GETTING THE STAIN IN: A QUANTITATIVE ANALYSIS OF DNA AND RNA STAINING WITH VARIOUS DYES

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ABSTRACT

Staining and other methods of distinguishing between deoxyribonucleic acid and ribonucleic acid in polyacrylamide gels tend to be dangerous or expensive. In addition, retrieving quantitative data from DNA and RNA bands to determine linearity between absorbance and sample size of nucleic acid using fiber optic technology to date has not been established. In an effort to find the dyes that most effectively address these concerns, eight dyes were tested with single stranded DNA and RNA by polyacrylamide gel electrophoresis. The gels were then analyzed with fiber optic spectroscopy to determine which dyes best distinguished between DNA and RNA bands. Of the eight dyes, only Methylene Blue displayed significant differences in absorbency between bands of DNA and RNA. Methylene Blue, Cresyl Violet, Thionin, and Azure B were used to stain gels containing increasing concentrations of nucleic acids to determine if there was a linear relationship between amount of nucleic acid and absorbance. Based on the results of two runs for each stain, Methylene Blue and Azure B showed the greatest linearity in RNA up to 100 pmole. Results for staining of DNA were inconclusive and the other stains lacked clear linear relationships.

INTRODUCTION

Due to the ability, through transcription and translation, to code for proteins necessary for cell function, DNA and RNA are generally considered to be the building blocks of life, and their study has been vital to numerous influential discoveries. Both DNA and RNA are stabilized by sugar-phosphate backbones, the difference being that DNA has a deoxyribose sugar, while RNA has a ribose sugar attached to the repeating phosphate groups [5]. Attached by N-glycosidic linkages to these backbones are purine and pyrimidine bases, the order of which determines the function of that strand of DNA or RNA. Pairing of these bases to create complementary sequences leads to the formation of a double helix. Though DNA has a naturally double-stranded structure, for the purposes of polyacrylamide gel electrophoresis (PAGE), single-stranded DNA was used.

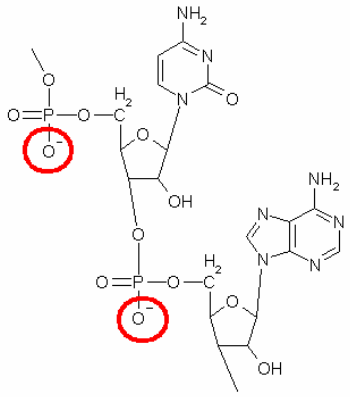


Fig. 1 Two Nucleotides of RNA with the negative charges of the phosphate groups highlighted

In PAGE, charged biological molecules are subjected to an external electrical field. For DNA and RNA separation, samples must be placed on the end of the gel closest to the cathode of the electrical apparatus, as both of these molecules carry negative charges due to the phosphate groups (Fig. 1). The opposite charges draw the molecules toward the positively charged bottom of the gel, causing the molecules to move down the gel [8]. Allowing for the separation of DNA and RNA is the

inverse relationship between the mobility of a molecule and molecular friction, which is the result of the molecule's size and shape. Polyacrylamide, a cross-linked polymer gel, is used to slow various molecules, depending on molecular size, thereby effectively separating the molecules. A tris buffer, glycine, ammonium persulfate, TEMED, and urea are other ingredients in the gel [24]. This gel's thermostability, transparency, strength, inert nature, and ability to withstand high voltage gradients are all advantages of using polyacrylamide.

For postelectrophoresis visualization, one of several techniques is needed to detect the various DNA/RNA bands. The first of these is radioactivity. Radioactivity is performed by producing DNA/RNA in an environment with radioactive phosphorus (32P), as opposed to stable phospohorus (31P). Afterwards, a phosphorimager may be used to analyze radioactivity in order to determine locations and properties of the bands in the gel. However, radioactivity analysis is comparatively expensive, requires a license, and is perceived as more dangerous than other methods. An alternative technique involves the use of specially prepared fluorescently labeled nucleic acids to detect fluorescence from the bands. Conversely, staining dyes are inexpensive and easy to use. Staining dyes bind to the nucleic acids rather than the gel by various methods, highlighting the bands in the gel (Fig. 2). Dyes are less sensitive, however, so larger samples are required. Furthermore, the most widely used dye, ethidium bromide, intercalates with nucleic acids, thereby having a mutagenic effect [8]. It is generally used to stain double-stranded DNA. Therefore, a safe and sensitive dye that retains accuracy is needed. Previous research has indicated that Cresyl Violet is effective, and other potential candidates are Methylene Blue, Azure A, Azure B, Azure C, Toluidine Blue O, Thionin, Nile Blue A, Brilliant Cresyl Blue, and Coomassie Brilliant Blue [20].

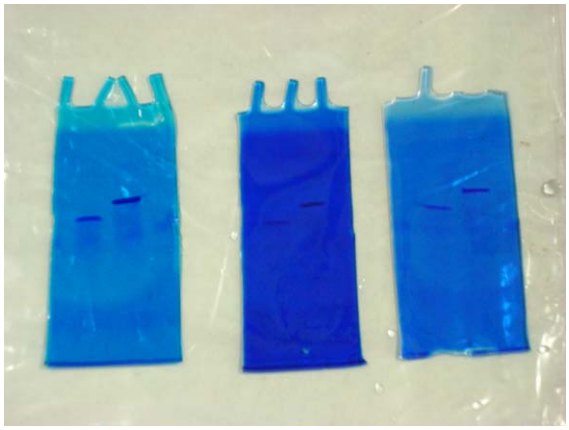


Fig. 2 Strips of Gel Stained with Methylene Blue, Azure A, and Azure C prior to destaining

Dyes

Cresyl Violet ($C_{18}H_{15}N_3O_3$) (Fig. 3a) has a chemical name of aminonaptho-aminophenoxazonium acetate and is used to stain neuronal tissues as it binds to the acidic portions of RNA containing ribosomes, nuclei, and nucleoli [14]. Cresyl violet acetate comes in green crystals and melts at 142 degrees Celsius.

Methylene Blue ($C_{16}H_{18}N_3SCl$) (Fig. 3b) is a dye that is commonly used to stain nuclei blue. It is a solid green powder that makes a blue solution when dissolved in water. It is strongly metachromatic causing it to appear as different colors when viewed under different wavelengths of light. This property allows for the demonstrations of mucins, cartilage, and mast cells.

Azure A ($C_{14}H_{14}N_3SCl$) (Fig. 3c), Azure B ($C_{15}H_{16}N_3SCl$) (Fig. 3d), and Azure C ($C_{13}H_{12}N_3SCl$) (Fig. 3e) are derived through the oxidation of methylene blue [8]. They bind ionically to the negatively charged phosphate groups of either DNA or RNA and thus can be used to stain both. Azure A and B are primarily used to make azure eosin stains for blood smear staining and all three Azure stains are metachromatic. These dyes are less sensitive than ethidium bromide and often heavily stain the gel. As a result, a lengthy destaining time is necessary. Azure B, however, has shown promise in staining DNA and RNA different colors [21].

Toluidine Blue O ($C_{15}H_{16}N_3SCl$) (Fig. 3f) is a metachromatic dye often used to demonstrate Nissl substance [2]. There is little documentation of the specificity of this dye but it is useful for the staining of mast cell granules and is also suitable for a variety of other histological studies. Moreover, past research [26] has indicated that under conditions of competitive staining with Mg^{2+}, Toluidine—and perhaps other thiazin and related dyes—tends to be highly metachromatic while staining RNA in histological samples, thereby providing a method to distinguish the two nucleic acids. The mechanism behind this is the Critical Electrolyte Concentration, the magnesium concentration at which metachromasy is suppressed in DNA (.2M - .3M). The difference in this value between DNA and RNA may provide a mechanism to distinguish the two.

Thionin ($C_{12}H_{10}N_3SCl$) (Fig. 3g) is a metachromatic dye useful in the staining of acid mucopolysaccharides. This common nuclear stain is often used to show Nissl substance in nerve cells of the central nervous system [25]. It stains acidic proteins and nucleic acids with the specificity determined by the pH of the final staining solution.

Nile Blue A ($C_{20}H_{20}N_3OCl$) (Fig. 3h) is used to stain the nuclei of cells a blue color. However, if Nile Blue A is boiled with dilute sulfuric acid, it forms Nile Red, which is a red-staining lysochrome. This conversion occurs as oxygen replaces an amino group. Nile Blue stains lipids red. Nile Blue A and Nile Red are useful in histology due to their ability to stain these specific substances in cells blue or red.

Brilliant Cresyl Blue (Fig. 3i) is normally used for the staining of microscopic reticulocytes, juvenile red blood cells, in peripheral venous blood. When used for this purpose, Brilliant Cresyl Blue reacts with basophilic ribonucleoproteins in reticulocytes; younger reticulocytes have higher amounts of basophilic ribonucleoproteins, thus staining darker [22]. This dye has been shown to stain both DNA and RNA through ionic binding to these molecules [4].

Coomassie Brilliant Blue R-250 (R for “reddish” and 250 for dye strength) (Fig. 3j) is a dye traditionally used for protein staining [6]. Through staining and destaining with PAGE, protein bands can

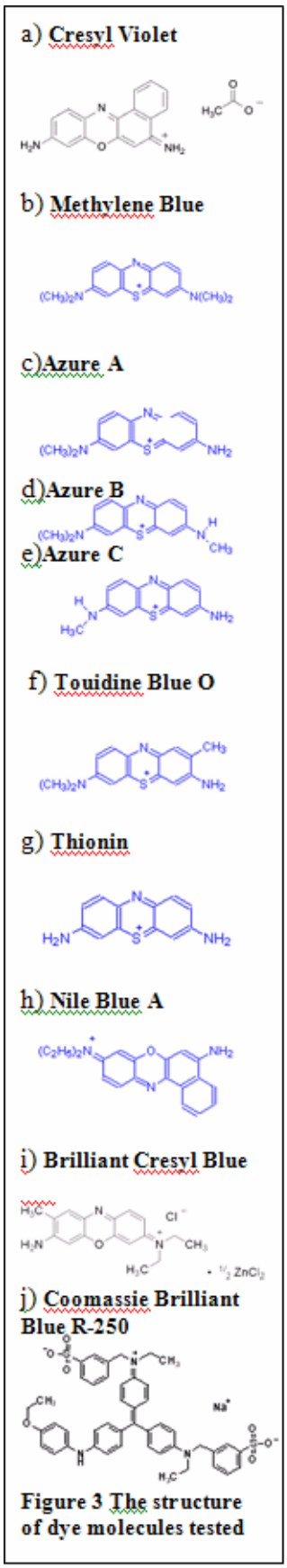


Figure 3 The structure of dye molecules tested

be visualized. Advantages to Coomassie Brilliant Blue R-250 are low cost and high visibility. Coomassie Brilliant Blue R-250 is not normally used for staining RNA and DNA, therefore its efficiency in doing so was tested in this experiment.

In order to accurately and efficiently determine whether the amount of DNA or RNA loaded into the wells (Fig. 4) affects absorbance in a quantitative and preferably linear nature, a method of in-situ (in-gel observation) must be developed. Fiber-optic probes were utilized for this method and through the use of a sliding microscope stage, the absorbance of the DNA and RNA could be measured in real time. A plot of absorbance, as determined by Beer's Law, versus amount of DNA and RNA loaded can be plotted to determine if the quantity of DNA and absorbance have a linear relationship. UV-Vis spectroscopy was used to provide a quantitative value establishing the difference in staining in addition to a potential qualitative difference in color. Polarizing filters crossed at about 85 degrees cut out 80% of the light, reducing background noise without affecting wavelength data. The stain cresyl violet has shown promising results in previous trials, with a nearly linear relationship between absorbance and DNA/RNA sample size [20]. A second trial of cresyl violet was run, in addition to the other dyes, in order to validate previous data.

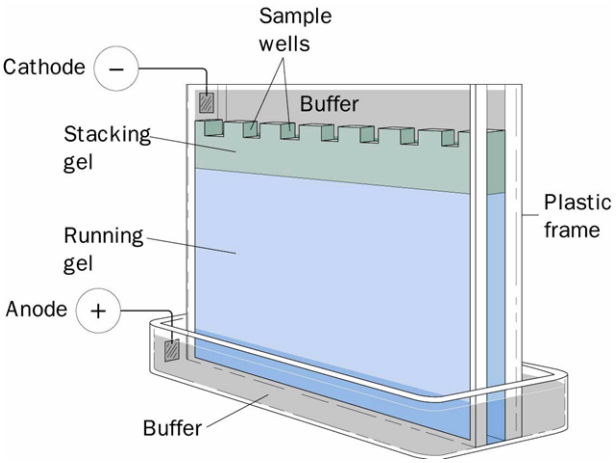


Fig. 4 Polyacrylamide Gel Electrophoresis apparatus

This research investigated small dye molecules and how they interact with nucleic acid molecules to find a dye that would be effective at staining single-stranded DNA while being non-mutagenic, less costly, and safe for common use. However, for a dye to be considered effective, it was required to have different levels of absorbance based on the sample size of DNA or RNA. Finally, the ideal dye needed to show a distinct color difference when staining DNA as opposed to RNA. This variation in color assists in analysis of results. Finding an effective dye with the aforementioned properties has the potential to make DNA and RNA staining more widely available and efficient.

MATERIALS AND METHODS

Table 1 explains the procedures used to formulate each dye. Listed are the concentration of the dye powder used in each solution along with the solvent this was dissolved into. If the solvent was a buffer, the pH necessary is noted. In addition, the stain times are included, but all dyes were destained in water for approximately six hours unless otherwise noted so this information is not specific to each dye. Finally, the wavelength at which the bands were viewed using spectroscopy is available for the four dyes used to stain full gels.

Table 1

Dye	Concentration	Solvent	pH	Stain Time (min)	Wavelength DNA/RNA (nm)
Methylene Blue	0.025%	Water	N/A	10	671/601 and 672
Azure A	0.08%	20% ethanol	N/A	4	N/A
Azure B	0.025%	0.1M Sodium Citrate	4.0	15	601/601
Azure C	0.04%	Water	N/A	4	N/A
Brilliant Cresyl Blue	0.04%	20% ethanol	N/A	5	N/A
Coomassie Blue *	0.2%	7.5% acetic acid 50% ethanol	N/A	5	N/A
Nile Blue A	0.0015%	20% methanol	N/A	15	N/A
Thionin	0.764%	20% 1M Acetic Acid 3.6% NaOH	4.0	5	560/560
Cresyl Violet	0.13%	0.01M Acetate	3.6	30	560/560
Toluidine Blue O 1)	0.03%	0.01M Acetate	N/A	5	N/A
2)	0.03%	0.01M Acetate	3.6	5	N/A
3)	0.03%	0.01M Acetate, 0.25M $MgCl_2$	3.6	5	N/A
4)	0.025%	0.1M Sodium Citrate	3.2	5	N/A
5)	0.025%	0.1M Sodium Citrate, 0.2M $MgCl_2$	3.2	5	N/A

* Coomassie Blue destained in 50% methanol and 10% acetic acid

Preliminary Running of Gels and Staining

Preliminary staining involved all fourteen dye formulations, including one solution of each gel and four additional solutions of Toluidine Blue O. A 0.045% solution of DNA and a 0.035% solution of RNA were created, both of which using bromophenol blue as the loading dye, the solution used to load the wells and track the running of the gel. Next, each solution was loaded into the wells of the gel used for PAGE with separate wells for RNA and DNA. Loading dye was used as a spacer between the paired lanes of RNA and DNA so that the gel could be cut into strips without damaging the DNA or RNA. Once PAGE was completed at 180V for 35 minutes, the gel strips were stained and destained according to the formulations in Table 1. These gels were placed into appropriate solutions and rocked for the times listed in Table 1. Destaining removed excess dye from the background to enhance the clarity of the DNA/RNA bands. After removal from the destaining solutions, the gels were scanned by a professional grade scanner in order to save images of the gels for further analysis.

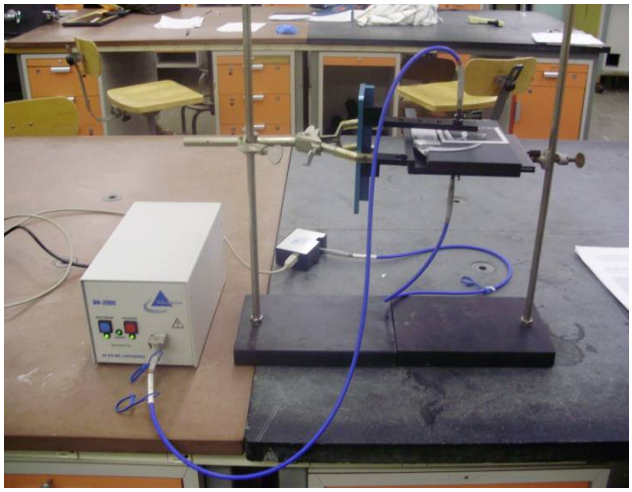
Quantitative Analysis

DNA and RNA amounts used in the first trial with full gels were as follows: 25, 50, 75, 100, 150, 200, and 250 picomoles. Because RNA runs faster than DNA during PAGE, solutions

holding both RNA and DNA for a given picomole value were made in order to test all quantities of both DNA and RNA in one gel per dye. The four dyes chosen for further testing were Methylene Blue, Cresyl Violet, Thionin, and Azure B. All dyes were scanned after approximately six hours, but Azure B was left to destain overnight, causing faded results.

The second quantitative trial included concentrations of DNA and RNA for trial one as follows: 10, 25, 50, 75, 100, 150, and 200 picomoles. The decision to decrease the picomole values at the lower end of the testing spectrum was due to the clarity of the 25 pm bands in the first trial. The purpose of repeated testing was to show that the results from trial one were reproducible.

Fiber Optic Spectroscopy



Fiber Optic Spectroscopy was performed using a Halogen light source connected to the positioner on top of the microscope stage, while the detector attached on the bottom (Fig. 5). The gels, resting between two glass plates, were placed on the microscope stage under the light source with a polarizing filter above the detector. Readings from the detector were directly transferred to a computer and analyzed using Ocean Optics® SpectraSuite™ software.

Fig. 5 The fiber optic spectroscopy apparatus

RESULTS

Preliminary Gels

Fig. 6 Partial Gel stained with Azure A

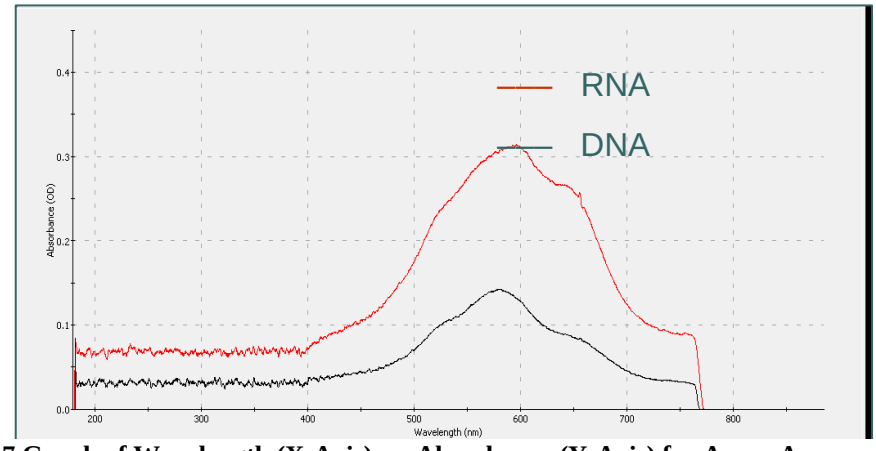


Fig. 7 Graph of Wavelength (X-Axis) vs. Absorbance (Y-Axis) for Azure A

Azure A (Fig. 6) stained DNA marginally darker than it stained RNA, but exhibited very little difference in color according to spectroscopic analysis (Fig. 7). While the DNA band appeared to be blue and RNA purple, this was invalidated by fiber optic testing which showed similarly shaped and located absorbance peaks. It is a member of the thiazin family of dyes

(which also includes Methylene Blue and Azures B and C), considered to be good prospects for this study, but quantitative analysis did not support this hypothesis.

Fig. 8 Partial Gel stained with Azure B

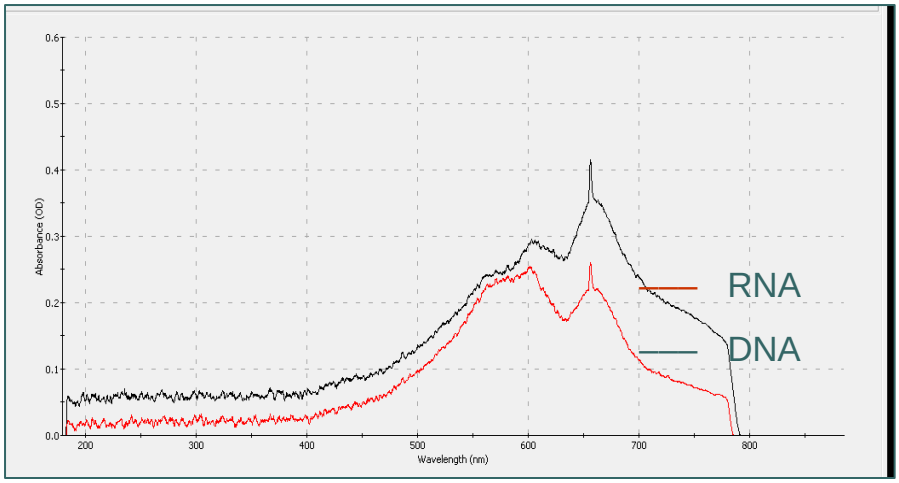


Fig. 9 Graph of Wavelength (X-Axis) vs. Absorbance (Y-Axis) for Azure B

Preliminary staining with Azure B showed promising results, exhibiting a slight difference in color between the RNA and DNA bands (Fig. 8). DNA, in the right lane, was stained a deeper blue-purple, while RNA was closer to pure cobalt. Quantitative analysis (Fig. 9) showed similar potential because the peak ratios for RNA and DNA are quite different, suggesting a difference in color in addition to variation in the depth of staining.

Fig. 10 Partial Gel stained with Azure C

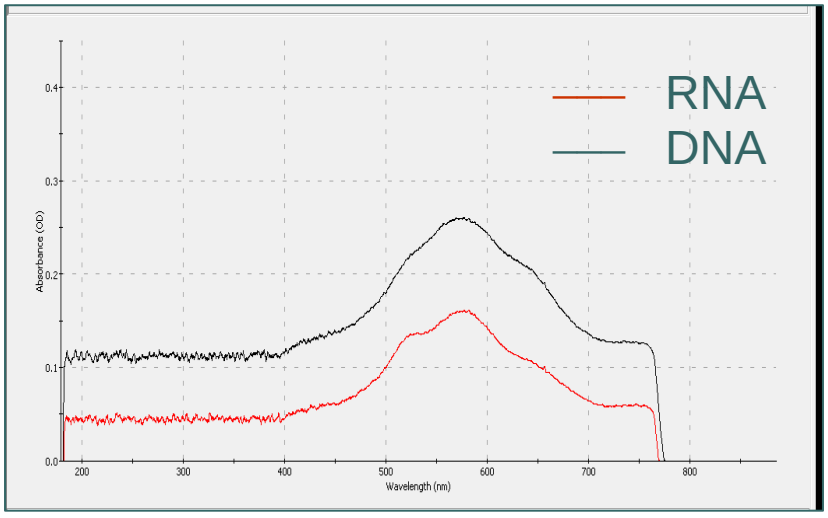


Fig. 11 Graph of Wavelength (X-Axis) vs. Absorbance (Y-Axis) for Azure C

Staining with Azure C resulted in varied depths of the same shade of purple for RNA and DNA bands. In the scan (Fig. 10), RNA can be seen in the left lane stained lighter than the DNA band present in the right lane of the gel. Though the ease of formulation was advantageous, the lack of variation in color was evident in the graph produced by spectroscopy. The nearly

identical locations of the peaks (Fig. 11) prove that the dye stains RNA and DNA bands the same color.

Fig. 12 Partial Gel stained with Brilliant Cresyl Blue

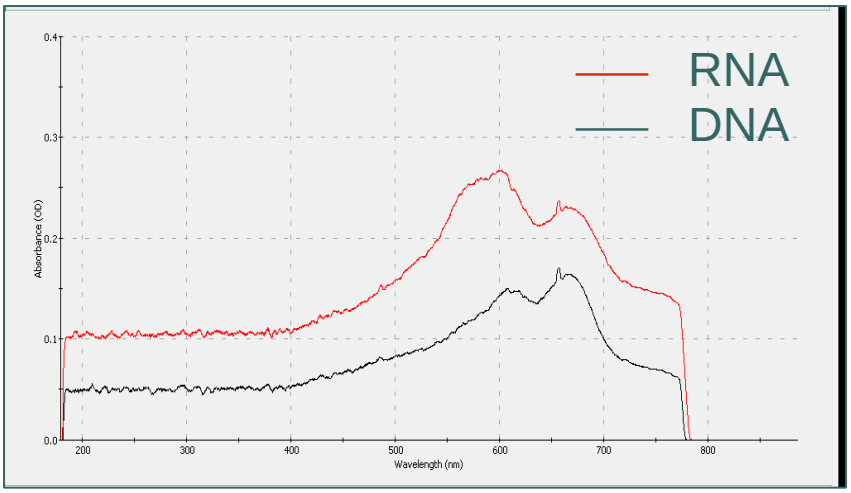


Fig. 13 Graph of Wavelength (X-Axis) vs. Absorbance (Y-Axis) for Brilliant Cresyl Blue

Rapid degradation of staining clarity following the completion of the scan shown in Fig. 12 may have affected the absorbance values of Brilliant Cresyl Blue. A second trial done with different staining and destaining times in an effort to decrease fading of the bands did show improved results, but Brilliant Cresyl Blue was not tested for absorbance vs. amount of nucleic acid. Fiber optic testing (Fig. 13) showed similar peaks of absorbance for RNA and DNA, though the ratio between these peaks is noticeably dissimilar. This was the only dye for which RNA was more deeply stained than DNA.

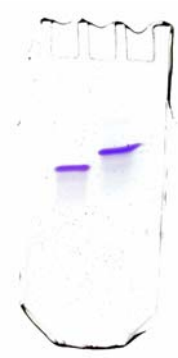


Fig. 14 Partial Gel stained with Cresyl Violet

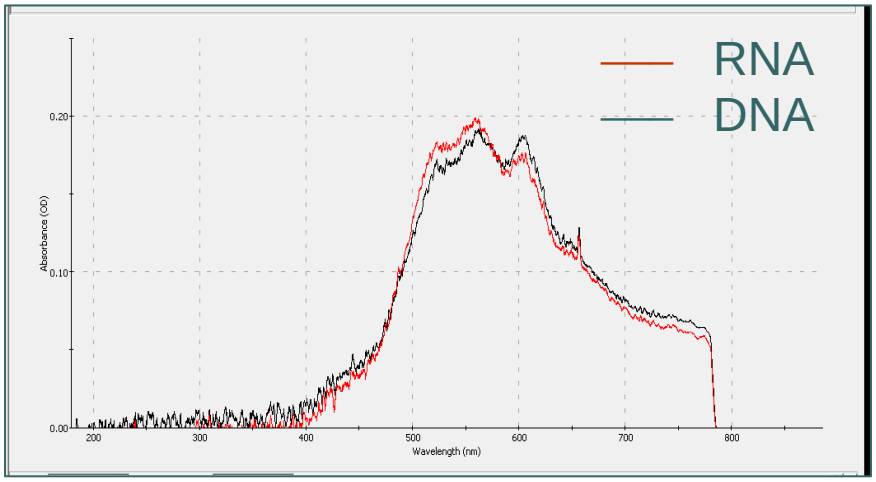


Fig. 15 Graph of Wavelength (X-Axis) vs. Absorbance (Y-Axis) for Cresyl Violet

Previous testing had shown Cresyl Violet (Fig. 14) to stain DNA and RNA different colors, and preliminary testing in this study confirmed these results with RNA stained violet and DNA stained blue-purple based on visual inspection. Spectroscopy, however, revealed little quantitative difference between the bands (Fig. 15). Both DNA and RNA had the same peaks,

and exhibited only slight variation in absorbance. The similar shape of the graphs was not optimal for this study.

Fig. 16 Partial Gel stained with Methylene Blue

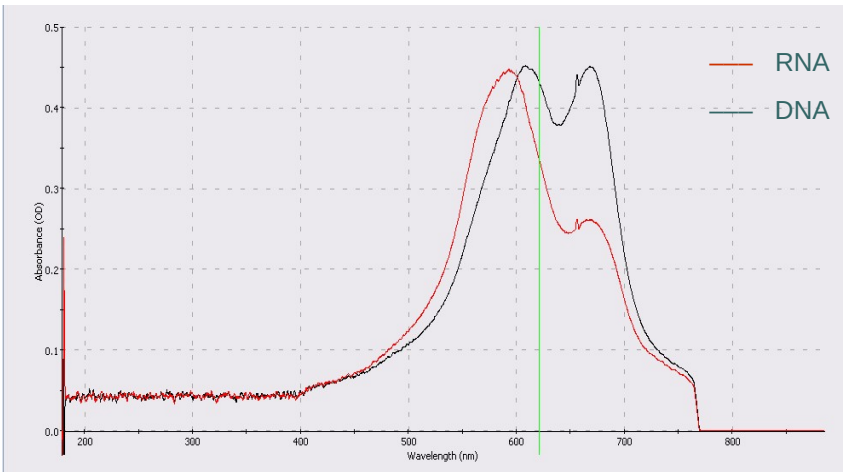


Fig. 17 Graph of Wavelength (X-Axis) vs. Absorbance (Y-Axis) for Methylene Blue

Initial visual analysis of the partial gel stained with Methylene Blue did not show an obvious difference in color between RNA and DNA bands, but further spectroscopic analysis would prove quite different. The scan (Fig. 16) picks up a light turquoise blue staining of RNA in the left lane when compared with the darker staining of DNA in the right lane of the gel. This difference was less visible to the naked eye, highlighting the importance of a quantitative analysis using spectroscopy. Once the gel was viewed using fiber optic spectroscopy (Fig. 17), the spectra of DNA and RNA were clearly different as evidenced by the plot of absorbance vs. wavelength. The distinct peaks of the two nucleic acids demonstrated a clear difference in color between the RNA and DNA when stained with methylene blue.

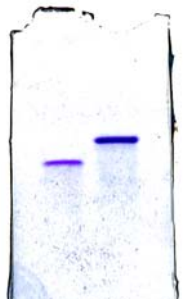


Fig. 18 Partial Gel stained with Thionin

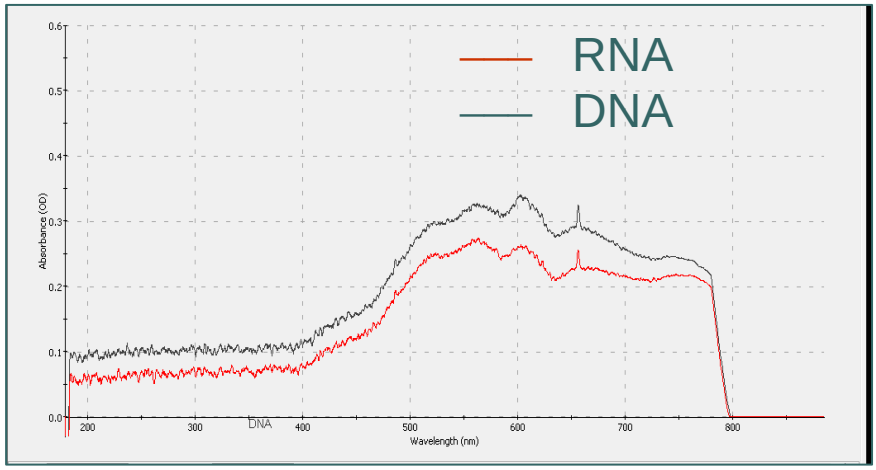


Fig. 19 Graph of Wavelength (X-Axis) vs. Absorbance (Y-Axis) for Thionin

Preliminary staining with Thionin (Fig. 18) showed great promise for the purpose of this study, exhibiting clear, dark bands of differing colors for RNA and DNA. The RNA band, seen

in the left lane in Fig. 18, stained lavender, while DNA staining resulted in a blue-purple band. The opacity of Thionin is beneficial for the formation of well-defined bands, but can result in residual background staining. Quantitative analysis (Fig. 19) revealed the same absorbance peaks and curve shape for DNA and RNA,

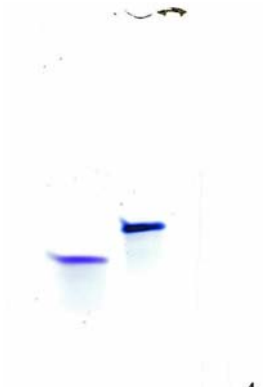


Fig. 20 Partial Gel stained in Toluidine Blue O at a concentration of 0.025% in sodium citrate at pH3.2

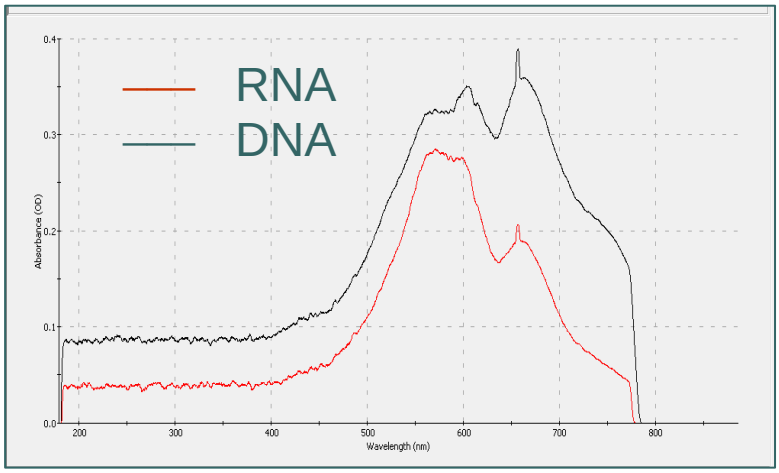


Fig. 21 Graph of Wavelength (X-Axis) vs. Absorbance (Y-Axis) for Toluidine Blue O in sodium citrate at pH 3.2

For Toluidine Blue O in sodium citrate at pH 3.2 (Fig. 20), the bands exhibited metachromasy, with RNA staining violet and DNA staining a deep blue. Spectroscopic analysis of bands stained with this formulation of Toluidine resulted in graphs with virtually identical peak locations, but the difference in peak ratios shows promise (Fig. 21). The difference in absorbance indicated darker staining of DNA.

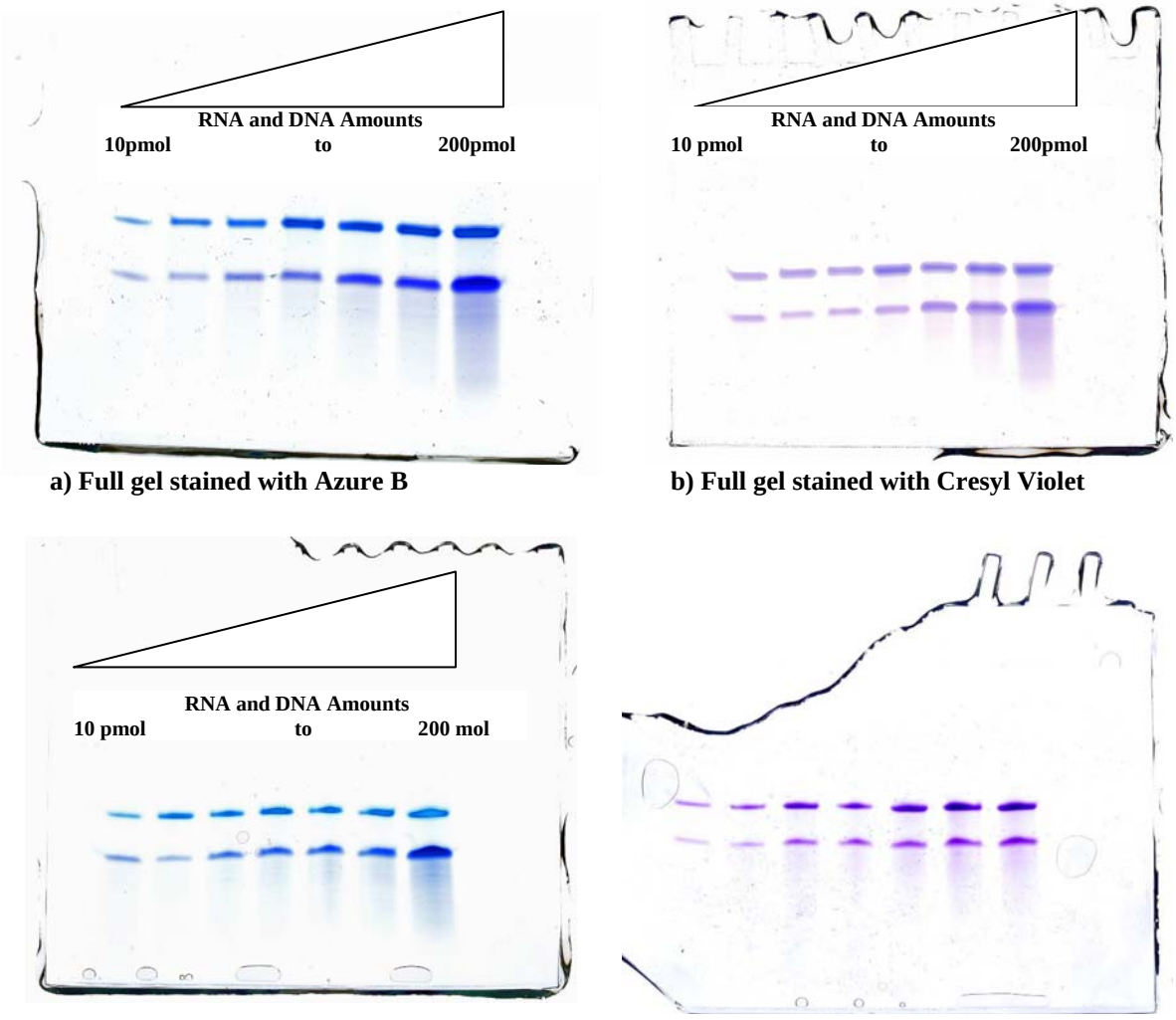
Quantitation Gels

Four dyes, including Azure B, Cresyl Violet, Methylene Blue, and Thionin were used to gather quantitative data regarding potential linearity between absorbance and amount of RNA (Fig. 23) or amount of DNA (Fig. 24). Azure B was stained for fifteen minutes in both trials, but different times for destaining led to remarkably dissimilar results (Fig. 22a). For the first trial, destaining occurred overnight which washed out the DNA bands almost completely. Therefore, spectroscopic testing could only occur on RNA bands and this run was not used in final quantitative analysis. The difference in color between RNA and DNA was fairly slight and appeared more strongly in the second trial. Lightening of the bands is apparent as the concentration of the DNA or RNA solution decreases. Spectroscopic analysis was implemented to determine whether changes in DNA and RNA concentration led to a linear change in the absorbance spectrum.

The first run stained with Cresyl Violet yielded satisfactory results. The second run (Fig. 22b) showed a much more consistent increase in intensity of color for RNA bands when compared with the similar, heavily stained bands at high RNA concentrations on the first gel.

Methylene Blue showed a distinct visible difference in color between bands of DNA and RNA (Fig. 22c). DNA stained a lighter turquoise blue, while RNA stained a deeper shade of blue. In both trials, increasing amounts of DNA and RNA exhibited greater depth of color.

The first and second trial using Thionin yielded similar results with RNA bands being visibly stained a lighter violet, while DNA was stained a deeper plum purple (Fig. 22d). The increase in intensity of color for increasing amounts of RNA and DNA was clear in both gels, especially in Trial 2, showing the consistency of the stain as an indicator of various concentrations of nucleic acids. Thionin is a dark dye so some residual background staining is possible depending on destaining time, as was evident in Trial 1.



a) Full gel stained with Azure B

b) Full gel stained with Cresyl Violet

c) Full gel stained with Methylene Blue

d) Full gel stained with Thionin

Fig. 22 Representative Gels stained using various dyes with increasing amounts of DNA and RNA at values of 10, 25, 50, 75, 100, 150, and 200pmol

DISCUSSION

The purpose of this experiment was to identify safe and inexpensive dyes that can differentiate between DNA and RNA and have linear relationships between sample size of nucleic acid and absorbance. All dyes tested already met the conditions of being safe and inexpensive, however, it was unclear whether they were effective at staining DNA and RNA and could clearly differentiate between the two. Out of the ten dyes tested, all but Coomassie and

Nile Blue A stained DNA and RNA successfully. Coomassie, a dye traditionally used to stain proteins, binds by Van der Waals attractions to the amino acids arginine and histadine [28]. Due to the fact that nucleic acids do not contain amino acids, Coomassie was unable to effectively stain DNA and RNA. Nile Blue, which intercalates into the interior of the double helix of DNA, may have been unable to stain the DNA and RNA due to the fact that single stranded DNA and RNA were used [30]. The dyes that did successfully stain the nucleic acids were: Methylene Blue, Azure A, B, and C, Thionin, Toluidine Blue O, Cresyl Violet, and Brilliant Cresyl Blue. All dyes with the exception of Thionin bind ionically to the negatively charged phosphate groups of DNA and RNA and therefore were able to stain the nucleic acids [4]. Thionin, which intercalates nucleic acids, binds tightly to DNA and RNA, enabling to effectively stain the nucleic acids [12].

In addition, of the dyed which suitably stain the nucleic acids, some of the dyes exhibit metachromatic qualities by staining the DNA and RNA different colors, thus meeting the purpose of identifying a dye that can differentiate between the two nucleic acids. Methylene Blue proved highly metachromatic, with distinctly different absorbance peaks for DNA and RNA. This distinct variance between the stain color of DNA and that of RNA makes Methylene Blue useful in distinguishing between the two nucleic acids. Additionally, although Methylene Blue was the only dye that exhibited strikingly disparate absorbance peaks, other dyes demonstrated visual differences in color. For example, the DNA and RNA bands stained by Azure A appeared blue and violet, respectively, even though the analysis of absorbance only revealed separate values for the difference in absorbance rather than different locations for the peaks. Therefore, Azure A may be useful for a quick, visual differentiation between RNA and DNA bands.

Analysis of Linearity

After determining absorptivities using the fiber optic spectroscopy apparatus, these values were plotted against amount of DNA or RNA as shown in Figures 23 and 24. Least-squares regression was used to compute a best-fit line and r-squared values for each quantitated dye in each nucleic acid.

The goal of this study was to identify linear patterns because the equations for these lines of best fit may be used to facilitate prediction of the sample size of a particular nucleic acid based on absorbance data. One hypothesis relating the equation to these qualities is that the y-intercept of the line of best fit represents the base-line absorptivity of the gel itself, regardless of the dye used. Conversely, the slope depends solely on the dye used, independent of the base-line absorptivities of the gels. Although some variation exists in the y-intercepts of the least-squares regression lines, they seem to cluster in localized ranges, thereby suggesting that the intercept is dependent on the gel rather than the dye. However, the same cannot be said for slopes.

DNA (Fig. 23)

For the three dyes run in the first trial for DNA (Methylene Blue- 670.54 nm, Cresyl Violet- 560 nm, and Thionin-560 nm), no significant linear correlation was observed with all r-squared values below 0.81. Moreover, no significant linear correlation (r-squared values below

0.87) was expressed for any of the four dyes (three from trial one, as well as Azure B at 601.43 nm).

Nonetheless, a multilinear pattern emerged for Thionin in both trials, demonstrating that this may be a phenomenon beyond simple experimental error. With regard to Cresyl Violet, although it exhibited a very low correlation (0.32) in the first trial, the r-squared value in run two was significantly higher (0.86), suggesting that the lack of linearity may be addressed with further repetition. In run one, Methylene Blue seemed to show a linear pattern up to 75 picomoles. However, this was not reproduced in run two, thereby precluding any conclusions on its efficacy in staining DNA linearly. Finally, while Azure B cannot be considered truly linear, it exhibited a relatively high r-squared value (0.87), once again suggesting that the dye indeed may behave linearly. Without further testing, it is impossible to draw any conclusions on the efficacy of Azure B in staining DNA.

RNA (Fig. 24)

No dye showed true linearity in either line of RNA over the full range from 10 to 250 picomoles. However, when certain dyes were plotted in the 0 to 100 picomole range from the first run, they exhibit a high degree of linearity with r-squared values ranging from 0.98 to 0.99. The two dyes that displayed this property were Methylene Blue at 601 nm and 672 nm, as well as Azure B at 600.85 nm. In the second trial, Azure B did show strong linearity over the entire range from 10 to 200 picomoles, with an r-squared value of approximately 0.98. Furthermore, both Azure B and Methylene Blue at 672 nm displayed strong linearity with r-squared values greater than 0.98. It was hypothesized that this absence of linearity past 100 picomoles is due to the dye reaching its saturation point in binding to the RNA, similar to the saturation point reached in enzyme kinetics. There are limited binding sites for these dyes to bind, and so it is probable that such a plateau in linearity is caused by the fact that there is more dye than binding sites. For Azure B in particular, excessive destaining in Trial 1 may have led to increased diffusion of dye in bands with high amounts of RNA, a potential explanation for the weaker results of this trial.

One significant inconsistency was that the slope and y-intercept of each respective dye differed between the two trials, even in the corrected versions of the graphs. The most probable cause for such an inconsistency was unavoidable experimental error (discussed further below), and could be eliminated with further repetition.

Finally, while both Azure B and Methylene Blue demonstrated limited success in linearly identifying RNA concentrations, no such behavior was observed when staining DNA. Due to the obvious structural similarities between ribonucleic acid and deoxyribonucleic acid, and considering that both dyes bind to the phosphate groups [5], which are found in both DNA and RNA, it seems probable that these two dyes should bind similarly to DNA as they did to RNA in terms of linearity. However, this was not observed, suggesting that further observation is required.

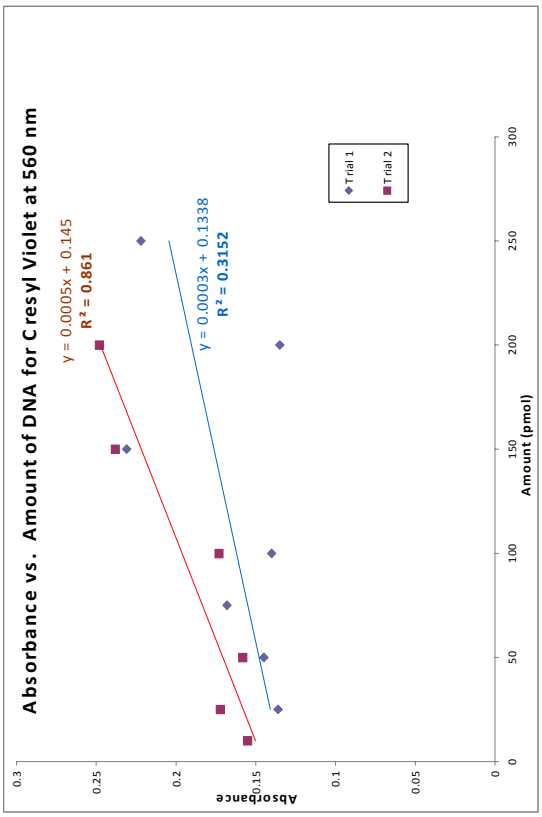


Fig. 23 a) Graph of Absorbance vs. Amount of DNA for Cresyl Violet

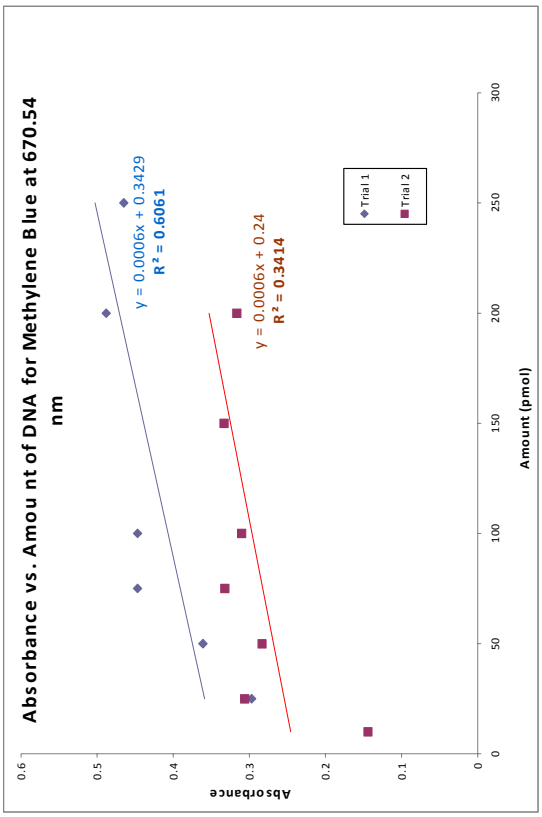


Fig. 23 b) Graph of Absorbance vs. Amount of DNA for Methylene Blue

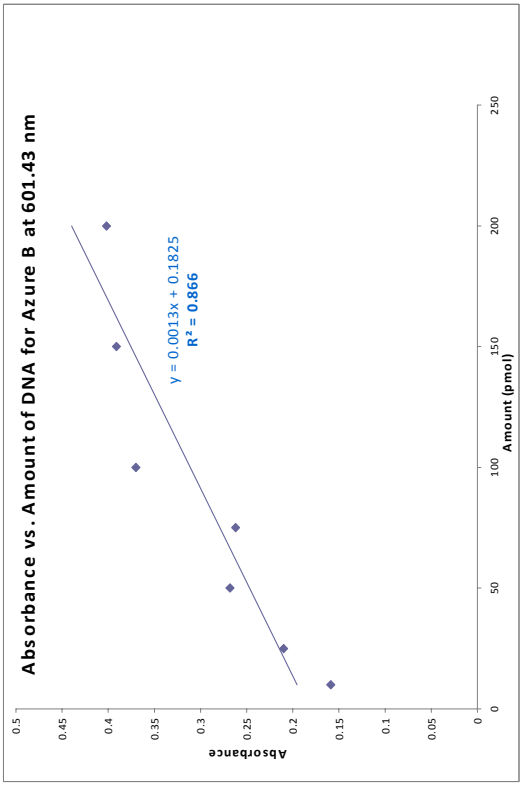


Fig. 23 c) Graph of Absorbance vs. Amount of DNA for Azure B

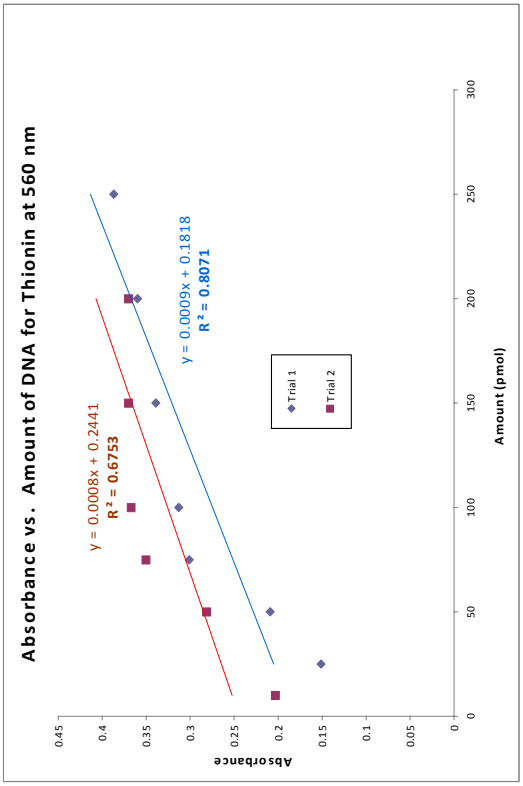


Fig. 23 d) Graph of Absorbance vs. Amount of DNA for Thionin

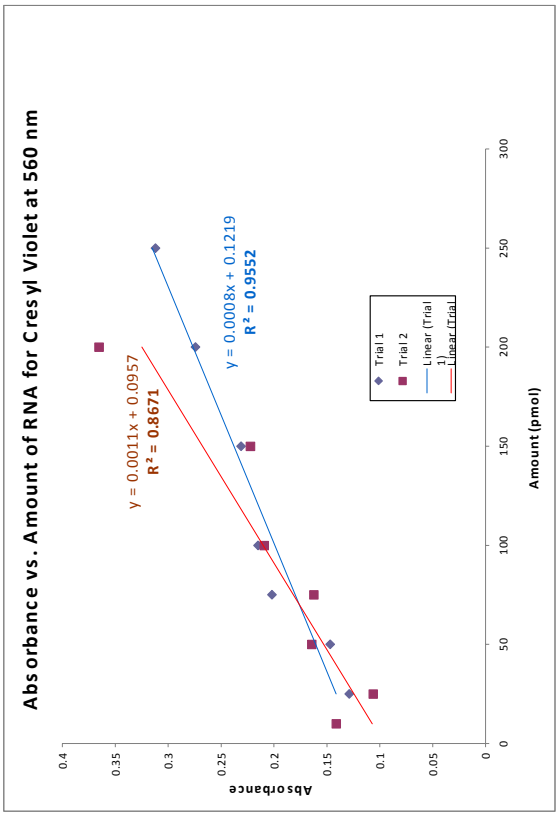


Fig. 24 a) Graph of Absorbance vs. Amount of RNA for Cresyl Violet

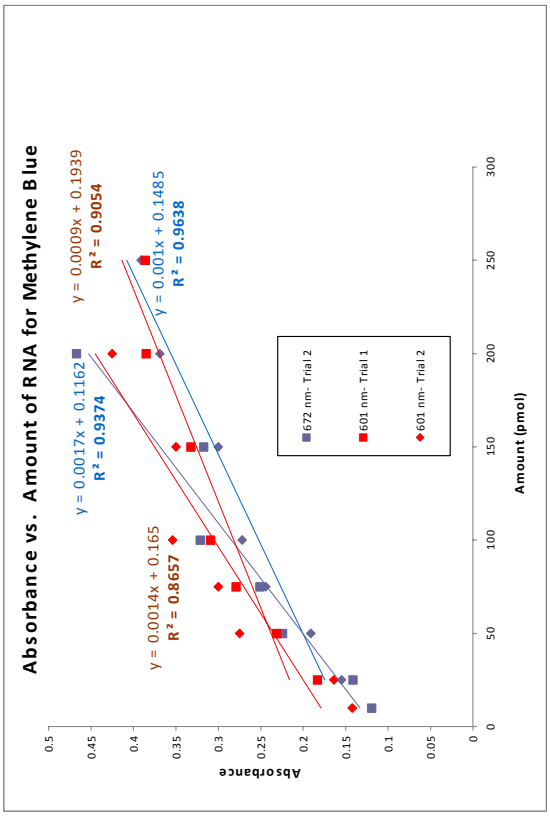


Fig. 24 b) Graph of Absorbance vs. Amount of RNA for Methylene Blue

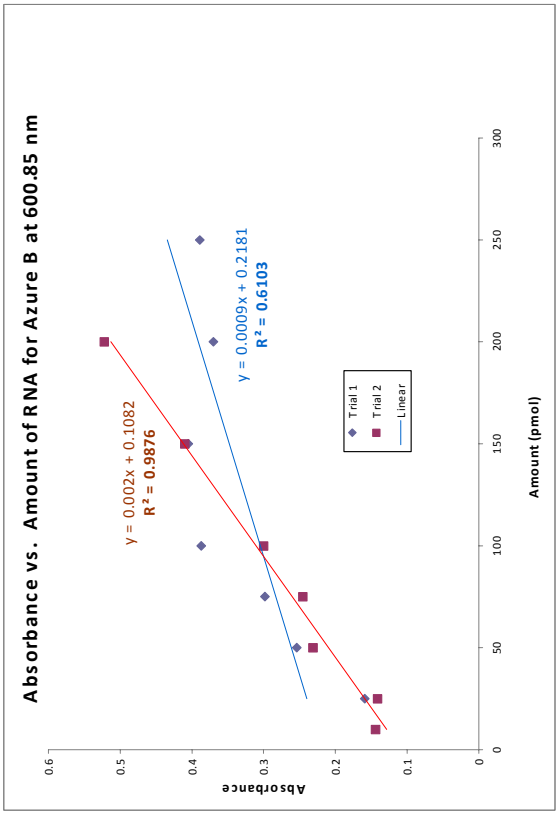


Fig. 24 c) Graph of Absorbance vs. Amount of RNA for Azure B

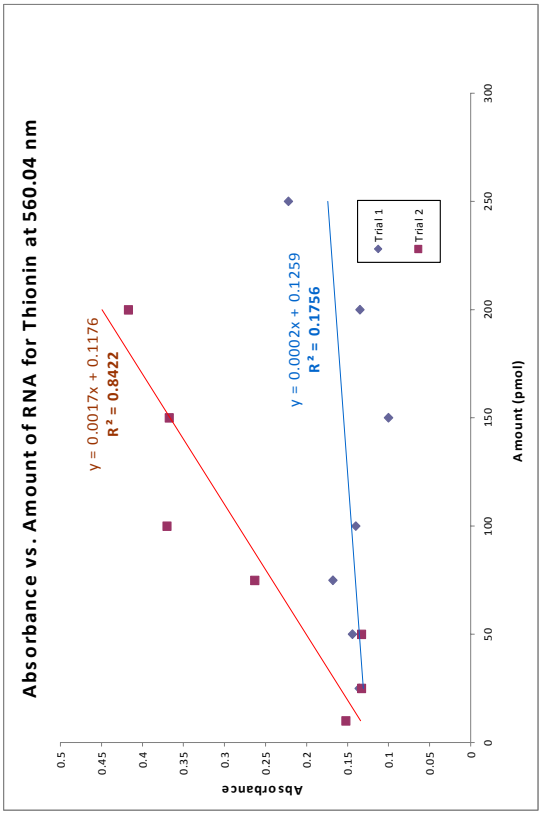


Fig. 24 d) Graph of Absorbance vs. Amount of RNA for Thionin

Experimental Error

Experimental error may arise from various sources, the first of which potentially occurred during pipetting. Due to the fact that this experiment depended heavily on pipetting while preparing gels, preparing DNA/RNA solutions, creating dye solutions, etc., small errors in pipetting may have significantly altered our results, thereby preventing the appearance of a linear pattern. This was especially true when preparing samples for gel electrophoresis, as the amount of DNA/RNA was at the picomole level. During the analyses, the team removed extreme outliers as standard procedure, but these outliers are indicative of errors caused by pipetting.

Fortunately, the majority of these errors can be corrected with simple replication. With a larger data set, future researchers would be able to demonstrate that the results are reproducible and, furthermore, demonstrate that deviations from expected linearity were caused by operator error. Finally, with a larger data set, one would be able to determine that population correlation between absorbance and concentration at a high level of statistical significance.

In addition, the time between the destaining of the gel and scanning of the gel represents a possible source of error because the time elapsed was not constant. As a result, different amounts of dye may have diffused from the nucleic acids leading to differences in absorbance values. In a similar way, the dye may have experienced degradation during the time period before scanning, leading to differences in absorbance values as well.

Future Avenues for Research

Due to limited resources in terms of both time and equipment, the group was not able to fully examine and investigate all of the dyes initially considered in this experiment. In order to more fully investigate the efficacy of these dyes, the following avenues for further research should be taken. First, since most if not all of the dyes showed some staining effect on the nucleic acids, one should determine the correlation of absorbance to sample size of nucleic acid for all dyes. In this study, the team was only able to determine absorbance vs. amount of DNA or RNA for Thionin, Cresyl Violet, Azure b, and Methlyene Blue, leaving five dyes uninvestigated.

Another potential phenomenon to research is the critical electrolyte concentration and competitive staining with various cations [26]. The research indicated that these cations may affect the metachromasy of various dyes when staining RNA and DNA such that distinguishing the two may become much easier. While this study did investigate this phenomenon with Toluidine Blue O, it is important to investigate these effects with other thiazin dyes and derivatives of Methylene Blue.

A third potential path would be to investigate the effects of mixing dyes. Several dyes exhibited strengths in staining nucleic acid at specific concentrations, and mixing such dyes could potentially create a solution that would be effective at a broader range of concentrations and for a wider repertoire of substances.

During this study, the determination of linearity was restricted to least-squares regression. However, it is a well-documented fact that the least-squares procedure is not robust and is highly

subject to small outliers [31]. In order to draw more robust conclusions of linearity from the quantitative data, in any future experiment either the least-absolute residuals or one-step sine estimator methods should be used to determine linearity [31].

While analyzing, the team restricted regression to simple linear regression. However, the study has not ruled out the possibility that certain dyes may exhibit multilinear patterns. Several of the dyes exhibited nonlinear patterns, which may or may not have been caused by error. Future research may be able to demonstrate that certain dyes exhibit true multilinear patterns by repeatedly testing dyes and performing multilinear regression analyses. Furthermore, certain dyes such as thionin exhibited piecewise linear behavior, as in up to a certain concentration, the dye exhibited almost linear behavior with one slope, and after this concentration exhibited almost linear behavior with a different slope. The reproducibility of this phenomenon must be demonstrated and further research be done in order to determine the mechanism behind such behavior.

A final avenue that should be explored in order to fully understand dye behavior with respect to concentration is the effect of dye band area on absorbance values. Due to various conditions during electrophoresis, the area of the bands in the polyacrylamide gel is not preserved across all samples. A greater area may reduce absorption values. In order to fully account for this source of variation, a correction for band area is required.

Conclusion

Due to time and resource constraints, this research is prospective rather than conclusive. While Azure A, Azure C, Toluidine Blue O (acidic), and Thionin showed visual promise, it was Methylene Blue that was able to distinguish DNA and RNA under fiber optic spectroscopy. Methylene Blue and Azure B had significant linear relationships for RNA up to 100 pmol, but no dye was successful in achieving a linear relationship for DNA. Our information could be used for future research into quantitative dye analysis.

REFERENCES

[1] Alvarado, O, Fellers, T.J., Davidson, M.W. (2008). Cresyl Violet. Retrieved July 20, 2008, from Polarized Light Gallery Web site: http://www.olympusmicro.com/micd/galleries/polarized/cresylviolet.html

[2] Amaral and Mello, 1989. M.J.L.V. Amaral and M.L.S. Mello, Critical electrolyte concentration of heterochromatin and euchromatin in cells of starved animals. *Cvtobios* 59 (1989), pp. 159–165.

[3] (2005, May). Azure A. Retrieved July 20, 2008, Web site: http://stainsfile.info/stainsfile/dyes/ 52005.htm

[4] Bioscience Explained, Retrieved July 20, 2008, Web site: http://www.bioscience-explained.org/EN1.2/stains.htm

[5] Chemical Differences between DNA and RNA. Retrieved July 20, 2008, from Nucleic Acid Structure Web site: http://www.biochem.uwo.ca/meds/medna/nachem.html

[6] (2008, November 7) Coomassie Blue: Destaining, Retrieved July 20, 2008, from Bionet Website: http://biowww.net/detail-98.html

[7] (2005) Coomassie Blue Solution Recipe, Retrieved July 20, 2008, from The Lab Rat Website: http://www.thelabrat.com/protocols/coomassieblue.shtml

[8] DNA Revealed, Retrieved July 20, 2008, from National Center for Biological Technology Education Website: http://www.ncbe.reading.ac.uk/ncbe/protocols/dna/pdf/dna14.pdf

[9] Ehrig, T., Abdulkadir, S.A., Dintzis, S.M., Milbrant, J., & Watson, M.A. (2001, February). Quantitative Amplifciation of Genomic DNA from Histological Tissue Sections After Staining With Nuclear Dyes and Laser Capture Microdissection. *Journal of Molecular Diagnostics*, *3*, Retrieved July 20, 2008, from http://jmd.amjpathol.org/cgi/reprint/3/1/22

[10] Erenpreisa, J., Evenpreiss, J., Freivalds, T., Slaidina, M., Krampe, R., Butikova, J., Ivanov, A., & Pjanova, D, (2004, October 24), Toluidine Blue Test for Sperm DNA Integrity and Elaboration of Image Cytometry Algorithm. Retrieved July 20, 2008, from Wiley InterScience Website: http://www3.interscience.wiley.com/cgi-bin/fulltext/102530748/pdfstart?cretry=1&sretry=0

[11] Experimental Molecular Biology of the Cell, Retrieved July 20, 2008, from Background on Acrylamide Gel Electrophoresis Website: http://www.udel.edu/biology/fschmieg/ 411acrylamide.htm

[12] Hecht, C, Friedrich, J., & Chang, T. (2004, January, 27). Interactions of Thionin with DNA Strands. *108*, Retrieved July 20, 2008, from http://pubs.acs.org/cgi-bin/article.cgi/jpcbfk/2004/108/i29/pdf/jp0372993.pdf

[13] (2006, September 23). Introduction to the Ocean Optics Spectrometer, Retrieved July 26, 2008, from Ocean Optics Spectrometer Setup Website: http://chemlab.truman.edu/instrumentation/ oceanoptics/oointro.htm

[14] Jafari, A., A., Ghanadzadeh, H. Tajalli, M. Yeganeh, & M. Mhogadam. (2006, April 21). Spectrochimica Acta Part A: Molecular and Biolmolecular Spectroscopy Retrieved July 20, 2008 from Science Direct Website: http://www.sciencedirect.com/science?_ob=articleurl&_udi=b6vng-4kgg4dp-2&_user=799667&_rdoc=1&_fmt=&_orig=search&_sort=d&view=c&_acct=c000038258&_version=1&_urlversion=0&_userid=799667&md5=85f146e7f7c15c93fa894856a40f8438#aff1

[15] Jameson, J.L. (1998). Principles of Molecular Medicine. Totowa, NJ: Humana Press, Inc.

[16] Mello and Vidal, 1973. M.L.S. Mello and B.C. Vidal, Linear dichroism and anomalous dispersion of birefringence on sperm heads. *Acta Histochem.* 45 (1973), pp. 109–114.

[17] Method for preparing slides for blood evaluation, Retrieved July 20, 2008 from Free Patents Online Website: http://www.freepatentsonline.com/3906120.html

[18] (2003, December 29). Methylene Blue Stain. Retrieved July 20, 2008, from Molecular Research Center Web site: http://www.mrcgene.com/met-blue.htm

[19] Orkin, S.H., Swan, D., & Leder, P. (1975, November). Differential expression of alpha- and beta-globin genes during differentiation of cultured erythroleukemic cells. *Journal of Biological Chemistry*, *250*, Retrieved July 20, 2008, from http://www.jbc.org/cgi/content/abstract/250/22/8753

[20] Patel, P., Brandolini, A.J., & Charlebois, A.F. (2007, May 17). Visible Spectrophotometric Study of the Interaction of Cresyl Violet with Nucleic Acid Molecules in Solution, Retrieved July 20, 2008, from Poster Session III Website: http://acs.confex.com/acs/marm07/techprogram/p43161.htm

[21] Regan, S.M., & Moffatt, B.A. (1990) Cytochemical Analysis of Pollen Development in Wild-Type Arabidopsis and a Male-Sterile Mutant, Retrieved July 20, 2008 from The Plant Cell Website: http://www.plantcell.org/cgi/reprint/2/9/877

[22] (2004, December 20). Restain Brilliant Cresyl Blue. Retrieved July 28, 2008, Web site: http://www.reagena.fi/www/en/products/pdf/instructions/REASTAIN_BrilliantCresylBlue_eng.pdf

[23] Rice, K.G., Rottink, M.K., & Lindhardt, R.J. (1987, February, 25). Fractionation of heparin-derived oligosaccharides by gradient polyacrylamide-gel electrophoresis. *Biochem Journal*, *244*, Retrieved July 20, 2008, from http://www.biochemj.org/bj/244/0515/2440515.pdf

[24] Sharma, S., Yadav, B.R., Bhardwaj, A., Jain, S., & Yadav, H. (2007, August, 27) Denaturing PAGE Based Microsatellite Analysis Retrieved July 20, 2008 from Protocol Online Website: http://www.protocol-online.org/prot/protocols/denaturing-urea-polyacrylamide-gel-electrophoresis--page--based-microsatellite-analysis-4787.html

[25] Thionin stain, Retrieved July 20 2008, Website: http://depts.washington.edu/rubelab/protocols/thionin-stain.html

[26] Vidal and Mello, 1989. B.C. Vidal and M.L.S. Mello, Critical electrolyte concentration of DNA and nucleoprotein complexes *in vitro*. *Acta Histochem. Cytochem.* 22 (1989), pp. 471–478.

[27] Wallman, S. (2001) Polyacrylamide Gel Electrophoresis (PAGE) retrieved July 20, 2008 from Technical Tutorials Website: http://biotech.nhctc.edu/bt210/tutoria l10.html

[28] Coomassie Blue, How it Works. Retrieved July 29, 2008, Web site: http:// 209.85.215.104/search?q=cache:h2EizeEo2zUJ:www.merck.es/servlet/PB/show/1288750/105235en.pdf+cresyl+violet+acetate&hl=en&ct=clnk&cd=1&gl=us

[29] Cresyl violet, How to formulate. Retrieved July 29, 2008, Web site: ht tp://209.85.215.104/search?q=cache:h2EizeEo2zUJ:www.merck.es/servlet/PB/show/1288750/105235en.pdf+cresyl+violet+acetate&hl=en&ct=clnk&cd=1&gl=us

[30] Chen, Q., Li, D., Zhao, Y., Yang, H., Zhu, Q., & Xu, J. (1999, May, 6). Interaction of a novel red-region fluorescent probe, Nile Blue, with DNA and its application to nucleic acids assay. *Analyst, 124*, Retrieved July 29, 2008, from http://www.rsc.org/ej/AN/1999/a901174i.pdf

[31] Hill, R. W., & Holland, P. W. (1977). Two Robust Alternatives to Least Squares Regression. *Journal of the American Statistical Association, 72*, Retrieved July 29, 2008, from http://www.jstor.org/stable/2286469.

Image Citations

Figure 1

Partially Adapted from http://www.steve.gb.com/science/prokaryotes_and_eukaryotes.html

Figure 3

a. Cresyl Violet- http://www.sigmaaldrich.com/structureimages/51/mfcd00013151.gif
b. Methylene Blue- http://stainsfile.info/StainsFile/dyes/graphics/52015.gif
c.Azure A- http://stainsfile.info/StainsFile/dyes/graphics/52005.gif
d. Azure B- http://stainsfile.info/StainsFile/dyes/graphics/52010.gif
e. Azure C- http://stainsfile.info/StainsFile/dyes/graphics/52002.gif
f. Toluidine Blue O- http://stainsfile.info/StainsFile/dyes/graphics/52040.gif
g. Thionin- http://stainsfile.info/StainsFile/dyes/graphics/52000.gif
h. Nile Blue A- http://stainsfile.info/StainsFile/dyes/graphics/51180.gif
i. Brilliant Cresyl Blue- http://www.sigmaaldrich.com/thumb/structureimages/01 mfcd00148901.gif
j. Coomassie- http://www.sigmaaldrich.com/structureimages/62/mfcd00041762.gif

Figure 4

PAGE - http://web.siumed.edu/~bbartholomew/images/chapter6/F06-21.jpg

Figure 5

Fiber optics http://chemlab.truman.edu/Instrumentation/OceanOptics/OOIntro.htm