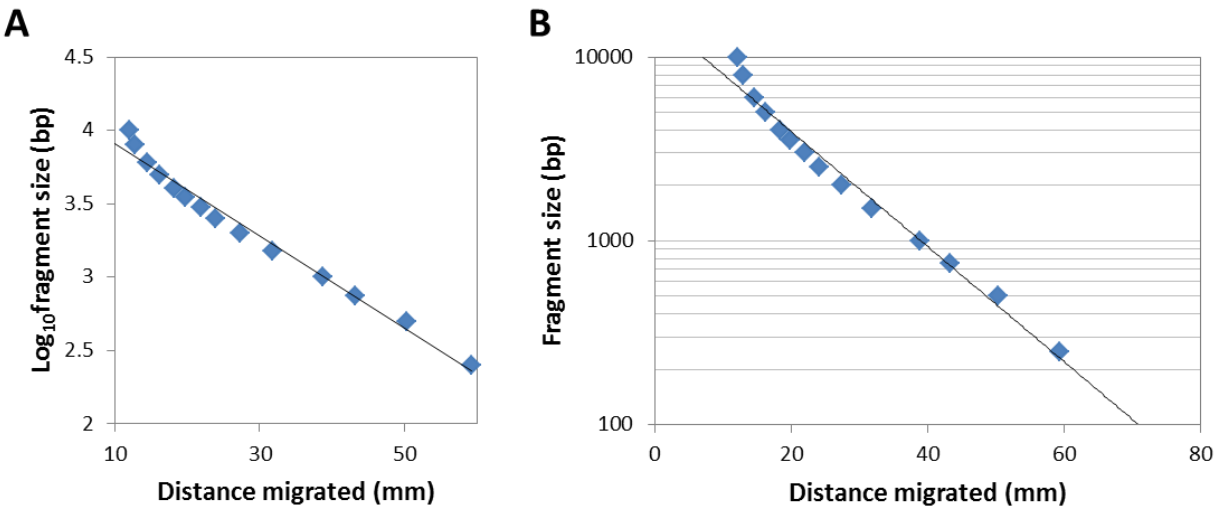


Figure 7.4. Two ways to generate a log vs. distance plot. (A) Log of the fragment size was plotted vs distance migrated. (B) Fragment size was plotted vs. distance in a semi-log graph. Note that the Y-axis uses a log scale while the X-axis uses a linear scale.

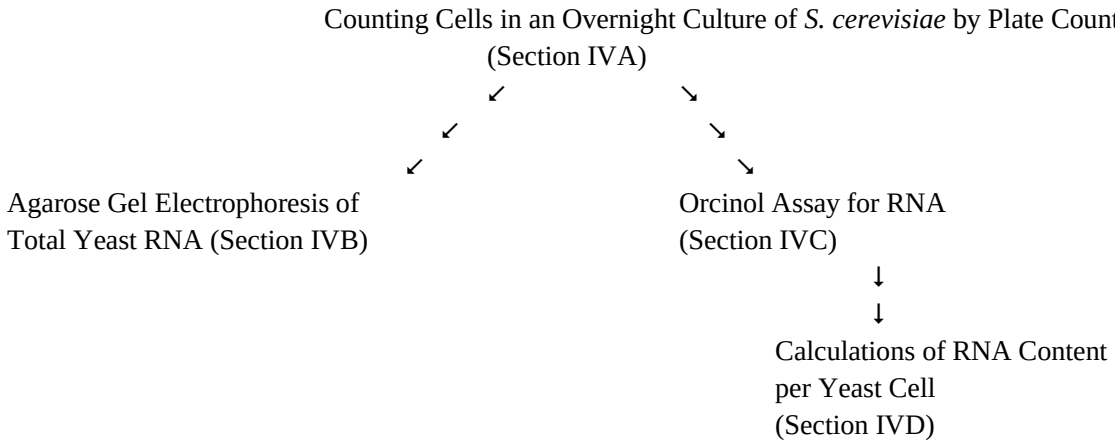
Note that in the semi log paper shown beginning at the bottom, the first log cycle goes log cycle goes from 100 to 1000 and the second from 1000 to 10000. When this data set is plotted, the points fall on what might be a straight line. The best-fit straight line to these points can be drawn with a ruler. Note: the paper you will use may be three-cycle semi log paper in which case you would start at 10, rather than 100.

The molecular mass of an unknown DNA or RNA fragments can then be determined from a standard curve such as the one noted on the previous page. In our example if a value on the X axis was known to be 50 mm, the corresponding value on the y axis would be ~500 bp if you draw a straight line through the points given.

IV. Experimental Procedures

This experiment has several parts. Because it take some time for the electrophoresis to occur, you should set that part of the experiment up early during the lab session and then do the orcinol assays for RNA.

The following is a flow chart for this laboratory session:



A. Counting Cells in an Overnight Culture of *S. cerevisiae* by Plate Counts

Last week, you determined the number of cells in an overnight YPD culture of S. *cerevisiae* using three different methods: a microscopic count for total cells/ml using hemocytometer, a count for viable cells/ml using quantitative dilutions and agar plates, and an indirect count based on turbidity or optical density. The agar plates that were made last week were incubated at 30°C for three days in order for the cells to form visible colonies. The objective of this part of the experiment is to count the plates and calculate the viable cells/ml.

1. Retrieve your set of YPD plates and arrange them in order of dilution: 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}. Look at the plates qualitatively and note the relative number of colonies on each plate. Some of the plates may have confluent growth, in which there are so many colonies that they merge together, but other plates may have no growth at all.

2. Select those plates that have somewhere between 20 and 500 colonies. Use the electronic colony counter to **count the colonies on these plates and record the data in your lab notebook**.

Dilution Used	# of colonies (plate 1)	# of colonies (plate 2)	Average # of colonies
10^{-4}			
10^{-5}			
10^{-6}			
10^{-7}			

3. Average the counts for the duplicate plates. **Record that information in the above table or in your lab notebook.**

4. Then calculate the viable cells/ml for each of usable dilutions in the following way:

$\frac{\text{colonies}}{0.1\text{ ml}}$	x	$\frac{1}{\text{dilution factor}}$	=	viable cells/ml

Since you spread 100 µl (0.1 ml) of a certain dilution on each plate, you should divide by 0.1 ml to get the number of colonies/ml. You should then multiple by the inverse of the dilution factor to get the number of viable cells in the original overnight culture.

For example, if a 10^{-4} (1/10,000) dilution has 243 colonies/ml, the original culture would have 10,000X this number or 2,430,000 colonies. Convert your results to scientific notation, as in 2.43×10^{6} viable cells/ml. **Summarize these data in the following table:**

Dilution	Viable Cells/mL
10^{-4}	
10^{-5}	
10^{-6}	
10^{-7}	

5. Finally, average the viable counts from the different dilutions to get a single number for the viable cells/ml in the original culture. Compare this value with the cell count determined from the hemocytometer counts and from the turbidity measurement.

Method	Cells/mL
Hemocytometer	
Turbidity	
Viable Cell Count	

B. Agarose Gel Electrophoresis of Total Yeast RNA

The objective of this part of the experiment is to cast a horizontal agarose gel and to use it to separate the different RNAs present in the spin column preparation made last week. The RNAs in the mini-prep will be compared to a series of size markers in a RNA "ladder." Because the gel takes some time to run, you should start this part of the experiment before beginning the orcinol assays for RNA.

1. Each group will be provided with an Owl horizontal mini-gel system for agarose gel electrophoresis. Remove the casting tray from the apparatus and rotate it 90° so that the ends will be sealed against the side of the gel box. Sealing the ends of the casting tray is essential in order to keep the molten gel mixture from leaking out until the gel has set. Place a 10-tooth comb in position near one end of the tray.

2. Heat 40 ml of a stock solution of 1.2% agarose in TBE (Tris-borate-EDTA) buffer in the microwave oven until the agarose has completely dissolved and a transparent gel solution has formed. Swirl the solution periodically to prevent it from boiling over. Cool the gel mixture to about 60°C. Add 3 µl of GelRed, swirl gently, and pour the entire 40 ml into the gel tray so that the teeth of the comb are embedded in the solution. The goal in this case is to make a thin gel with fairly small wells that can be run quickly within the lab period. Allow the gel to set for at least 20 minutes. **GelRed is a potential carcinogen so wear gloves when handling the gel and this solution.**

3. Retrieve the RNA sample that you made last week from the freezer box. Each group will also be provided with samples of a standard RNA ladder mixture from New England BioLabs. (Figure 7.5)

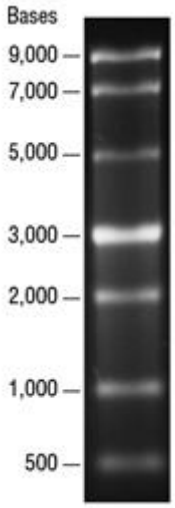


Figure 7.5 The standard RNA ladder is a mixture of 7 RNA pieces with sizes of 500, 1000, 2000, 3000, 5000, 7000, and 9000 nucleotides. The mixture contains more of the 3000 nucleotide piece so that when the RNAs have been separated, the band corresponding to this RNA is more intense. A 1.0% TBE Gel was used to separate the RNA fragments and RNA was visualized by ethidium bromide staining.

4. Set up the following samples in sterile 1.5 ml microcentrifuge tubes. Note that all volumes are in **µl**. Carefully use a P-10 micropipetter and small sterile tips to dispense the samples, water, and 2X sample buffer. **NOTE: IN ADDITION TO USING YOUR OWN RNA MINI-PREP SAMPLES, YOU WILL ALSO USE SAMPLES FROM TWO OTHER GROUPS**. Be sure to find out from each of the other groups which type of yeast they used to make their miniprep and write them down here: (you need to use both types of yeast)

Type of yeast from Group X: _____
Type of yeast from Group Y: _____

Tube	Sample	Volume (µl)	Water (µl)	2X RNA Sample Buffer (µl)
1	---	0	5	5
2	RNA ladder	2	3	5
3	Your RNA prep	2	3	5
4	Your RNA prep	5	0	5
5	Group X RNA prep	2	3	5
6	Group X RNA prep	5	0	5
7	RNA ladder	2	3	5
8	Group Y RNA prep	2	3	5
9	Group Y RNA prep	5	0	5

5. Briefly centrifuge the samples in a microcentrifuge to collect the liquid at the bottom of the tubes. Place the tubes in a foam float and heat the samples at 65^{o}C for five (5) minutes to denature the RNA. Allow the tubes to cool and centrifuge the samples again in a microcentrifuge to collect the liquid at the bottom of the tubes.

6. Remove the comb from the hardened agarose gel. Remove the casting tray and rotate it 90^{o} so the ends are open to the buffer compartments. Add enough TBE buffer to cover the gel completely but do not overfill the buffer chambers. The gel should be submerged just about 2 mm under the buffer.

7. Using a P-10 micropipetter, carefully load the entire 10 µl of each sample into the wells of the agarose gel. The instructor will demonstrate the procedure. Be sure to use a separate plastic tip for each sample.

8. Place the lid on the electrophoresis chamber and connect the leads to the power supply. The Black lead goes to the Negative Pole and the Red lead goes to the Positive Pole. Be sure that the power supply is set to 150 Volts, 400 mA, and a time of 60 minutes.

9. Subject the samples to electrophoresis at 150 Volts for about 60 minutes or until the bromophenol blue in the sample buffer has moved about 5 cm into the gel. Note the movement of the bromophenol blue tracking dye during this time.

10. Put on a pair of gloves and remove the lid from the electrophoresis chamber. Carefully lift out the gel tray and pour the buffer into the designated container.

11. Place your gel on a UV light box to visualize the bands. You may draw what you see or take a picture of the gel with your phone or tablet to print later.

12. Examine the picture of the gel carefully. Look first at the standard RNAs in the RNA ladder. The 3,000 nucleotide standard band will be brighter than the others for orientation. **Measure the distance in mm from the bottom of the well to each of the RNA bands**. This is the mobility of that size of RNA fragment.

Size (bp)	Distance migrated (mm)
9,000	
7,000	
5,000	
3,000	
2,000	
1,000	
500	

13. Now make a standard curve in which you **plot the sizes of the standard RNA molecules in the ladder on the Y axis and mobility on the X axis on linear graph paper**. Draw the best fit line to the data points. This line will probably be a curve because mobility is actually related to the log of the size of the RNA molecules. Now **make another standard curve in which you plot the sizes of the standard RNA molecules in the ladder on the Y axis and mobility on the X axis on semi-log graph paper.** Draw the best fit line you can to the data points. This graph should approximate a straight line.

14. Now look at the RNA bands that are visible in your RNA mini-prep. How many different bands of RNA can you see? **Measure the distance in mm from the well to each of the major RNA bands. Record the distance migrated for each band here:**

Sizes of fragments observed in **your RNA Prep**

Band	Distance migrated	Estimated Size
1		
2		
3		
4		
5		

**Note: you might only see 2-3 distinct bands.*

15. Now use the linearized standard curve to **estimate the size of each band in nucleotides**. Because you loaded several different volumes of the same sample, use the bands that are most clear for your estimates. Now **repeat the process with the RNA bands in the mini-preps from the other groups**.

Sizes of fragments observed in **Group X RNA Prep**

Band	Distance migrated	Estimated Size
1		
2		
3		
4		
5		

**Note: you might only see 2-3 distinct bands.*

Sizes of fragments observed in **Group Y RNA Prep**

Band	Distance migrated	Estimated Size
1		
2		
3		
4		
5		

**Note: you might only see 2-3 distinct bands.*

16. How do the sizes of the RNAs you observed compare with the yeast ribosomal RNAs described in the introduction to lab 6? How does your yeast RNA preparation compare with that of the other groups?

C. Orcinol Assay for RNA

The purpose of this part of the experiment is to determine the RNA content of the PCA extract of *S. cerevisiae* prepared last week. To do this, you will need a standard curve showing the relationship between absorbance and amount of RNA (Figure 7.6) You will carry out an orcinol assay using your PCA extract. Because the RNA content of the extract is unknown, you will need to test varying volumes of several dilutions of the PCA extract.

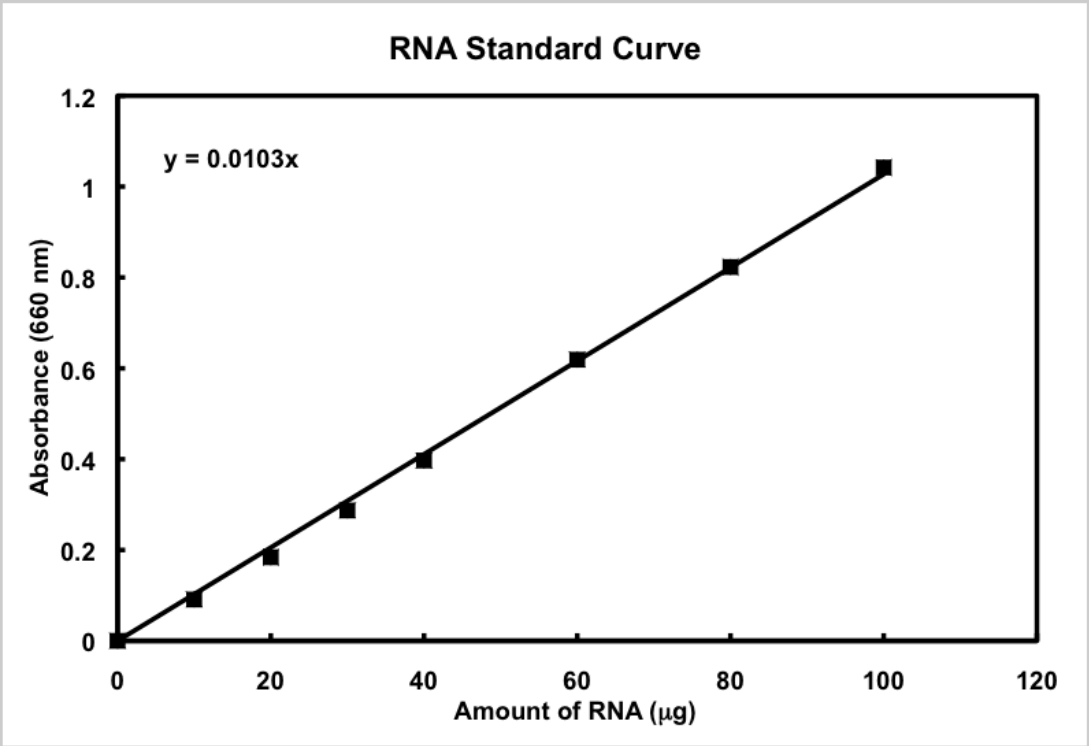


Figure. 7.6 Standard curve showing the quantity of RNA in micrograms per unit of absorbance at 660 nm, using the Orcinol reagent. Two point five milliliters of reagent were used per mL of sample, and samples were done in replicate.

1. Retrieve your PCA extract from the refrigerator. Record the volume below you will need this later. Prepare two serial 1/10 dilutions (10^{-1}, 10^{-2}) of this extract as follows. Using a P-1000 micropipetter and a large tip, add 1800 µl of **5% PCA** to each of two 13 x 100 mm test tubes. Mix the concentrated PCA extract, and using a P-100 micropipetter and a small tip, add 200 µl to the first dilution tube. Mix the solution by drawing the liquid up and down and discard the tip. Then add 200 µl of the first dilution to the second dilution tube and mix the solution. Note that 200 µl + 1800 µl = 2000 µl total, so each dilution is a 1/10 dilution.

Total Volume of PCA Extract _____

2. Set up the following reactions in new glass 13 x 100 mm test tubes. Note that all of the volumes are in ml and you will test four different volumes of the concentrated PCA extract and each of the dilutions.

Tube	Sample	Sample Volume (mL)	Water (mL)	Absorbance
1	---	0	1.0	
2	Concentrated Extract	0.1	0.9	
3		0.2	0.8	
4		0.3	0.7	
5		0.5	0.5	
6	10^{-1} Dilution	0.1	0.9	
7		0.2	0.8	
8		0.3	0.7	
9		0.5	0.5	
10	10^{-2} Dilution	0.1	0.9	
11		0.2	0.8	
12		0.3	0.7	
13		0.5	0.5	

3. Carefully add 2.5 ml of the orcinol reagent to each tube using the Repipetter. Vortex the solutions carefully. Heat the tubes **in the hood** in a heat block set at 95°C for 15 minutes. Cool the tubes in a pan of water to room temperature and read the absorbance of each solution at 660 nm. Use tube #1 to set the instrument to 0. You will probably find that some tubes are too dark to read and others show no reaction at all.

4. Use just those samples that do give absorbance values within the range of the standard curve for the rest of the calculations. Use the conversion factor based on the RNA standard curve to convert those absorbance values within the range of the standard curve to µg of RNA. Then divide each amount by the volume used in the assay to calculate the RNA content in µg/ml. Finally, correct for any dilution factors to get the RNA content in mg/ml in the original PCA extract. You will probably find that several different absorbance values can be used, so take an average to get a single value for the RNA concentration. **Record those calculations here or in your lab notebook.**

RNA Content _____ mg/ml

D. Calculations of RNA Content per Yeast Cell

The objective of this part of the experiment is to complete the calculations of the RNA content per yeast cell. To do this, you will need to know the volumes of the yeast culture and the PCA extract, the number of total yeast cells/ml and viable yeast cells/ml from **Laboratory 6 (Analysis of the RNA Content of Yeast Cells, Part A)** and the RNA content of the PCA extract in mg/ml from **Laboratory 7 (Analysis of the RNA Content of Yeast Cells, Part B).**

Method	Cells/mL
Hemocytometer	
Turbidity	
Viable Cell Count	

Volume of Culture _____
Volume of PCA Extract _____

1. Look first at the cell count data. Is the total number of cells/ml from the direct microscopic count the same as the number of cells/ml value from the viable cell count? **If the viable cell count is smaller than the total cell count, calculate the percentage of the cells in the overnight yeast culture that are viable**. How could you explain an observation that the total counts is less than the viable cell count? Use the total cells/ml value for the following calculations. Even if a cell is dead, it is likely to contain RNA and protein if it is visible microscopically as an intact cell.

2. You will need to use all of the values you have found before. You will need to determine the total mL of the PCA extract and the total mL of yeast culture. Do the calculation in two ways: once using the total yeast cells/mL from the hemocytometer count and once using the viable yeast cells/mL from the plate counts.

To determine the amount of RNA per cell, carry out the following calculations:

$$\frac{\text{mg RNA}}{\text{ml PCA extract}} \times \frac{\text{total ml of PCA extract}}{\text{total ml of yeast culture}} \times \frac{\text{1 ml of yeast culture}}{\text{yeast cells}} \times 10^9 \frac{\text{pg}}{\text{mg}}$$

RNA Concentration from orcinol assay *(Lab 7, Part IVC)*

Total Volume of PCR Extract *(Lab 7, Part IVC)*
Total volume of INITIAL yeast culture *(Lab 6, Part IVB)*

Cell Concentration from Hemocytometer count *(Lab 6, Part IVD)* OR from Plate count *(Lab 7, Part IVA)*

Summarize these calculations here:

Method	RNA (pg/cell)
Hemocytometer	
Plate count	

3. Once you have obtained the content data for your sample, enter the mg/mL of RNA in your PCA, hemocytometer count, and pg RNA per cell into the following chart. If time does not allow you to complete this you will need to send these values to your teacher within 24 hours.

Yeast Strain	RNA Concentration (mg/mL)	Hemocytometer Counts (Cells/mL)	RNA (pg/cell)
SEY6210			
Red Star			

How do the different types of yeast compare?