PURDUE UNIVERSITY INSTRUMENT VAN PROJECT

QUANTIFICATION OF NUCLEIC ACIDS USING THE ULTRAVIOLET/VISIBLE (UV/VIS) SPECTROPHOTOMETER

(Revised 5/14/96)

OBJECTIVE

You will use a UV/VIS spectrophotometer and graphical analysis to determine the purities and concentrations of several samples of deoxyribonucleic acid (DNA).

BACKGROUND

The DNA molecule contains the blueprint for what a cell is or what it will become. The DNA molecule consists of two strands of nucleotides that are coiled about each other in the form of a double helix. These nucleotides consist of three parts: a sugar group, a phosphate group, and a base. The chains or nucleotide polymers of these molecules are known as nucleic acids. In DNA, the nucleotides that are linked together are all exactly the same except for the base in the molecule. It is the bases that are linked together by hydrogen bonding that give DNA its unique structure. There are four bases involved in the DNA molecule: Adenine (A), Guanine (G), Thymine (T), and Cytosine (C). Due to the structure of these bases Adenine and Thymine always pair and Guanine and Cytosine always pair. These groups are known as base pairs. In summary, the two strands of DNA are made of nucleic acids that are linked together by base pair hydrogen bonding.

The order in which the nucleotides are linked together, known as the genetic sequence, determines what proteins will be built within a cell. Your genetic sequence determined your hair color, eye color, shape of nose,height and every other physical trait about you. DNA contains the information that determines the very nature of a cell or group of cells. This is one powerful molecule!

The quantification of nucleic acids is very important in current genetic engineering. The amount of DNA present in solution can be determined using an ultraviolet spectrophotometer. The amount of DNA extracted from cells can be calculated using this method. This technique is routinely applied in biotechnology laboratories. A piece of DNA that codes for an important protein is called a gene. Genes are studied in molecular biology laboratories to uncode the gene's nucleotide sequence and protein production. Large amounts of DNA are necessary to carry out these tests. Changes in the DNA sequence of a gene, mutations, can be found through careful analysis. To get large amounts of the DNA of interest a gene is inserted into a circular pieces of DNA called plasmids. Plasmids are introduced into bacterial cells and these newly transformed bacterial cells are allowed to reproduce. When the bacterial cells divide each new daughter cell contain a copy of the plasmid that was introduced in the parent cells. In order to successfully insert a gene of interest into a plasmid, the molecular concentrations of both the gene and the plasmid must be calculated.

Another type of nucleic acid, RNA can be quantitated with this method. Messenger RNA is single-stranded and more difficult to study. However, it is studied in molecular biology labs that are focusing on the second step of protein synthesis called translation. Translation can be carried out in a test tube using special buffers and enzymes. The concentration of RNA must be known in these systems and is calculated by the same method used in this lab.

One characteristic that often differentiates one molecule from another is the type or wavelength of light that the molecule absorbs. DNA's characteristic absorbance is in the ultraviolet region of the electromagnetic spectrum. Light or electromagnetic radiation is characterized in terms of wavelength. The ultraviolet region of the spectrum falls between the visible range and the portion of the spectrum associated with X-rays. UV light has wavelengths ranging from 400 nm to 10 nm.

A UV spectrophotometer is an instrument that is often used to tell how much of a compound is in a sample. The instrument can read the amount of ultraviolet light that is absorbed by a sample. This amount is known as the sample's absorbance. The absorbance of a sample is directly related to the amount of the compound of interest that is contained in the sample. The UV spectrophotometer may be set to emit light at a particular wavelength or range of wavelengths. Since compounds have a characteristic wavelength that they absorb and the amount of light that they absorb is directly related to how much of the compound is the sample, the UV spectrophotometer is a useful tool for scientists to quantify how much of a particular substance is a sample of material.

SAFETY CONSIDERATIONS

You should **NOT** look at the ultraviolet light. Ultraviolet light can be harmful to the eyes if stared at for any length of time! Quartz cuvettes are **very expensive** so handle them carefully. DNA can be degraded by enzymes present in our saliva and on our skin. Be careful not to sneeze into, talk over, or touch the insides of the cuvettes.

MATERIALS NEEDED

UV/VIS Spectrophotometer
1 mL quartz cuvette
DNA sample(s)
TE Buffer
Disposable 1 mL polyethylene Transfer Pipettes (Berol) [2 per group]
Eppendorf tubes (1.5 mL) [2 per group]
Ruler
Kimwipes

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PROCEDURE

I. Setting Up the Spectrophotometer (Beckman DU64)

1. Turn on the spectrophotometer at the power strip. Check that the printer is on line and ready.
2. Turn on the UV lamp source and allow it to warm up for 5 minutes.
3. Select the absorbance reading mode (ABS key).
4. Press the SCAN key "Edit" will be displayed.
5. Enter the starting wavelength as 280 nanometers (nm) and press enter.
6. Enter the ending wavelength as 260 nm and press enter.
7. The speed for the scan of the sample will be displayed. It should read 750 nm/min. If it does not, press the STEP key and scroll through the options until 750 nm/min is displayed. Press enter.
8. Upper limit will be displayed. Set the upper limit at 2,000 absorbance. Press enter.
9. Lower limit will be displayed. Set the lower limit at .000 absorbance. Press enter. The starting wavelength will reappear.
10. The instrument is now ready to be calibrated against a control solution. The purpose of the calibration is to measure and then subtract from the samples absorbance any absorbance from the buffer solution.
11. Place 200 microliters (μL) of the TE Buffer into the quartz cuvette. This is the solution you will use to calibrate the instrument.
12. Open the sample compartment lid on the instrument.
13. Carefully wipe the cuvette with a Kimwipe and be careful not to get fingerprints on the quartz panels. Place the cuvette into the cuvette holder so that the quartz sides are in the path of the light source (left to right).
14. Close the sample compartment lid.
15. Press CALB. The absorbance of the TE Buffer solution will now be recorded in memory as the "background" and "Bkg" will be displayed.
16. Press READ, Calibration is complete when "Scan" is displayed. The instrument is now ready to measure DNA samples.
17. Open the sample compartment lid and remove cuvette.
18. Discard the 200 μL of TE Buffer.
19. Rinse the cuvette twice with the TE Buffer solution and drain the cuvette onto a Kimwipe.

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II. Sample Preparation

20. Place 200 µL of the DNA sample in the cuvette and place the cuvette in the sample holder.
21. Press READ. The absorbance of the sample between 260 and 280 nm will be measured and plotted as a graph on the printer.
22. Repeat steps 20 and 21 for any other DNA samples that you have been assigned.

PRE-LAB QUESTIONS

1. List the four bases that make up the DNA molecule.
2. What are plasmids and how do they help molecular biologists study genes?
3. What is the range of wavelengths of ultraviolet light?
4. How can a UV/VIS spectrophotometer help a scientist determine how much of a compound is in a sample?

DATA

Collect the graphs and label them with the names of your group members and the DNA samples that were measured.

ANALYSIS

1. For each DNA sample that you analyzed, determine the absorbance at 280 nm and at 260 nm. To do this use your ruler and estimate the absorbance to the nearest hundredth from your graphs.
2. Divide the absorbance at 260 nm by the absorbance at 280 nm. This value provides information about the purity of the nucleic acid in the sample. A very pure sample falls within the range of 1.8 to 2.0.
3. Determine the concentration of the DNA sample by multiplying the value for the absorbance at 260 nm by the known constant:

$$\frac{50\ \mu g/mL}{1.0\ (260\ nm\ absorbance)}$$

4. Your answer should be in units of µg/mL.

CONCLUSIONS

1. What are the purities of your DNA samples?
2. What are the concentrations of your DNA samples?
3. What are some possible explanations for a purity ratio that falls outside of the given range?
4. Why is it necessary to measure the background absorbance before measuring a DNA sample?
5. How is this procedure useful in biotechnology today?
6. Why is it important that all citizens understand the basics of biotechnology?
7. What issues may arise from this new technology?

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QUANTIFICATION OF NUCLEIC ACIDS
THE ULTRAVIOLET/VISIBLE (UV/VIS) SPECTROPHOTOMETER

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TEACHERS GUIDE

TYPICAL CLASSROOM USAGE

The quantitation of DNA can be performed at the general biology level, advanced biology level, and it has also been adapted for chemistry. The lab can be tailored to the level of the students quite easily. Several options and alternatives are provided below which can be used in whatever manner the instructor deems is appropriate for their class.

CURRICULUM INTEGRATION

General Biology this lab would fit very well into a unit on genetics. An introduction to basic DNA composition and structure may precede or follow the lab depending on the approach (professional term for schedule adjustments) the teacher would like to take. Give the students the background language first or give them the hands on experience first to give more meaning to the sometimes very detailed lecture on structure that follows. This lab also fits very well with a DNA isolation method developed by another teacher in the Purdue Van Project ("DNA Spooling of Calf Thymus DNA" by Garland Howard). The questions students often have after Mr. Howard's lab are: "Is this really DNA?" and "How much DNA is this?" This lab can help to address the second question. A Gel Electrophoresis developed by John Andrews (also associated with the project) can help students visualize the DNA on a gel and make some comparisons between DNA from different sources.

Advanced Biology This simple quantitation of DNA lab may be very helpful for increasing students preparation for Advanced Placement Biology courses. For example, transformation of bacterial cells is a requirement in recognized Advanced Placement Biology courses.

Chemistry This activity could be used in a unit covering organic chemistry and/or biochemistry. DNA is an organic structure for which most, if not all, of the students would have prior knowledge, and this laboratory would connect biochemistry to their world. The lab could also be incorporated into a unit dealing with concentration and/or percent composition. Finally, the lab might be used in introductory analytical chemistry.

SCIENCE PROFICIENCIES

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This lab incorporates many aspects of the science proficiencies as outlined by the state of Indiana. For example, students will:

a. be working cooperatively to accomplish a task.
b. use basic science process skills of observing, inferring, measuring, and communicating.
c. use the integrated science process skills of interpreting data from graphs, formulating operational definitions, and experimenting.
d. obtain scientific/technical information from various reliable and relevant sources.
e. demonstrate comprehension of scientific/technical material.
f. consider the ethical implications when applying scientific knowledge.

PREPARATION

Tris-Acetate-EDTA buffer (TE pH 8.0)

Standardized samples of DNA. This may be obtained from Modern Biology (1-800-733-6544). The product needed is Plasmid DNA-pUC18. From Modern Biology it is catalog #3-14-120. The concentration of this stock solution is 120μg/400μL. This must be diluted 1:10 in the TE buffer. (Add 3600 μL of TE to the 400 μL of stock DNA contained in the vial. This will give the teacher enough DNA for 20 groups)

TIME

Preparation: 15 Minutes
Student time: 60 Minutes
Stopping points: after completing the data collection portion of the lab which is before graphical interpretation.
One time consideration: is not necessary that each lab group go through the "Setting up the Spectrophotometer" section of the experiment. This section will take about 5 minutes per group. Once a background correction has been obtained, it will be good for the entire class period. Therefore, if time is short, the students may go directly to Part II of the procedure once the setup has been completed.

PRELAB ANSWERS

1. Adenine, Guanine, Cytosine, and Thymine
2. Plasmids are small, circular, extrachromosomal DNA that carry genes which provide some kind of selective advantage for the bacterial cell. A tool of genetic engineering. This answer would be difficult to get given the information in the BACKGROUND. I suggest removing this particular question. I am not sure it can be answered in a brief way.
3. 10 nm to 400 nm
4. The amount of a compound can be determined by its absorbance. The higher the concentration, the more light is absorbed.

OPTIONS/ALTERNATIVES

Calf thymus DNA can be prepared with a few minor changes according to the Promega Protocol. The TE Buffer that the company recommends is pH 7.6 however, TE Buffer pH 8.0 can be used without affecting the purity or yield significantly. The columns provided in this kit on top of Eppendorf tubes during the microcentrifuge spin. 3 mL disposable syringes and small test tubes are also needed to collect the eluent from the column. The Calf thymus DNA is usually too concentrated to be effectively purified with the column. We suggest diluting the DNA in TE buffer before the purification. Since the concentration is not yet known, several dilutions must be run. We suggest assigning various dilutions to different groups in each class. A range such as 1:1000, 1:500, 1:100, and 1:10 should place at least one of the groups within the limits of the UV/VIS spectrophotometer. Prior to extracting the DNA from the thymus, weight the amount of starting tissue. Quantitation of DNA can then be related back as a percentage of total cell mass. Additional items and precautions will be required to use the "Wizard Purification System". The Purdue Van Project will supply the purification kit, 3 mL syringes, and a microcentrifuge. Isopropyl alcohol and disposable Berol pipets will need to be supplied by the teacher. Students must wear goggles and aprons because of the alcohol.

Another possible extension to further investigate Calf thymus DNA would be to digest the DNA with restriction enzymes and run the digested DNA out on an agarose gel along with known DNA samples. The reasons for large smear should be discussed. Many fragments of various lengths will migrate in the gel. Be sure to include DNA markers of known lengths.

Another extension might be to assign a paper in which the students must search the Internet for current information on genetic engineering and/or ethical issues surrounding biotechnology and genetics.

SAFETY AND DISPOSAL

DNA and the other substances used in this experiment are safe for your students to use. (We would not, however, drink or otherwise ingest any of the solutions). All solutions may safely be disposed of down the drain. All plastics may be thrown in the trash.

ASSESSMENT

A pre-lab V-diagram may be helpful to evaluate the students understanding before the lab begins. Completion of the diagram after the lab has been completed can give an indication of the knowledge gained from this lab. Graphical interpretation and mathematical manipulation of formulas is also stressed in this lab. If desire, the teacher may reproduce a graph for the entire class to interpret. Group work is also practiced in this lab and some division of labor and collaboration on the analysis may be beneficial.

REFERENCES

Ebbing, Darrell D. General Chemistry Houghton Mifflin, Co., Boston: 1984.

Maniatis, T.; Fritsch, E.F.; Sambrook, J. Molecular Cloning: A Laboratory Manual Cold Spring Harbor Press, Cold Spring Harbor: 1982.

Promega Technical Bulletin, "Wizard DNA Clean-Up System" Madison: 1993.