Spectrophotometry of DNA and RNA

Many of the techniques used to study cells are focused on characterization of the molecules that make up cells. Since the molecules are invisible, they are studied using techniques that recognize their inherent physical properties. Nucleic acids are often characterized and quantified using their *absorption spectra*, as measured by spectrophotometry. An absorption spectrum is generated by measuring the amount of light the nucleic acids absorb at various wavelengths.

In addition, spectrophotometry can be used in a quantitative manner, since there is a relationship between the amount of light absorbed by a solution and the concentration of the nucleic acids in the solution. For many substances, the amount of light absorbed depends on the solute concentration and the length of the light path. This relationship is known as the *Beer-Lambert Law* and is expressed as:

$$A = \alpha c l$$

where A is the absorbance, the constant α is the absorption coefficient ($cm^2/\mu g$), c is the solute concentration ($\mu g/ml$), and l is the length of the light path (cm). A plot of absorbance (ordinate) versus concentration (abscissa) gives a straight line with slope α. In this exercise, a standard curve will be made using DNA and RNA solutions of known concentrations. This standard curve will be used to determine the concentrations of unknown solutions of DNA and RNA isolated from liver cells.

Nucleic acids are isolated by homogenizing fresh bovine liver, disrupting the cells, and precipitating the DNA and RNA with trichloroaceitc acid and ethanol. To determine concentrations a calorimetric assay is used. *Diphenylamine* is specific for 2'-deoxypentoses, including the deoxyribose characteristic of DNA. When a solution of diphenylamine and DNA is heated, the solution turns blue. The intensity of the blue color, measured at 600 nm, is directly proportional the concentration of the DNA. *Orcinol* reacts with the ribose of RNA to produce a green-colored solution. However, the orcinol reaction is not as specific as the diphenylamine reaction. To some extent orcinol reacts with the deoxyribose of DNA as well, but the color intensity of this reaction is only about 10% as that of the reaction with ribose of RNA. Therefore, it is necessary to subtract the contribution to the orcinol color reaction made by DNA. The standard curve for the orcinol reaction is generated from absorbance readings taken at 660 nm.

The objectives for this exercise are: (i) To construct a standard curve of absorption spectra for known concentrations of DNA and RNA solutions; (ii) to determine the concentrations of DNA and RNA found in liver cells; (iii) to determine a value for α; (iv) to determine the ratio of RNA to DNA in liver cells and propose an explanation for this ratio.

NOTE: All students must wear safety glasses and a lab coat during this exercise.

Experimental Procedures

A single homogenization of liver tissue will provide enough material for the entire class. This solution will be aliquoted to each of the teams in the class and each team will proceed with its own extraction of nucleic acids. *Wear safety goggles when performing the following steps*.

Homogenization.

1. Obtain 30 g of frozen calf liver which has been cut into small (1 - 2 cm) cubes.

2. Add 120 ml ice-cold distilled water to the pre-chilled jar of an electric blender. With the blender on at its highest speed, add the pieces of liver one at a time. After all of the pieces have been added, continue to homogenize for one minute.

3 Dispense 2.0 ml of the homogenate to chilled 15 ml centrifuge tubes. (Each team will get a single tube).

Extraction.

4. Add 5.0 ml of ice-cold 10% TCA to the centrifuge tube and mix well by pipetting up and down.

5. Centrifuge at 1300 x*g* for two minutes. *Make sure that the centrifuge rotor is balanced*.

6. With a Pasteur pipet, remove and discard the supernatant. (If there is a layer of solid material floating on top of the supernatant, save it with the pellet).

7. Add 5.0 ml of fresh ice-cold 10% TCA to the pellet and resuspend by pipetting up and down.

8. Centrifuge at 1300 x*g* for two minutes. *Make sure that the centrifuge rotor is balanced*.

9. Remove and discard the supernatant leaving behind one or two drops of liquid above the pellet. Using a Pasteur pipet, resuspend the pellet in the liquid.

10. Add 10 ml 95% ethanol (room temperature) to the resuspended pellet and disperse well with the pipet.

11. Centrifuge at 1300 x*g* for two minutes. *Make sure that the centrifuge rotor is balanced*. Decant and discard the supernatant.

12. Wash the pellet with 10 ml fresh 95% ethanol and centrifuge and decant as in step 11. above.

13. Add 5.0 ml of 5% TCA (room temperature) and disperse the pellet with a Pasteur pipet.

14. Place the tube in a 90°C water bath and incubate for 15 min. Agitate the tube carefully every few minutes.

15. Centrifuge at 1300 x*g* for two minutes. Carefully decant *and save* the supernatant to a fresh tube. *The supernatant contains the nucleic acids*.

16. Wash the pellet with an additional 5.0 ml TCA, and centrifuge as in step 15. above. Remove the supernatant and combine with the nucleic acid solution saved in step 15. *This nucleic acid extract will be used in the colorimetric assays*.

Colorimetric Assays.
A. Diphenylamine Reaction.

Label six test tubes as shown in the table below and add to each the appropriate volume of each solution. Tubes 1-4 are the standards, which contain known concentrations of DNA; tube 5 contains the extract prepared above with an unknown concentration of DNA. *Make sure that you use the correct concentration of each stock solution. Wear safety goggles when performing the following steps*.

Tube	[DNA] (μg/ml)	Volume of DNA stock solution	Nucleic acid extract	5% TCA
Blank	0	—	—	2.0 ml
1	100	2.0 ml (100 μg/ml)	—	—
2	200	2.0 ml (200 μg/ml)	—	—
3	300	2.0 ml (300 μg/ml)	—	—
4	400	2.0 ml (400 μg/ml)	—	—
5	?	—	2.0 ml	—

1. To each tube, add 4.0 ml diphenylamine reagent, dispensed from a buret. Cover each tube with parafilm and invert two or three times to mix. *This reagent contains concentrated acetic acid and sulfuric acid. If it comes in contact with your skin, wash it off immediately with water*.

2. Place the tubes in a beaker of boiling water (with boiling chips) and incubate for 10 minutes. Monitor your tubes carefully so that they do not spill.

3. After 10 minutes, transfer the tubes to ice. Label six cuvettes as in the table above.

4. When cool, transfer the contents of the tubes to labeled cuvettes.

Your instructor will demonstrate the proper use of the spectrophotometer. Be sure to wipe the outside of each tube with a laboratory tissue before inserting it into the instrument.

5. Prepare the spectrophotometer for measurement at 600 nm.

6. Insert the reference Blank and adjust the controls so that the absorbance, *A*, reads zero.

7. Read the absorbance of each of the four standard tubes (tubes 1-4) and of the unknown extract (tube 5); it is not necessary to zero the spectrophotometer between readings. Record your readings on a data sheet.

B. Orcinol Reaction.

Label six test tubes as shown in the table on the next page and add to each the appropriate volume of each solution. Tubes 1-4 are the standards, which contain known concentrations of DNA; tube 5 contains the extract prepared above with an unknown concentration of RNA. *Make sure that you use the correct concentration of each stock solution. Wear safety goggles when performing the following steps.*

Tube	[RNA] (μg/ml)	Volume of RNA stock solution	Nucleic acid extract	5% TCA
Blank	0	—	—	3.0 ml
1	100	0.4 ml (100 μg/ml)	—	2.6 ml
2	200	0.4 ml (200 μg/ml)	—	2.6 ml
3	300	0.4 ml (300 μg/ml)	—	2.6 ml
4	400	0.4 ml (400 μg/ml)	—	2.6 ml
5	?	—	0.4 ml	2.6 ml

1. To each tube, add 3.0 ml of the orcinol reagent, dispensed from a buret. Cover each tube with parafilm and invert two or three times to mix. *This reagent contains concentrated hydrochloric acid. If it comes in contact with your skin, wash it off immediately with water.*

2. Place the tubes in a beaker of boiling water (with boiling chips) and incubate for 20 minutes. Monitor your tubes carefully so that they do not spill.

3. After 20 minutes, transfer the tubes to ice. Label six cuvettes as in the table above.

4. When cool, transfer the contents of the tubes to labeled cuvettes.

5. Prepare the spectrophotometer for measurement at 660 nm.

6. Insert the reference Blank and adjust the controls so that the absorbance, *A*, reads zero.

7. Read the absorbance of each of the four standard tubes (tubes 1-4) and of the unknown extract (tube 5); it is not necessary to zero the spectrophotometer between readings. Record your readings on a data sheet.

Post-lab Assignment:

Preparing and Interpreting the Data

Prepare two separate graphs, one for the diphenylamine reaction (DNA) and one for the orcinol reaction (RNA). Plot the values of the respective nucleic acid standards and draw a best-fit line connecting the points on each graph.

From the respective standard curves and the absorbance reading of the unknown, determine the DNA and RNA concentrations of the extract. Since the orcinol reaction is not entirely specific for RNA, DNA contributes to some of the absorbance seen in the orcinol reaction. Assuming that about 10% of the absorbance is due to DNA, simply subtract 10% from the absorbance value of the orcinol reaction to determine the final RNA concentration in the extract.

If the length of the light path, c, is 1 cm, use the Beer-Lambert equation to calculate the value of α for the diphenylamine reaction. Calculate the ratio of [RNA]/[DNA] for the extract.

Points to Consider

What is your value of α? Can you derive the units of α ($cm^2/\mu g$) from the Beer-Lambert equation? (Hint: a ml is equivalent to a cm^3)

Would you expect the ratio of [RNA]/[DNA] for the extract to be greater than or less than 1.0? How does this compare with the value you calculated? Would you expect this ratio to be constant? Why or why not?