

# Laboratory Exercise

## Applied Spectrophotometry: Analysis of a Biochemical Mixture

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### Abstract

Spectrophotometric analysis is essential for determining biomolecule concentration of a solution and is employed ubiquitously in biochemistry and molecular biology. The application of the Beer-Lambert-Bouguer Law is routinely used to determine the concentration of DNA, RNA or protein. There is however a significant difference in determining the concentration of a given species (RNA, DNA, protein) in isolation (a contrived circumstance) as opposed to determining that concentration in the presence of other species (a more realistic situation). To present the student with a more realistic laboratory experience and also to fill a hole that we believe exists in student experience prior to reaching a bio-

chemistry course, we have devised a three week laboratory experience designed so that students learn to: connect laboratory practice with theory, apply the Beer-Lambert-Bouguer Law to biochemical analyses, demonstrate the utility and limitations of example quantitative colorimetric assays, demonstrate the utility and limitations of UV analyses for biomolecules, develop strategies for analysis of a solution of unknown biomolecular composition, use digital micropipettors to make accurate and precise measurements, and apply graphing software. © 2013 by The International Union of Biochemistry and Molecular Biology, 41(4):242–250, 2013

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### Introduction

Spectrophotometric analysis is essential for determining biomolecule concentration of a solution and is employed ubiquitously in biochemistry and molecular biology. The application of the Beer-Lambert-Bouguer Law [1,2] is routinely used to determine the concentration of DNA, RNA or protein [Eq. (1)].

$$A = kcl \quad (1)$$

There is however a significant difference in determining the concentration of a given species (RNA, DNA, protein) in isolation (a contrived circumstance) as opposed to determining that concentration in the presence of other species (a more realistic situation). The more generalized equation that would apply in this case is:

$$A_t = A_1 + A_2 + A_3 + \dots = k_1c_1l + k_2c_2l + k_3c_3l + \dots \quad (2)$$

The total absorbance  $A_t$  is the sum of the absorbances of various species that absorb at a given wavelength which is dependent on the extinction coefficients and concentrations of that species.

In order to present the student with a more realistic laboratory experience and also to fill a hole that we believe exists in student experience prior to reaching a biochemistry course, we have devised a three week laboratory experience that is described in this communication.

In reviewing freshmen general chemistry texts [3–5] we find that most do not cover this important analytical tool.

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Students are typically exposed to the Beer-Lambert Law in freshmen general chemistry laboratories either through lab manuals or through homegrown lab exercises. Most organic texts [6–8] also do not cover this technique. Biology degree students are rarely required to take an analytical course that covers this concept in detail. Biochemical texts [9–12] typically offer limited coverage of the laboratory technique and biochemistry lab manuals provide detailed spectrophotometry theory but vary in the practical applications [13,14]. We therefore believe it important that the students have a thorough exposure to this analytical tool so as to apply it to future experiments dealing with the isolation of RNA, DNA and protein; enzyme kinetics; and biochemical analyses.

The first multi-week experiment of our Biochemistry 1 course are designed to accomplish the following student learning objectives:

- Connect laboratory practice with theory
- Apply the Beer-Lambert-Bouguer Law to biochemical analyses.
- Demonstrate the utility and limitations of example quantitative colorimetric assays: Bradford, Dische, and Bial.
- Demonstrate the utility and limitations of UV analyses for biomolecules.
- Develop strategies for analysis of a solution of unknown biomolecular composition.
- Use digital micropipettors to make accurate and precise measurements.
- Apply graphing software.

In order to understand how the biomolecules affect the absorbance of a solution, students take UV spectra of pure DNA, RNA, and protein (as bovine serum albumin, BSA) solutions, as well as mixtures of these three components. The absorbance interactions among the various biomolecules are analyzed by the shape of the spectra, wavelength maxima, Warburg-Christian equation and nomograph [15], and changes in the  $A_{260}/A_{280}$ ,  $A_{280}/A_{260}$ , and  $A_{230}/A_{260}$  ratios.

In addition to the UV spectroscopy, colorimetric assays are performed for pure DNA, RNA, and BSA solutions using the Dische assay [16], Bial assay [17], and Bradford assay [18], respectively to determine the individual extinction coefficients for each biomolecule. Technically, the Bradford assay, being a binding assay, the accepted term is “color response” instead of extinction coefficient [19]. In order to simplify comparisons with true extinction coefficients, we will use “extinction coefficient” for the Bradford assay. To demonstrate the limitations, special attention is given to interferences in the assay. After the preliminary experiments are completed, the students take what they have learned and apply their newfound understanding to determine the concentrations of each component in a mixture.

To accomplish the student learning objectives in such a short period of time (three 4 h lab sections), students work in teams of 2-3 where they must divide the tasks and develop a strategy for completing the analyses. Teams must also hone communication skills, both among team members and through writing a common, comprehensive laboratory report. Evaluation of student performance occurs primarily through a journal-style written lab report.

## Laboratory Methods

We use a lab manual written and published in-house. The manual contains reference sections on spectrophotometry and dilution mathematics that students are asked to read before lab. The students are also instructed to read the lab introduction and procedures and then divide the tasks among members of the team. The pre-lab discussion each week covers only safety and gives the students opportunities to ask general questions.

### Overview

- Period 1-conduct UV scans of standard solutions; calculate absorbance ratios; make samples for colorimetric standard curves; begin to construct computer generated standard curves.
- Period 2-complete any UV and colorimetric work on standards; isolate an unknown (optional); perform initial UV scan of the unknown in order to develop an analysis strategy; preliminary analysis of the unknown
- Period 3-complete colorimetric analysis of unknown.

### Safety and Waste Disposal

Bradford, Dische, and Bial reagents all contain strong acids. Latex or nitrile gloves, safety eyewear with side shields, and normal lab attire, should be used when preparing or handling reagents/samples. Dische and Bial samples should be incubated in the hood or other well-ventilated area to avoid accumulation of irritating fumes.

Samples of DNA, RNA, and protein and mixtures are disposed in the sink. Bradford, Dische, and Bial samples are disposed as acids. Alconox, or similar detergent, and water are sufficient for cleaning glassware. Bradford reagent stains on glassware are readily removed with acetone or aqueous ethanol.

### Week by Week Detail

#### *Period 1 and 2 UV Analyses*

Individual standard samples of BSA (~1.00 mg/mL), baker's yeast rRNA (~0.0500 mg/mL), and salmon testes DNA (~1.000 mg/mL) in TE Buffer (10 mM Tris, 1 mM EDTA, pH



TABLE 1

*Samples to create a standard curve and determine interference extinction coefficients for the Dische assay*

Sample #	1	2	3	4	5	Curve Check Sample	Protein Standard	RNA Standard
0.500 mg/mL DNA (mL)	0.00	0.25	0.50	1.00	1.50	0.50	–	–
1.00 mg/mL BSA (mL)	–	–	–	–	–	–	0.25	–
0.0500 mg/mL RNA (mL)	–	–	–	–	–	–	–	1.00
TE Buffer (mL)	1.5	1.25	1.00	0.50	0.00	1.00	1.25	0.50
diphenylamine reagent (mL)	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00

7.5) are scanned from 200–350 nm in quartz cuvettes. Students are given concentration values of each standard solution [20]. The DNA solution purposely produces a scan that is off scale on the spectrophotometer such that the solution must be diluted 1:20 (~0.0500 mg/mL) to produce an absorbance that is on-scale. This provides the students with an opportunity to learn about the trial and error process in science by finding their own DNA dilution that will fall within the spectrophotometer absorbance parameters. Spectra are either exported to Excel or are opened in spectrophotometric analysis software for processing. Absorbance values at 230, 260, and 280 nm are recorded and used to calculate absorbance ratios. The Warburg-Christian Equation and Warburg-Christian nomograph [15] are used to estimate the concentration of protein and nucleic acid in each solution. With the known concentrations and absorbances, extinction coefficients are calculated for BSA, RNA, and DNA at 260 and 280 nm. The extinction coefficients are posted for comparison among the teams. The pure solutions are then heated for 5 min in a water bath at 85°C. In order to observe the effect of denaturing, as a hyperchromatic shift, absorbances are quickly acquired at 260 and 280 nm while the solutions are still hot and again after the solutions have cooled to room temperature.

Mixtures are made using equal volumes of the standard samples to produce solutions of: DNA and BSA, DNA, and RNA, BSA, and RNA, and all three. In this instance 0.05 mg/mL DNA must be used. The mixtures are each scanned from 200 to 350 nm and are evaluated as previously described.

#### *Period 1 and 2 Colorimetric Analyses*

For the three colorimetric assays a standard curve of absorbance versus concentration of the target biomolecule is made and the extinction coefficient is determined by linear regression analysis. If a coefficient of determination ( $R^2$  value) is less than 0.990, indicating poor pipetting technique or contaminated glassware, a new set of experimental solutions are prepared in order to obtain a better standard curve. In each assay, extinction coefficients for nontarget molecules are calculated from the single absorbance readings for a defined concentration of this molecule. A curve check sample is made for each assay to facilitate student discussion of accuracy. The extinction coefficients for both target and non-target biomolecules are posted for comparison among the teams in the class. As in the proverbial real world, no correct answers are provided.

TABLE 2

*Samples to create a standard curve and determine interference extinction coefficients for the Bial assay*

Sample #	1	2	3	4	5	Curve Check Sample	Protein Standard	DNA Standard
0.0500 mg/mL RNA (mL)	0.00	0.25	0.50	1.00	1.50	1.00	–	–
1.00 mg/mL BSA (mL)	–	–	–	–	–	–	0.25	–
0.500 mg/mL DNA (mL)	–	–	–	–	–	–	–	0.50
TE Buffer (mL)	1.50	1.25	1.00	0.50	0.00	0.50	1.25	1.00
Acid orcinol reagent (mL)	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Alcoholic orcinol (mL)	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20

TABLE 3

Samples to create a standard curve and determine interference extinction coefficients for the Bradford assay

Sample #	1	2	3	4	5	Curve Check Sample	RNA Standard
1.00 mg/mL BSA (mL)	0.000	0.0050	0.0125	0.0200	0.0250	0.0250	–
0.0500 mg/mL RNA (mL)	–	–	–	–	–	–	0.0500
TE Buffer (mL)	0.0500	0.0450	0.0375	0.0300	0.0250	0.0250	0.000
Bradford reagent (mL)	1.95	1.95	1.95	1.95	1.95	1.95	1.95

#### DNA Colorimetric Analyses

Diphenylamine reagent is prepared beforehand: 5.0 g diphenylamine, 500 mL glacial acetic acid, and 13.75 mL concentrated  $\text{H}_2\text{SO}_4$ . Samples are made for the Dische (diphenylamine) Assay standard curve as shown in Table 1. The samples are thoroughly vortex mixed, incubated in a boiling water bath for 20 min; allowed to cool to room temperature after which the contents are transferred to glass cuvettes; and absorbance readings at 595 nm are obtained.

#### RNA Colorimetric Analysis

Acid orcinol reagent is made in advance: 0.030 g anhydrous  $\text{FeCl}_3$  in 0.50 mL dI  $\text{H}_2\text{O}$ , diluted to 100 mL with concentrated HCl. Alcoholic orcinol is also made in advance and stored in a brown bottle in the refrigerator: 0.60 g orcinol dissolved in 10 mL of 95% ethanol. Samples are made for the Bial (orcinol) Assay standard curve as shown in Table 2. The samples are thoroughly vortex mixed; incubated in a boiling water bath for 25 min; allowed to cool to room temperature after which the contents are transferred to glass cuvettes; absorbance readings at 660 nm are obtained.

#### Protein Colorimetric Analysis

5X Bradford Reagent is made in advance (dissolve 100.0 mg of Coomassie Brilliant Blue G-250 in 100 mL of 95% ethanol; add 100 mL of 85% (w/v)  $\text{H}_3\text{PO}_4$  and dilute to 1 L with d $\text{H}_2\text{O}$ ; filter) then diluted by the instructor to 1X before use. Samples are made for the Bradford Assay standard as shown in Table 3. The samples are thoroughly vortex mixed; incubated at room temperature for 10 min after which the contents are transferred to glass cuvettes; then absorbance readings at 595 nm are obtained.

To illustrate the behavior of DNA in the Bradford Assay a separate set of samples are made, as shown in Table 4. The samples are treated as previously described.

#### Nature of the Unknown

The students may either prepare their unknown or the instructor may construct an unknown. In the work presented here, the students isolated DNA from bovine thymus gland in a method described by Miller *et al.* [21].

#### Period 2 UV Analysis of the Unknown

A sample of the unknown is scanned from 200 to 350 nm. If the scan exceeds an absorbance of 1.0 at 260 or 280 nm a dilution must be made and another scan performed. Absorbances at 230, 260, and 280 nm are recorded from the scan and absorbance ratios are calculated. The absorbance ratios and shape of the scan are employed to develop a strategy to accomplish the colorimetric analyses of the unknown in the final lab period.

#### Period 3 Colorimetric Analyses of the Unknown

The concentration of each biomolecule in the unknown is determined by colorimetric analyses. Absorbances are acquired for no fewer than three samples in each assay. Each absorbance is used with previously determined extinction coefficient to calculate the concentration of the biomolecule in the unknown solution. Extrapolation is not allowed; therefore dilution in sample-making must be considered. Where necessary, correction of the absorbance for additional absorbance produced by interfering molecules is employed. The reported concentration is the

TABLE 4

Samples to determine interference extinction coefficient for DNA in the Bradford assay

Sample #	1	2	3	4	5
0.500 mg/mL DNA (mL)	0.00	0.0125	0.0250	0.0375	0.0500
TE Buffer (mL)	0.0500	0.0375	0.0250	0.0125	0.000
diphenylamine reagent (mL)	1.95	1.95	1.95	1.95	1.95

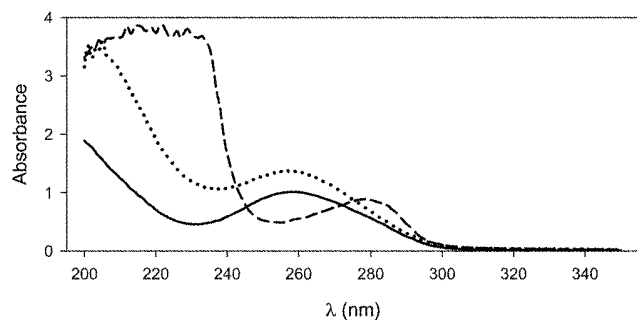


FIG 1

Representative student scans of pure DNA, RNA, and BSA. Spectra for DNA (—) and RNA (...) have similar shapes and  $\lambda_{max}$ s, making it difficult to distinguish them by UV scan alone. As a protein, the BSA (---) spectrum has a distinct shape.

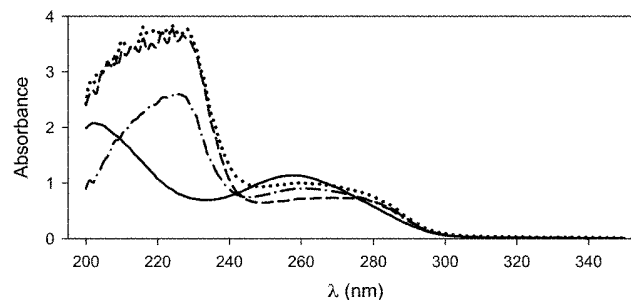


FIG 2

Representative student scans for mixtures of DNA, RNA, and BSA. The DNA/RNA spectrum (—) has a shape that is indistinguishable from those for the pure DNA and pure RNA. By contrast, the DNA/BSA (---), RNA/BSA (...), and DNA/RNA/BSA(- · -) spectra all exhibit the influence of an increased absorbance at 280 nm compared with the pure nucleic acids.

average of at least three values. The relative standard deviation must be less than 10%.

#### Evaluation of Student Performance

In this junior-level writing-intensive class, students are asked to write a full lab report as a group, often for the first time. To aid in writing, they are provided with a set of points for discussion and a template for formatting. They must thoroughly analyze the results with an emphasis on comparing and contrasting the evidence. Rough drafts are required. Reports are graded. Grammar and construction are also evaluated. The grade leans heavily on interpretation of the results.

### Assessment of Student Learning

A major recent initiative at institutions of higher education has been an assessment of whether learning has occurred. Therefore we developed an instrument to measure this. Students are given the following questions as a pre-lab quiz:

1. In Beer's Law, for what does the "l" stand?
2. What is the extinction coefficient for this assay? (given a plot with linear regression and units)
3. Your group determined the extinction coefficients for the Bial Assay in mL/mg cm. What conclusions may be drawn?
4. A sample of DNA has an  $A_{260}/A_{280}$  of 2.2. What does this indicate?
5. What can be said about the different assays (Dische, Bial's, and Bradford) you will/have performed?
6. For which assay is this a standard curve? (given a plot with linear regression and units)
7. For which solution is the indicated spectrum a scan?
8. The presence of protein would cause the experimental  $A_{260}$  of a presumed DNA solution to be \_\_\_\_, thus \_\_\_\_ the true DNA concentration.

The questions were given again as a post-lab quiz. The outcome of each question was analyzed.

### Student Results and Instructor Observations

#### UV Analysis with Known Solutions

Students first acquire and analyze the spectra of pure DNA, RNA and BSA solutions and four different mixtures of these solutions. Figures 1 and 2, respectively, show student-generated representative 200–350 nm scans for the individual pure biomolecules and mixtures. Students observed that spectra for pure DNA and RNA had similar shapes, making it difficult to distinguish them by UV scan alone. The spectrum for BSA, as a protein, had a different shape with a distinguishing  $\lambda_{max}$  at 280 nm and broader peak at low UV wavelengths.

After noting the effect that mixing the biomolecules had on the shape of the curves of the pure biomolecules, the students determined the extinction coefficients,  $k$ , at 260 nm and 280 nm. Typical average results are shown in Table 5. Students compared their calculated theoretical absorbance at 260 nm and 280 nm from their extinction coefficients with their experimental values. The  $A_{260}/A_{280}$  absorbance ratios were also compared to expected ranges [22–24] for pure and mixed solutions. They indicated that the ratios could be used for a qualitative prediction of biomolecular content. Students used the Warburg-Christian Equation [15] to estimate the protein concentration in the pure and mixed samples. Finally, pure solutions were heated to observe changes in absorbance due to hyperchromatic shift.

#### UV Analysis-Points for Instructors

The most common error in determining single-point extinction coefficients for pure solutions was that many forget the

TABLE 5

UV extinction coefficients for pure DNA, RNA, and BSA

	DNA		RNA		BSA	
	260 nm	280 nm	260 nm	280 nm	260 nm	280 nm
$k$ (mL mg <sup>-1</sup> cm <sup>-1</sup> )	19.4	10.4	23.4	10.8	0.409	0.662
SEM <sup>a</sup>	0.1	0.2	0.1	0.2	0.003	0.004
Range	19.4–19.9	9.90–11.1	23.2–24.0	10.3–11.8	0.397–0.421	0.648–0.680

<sup>a</sup> SEM, standard error of the mean,  $n = 6$ .

dilution factor for the concentration component of the  $A = kcl$  equation. They experienced the same issue with the Warburg-Christian Equation. For example, a 1:20 dilution is typical to get the spectrum on scale but students would forget to divide the given concentration by 20 in the calculation. Once they corrected for this, the experimental and theoretical results were extremely close.

A second common error that occurs was incorrect cuvette selection for a particular analysis. Quartz cuvettes are used for UV analyses and glass cuvettes are used for colorimetric analyses. In the week prior to this lab, students observe the spectroscopic properties of glass versus quartz cuvettes to determine the optimal wavelength ranges for each cuvette. Student then apply that knowledge for this lab in choosing the correct cuvette. This lesson also serves to teach students to consider the cost when choosing analytical methods, as quartz cuvettes are easily stained by the products of colorimetric assays and are more expensive than glass cuvettes. Although this concept is continually reinforced during lab, the most common mistake is using a glass cuvette for UV analysis; in which case an indiscernible spectra results. This mistake is generally remedied after the first instance and is not a problem thereafter.

Students readily discerned the effect that mixing the biomolecules has on the absorbance ratios for the mixtures

when compared to the pure biomolecules. However, many students had trouble explaining why the Warburg-Christian equation gave a lower than expected result for the [BSA] even though the basis for this equation (absorbance at 280 nm due to number of aromatic amino acid residues present in the protein) was described in the introduction section of their lab manual.

When analyzing the effect of temperature, the greater hyperchromatic shift, and the reason for it, for DNA versus RNA was easily understood by the students. However, some students mistook the light scattering effect for the denatured BSA as a hyperchromatic shift. Overall, many students realized that data from UV determinations alone may not be the best way to determine the composition and concentration of a biomolecule mixture.

### Colorimetric Analysis with Known Solutions

After performing the Dische, Bial, and Bradford colorimetric assays with separate DNA, RNA, and BSA of known concentrations, the students constructed standard curves and determined the extinction coefficients,  $k$ , from the slopes or from a single absorbance and algebraic solution to Beer's Law. From this experiment the student were able to evaluate the interference, or lack of, of each biomolecule within an assay. The results obtained by the students are shown in Table 6.

TABLE 6

Extinction coefficients for DNA, RNA, and BSA in colorimetric assays

Biomolecule	Dische	Bial		Bradford	
	DNA	RNA	DNA	BSA	DNA
Wavelength (nm)	595	660	660	595	595
$K$ (mg <sup>-1</sup> cm <sup>-1</sup> )	0.800	3.47	0.443	8.97	0.872
SEM <sup>a</sup>	0.018	0.070	0.042	0.38	0.098
Range	0.688–0.866	3.04–3.77	0.102–0.574	7.40–11.07	0.280–1.52

<sup>a</sup> SEM, standard error of the mean,  $n = 6$ .



TABLE 7

*Student-determined concentrations of DNA, RNA, and BSA in an instructor-made unknown bio-mix solution*

	DNA	RNA	BSA
Concentration (mg mL <sup>-1</sup> )	0.195	0.198	0.31
SEM <sup>a</sup>	0.013	0.013	0.031
Range	0.150–0.299	0.125–0.270	0.230–0.592
% error	2.5	1.0	22.5

<sup>a</sup> SEM, standard error of the mean, n = 6.

They found that neither RNA nor protein interfere with the Diche assay. DNA produces some color in the Bial assay, but with only 1/8<sup>th</sup> the sensitivity. They further discovered that DNA exhibits a response in the Bradford assay at about 1/10<sup>th</sup> of the sensitivity exhibited with BSA.

### Colorimetric Analysis-Points for Instructors

The greatest variability of results, as denoted by the Standard Error of the Mean (SEM) and Range in Table 6, occurred with the Bradford assay. We believe that this is due to the students' lack of experience in manipulating microliter quantities with the digital micropipets. We have observed that, as the semester proceeds, their overall technique improves dramatically.

The Bradford standard curve has proven to be problematic over the years. As the Bradford assay is extremely sensitive for protein, we believe that the major source of variability is due to contaminated glassware when generating the standards. We have given extensive guidelines for the proper preparation of glassware for use but have found that students do not initially follow them. This provides an excellent learning opportunity. When students with insufficient coefficients of determination (R<sup>2</sup> values) have to repeat preparation of the samples, some groups need three or four attempts; they find that it would be more efficient to clean the glassware properly the first time. When warned of sensitive assays later in the semester, the same students take more care in glassware preparation.

During the initial phase of developing this experiment, results obtained by students for a single sample of DNA in the Bradford Assay were inconsistent, at best. There were no previously published results to indicate that nucleic acids should have an interaction and so we suspected protein-contaminated glassware. The experiment was amended to include a DNA interference curve. Student-generated data remained scattered but, when averaged, the slope was consistent. In their recently-published study,

Wenrich, *et al.* [25] found that DNA does bind Coomassie Blue G-250 in the Bradford Assay. We now know to warn the students about careful cleaning of test tubes and cuvettes. At least one group per lab has to repeat the experiment multiple times before they take the cleaning seriously. Student-generated results have become much more consistent.

### Analysis of Instructor-Made Unknown Solutions

After completing all of the tasks associated with the known solutions, the students were given an unknown solution containing 0.20 mg/mL DNA, 0.20 mg/mL RNA and 0.40 mg/mL BSA. They were not told that they all had the same unknown as each unknown given had a separate number. The students were asked to think through the problem. The hope was that students would conduct a UV scan for comparison, and calculate absorbance ratios, to get a rough idea of the content. Typically, they needed some prodding. One bit of advice that we offered was how to establish an approximate upper concentration for each biomolecule. They were advised to take the 260 nm and 280 nm absorbance readings and use those values, appropriate extinction coefficients, and any dilution factors to calculate a concentration assuming that the absorbance was due to just a single biomolecule. Thus, they had an upper concentration value for DNA, RNA and BSA. This then allowed them to determine if dilutions were necessary to establish appropriate volumes to use in the colorimetric assays. After obtaining the data the students made the appropriate calculations using the concept of Equation 2 to determine the concentrations of the biomolecules in their unknown. The results obtained by the students are shown in Table 7.

### Analysis of Bovine Thymus DNA Isolates

Alternative to the instructor making the unknown, students can analyze a true unknown. Isolation of DNA from bovine thymus gland was completed during period 2 in parallel with finishing work on the standards. The students were asked to consider how to characterize the isolate using the tools they developed with the standards. The hope was that

TABLE 8

*Student-determined concentrations of DNA, RNA, and BSA in bovine DNA isolates*

	DNA	RNA	BSA
Concentration (mg mL <sup>-1</sup> )	0.57	0.016	0.032
SEM <sup>a</sup>	0.148	0.008	0.026
Range	0.183–1.87	0–0.059	0–0.290

<sup>a</sup> SEM, standard error of the mean, n = 11.

TABLE 9

Student learning assessment quiz: % responding correctly

	Pre-Lab	Post-Lab	$\chi^{2a}$
1. In Beer's Law, for what does the "l" stand?	75.8%	90.9%	Yes
2. What is the extinction coefficient for this assay? (given a plot with linear regression and units)	45.5%	100.0%	Yes
3. Your group determined the extinction coefficients for the Bial Assay in mL/mg cm. What conclusions may be drawn?	45.5%	66.7%	Yes
4. A sample of DNA has an $A_{260}/A_{280}$ of 2.2. What does this indicate?	36.4%	57.6%	Yes
5. What can be said about the different assays (Dische, Bial's, and Bradford) you will/have performed?	81.8%	97.0%	Yes
6. For which assay is this a standard curve? (given a plot with linear regression and units)	33.3%	97.0%	Yes
7. For which solution is the indicated spectrum a scan?	36.4%	78.8%	Yes
8. The presence of protein would cause the experimental $A_{260}$ of a presumed DNA solution to be ____, thus ____ the true DNA concentration.	57.6%	48.5%	No
Cumulative results	51.5%	79.5%	Yes

<sup>a</sup>  $\chi^2$  Analysis for significant increase. One-tail Analysis,  $p \leq 0.05$ ,  $n = 34$ .

students would conduct a UV scan for comparison, and calculate absorbance ratios, to get a rough idea of the content. Typically, they needed some prodding. Before period 3, students obtained a UV scan of their isolate. Students then devised a procedure, with instructor advice as stated in the preceding section.

Results varied, depending on the selection of tissue and the care students took during the isolation procedure. Results from one semester are shown in Table 8. In contrast to the instructor-made unknown, there was no real "answer." Students were evaluated solely on discussion, with a thorough "compare and contrast" of UV and colorimetric results especially with respect to the concept of purity of the isolation. <-- what is the "concept of purity of isolation". They were also required to discuss the effectiveness of incubation with proteinase K and RNase A.

### Analysis of Unknowns-Points for Instructors

The analysis for DNA is straightforward as neither RNA nor BSA interferes with the Dische assay. However, DNA interferes with both the Bial and Bradford assays and thus the astute student realized that the absorbance due to the DNA must be factored out due to Beer's Law consideration as seen in the following equations (Eq. 3):

$$A_{\text{RNA}} = A_{\text{total}} - A_{\text{DNA}} \text{ or } A_{\text{BSA}} = A_{\text{total}} - A_{\text{DNA}} \quad (3)$$

Two common problems have arisen with the analysis of the Bial and Bradford data by the students.

1. The first involves the improper use of dilutions which was mentioned previously for the UV analysis. If they make a dilution for the Bial assay, they forget to apply the dilution factor to the concentration of the DNA when accounting for its interference in the assay. Typically the students will substitute the [DNA] obtained from the Dische assay thus leading to an underestimation of the [RNA].
2. The second involves the improper use of extinction coefficients. The students have a tendency to use the Dische extinction coefficient for DNA instead of the Bial or Bradford extinction coefficient for DNA when determining the DNA absorbance contribution for those assays.

As evident from Table 7, the students on average obtain very good results for DNA and RNA. Over the years the [BSA] has always been low. At first we attributed this to the BSA adhering to the glassware. For several semesters, the experiment was performed in plasticware except for the final absorbance readings done in glass cuvettes with no discernible difference in the BSA results. It appears that perhaps some other factors are at play as noted previously.

### Assessment of Student Learning

This laboratory exercise was conducted with hundreds of students, over a number of semesters, before formal





assessment was put in place. During that time, informal assessment occurred through lab reports and lecture exam questions. The instructors were able to glean from the reports which concepts were eluding students. Changes to the pedagogical approach were made in response to the outcome of the informal assessment. For example, we initially included in the introduction UV scans of the pure biomolecules. When the reports indicated that students used the scans for matching to the “right answer” instead of correlating features in the scans with functional groups, the scans were removed and questions were added to points for discussion. The students are now guided towards an analysis of the scans, as is appropriate for students at the junior level.

Formal assessment is now under development in all programs at our university. Toward this effort, we devised an assessment quiz for this laboratory exercise. The quiz was administered to 34 students in two sections of Biochemistry 1. Results from the assessment quiz are illustrated in Table 9. In all but one of the questions, there was a statistically significant increase in correct answers. Students in this sample and, we believe, overall have difficulty predicting results based upon previous experience, as illustrated by no significant change in the percent of correct answers for question 8: “The presence of protein would cause the experimental A260 of a presumed DNA solution to be \_\_\_\_, thus \_\_\_\_ the true DNA concentration.” Given this result, more points for discussion will be added to the lab report that asks students to make predictions. For example, “What would protein contamination do the A260 of your DNA isolate? How would the measured DNA concentration compare to the true concentration?”

Significant increases in percent correct occurred with all other questions in the assessment. The greatest increases occurred with questions 2, 5, and 6, all questions assessing graphical analysis. It is clear that skills involving interpretation of graphs have developed through the experiment. It has been often lamented by our STEM colleagues that students possess insufficient graphical analysis skills, and so we are very pleased with this outcome. We continue to develop and employ formal assessment.

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