University of Toronto

Faculty of Applied Science and Engineering

BME440 Biomedical Engineering Technology and Investigation

Lab 1: Pipetting Techniques & Spectrophotometric Assay

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1.0 Introduction & scientific objective

DNA and proteins are the essential elements for cell function and therefore they provide critical information about the cell to biologists. Since DNA and proteins are quite small molecules, measured in nm, spectroscopy-based analysis methods that are based on observing their light absorbance and emissions, are often used to determine their presence and concentration in a sample. However, measuring the direct absorbance is not sufficient to determine the concentration of proteins and DNA in many scenarios, therefore adding secondary substances that have high specificity to the molecule of interest, are necessary to amplify their absorbance[1].

The objective of this lab is to determine DNA and protein (BSA) concentrations in an unknown sample. This is done by using BCA Assay to amplify the protein signal and measuring the absorbance, and using SYBR Green Assay to amplify the DNA signal and measuring the fluorescence of the sample. Through our experiments, we expect to observe an absorbance plot that is an average of DNA and BSA absorbance spectra, to determine protein concentration using BCA Assay and to determine DNA concentration by using SYBR Green Assay to enable us to calculate the concentrations of DNA and protein in the unknown sample.

2.0 Materials and Methods

In Part A of this lab, we used Serological and Single-Channel pipettes to transfer liquids such as water, ethanol and Tween-20.

In Part B, Task 1, to determine the DNA and protein content, we transferred solutions into a Nanoquant plate and took it to an absorbance plate reader to measure the spectrum at each point. In Task 2, to determine the protein content, we prepared dilutions and added BCA to each sample and placed them in a 60° water bath for an hour. We then added the samples to a 96 well plate and measured the absorbance. In Task 3, to determine DNA content of unknown samples, we added SYBR Green 2X stock to each dilution and transferred them into a 96 well plate and measured the fluorescence. For detailed information on materials and methods, please refer to the lab manual.

3.0 Results

1. Provide absorbance plot of DNA standard, protein standard and unknown sample. Choose x-axis and y-axis limits appropriately to show the peaks in each case. (See Figure 3 as an example)

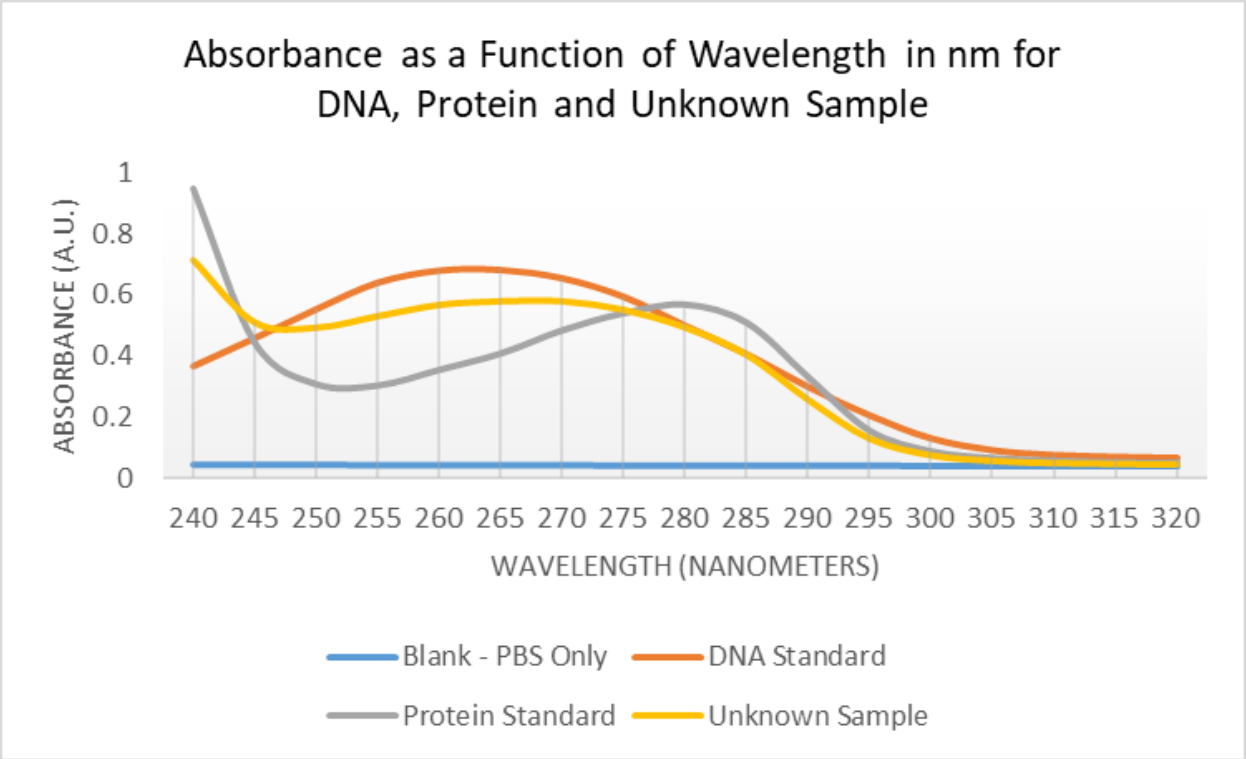

Figure 1: Direct absorbance spectrum plot of DNA standard, protein standard and unknown sample for the 240-320 nm wavelength range. This plot was generated based on the direct absorbance plate readings of the Nanoquant plate.

3.1 Graphs & Collected Data

Plots of all collected data points in Tasks 1, 2 and 3 are provided in the figures below. Tables that include the raw measurements are also included in this section.

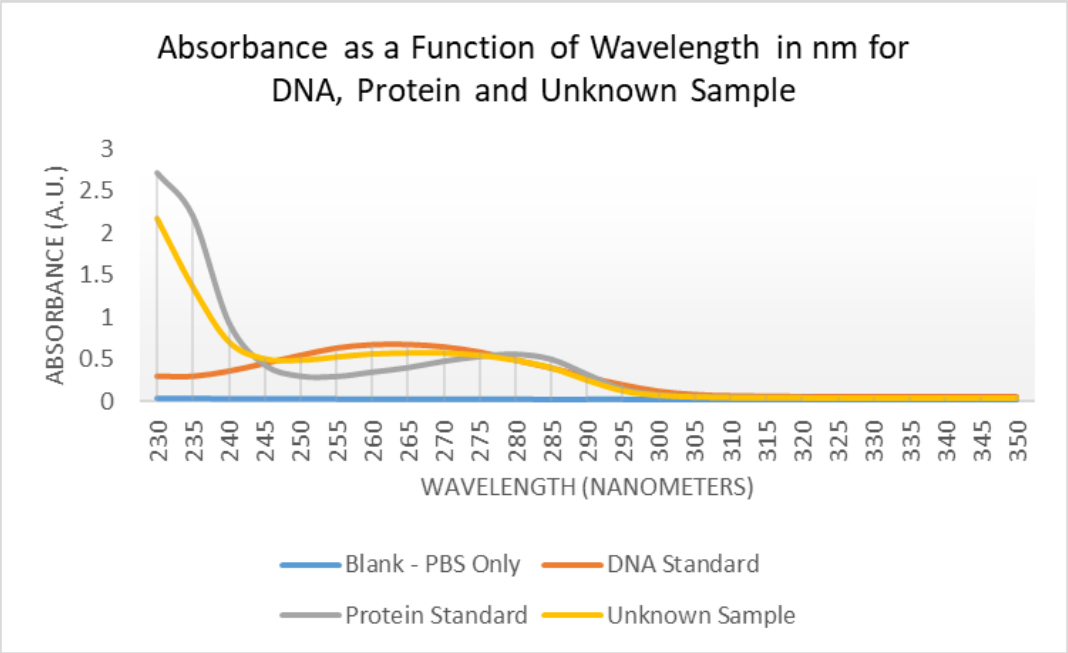

Figure 2: Direct absorbance spectrum plot of DNA standard, protein standard and unknown sample which includes all collected data points in the 230-350 nm wavelength range.

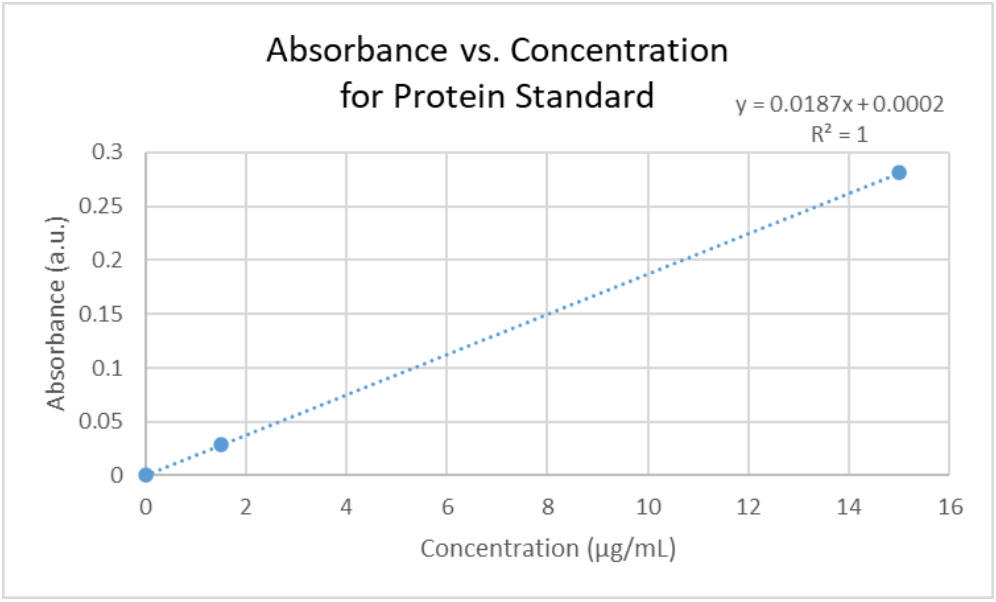

Figure 3: BCA Assay - Standard curve of absorbance vs. concentration for the protein standard sample.

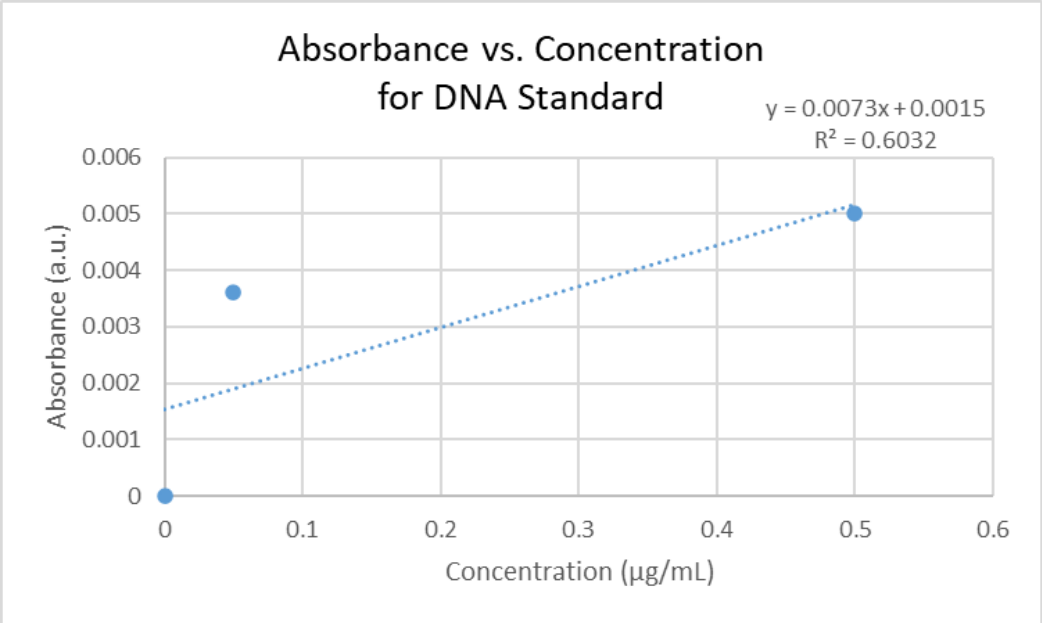

Figure 4: BCA Assay - Standard curve of absorbance vs. concentration for the DNA standard sample.

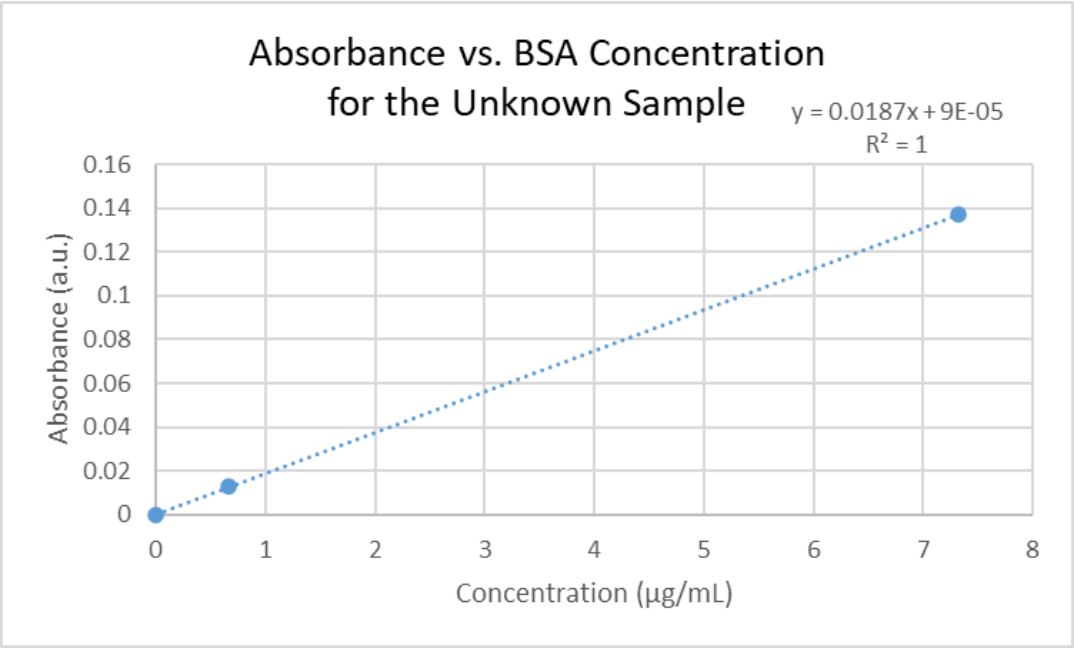

Figure 5: BCA Assay - Standard curve of absorbance vs. BSA concentration for the unknown sample. Concentration values are based on the calculated values in Table 3.

Table 1: BCA Assay - Absorbance measurements of the protein standard sample with respect to concentrations.

Dilution Factor	*Protein Standard Concentration (µg/mL)*	*Raw Absorption Values Measured at 562 nm Wavelength*	*Background Corrected Absorption Values Measured at 562 nm Wavelength*
0.001 X	*15*	*0.3916*	*0.2812*
0.0001 X	*1.5*	*0.1438*	*0.0286*
0	*0*	*0.1104*	*0*

Table 2: BCA Assay - Absorbance measurements of the DNA standard sample with respect to concentrations.

Dilution Factor	*DNA Concentration (µg/mL)*	*Raw Absorption Values Measured at 562 nm Wavelength*	*Background Corrected Absorption Values Measured at 562 nm Wavelength*
0.001 X	*0.5*	*0.1154*	*0.005*
0.0001 X	*0.05*	*0.114*	*0.0036*
0	*0*	*0.1104*	*0*

Table 3: BCA Assay - Absorbance measurements of the unknown sample with respect to calculated diluted concentrations of BSA.

Dilution Factor	*Calculated BSA Concentration in Unknown Sample (µg/mL)*	*Raw Absorption Values Measured at 562 nm Wavelength*	*Background Corrected Absorption Values Measured at 562 nm Wavelength*
0.001 X	*7.32086*	*0.2475*	*0.1371*
0.0001 X	*0.66845*	*0.1231*	*0.0127*
0	*0*	*0.1104*	*0*

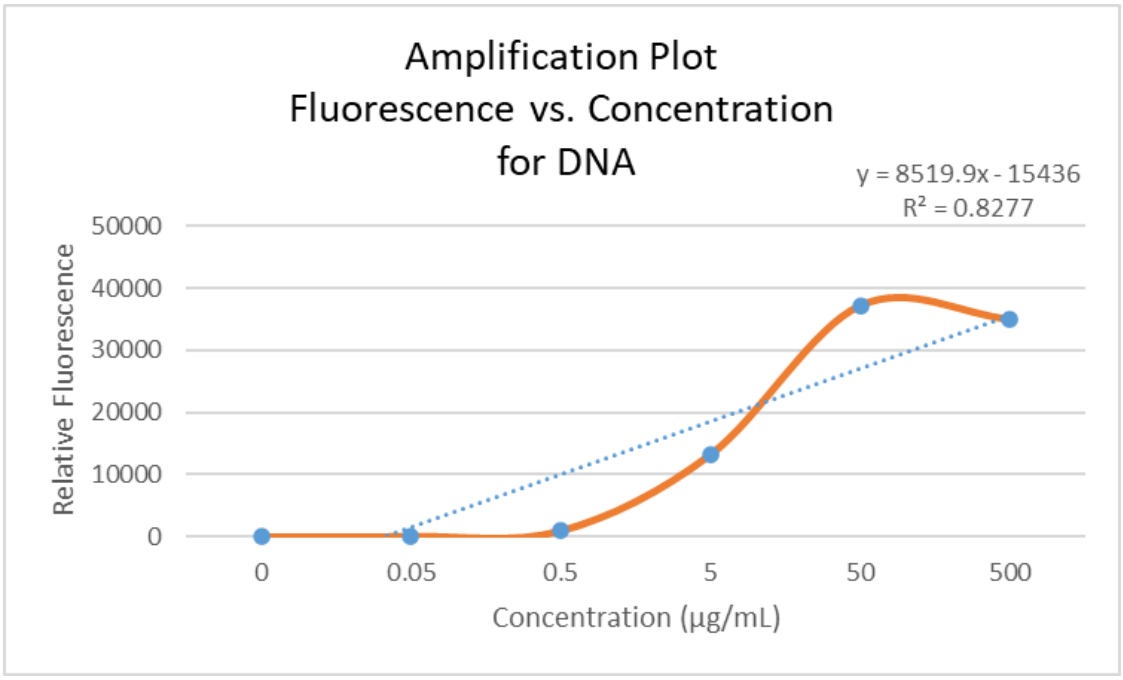

Figure 6: SYBR Green Assay - Amplification plot of fluorescence vs. concentration for the DNA sample.

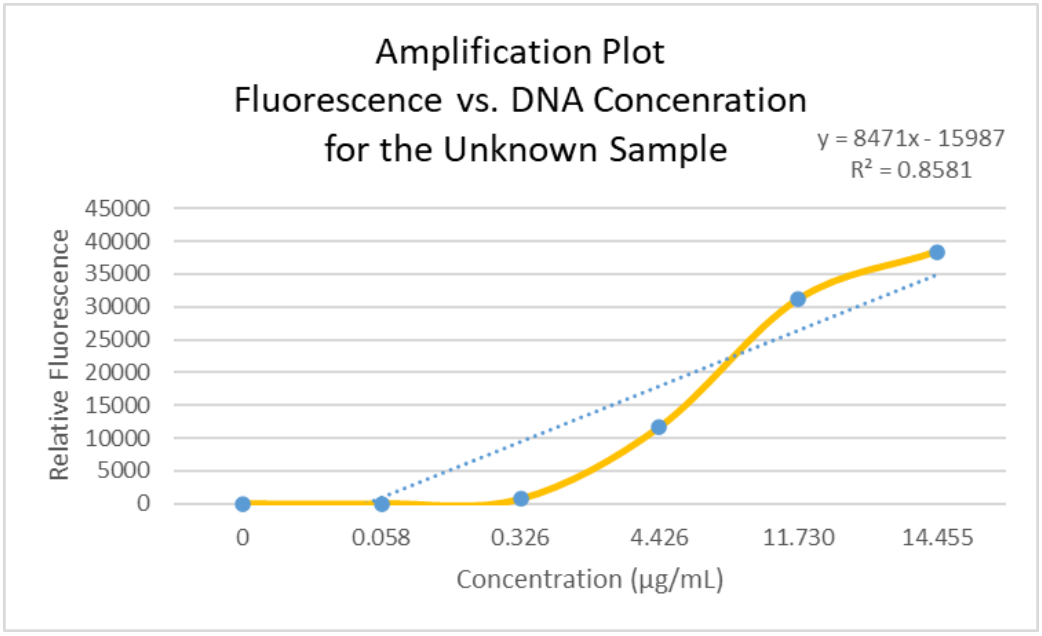

Figure 7: SYBR Green Assay - Amplification plot of fluorescence vs. concentration for the unknown sample.

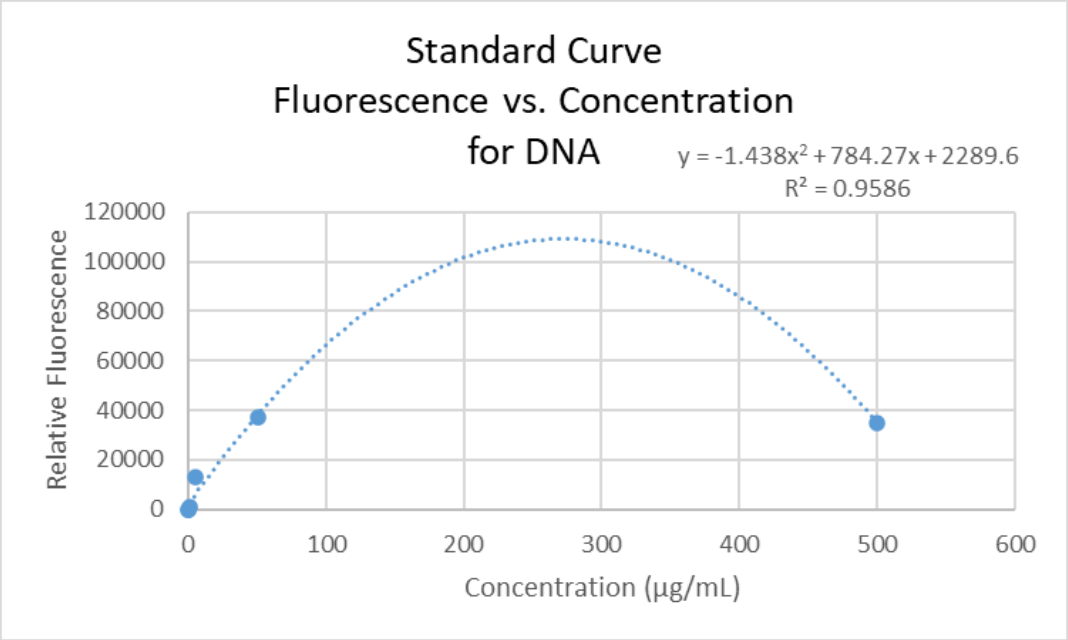

Figure 8: SYBR Green Assay - The Standard curve of fluorescence vs. concentration for the DNA sample.

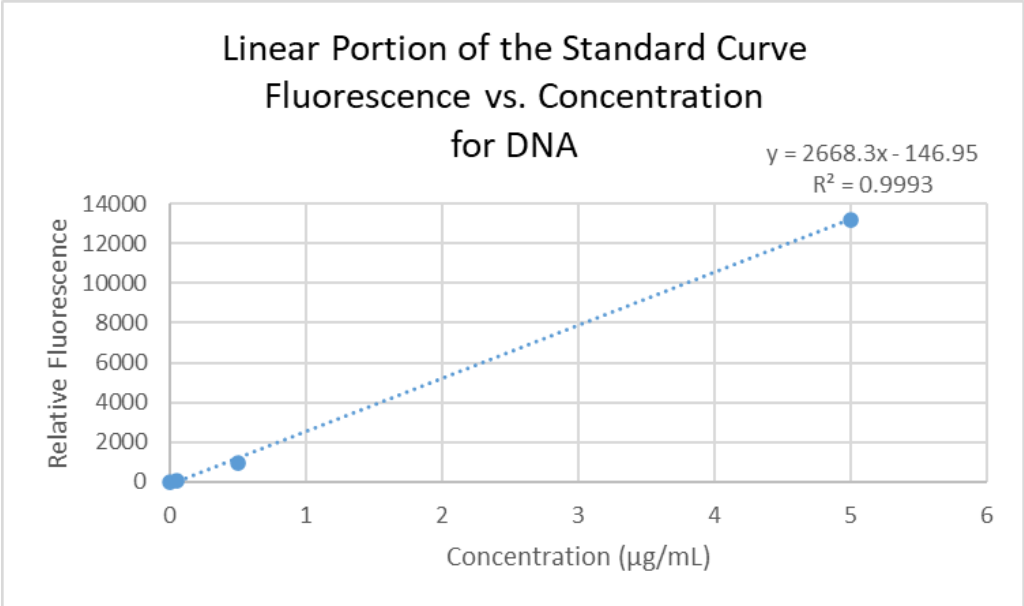

Figure 9: SYBR Green Assay - Linear portion (from 0 to 5 µg/mL concentration) of the standard curve of fluorescence vs. concentration for the DNA sample.

Table 4: SYBR Green Assay - Fluorescence measurements of the DNA standard sample with respect to diluted concentrations.

Dilution Factor	*DNA Concentration (µg/mL)*	*Raw Fluorescence Values*	*Background Corrected Absorption Values*
1 X	*500*	*35095*	*34927*
0.1 X	*50*	*37323*	*37155*
0.01 X	*5*	*13386*	*13218*
0.001 X	*0.5*	*1111*	*943*
0.0001 X	*0.05*	*228*	*60*
0	*0*	*168*	*0*

Table 5: SYBR Green Assay - Fluorescence measurements of the unknown sample with respect to calculated diluted DNA concentrations.

Dilution Factor	*DNA Concentration (µg/mL)*	*Raw Fluorescence Values*	*Background Corrected Absorption Values*
1 X	*14.455*	*38590*	*38422*
0.1 X	*11.730*	*31321*	*31153*
0.01 X	*4.426*	*11832*	*11664*
0.001 X	*0.326*	*890*	*722*
0.0001 X	*0.058*	*177*	*9*
0	*0*	*168*	*0*

4.0 Discussion

2. Determine the concentration of proteins and DNA in the unknown sample you were provided using the results from Task 1, 2 and 3. Do these match the values you were provided? Discuss for each case. (Include raw measurements)

In Task 1, as seen on Figure 1, we observe peaks in the absorbance plot for DNA standard at 260 nm, for protein (BSA) standard at 280 nm and for our unknown sample at 270 nm wavelength. This result confirms that the unknown sample contains both DNA and proteins. At 260 nm, the absorbance of the DNA sample is 0.681 and of the unknown sample is 0.567. Since we know the DNA concentration is 0.5 mg/mL, we can estimate the DNA concentration of the unknown sample to be $(0.567/0.681) * 0.5$mg/mL = 0.416 mg/mL, with a 66.4% error. Similarly for BSA, at 280 nm, we can estimate the BSA concentration to be (0.493/0.568) * 15 mg/mL = 12.9 mg/mL, with 72% error. Therefore we can conclude, since the error is quite high, that this method does not provide results with high accuracy when determining the value of concentrations.

In Task 2, using the Beer-Lambert Law, $A = \varepsilon cl$, and the standard curve of the BSA sample, given in *Figure 3*, we calculate the protein concentration of the unknown sample. To do so, we first remove the background signal by subtracting the absorbance measured by the blank (0.1104 a.u.) from each value (see Table 1). We then create a linear standard curve and using the equation of the line y=0.0187x+0.0002 from *Figure 3*, where y corresponds to A, 0.0187 to εl, and x to c from the Beer-Lambert Law, we calculate x for each value of background corrected absorbance measurement (see Table 3) of the unknown sample. By doing so, we get 0.66845 µg/mL for 0.0001X dilution and 7.32086 µg/mL for the 0.001X dilution. In conclusion, based on the absorbance of the sample with 0.001X dilution, we can approximate the concentration of the unknown sample (1X) to be 7.32 mg/mL. With a small error of 2.4%, our calculated value is quite close to the actual protein concentration of the unknown sample, 7.5 mg/mL.

In Task 3, we follow the same steps of background signal removal and plotting for the DNA sample. By plotting the linear portion (see *Figure 9*) of the standard curve (see *Figure 8*), we obtain the line equation of y=2668.3x-146.95. By plugging in the absorbance values obtained from the unknown sample, we get the concentrations given in *Table 5*. We take the concentration of the 0.001X dilution, since it is closer to the middle of the linear standard curve, which equals 0.326 µg/mL. Taking the dilution factor into account, we determine the DNA concentration in the unknown sample to be 0.326 mg/mL. We can consider this value to be close to our expected result despite %30.4 error, since the concentration is quite small and it's harder to measure to a high level of accuracy.

4.2 Post-Lab Questions

1. Define "saturation" and "dynamic range" in reference to biological assays. Comment on the dynamic range of the assays used in this lab.

Saturation is the state that is achieved when all of the binding sites of a biological assay are occupied and it can no longer bind to more molecules. Dynamic range is the range between lowest and highest concentrations where the output is measurable with precision and accuracy. In this lab, the dynamic range of the Micro BCA assay covers the 1.5-15000 µg/mL range since we can obtain high accuracy results within that range and as seen in *Figure 3,* as the plot is linear and does not saturate. However, the amplification plot of the DNA saturates after 50 µg/mL as seen in *Figure 6,* therefore we conclude that the dynamic range of the SYBR Green Assay covers 0.05-50 µg/mL.

2. Assume that the instrument noise prevents you from measuring absorbance of samples below 0.02 absorbance units. What are the limits of detection of absorbance for detection of DNA and proteins?

Using the Beer-Lambert Law $A = \varepsilon l c$ and the extinction coefficients of DNA and BSA given in the lab report, for 260 nm and 280 nm wavelengths, respectively:

$0.02 = 20(mg/ml)^{-1} cm^{-1} * 0.05\ \text{cm} * c_{(\text{LoD-DNA})}$

$0.02 = 0.66(mg/ml)^{-1} cm^{-1} * 0.05\ \text{cm} * c_{(\text{LoD -BSA})}$

The limit of detection (LoD) of DNA at 260 nm would be $\mathbf{c_{(LoD\text{-}DNA)} = 0.02\ mg/mL}$ and LoD of BSA at 280 nm would be $\mathbf{c_{(LoD\ -BSA)} = 0.606\ mg/mL}$, assuming that the lowest measurable absorbance is 0.02 units.

3. Why is Micro BCA specific for proteins over DNA?

Micro BCA is an assay that contains copper(II) sulfate. At 60(degree)C the peptide bonds in the proteins reduce to Cu(II)2+ ion to Cu(I)+, allowing the copper ion to bind to the BCA reagent to form a complex that has a very strong absorbance at 562 nm.[2] Since peptide bonds are not present in nucleic acids, BCA cannot bind to DNA, in contrast to proteins which are made of long amino acid chains linked by peptide bonds.

4. Why is SYBR Green specific for DNA over proteins?

SYBR Green interacts with the hydrophobic pocket in the center of the double-stranded DNA specifically, by binding to DNA bases through intercalation [3]. Since the double helix structure is specific to the DNA and proteins do not portray such well-established structure, SYBR Green does not bind to proteins.

5.0 References

1. Chan, W.; Syed, A.M; Hoang, T.G.; Poon, W; Fiddes, L; Mohamed, M., BME 440 – Lab 1: Pipetting techniques & Spectrophotometric assay, Lab I, University of Toronto, Department of Biomedical Engineering, 2023.

2. Smith, P. K.; Krohn, R. I.; Hermanson, G. T.; Mallia, A. K.; Gartner, F. H.; Provenzano, M. D.; Fujimoto, E. K.; Goeke, N. M.; Olson, B. J.; Klenk, D. C., Measurement of Protein Using Bicinchoninic Acid. Anal Biochem 1985, 150 (1), 76-85.

3. Zipper, H.; Brunner, H.; Bernhagen, J.; Vitzthum, F., Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications. Nucleic Acids Res 2004, 32 (12).