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# Measuring Bacterial Growth Using a 3D-Printable Spectrometer

Samuel R. Bunting

*The University of Akron*, [srb99@zips.uakron.edu](mailto:srb99@zips.uakron.edu)

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## Measuring Bacterial Growth Using a 3D-Printable Spectrometer

### 1. Introduction

Spectroscopy is a broadly applicable laboratory technique and an essential part of quality education in the biological and physical sciences at early education levels. However, traditionally high costs and sophistication have relegated the necessary equipment to industry and higher education. Commercial spectrometers are ‘black boxes,’ with their inner workings and physics hidden from the student.

We have previously reported on an inexpensive, 3D-printable spectrometer, called the SpecPhone (Grasse, Torcasio & Smith, 2016). This apparatus uses an iPhone as the camera, along with several other inexpensive additions to make a fully functioning spectrometer for a fraction of the cost of a commercial instrument. The overall

purpose of the SpecPhone project is to provide laboratory experiences with spectroscopy in low-resource educational environments. The simplicity of the design, and widespread availability of the components make the SpecPhone particularly apt for this goal.

Previous protocols have only applied the SpecPhone to colorimetric assays (Smith, 2015), and simple protein quantification experiments (Smith, 2017). In practice, spectroscopy extends far beyond these two applications. Here, two protocols using the SpecPhone to measure bacterial growth are discussed. Both protocols provide instructions for the creation of a bacterial growth curve. One outlines a conventional procedure: inoculating a flask of blank media with a stock *Escherichia coli* culture, and taking growth measurements at regular



**Figure 1.** The SpecPhone apparatus is pictured. The housing is 3D-printed, and additional components are added in to complete the apparatus (Grasse, Torcasio & Smith, 2016).

intervals. The second describes a smaller-scale version of this concept, with the entire culture being grown in a 4.5-mL cuvette. Designing and testing these protocols broadens the applications of the SpecPhone to a new realm: measurement of living systems, thus extending its educational potential.

Bacterial growth curves are a widely-used laboratory measurement. Their basic underlying principle depends on the relationship between concentration and the amount of light absorbed by a sample, as represented by the Beer-Lambert Law:  $A = \epsilon \ell C$ . In this equation,  $A$  represents the light absorbed by the sample,  $\ell$  is the path length (1 cm. in these experiments), and  $C$  is the sample concentration. The molar absorptivity ( $\epsilon$ ) is a constant that depends on the solution. Following inoculation of sterile media, the increase in bacterial cells will proportionally increase the degree of light scatter caused by the turbidity of the culture. Regular absorbance measurements are used to monitor the growth of bacteria in the liquid culture. These absorbance measurements can be correlated with diluted, bacterial plates made at the same time points. From this correlation, the relationship between culture absorbance (turbidity) and cell number, determined by the number of colonies growing on the plates, can be established. This linear relationship represents the standard curve, and it can be used to determine the cell number in that culture, as well as in unknown cultures of the same organism.

Bacterial growth curves are broadly applicable in education, research and industry (Hall, Acar & Nadipati, 2014). Growth of bacteria under varying conditions can be monitored and compared using growth curves, a procedure which is used extensively in development of food safety guidelines (Zwietering, et. al., 1990). Effects of environmental factors (Hall, Acar & Nadipati, 2014), and antibiotics (Yourassowsky, et al., 1985) are also measurable using growth

curves. Growth rates of varying species, including different phenotypes of the same species can be compared using this kind of measurement as well.

## **2. Materials & Methods**

### *2.1 Growth of Liquid Bacterial Cultures*

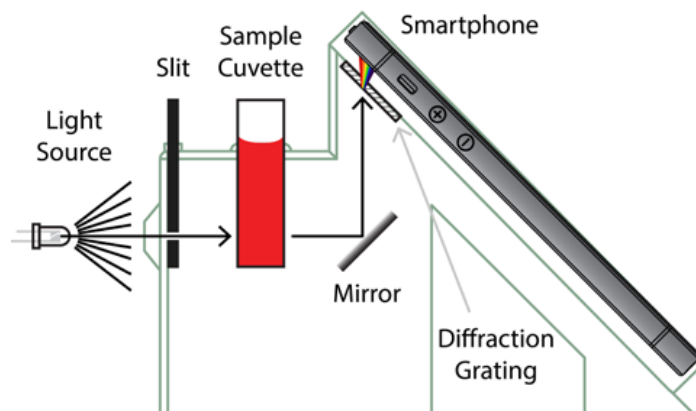
To begin, a stock culture containing a standard laboratory strain (K12) of *E. coli* with Kanamycin resistance was grown. Sterile trypticase soy broth (TSB) containing Kanamycin was inoculated with a single *E. coli* colony. The culture was incubated at 37°C for 24 hours in a shaking incubator (VWR, Radnor PA) at 265 rpm. The same procedure for creating the stock *E. coli* culture was followed for all trials, regardless of the vessel used for growth.

For the flask cultures, a 125-mL Erlenmeyer flask, containing 95 mL of sterile TSB was inoculated with 5 mL of the stock *E. coli* culture. This flask was placed in the shaking incubator at the same specifications as before. For the cuvette growth protocols, two plastic, 4.5-mL cuvettes were filled with 3 mL of sterile TSB containing Kanamycin. Both were inoculated with 100  $\mu$ L of the stock *E. coli* culture. One cuvette was secured in the shaking incubator at the same settings as the previous trial (mechanically-shaken cuvette). The second cuvette was placed in a stationary incubator at 37°C. This cuvette was removed from the incubator every 5 minutes, covered, vigorously shaken by hand, and then returned to the incubator (hand-shaken cuvette). The flask culture trials were replicated four times, and the each of the cuvette cultures was replicated three times.

### *2.2 Assembling the SpecPhone Apparatus*

Before measurements were taken with the SpecPhone, the components of the device were assembled. As mentioned, the housing of the SpecPhone is a 3D-printable file designed

using SolidWorks (Dassault Systèmes SOLIDWORKS Corp., Waltham MA). After printing, the remaining components are added. A 1 in.<sup>2</sup> aluminum mirror is inserted to reflect light coming in through the opening in the rear of the housing. Light passes



**Figure 2.** A schematic of the completed SpecPhone apparatus, including the 3D-printed housing, light source, slit insert, mirror and diffraction grating (Grasse, Torcasio & Smith, 2016).

through a removable, 3D-printed slit insert, with a slit width of 1 mm. The final component is a small piece of diffraction grating, containing 1,000 lines/mm. Light passes through the slit and then through the sample solution in the cuvette, which is inserted in the housing of the SpecPhone. This light is reflected off the mirror, and then passed through the diffraction grating before reaching the iPhone camera, where an image of the spectrum is captured. A schematic of the fully-assembled SpecPhone apparatus is shown above in **Figure 2**.

### 2.3 Recording Absorbance Measurements with the GeneSys20 and the SpecPhone

Absorbance measurements were taken with a GeneSys20 Visible Spectrophotometer (ThermoFisher Scientific, Waltham MA), and compared to results obtained with the SpecPhone outfitted with an iPhone 5. Before any absorbance measurements were taken, a cuvette containing sterile TSB was used to blank the GeneSys20. To blank the SpecPhone, three images of the spectrum for the blank TSB cuvette were captured.

The wavelength was set at 600 nm for the GeneSys20 for all measurements, and the same wavelength was when analyzing the SpecPhone results as well. For the SpecPhone, the light source was a desk lamp with a 40 W light bulb. For the flask cultures, absorbance

measurements were taken every 20 minutes with both devices over a period of 4 hours, following the initial ( $T_0$ ) measurement. A 3-mL aliquot of the culture was transferred to the sample cuvette for measurements. Between measurements, the culture flask was kept in the shaking incubator under the previously specified conditions. Spectrum images were taken in triplicate with the SpecPhone at each time point.

In the cuvette culture growth protocols, measurements were taken with the same two devices, however there was no transfer of a sample of the culture from a larger vessel. Cultures were grown over a period of 2.5 hours, with absorbance measurements taken every 10 minutes for both the mechanically-shaken and hand-shaken cuvettes, removing them from their respective incubators. Images of each bacterial culture spectrum were captured in triplicate using the SpecPhone.

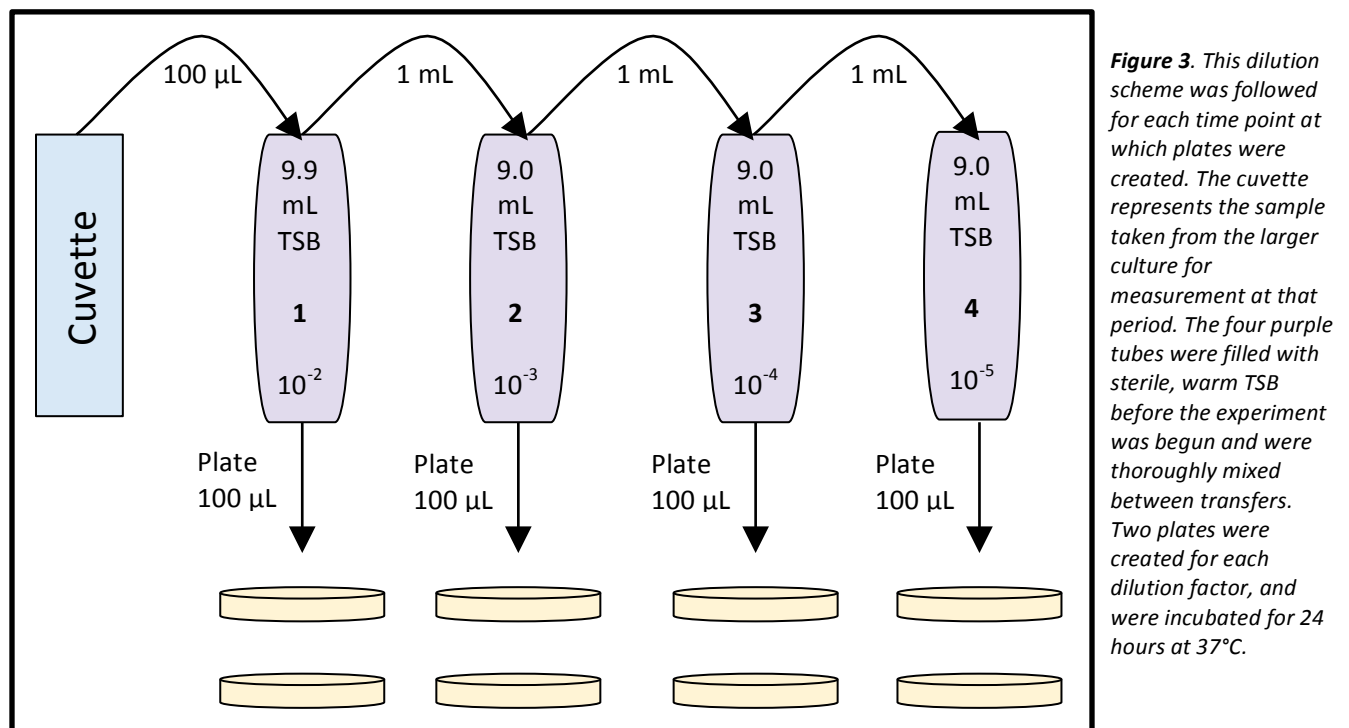
#### *2.4 Plating from Bacterial Culture*

Bacterial plates were also created from three of the four flask growth cultures. The initial flask culture trial was only performed to determine the feasibility of measuring culture turbidity, and plates were not constructed. For the remaining flask cultures, dilution tubes and plates were made beginning at  $T_{20}$ . Samples of the flask culture were plated at 40-minute intervals. The first three time points ( $T_{20}$ ,  $T_{60}$ ,  $T_{100}$ ) were diluted by factors of  $10^{-2}$ ,  $10^{-3}$ , and  $10^{-4}$ . The latter three time points ( $T_{140}$ ,  $T_{180}$ ,  $T_{240}$ ) were diluted up to a factor of  $10^{-5}$ .

After absorbance measurements had been recorded, a 100- $\mu$ L aliquot of the culture sample was transferred from the sample cuvette to the first dilution tube, which contained 9.9 mL of sterile TSB. From this initial tube, three subsequent transfers of 1 mL of culture were made to perform serial dilutions. Each subsequent dilution tube contained 9.0 mL of sterile TSB.

These tubes of blank media were created before the experiment was begun. After each transfer, the tubes were adequately shaken to ensure proper suspension of the bacteria.

From each dilution tube, a 100- $\mu$ L aliquot was plated. Two plates were created from each dilution tube. The plates were incubated at 37°C for 24 hours. A schematic of this dilution process is below in **Figure 3**.



Only those plates with colony counts between 30-300 were counted, as this is the established range for statistical robustness (Sutton, 2011). Confluent plates were not counted, and their colony count data was not included in any analyses. The colony counts from each time point were then correlated with the absorbance measurements from each device at the same time point to create a standard curve.

## 2.5 Image and Data Analysis

Spectral images captured by the SpecPhone were analyzed using ImageJ, the free image analysis software available from the U.S. National Institutes of Health (Rasband, 2016). A protocol detailing the steps for image analysis with ImageJ has been previously published (Smith, 2015). These steps were followed for analysis of all spectrum images collected from the SpecPhone. All subsequent data analysis and representation were done using Microsoft Excel and MiniTab statistical software (Minitab Inc., State College PA).

To correlate the absorbance and time data, time was plotted on the X-axis, and absorbance on the Y-axis, and the equation of a line of best fit was calculated. This was adequate for description of the growth curve data from both the flask and cuvette culture protocols. As mentioned, multiple repetitions were performed, and the absorbance values for each time point were averaged together to yield the values used to construct the growth curves for both the cuvette and flask cultures, plotted with the standard error of the mean (SEM).

For the standard curve data, which involved absorbance and colony forming units per milliliter (CFU/mL) data points, more advanced analysis was done. Each time point that included plating produced two plates for each dilution factor, and as such the number of colonies from each were averaged together. Using the average number of colonies, the dilution factor, and the amount plated, the number of CFU/mL were calculated using the formula:

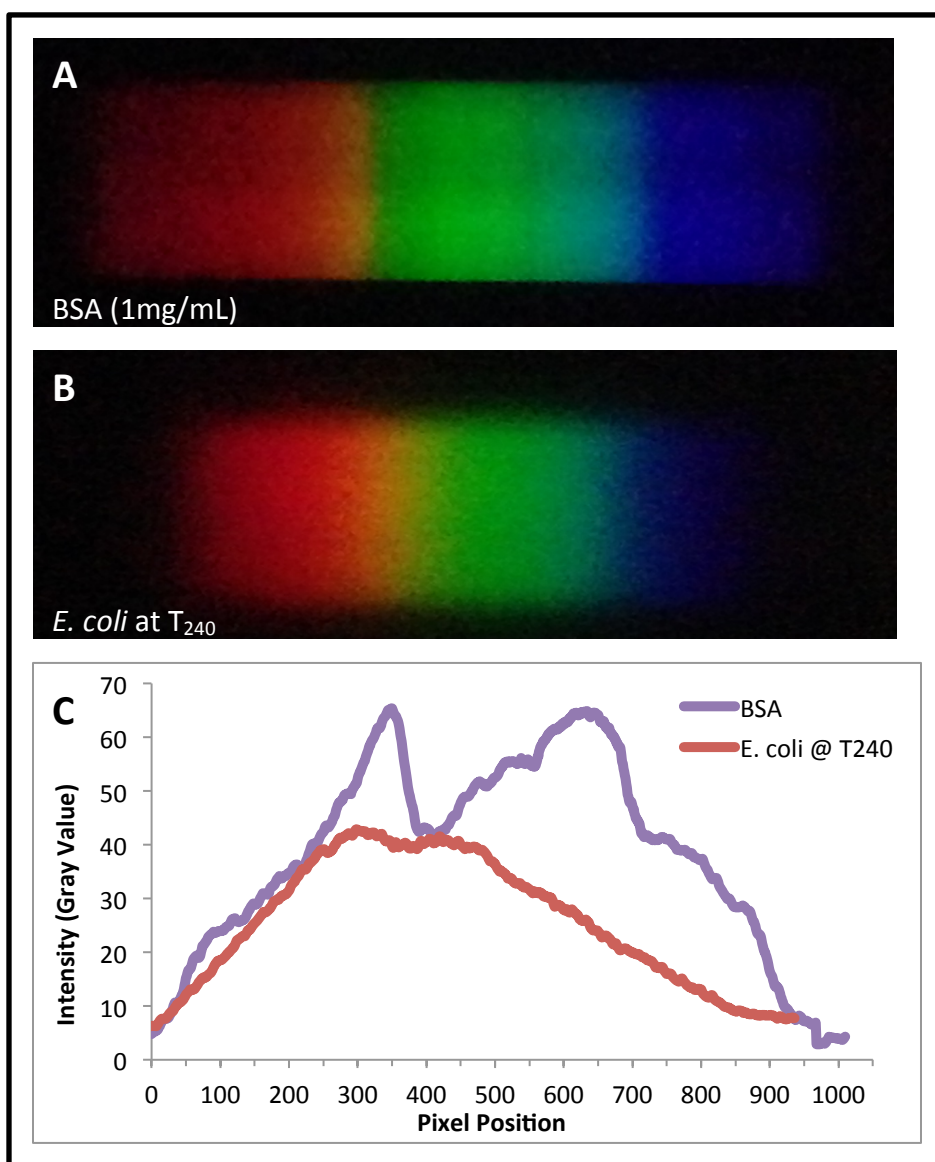
$$\frac{CFU}{mL} = \frac{\text{Average Colony Number}}{(mL \text{ Plated}) \times (\text{Dilution Factor})}$$

Correlating the average number of CFU/mL for each time point with the average absorbance at the same time points plotted the created curve. The significance of the correlation was determined, by calculating a 95% confidence interval and prediction interval for the data from both the GeneSys20 and SpecPhone. Finally, the statistical



difference between the equations of the lines of best fit for the GeneSys20 and the SpecPhone data was calculated.

The GeneSys20 reported percent transmission values (%T), which were converted to absorbance values using the formula  $A = -\log(\%T)$ . A sample spectrum of *E. coli* is shown in **Figure 4B**, compared to a highly concentrated sample of BSA in **Figure 4A**, with an overall comparison of pixel intensity in **Figure 4C**. This comparison shows the difference in spectrum appearance with a turbid culture, compared to measurement of a simple, colored solution.



**Figure 4.** Image 4A represents a spectrum of Bovine Serum Albumin (BSA) at a concentration of 1 mg/mL with the SpecPhone using the iPhone 5 camera. The horizontal edges are clearly defined and there are somewhat sharp, vertical divisions between the individual colors of the spectrum. Image 4B is a spectral image of the bacterial culture at the T<sub>240</sub> time point. The borders are less defined and the overall color intensity is reduced. The plot in Image 4C shows the overall pixel intensity for the entire spectrum for both images. The introduction of a turbid culture caused more light scatter, and this causes less light to reach the camera for measurement. However, in the colored solution (BSA), there is a reduction in the amount of light recorded due to more light being absorbed by the sample, rather than being scattered.

Full protocols are attached to this report, with the Flask Culture Protocol in **Appendix 2** and the Cuvette Culture Protocol in **Appendix 3**. Both protocols include detailed laboratory procedures, as well as data analysis instructions, and safety guidelines. These appear in the same format as will be distributed with the SpecPhone for educational purposes.

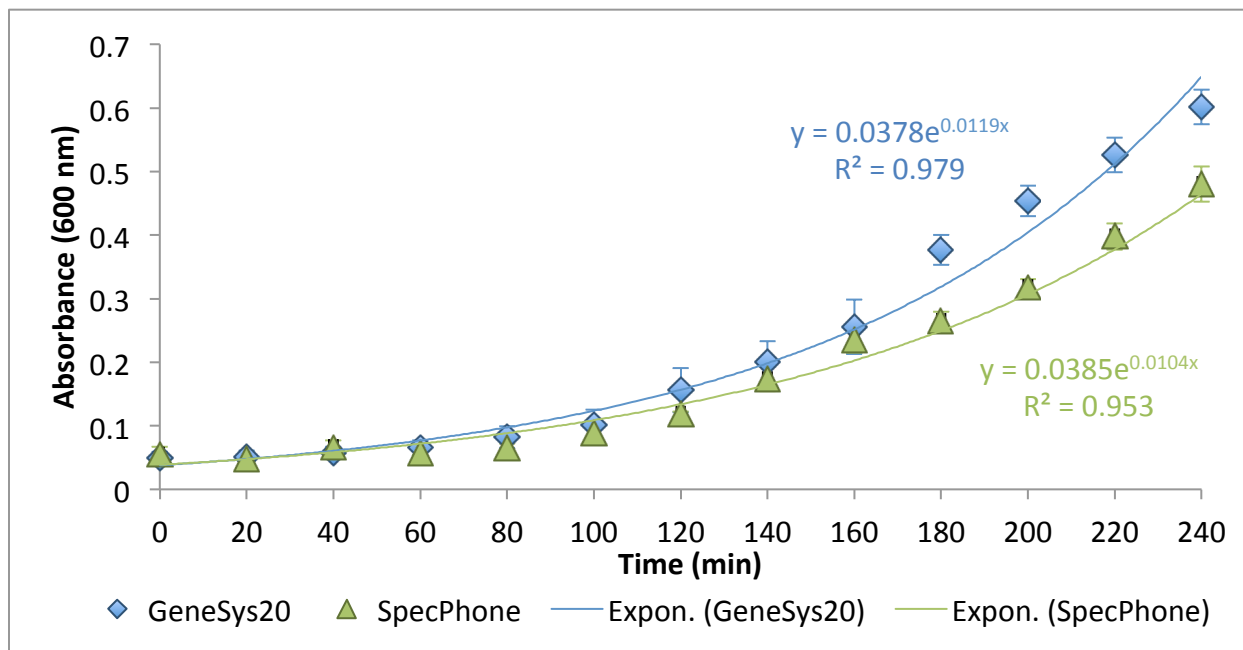
### **3. Results**

Two protocols centered on the same basic principle, using spectroscopy to measure bacterial growth, were designed and tested in this project. Multiple trials of each experimental design were conducted, and the data was combined. The first flask culture growth trial was conducted only to determine if the SpecPhone was capable of measuring culture turbidity (data not shown). Based on satisfactory results from this initial experiment, the remaining trials were conducted. Three additional flask cultures were grown and measured, and averaged with the data from the first. Plate counts were also averaged to create the standard curve. Similarly, three hand-shaken cuvette cultures and three mechanically-shaken cuvette cultures were grown, and the results averaged.

#### *3.1 Flask Culture Growth Curves*

Absorbance values from all four flask growth cultures recorded by the GeneSys20 and the SpecPhone were averaged and plotted with relation to time. An exponential trend line was fitted, which shows a positive correlation between increasing absorbance and turbidity. The GeneSys20 and SpecPhone absorbance values were very similar at the early time points. However, as turbidity increased the GeneSys20 values began to trend higher than those recorded by the SpecPhone, beginning around  $T_{120}$ . There is strong correlation between the data points for both devices, with  $R^2$  values of 0.979 and 0.953 for the GeneSys20 and the

SpecPhone, respectively. The GeneSys20 yielded an equation of  $y=0.0378e^{0.0119x}$ , and the SpecPhone yielded the equation  $y=0.0385e^{0.0104x}$ . Both equations have similar coefficients, which is an important characteristic for verifying the accuracy of the SpecPhone as compared to a commercial device. The Absorbance  $\pm$  SEM values are presented in **Figure 5**, along with the lines of best fit and the correlation values.

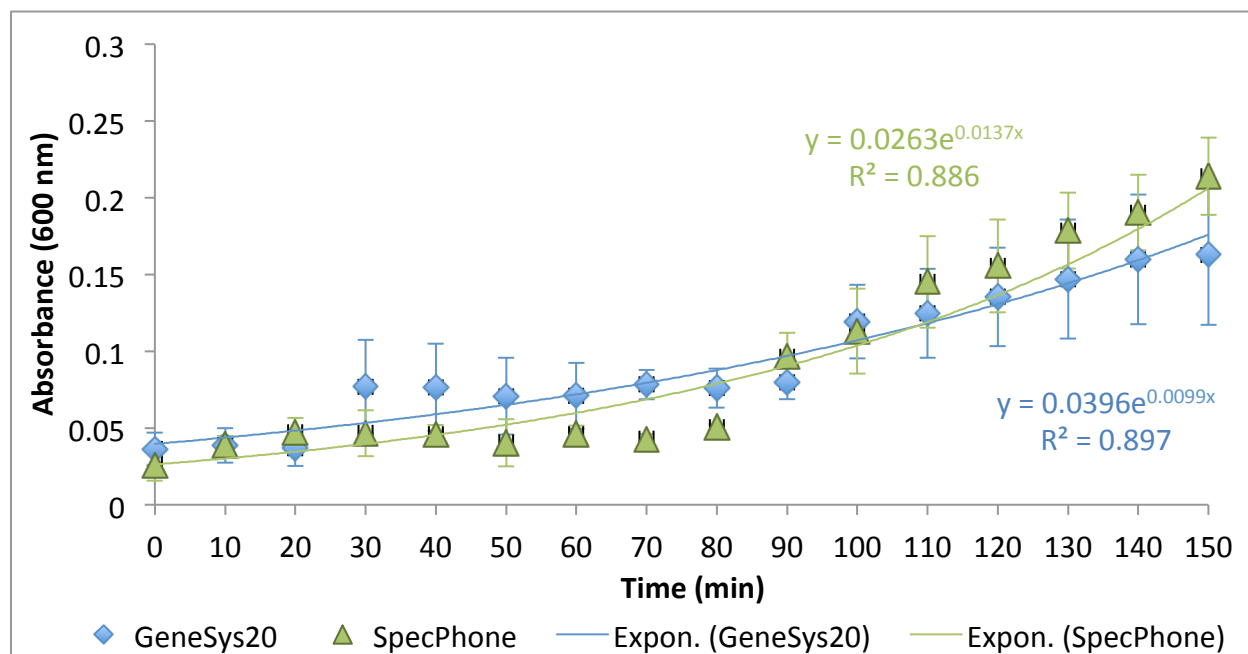


**Figure 5.** The combined results from the flask growth cultures. There is a strong trend upward corresponding to increased culture turbidity, as represented by the lines of best fit and their equations. Values are present as Absorbance  $\pm$  SEM.

### 3.2 Cuvette Culture Growth Curves

Following the initial flask culture, which demonstrated that the SpecPhone was capable of measuring turbidity, the smaller-scale cuvette culture protocols were tested. The results from the mechanically-shaken cuvette cultures are presented first. Overall, there is similarity between the values recorded by the SpecPhone and the GeneSys20, with minor disparities, particularly between  $T_{30}$ - $T_{80}$ , where the GeneSys20 recorded slightly higher values, and between  $T_{120}$ - $T_{150}$  where the SpecPhone recorded slightly higher values. Generally, there is a much more pronounced margin of error in these values, as represented by the more dramatic

error bars. When looking at the shape of the curves, the SpecPhone data follows a smoother upward trajectory, even though the correlation is slightly less pronounced than that of the GeneSys20, with  $R^2$  values of 0.886 and 0.897, respectively. The growth curve from the mechanically-shaken cuvette culture is below as **Figure 6**.

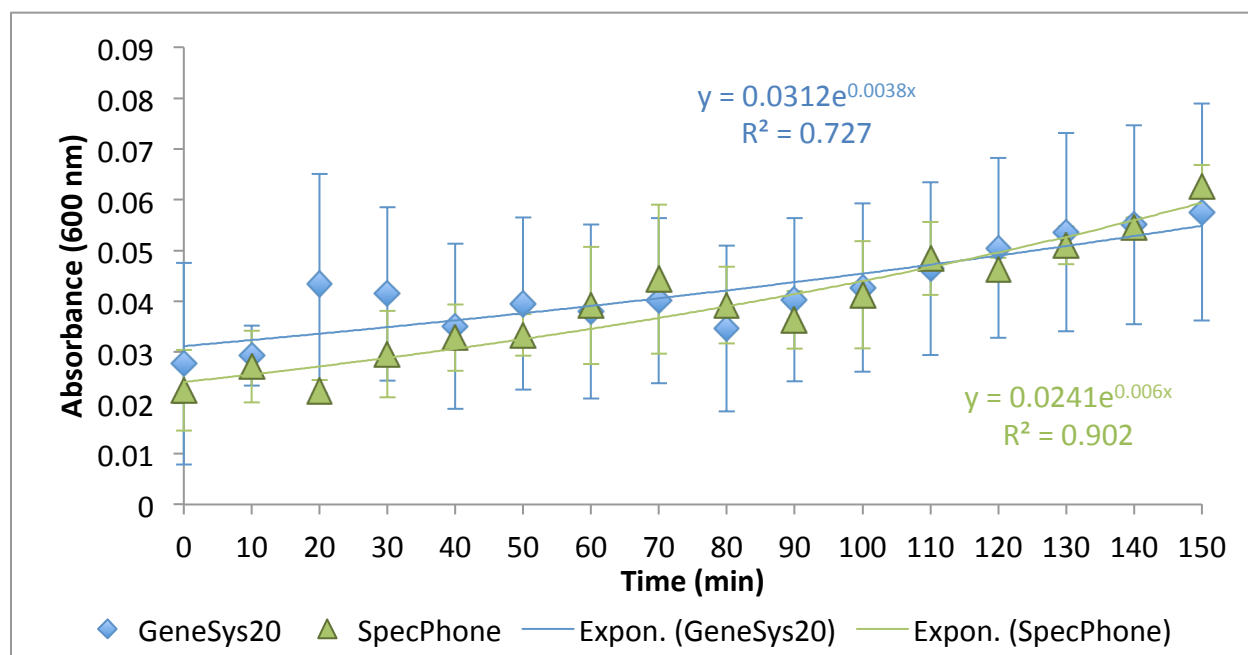


**Figure 6.** Growth curve from the mechanically-shaken cuvette cultures. Values are present as Absorbance  $\pm$  SEM.

The next trials were the hand-shaken cuvette cultures, which were removed from the incubator and vigorously shaken by hand at 5-minute intervals. Between shaking, these cuvettes were kept at 37°C. However, the absorbance values from this culture are markedly different from those of the mechanically-shaken culture.

The final, average absorbance values at  $T_{150}$  for the mechanically-shaken cultures were  $0.163 \pm 0.0456$  and  $0.214 \pm 0.0252$  for the Genesys20 and SpecPhone, respectively. At that same time point in the hand-shaken cultures, the absorbance values were  $0.0576 \pm 0.0214$  and  $0.0626 \pm 0.00434$  for the Genesys20 and SpecPhone, respectively, representing a substantial

reduction in the total absorbance of the hand-shaken cultures. This data is also much noisier than that of the mechanically-shaken cuvettes, particularly for the data from the GeneSys20, which has an  $R^2$  value of 0.727. The SpecPhone data has a stronger correlation, with an  $R^2$  of 0.902, however the upward trend in absorbance as a function of time is much less robust for both devices. The growth curve from the hand-shaken cuvette culture is below as **Figure 7**.



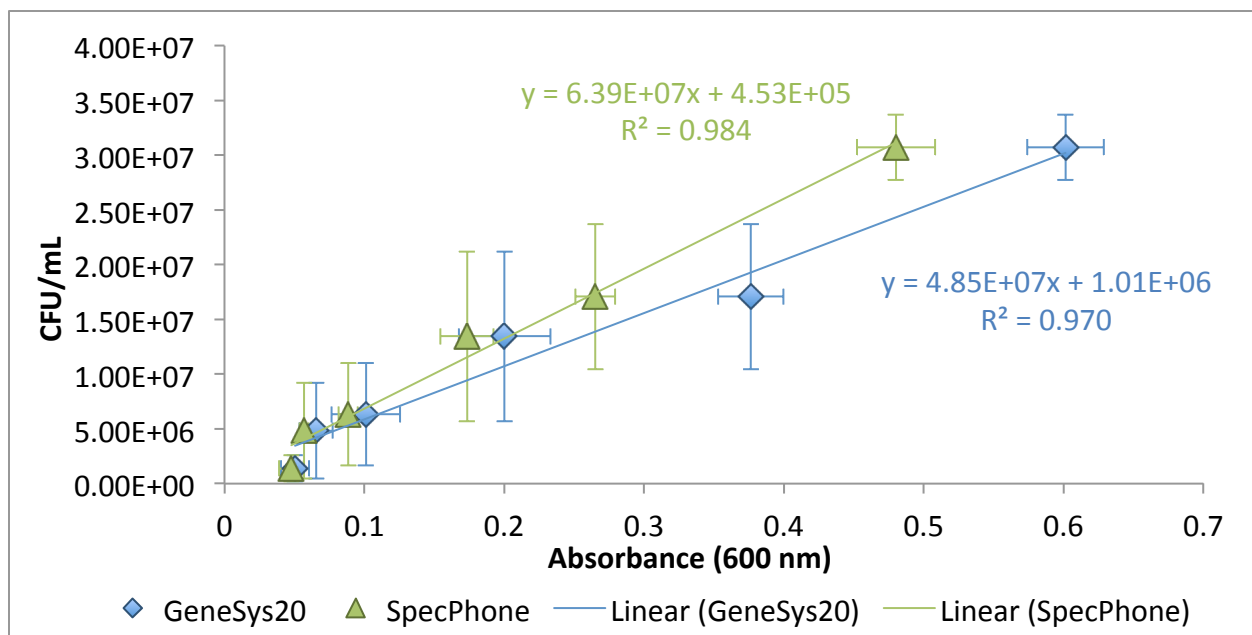
**Figure 7.** Growth curve from the hand-shaken cuvette cultures. Values are present as Absorbance  $\pm$  SEM.

### 3.3 Bacterial Plating and Standard Curve Construction

Three of the four flask culture trials involved bacterial plating. From the bacterial plates, a standard curve relating absorbance to the number of CFU/mL was constructed. Each colony growing on a plate was deemed to have begun from a single bacterium, and thus was counted as a CFU (Sutton, 2011). Correlating the number of colonies with the absorbance at the same time point, the relationship between absorbance and the number of cells present in the culture was determined. Using the equation of the line of best fit of the standard curve, the bacterial

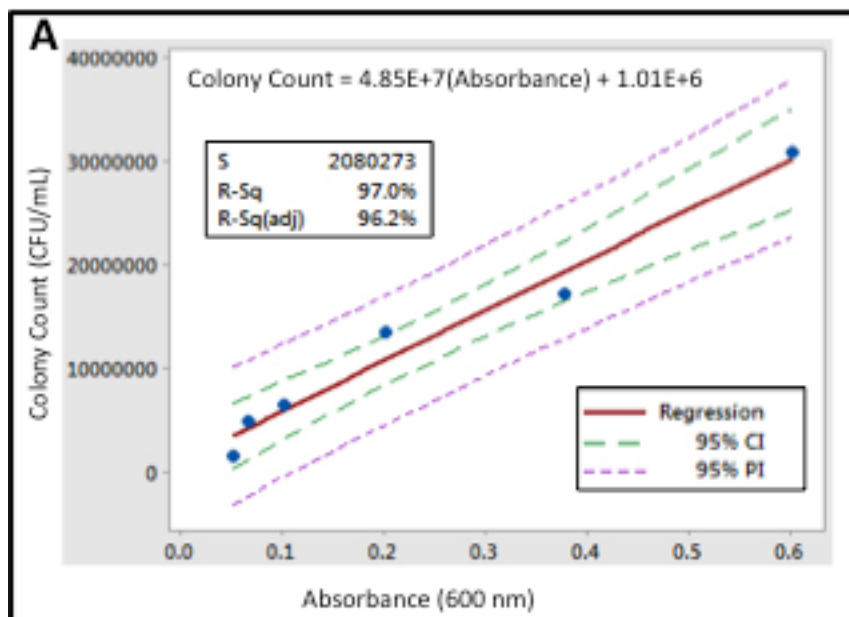
concentration of an unknown K12 *E. coli* culture can be calculated, provided an absorbance value is known.

The equation of the line of best fit for the standard curve created from the absorbance values recorded from the SpecPhone is  $y = 6.39E+07x + 4.53E+05$ , with an  $R^2$  value of 0.984. This line represents the data with statistical significance when subjected to analysis by linear regression ( $p < 0.05$ ). Similarly, the same analysis was performed on the line of best fit describing the data recorded from the GeneSys20, yielding an equation of  $y = 4.85E+07x + 1.01E+06$ , with an  $R^2$  value of 0.970 ( $p < 0.05$ ). Furthermore, the correlations are not statistically different from each other ( $p = 0.741$ ). The standard curve is presented as **Figure 8**.

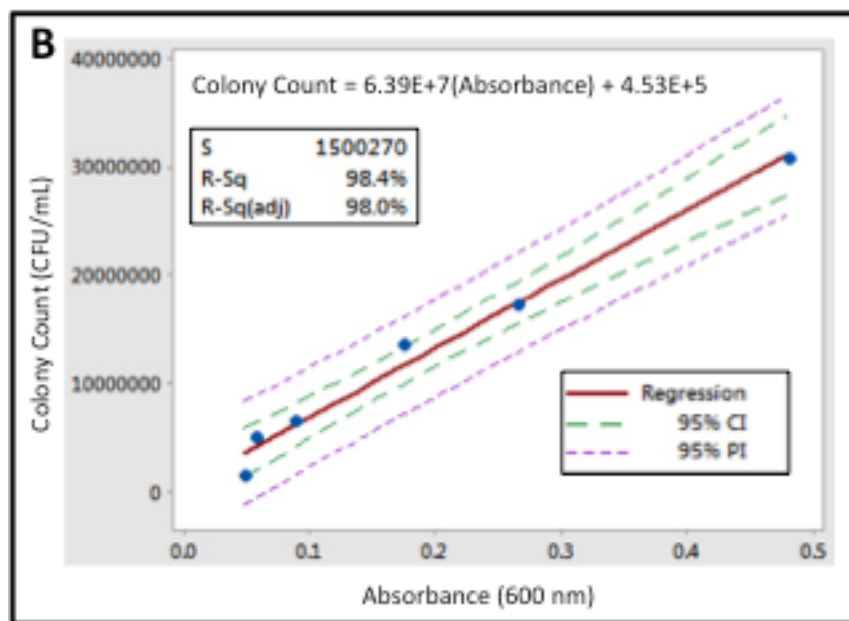


**Figure 8.** The standard curve relating absorbance and the number of CFU/mL. Values are present as Absorbance  $\pm$  SEM in the X-direction and CFU/mL  $\pm$  SEM in the Y-direction.

The lines of best fit for the standard curve in **Figure 8** were subjected to statistical analysis by linear regression, followed by plotting within a 95% confidence interval. These annotated graphs appear below, with the data from the GeneSys20 represented in **Figure 9A** and that from the SpecPhone in **Figure 9B**.

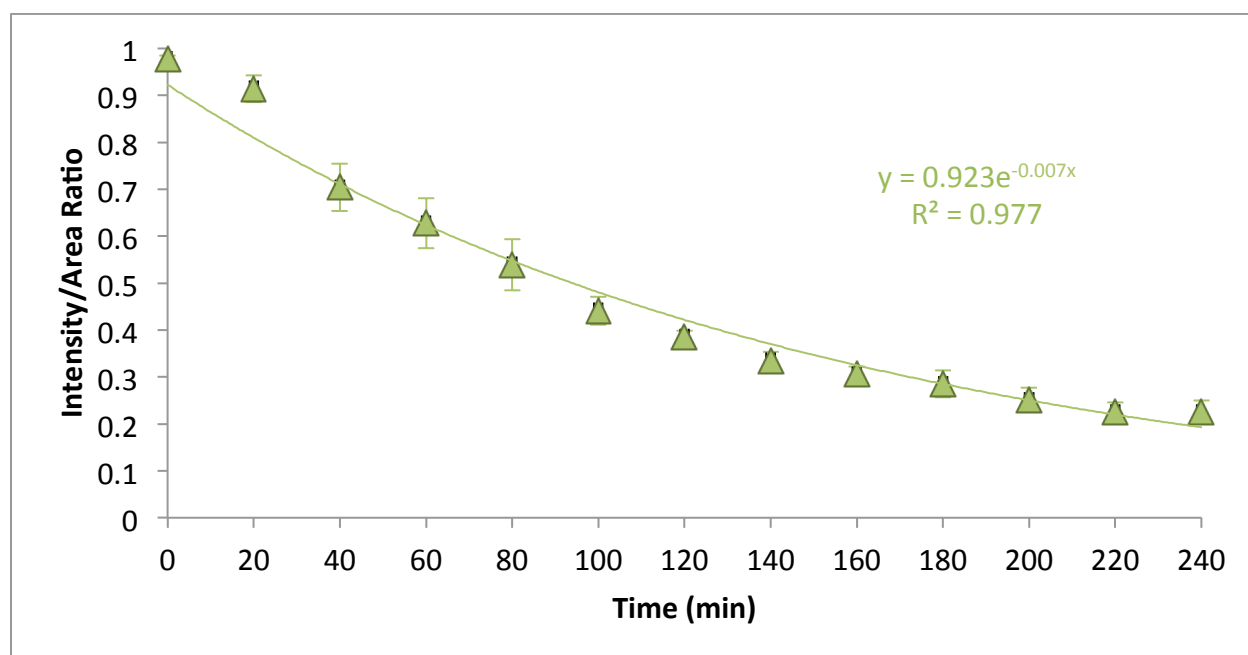


**Figure 9. 9A** Shows the annotated standard curve created from the data of the GeneSys20. All data points fall within a 95% confidence interval, as well as the 95% prediction interval. The resulting equation of the line of best fit is also shown. **9B** shows the same metrics for the data collected from the SpecPhone. The coefficients of the equations are different, however both have good  $R^2$  values.



Additional analysis was performed on the data from the flask culture trials as an alternative to measuring absorbance using the SpecPhone. The overall intensity of the spectrum images from the blank media and the cultures were compared as a ratio termed the intensity/area ratio (IR). For each blank and each sample, the average pixel intensity per unit of area (PIA) was measured using ImageJ and the following formula: 
$$\text{PIA} = \frac{\text{Intensity (Gray Value)}}{\text{Spectrum Area}}$$
.

The PIA value was calculated for each blank and each sample, and these values were used to calculate the IR using the following formula:  $IR = \frac{PIA_{sample}}{PIA_{blank}}$ . This ratio is intended to show the difference in the overall intensity of the light transmitted through the sample and the blank media. As the culture grew more turbid, the amount of light that passed through the sample decreased as the amount scattered by the bacteria increased. When correlated with the time points of measurement, the IR serves as an alternative measurement to absorbance. This data is presented below as **Figure 10**.



**Figure 10.** This relationship represents the decrease in the intensity/area ratios between the blank media and the bacterial culture at 20-minute intervals of measurement. As the number of bacteria in the culture increased, the amount of light scattered by their turbidity proportionally increased, decreasing amount of light that reached the camera. Values are present as  $IR \pm SEM$ .

#### 4. Discussion

The results of this project support the hypothesis that the SpecPhone can be used to measure bacterial growth. Broadening the scope of the SpecPhone to measurement of living bacterial systems opens new educational opportunities, thus advancing the overall goal of the SpecPhone project. Further, two distinct bacterial growth protocols have been designed, which



outline detailed instructions for use in a variety of settings. The distribution-ready versions of these protocols are included in **Appendices 2 and 3** of this report, representing the flask cultures and cuvette cultures, respectively.

A major concern when developing protocols for the SpecPhone is the accuracy and reproducibility of the results. This was especially pertinent for this project, which focused on determining the plausibility and practicality of using the SpecPhone to quantify actively dividing bacteria. Regarding the growth curves that resulted from the flask culture experiments, the absorbance values recorded from the SpecPhone and GeneSys20 are closely correlated with each other, as shown by the similarity of the coefficients of the respective lines of best fit. SpecPhone values are represented by the equation  $y = 0.0385e^{0.0104x}$ , and the GeneSys20 values by the equation  $y = 0.0378e^{0.0119x}$ . The error bars for both sets of data are also small, indicating the standard error of the average absorbance values was small. Again, this points to the reproducibility of the data obtained from these protocols.

In an effort to simplify use of the SpecPhone to measure bacterial growth, the cuvette culture growth protocols were designed and tested. The goal of these protocols was to decrease the time necessary for completion. Results from the cuvette culture trials were not as robust as those from the cultures grown in the flasks. Given the smaller vessel and the shortened duration of measurement, cuvette culture absorbance measurements are much lower than those recorded in the flask culture trials. At the last point of measurement,  $T_{150}$ , the absorbance value recorded by the SpecPhone from the flask growth protocols was  $0.480 \pm 0.0281$ . Conversely, the maximum absorbance values recorded by the SpecPhone were  $0.214 \pm 0.0252$  and  $0.0626 \pm 0.00434$  for the mechanically-shaken and hand-shaken cuvette

cultures, respectively. But, when comparing the maximum values recorded by the SpecPhone to those recorded with the GeneSys20, they are similar. Absorbance values recorded from the GeneSys20 were  $0.163 \pm 0.0456$  and  $0.0576 \pm 0.0214$  for the mechanically-shaken and hand-shaken cuvette cultures, respectively, and  $0.601 \pm 0.0274$  for the flask cultures.

There is a dramatic difference in the maximum absorbance value of the culture grown in the hand-shaken cuvette when compared to the mechanically-shaken cuvette. This is likely due to inadequate aeration of the *E. coli*, which is necessary to observe growth in a short period of time (Juergensmeyer, Nelson, & Juergensmeyer, 2007). Without adequate access to oxygen, the growth rate of the bacteria is slowed, and thus there was likely not enough cell division to produce a culture with noticeably increased turbidity. This represents the trade-off of using the hand-shaking protocol. Cost and equipment requirements are reduced, and the protocol can be completed in a shorter period of time. But, the results are naturally not as strong as those completed using more advanced equipment, larger culture vessels, and over a longer time period.

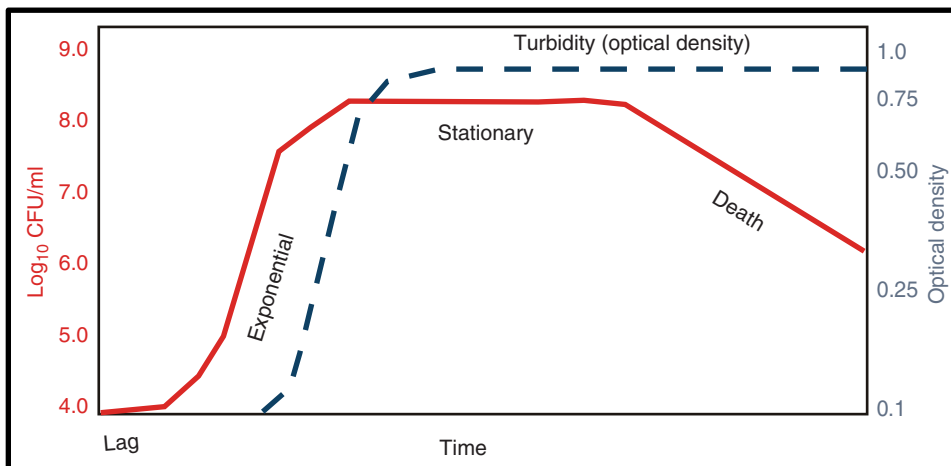
The cuvette culture protocols also do not result in the creation of a standard curve relating absorbance and colony number, as this requires removal of a sample of the culture for plating. Given the small volume of the cuvettes and the limited amount of culture available for analysis, periodic removal of culture for plating would interfere with growth. However, if a standard curve from another experiment is available, the equation of the line of best fit for this plot can be used to calculate CFU/mL of the cuvette cultures. Ultimately, the purpose of the cuvette growth protocols is to introduce the topic of measuring bacterial growth, and to do so in a shorter duration of time. Despite these limitations, this goal was accomplished overall.

Plating was also done in this experiment series to establish the relationship between absorbance and bacterial colony number, and to create a standard curve of bacterial growth (**Figure 8**). The standard curve correlates absorbance values with the number of bacterial colonies that grew on the plates from culture samples diluted at the same time point. The equations of the lines of best fit from the standard curve constructed in this experiment can be used to determine the number of CFU/mL of an unknown K12 *E. coli* culture, if the absorbance value is known. Rewriting these equations to include the variables of this experiment, the GeneSys20 equation becomes:  $CFU/mL = 4.85E+07(Absorbance) + 1.01E+06$ , and the SpecPhone equation becomes:  $CFU/mL = 6.39E+07(Absorbance) + 4.53E+05$ . As mentioned, these lines are statistically significant in their representation of the data for both the GeneSys20 ( $p < 0.05$ ) and the SpecPhone ( $p < 0.05$ ) when analyzed by linear regression, and are not statistically different from each other ( $p = 0.741$ ). And, as shown in **Figures 9A** and **9B**, the data points are aligned within a 95% confidence interval. Again, this points to the reproducibility and accuracy of the SpecPhone results, when judged against a commercial device.

Little variation exists in the absorbance values (X-axis error bars) recorded from each instrument, however; there is significant variation in the calculated CFU/mL values (Y-axis error bars) at each data point of the standard curve. There are a number of potential explanations for this variation, the first of which has to do with the unique growth patterns of each individual culture. All stock cultures were created with the same K12 strain of *E. coli*, however there were still differences in growth, and subsequently absorbance measurements, and ultimately fewer viable cells available to establish colonies upon plating. This could have to do with the age of the cells used to create the stock culture. In some of the trials, the plates of K12 *E. coli* used to

grow the stock culture were several days old. It is possible that the number of viable cells contained in each colony on the plate had decreased by that point, and thus fewer viable cells were available when the blank media was inoculated.

Correlated with this could be a second possible explanation: an increase in the time necessary for synthesis of growth enzymes and substrates. As shown in **Figure 11**, there is a distinct phase at the beginning of the bacterial growth trajectory in which the cells are not rapidly dividing, and instead are synthesizing the needed substrates and molecular machinery required for division. Depending on the number of viable cells at the beginning of the trial, this period may have been longer for some cultures than others as the bacteria prepared for growth. Beginning with fewer cells would naturally lead to a longer time necessary to visualize an increase in turbidity as increased absorbance.



**Figure 11.** An example of a standard bacterial growth curve is represented, with labeled phases of growth. In these experiments, only the initial phases were visualized in the results. However, allowing the trials to grow for a longer period of time may allow for visualization of later phases (Maier, Pepper, & Gerba, 2009).

Finally, when looking at the number of colonies present at the  $T_{240}$  time point in the standard curves, there is a large increase in Absorbance as well as CFU/mL. This difference may be explained by the fact that the final plates were created from the  $T_{240}$  time point, which is actually 60 minutes following the  $T_{180}$  point, instead of the 40-minute intervals at which the previous plates were created. This was done to ensure there were plates available for the final

point of the culture growth, rather than plating from the culture at  $T_{220}$  and neglecting the growth that occurred in the final 20 minutes. In a future revision, it may be beneficial to extend the trial to 260 minutes, allowing for plates to be constructed at the  $T_{220}$  and  $T_{260}$  time points, and adding an additional absorbance measurement at  $T_{260}$ .

Within these protocols, there is educational value and potential for extending that value. One potential area would be extension of the protocols to include more plating, with the hope of visualizing more phases of bacterial growth. A hallmark characteristic of bacterial growth curves is their shape, showing four, distinct phases of growth: lag, exponential, stationary and cell death (**Figure 11**). These phases of growth will not be represented as changes in turbidity, but instead can be seen when plotting CFU/mL. After the plateau phase has been reached, turbidity will remain constant as long as the culture is continually shaken to keep the cells in suspension. However, active cell division will no longer be occurring. If samples are taken from the culture at regular intervals and plated, the calculated number of CFU/mL will remain constant throughout the stationary phase, and will eventually begin to decline as the cells deplete the nutrients of the media and begin to die. If absorbance measurements are taken in concurrence with these plates, a growth trajectory similar to that of **Figure 11** will result, with absorbance remaining constant and the CFU/mL eventually declining. This could represent an interesting concept to broaden the educational prospects of these protocols.

A second option to visualize these same effects would be to grow a control flask culture along with the one to be measured (sample flask). A second flask of media could be inoculated with the stock culture at the same time as the sample flask. The control flask culture would grow along with the sample culture, and absorbance measurements would be taken at the

same time points. However, after the  $T_{240}$  measurement, absorbance measurement of the control flask would continue for approximately four additional hours. Eventually, the absorbance measurements would be expected to reach a plateau, pointing to cessation of bacterial growth. However, the number of cells is not decreasing, as they are still in suspension because of shaking in the incubator. But, this presents a tradeoff in that this extended measurement would increase the time necessary for completion. To decrease the amount of active laboratory time required, absorbance measurements for the control culture could be taken at 40-minute intervals instead of 20-minutes. The point of this modification would be to show the general shape of a standard bacterial growth curve, rather than close monitoring of culture turbidity.

Finally, further educational opportunities are implicit within these protocols by the addition of various other agents to the cultures. For example, antibiotics could be added to one culture, grown alongside one without them to visualize and teach the effects of these drugs. For a further nuanced comparison, a bactericidal antibiotic, one that kills the cells could be compared with a bacteriostatic drug, one that does not kill the existing cells but prevents further division. The hands-on nature of the SpecPhone, coupled with the ability to grow and visualize bacterial cultures in a relatively short amount of time presents a powerful teaching opportunity. Similarly, one culture could be supplemented with a glucose solution, and compared to a culture grown in un-supplemented media. The culture with glucose supplementation would be expected to grow at a faster rate, as glucose is the preferred carbon source for *E. coli* and could enter directly into central metabolism.

These protocols could also be extended to compare the growth of different bacterial species. For example, the growth rate of a Gram-positive species could be compared to that of a Gram-negative species. Importantly, these modifications would involve bacterial strains that may have different safety recommendations than the ones presented here. This should be carefully considered before any additional trials are performed, and before any protocol modifications are made. Importantly, these modifications could be applied to either the flask or cuvette culture protocols, depending on the resources and time restraints of the individual setting in which they will be implemented.

Despite the work presented here to increase the applications of the SpecPhone, several limitations persist regarding these protocols. The largest of these issues revolve around the conditions that are necessary for *E. coli* growth in the laboratory. While agitation and 37°C incubation are not absolutely required for *E. coli*, the rate of cellular growth is dramatically increased under these conditions. Without agitation, the bacteria will grow; however, the rate will be dramatically slower because they will be forced into anaerobic conditions and rely on fermentation rather than oxidative metabolism (Juergensmeyer, Nelson, & Juergensmeyer, 2007). In order for these protocols to be performed in a reasonably brief period of time, instruments to ensure agitation and heat were relied upon. Availability of this kind of equipment may not be a reality for educational settings that would benefit from teaching spectroscopy using the SpecPhone, thus presenting a major limitation for these protocols that did not affect the formerly designed ones. As previously stated, a potential extension of the protocols would be the supplementation of the media with a glucose solution. This is predicted to increase the growth rate, however it does not solve the issue of the need for constant

agitation to aerate, and to keep the cells in suspension for measurement. Efforts to shake the culture and suspend the cells without advanced equipment produced subpar results, as shown by the hand-shaken cuvette cultures.

One potential avenue that could solve the issue of agitation, which was not explored in the present experiment, would be the use of a magnetic stir bar. For the flask cultures, a pill-sized stir bar placed atop a magnetic stir plate could produce sufficient agitation for bacterial aeration. For the cuvette cultures, a rice grain-sized stir bar could be employed, with the cuvettes placed atop the stir plate. To provide heat, the flask or cuvette could be placed in a water bath that would be heated and kept in the range of 30-37°C to prevent cell damage and death. The benefits here are twofold in that the need for an expensive shaking incubator would be eliminated, and it would also allow these protocols to be performed in a more practical duration of time. While this option was not pursued in this experiment, it does present an interesting future option, and warrants further investigation.

Another issue surrounding these protocols, specifically those that were conducted in the flasks, is the amount of time required. Currently, the flask culture protocols are designed for completion in 4 hours, and the cuvette culture protocols in 2.5 hours. Neither estimate includes the necessary preparation time to create the stock culture, nor the additional time required for plate counting. While this is a relatively short period of time overall, it does limit the potential for visualization of all four phases of bacterial growth. Full phase visualization is not an absolute necessity for using these protocols to teach the principles of bacterial growth, but it would add an additional layer of depth to the experience. As mentioned, this may be compensated for by the addition of a control culture that is grown over a longer period of time. Extending the



amount of time given to complete each is a potential solution, yet this furthers the challenge of using these protocols in settings with inflexible schedules or limited laboratory time. Even with visualization of the exponential growth phase only, there are still valuable teaching opportunities for learning about bacterial growth.

Turning now to issues with image analysis, the ImageJ analysis was more complicated for these experiments than in the previous SpecPhone protocols, which dealt with simpler, colored solutions. Bacterial cultures are turbid, and the spectra became increasingly dispersed and dim as the culture grew. This is shown in the comparison between BSA and *E. coli* in **Figure 4A** and **4B**. Overall light intensity is diminished as the number of individual photons reaching the camera is reduced because of light scattering from the bacteria. This overall decrease in intensity is represented in the comparison plot of **Figure 4C**. The spectra area also became larger as the culture grew, and analysis with ImageJ relies on comparing intensity at specific pixel positions. Ensuring the areas of analysis between the culture and blanks were aligned became more complicated with increasing culture turbidity.

A potential alternative for this method of measurements is to use overall spectrum intensity, rather than intensity at a particular pixel position, which corresponds to a specific wavelength. ImageJ allows for measurement of intensity (Gray value) over a unit of area, or PIA. The PIA of a sample and blank can be compared to calculate the IR. At the beginning, the sample and blank spectrums will be nearly identical because there has been limited bacterial growth, meaning  $IR \approx 1$ . However, as the culture grows and becomes increasingly turbid, the intensity of light passing through the culture samples will begin to globally decrease, rather than solely at a specific wavelength. IR values will continually decrease below 1, resulting in a

downward trend. This decrease in overall intensity can be plotted as a function of time, which was explored and is presented in **Figure 10**. This correlation would not be verifiable by a commercial instrument, such as the GeneSys20, which only reports the absorbance value, not overall intensity. Further, when attempting to use the IR values to create an alternative growth curve for the data, the correlation was subpar, with an  $R^2$  value of 0.676 (data not shown). While IR may not be a useful for quantifying an unknown K12 *E. coli* culture, it does have potential for use as a teaching tool to demonstrate the physics of light scattering with increasing culture turbidity.

Finally, there are several issues that are related to the larger SpecPhone project, that are not unique to these protocols. The first is the image analysis process using ImageJ. While the aforementioned protocol is available (Smith, 2015), analysis is still not an overtly streamlined process. For beginners, ImageJ may not be an inherently user-friendly program, and it presents a significant learning curve for someone using it for the first time. That being said, ImageJ is commonly used software in many areas of research, and early introduction may be an advantage. An area of further research could be centered around development of a mobile application that could perform the data analysis on the phone, eliminating the need for import and analysis with ImageJ. A second issue centers on the design of the SpecPhone, which currently only fits the iPhone 5/5s. This places a significant limitation on the application of the apparatus, and efforts toward a redesign are ongoing.

## **5. Conclusion**

The work presented here shows that the SpecPhone can be consistently and accurately used to quantify an actively dividing K12 *E. coli* culture. From this project, three protocols were

designed and tested. One protocol provides steps for growth of *E. coli* in an Erlenmeyer flask with routine measurement of absorbance and CFU/mL via plating. The others give instructions for smaller-scale cultures grown entirely in plastic cuvettes, with either mechanical or hand-shaking. The results obtained from the cuvette culture protocols were not as robust as those from the flask cultures, however they still present valuable, educational opportunities. When comparing the results obtained from the SpecPhone to those of the GeneSys20, there is some deviation but overall the trends are consistent for all protocols. This reproducibility and consistency make the SpecPhone a compelling and useful tool for teaching the use of spectroscopy to measure actively dividing bacterial cultures. Combined with the relative inexpensiveness of the SpecPhone components, this body of work makes the ubiquitous and powerful technology of spectroscopy more hands-on and accessible.

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### **Safety Precautions & Guidelines**

Given that this protocol deals with bacteria, safe laboratory practice is of a more concern compared to the previous SpecPhone protocols. All work was completed in accordance with the CDC's guidelines for a BSL-1 laboratory (Centers for Disease Control and Prevention, 2009). Gloves, eye protection and a lab coat were worn throughout the entirety of all trials, none of which left the laboratory.

## Creating a Standard Bacterial Growth Curve

### Materials:

- Laboratory strain (K-12) of *Escherichia coli*
- Erlenmeyer Flask, 125 mL capacity
- Trypticase Soy Broth (TSB) (~400 mL)
- Trypticase Soy Agar (TSA) plates (48)
- Ethanol
- Culture tubes, sterile (25)
- p5000 & p1000 Pipettes and pipette tips
- Bunsen burner or other flame
- Shaking incubator, capable of reaching 37°C and 265 rpm
- Bacteria spreader
- Plastic cuvettes (2), 4.5 mL 1 cm. x 1 cm.
- Lens Cloth (KimWipes)
- SpecPhone apparatus with necessary additions
- Light Source (desk lamp)
- iPhone or another camera-containing mobile device
- Laboratory notebook
- Commercial spectrometer (if available)



### Introduction:

This protocol provides the steps for quantifying bacterial growth using the SpecPhone. A stock culture of *E. coli* is created and then used to inoculate sterile media to grow a new culture. At regular intervals along the process, a sample of the growing culture is removed and an absorbance measurement is taken. Plating is performed at regular intervals, and these plates are used to determine the number of bacteria in the culture along the growth process. When all of the data has been collected, a standard curve can be plotted relating absorbance to the number of bacteria, which can be used to assess the number of cells in an unknown culture.

As the culture grows, it becomes increasingly turbid. This increasing turbidity causes more light to be scattered by the culture, resulting in a decrease in the intensity of the light recorded by the SpecPhone. Light scatter is directly dependent on the density of bacterial cells present in the culture. The amount of light allowed to pass through the culture is the percent transmission (%T), which can be converted to an absorbance value using the equation:  $A = -\log(\%T)$ . The convention is to measure bacterial cultures at 600 nm.

A special note should be made that this protocol requires up to three days for completion. One day is needed to create the stock bacterial culture and allow it to incubate. A second day will be spent taking the actual measurements and the third will be devoted to counting bacterial plates.

## Procedure:

### Day 1:

#### **Creation of Stock *E. coli* Culture:**

1. Fill a culture tube with 10 mL of sterile TSB, and bring to a temperature of 37°C.
2. If the *E. coli* are initially on a bacterial plate, select a single colony from the plate with the loop and inoculate the TSB. Flame the loop before touching the plate to ensure sterility. If the *E. coli* are initially in broth, pipette 100  $\mu$ L of the culture into 9.9 mL of warm TSB.
3. Incubate this culture for 24 hours in the shaking incubator, shaking at a speed of 265 rpm, and at a temperature of 37°C.

#### **Preparation for Measurements:**

1. Plating and dilutions will be done in this experiment to create the standard curve relating colony number to absorbance. Blank tubes of TSB will be required for this, and should be filled ahead of time. Create six sets of the following tube volumes: 3 filled with 9.0 mL of TSB, and 1 filled with 9.9 mL of TSB. Ultimately, there will be a total of 24 culture tubes; six sets of four tubes.
2. Fill the Erlenmeyer flask with 95 mL of TSB. Place the flask along with the culture tubes in an incubator at 37°C and allow them to come to temperature.
3. Prepare the SpecPhone apparatus. Ensure the surroundings are free from outside light as possible, and that the light source being used is bright enough. Place the mobile device in the SpecPhone and launch the camera app.
4. None of the camera pixels should be saturated by the light source, and the spectrum should have even intensity across the color range. Once adequate and appropriate lighting is found, tape the device in place or make marks on the surface to denote the proper positioning. Take great care to keep the apparatus stationary throughout the trials to ensure accuracy across all measurements.
5. Fill one cuvette with TSB, this will serve as the blank.

### Day 2:

#### **Taking Absorbance Measurements:**

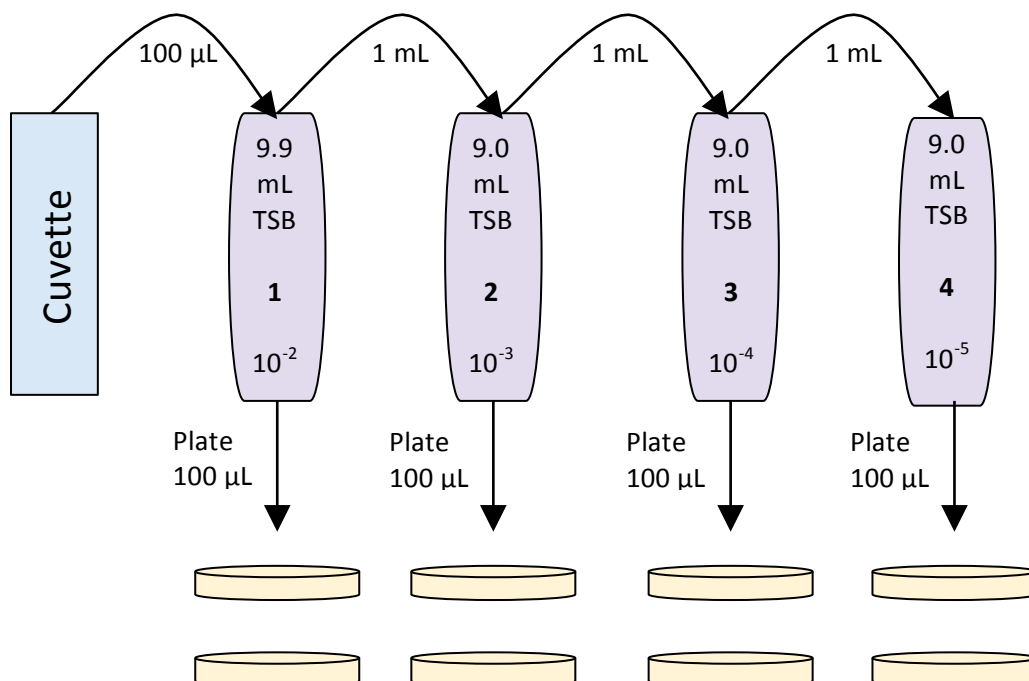
1. Inoculate the Erlenmeyer flask of warm TSB with 5 mL of the stock *E. coli* culture, transferring using the p5000 pipette.
2. Vortex to ensure mixing and immediately transfer 2.5 mL of the freshly inoculated culture to a cuvette using the p5000 pipette.
3. Place the blank cuvette in the SpecPhone and capture three spectrum images, and then replace the blank with the culture cuvette and capture three more spectrum images. Record the exact time of measurement in the lab notebook, and record which pictures in the camera roll correspond to the blank or culture samples. This is the  $T_0$  measurement.

*Tip: It may be useful to photograph some object at the beginning and end of the series of photos to denote the sequence in the phone's camera roll.*

- Place the Erlenmeyer flask in the shaking incubator at 37°C and 265 rpm, and set a timer for 20 minutes.
- At 20-minute intervals, pipette 2.5 mL of the culture in the Erlenmeyer flask to the sample cuvette and repeat the measurement process, remembering to blank the SpecPhone before taking each measurement. Keep detailed records in the laboratory notebook, or using the table provided in the **Appendix**.
- This process will continue over a period of 4 hours, with absorbance measurements being taken at the following time points: T<sub>0</sub>, T<sub>20</sub>, T<sub>40</sub>, T<sub>60</sub>, T<sub>80</sub>, T<sub>100</sub>, T<sub>120</sub>, T<sub>140</sub>, T<sub>160</sub>, T<sub>180</sub>, T<sub>200</sub>, T<sub>220</sub>, T<sub>240</sub>.
- If a commercial spectrometer is available, take measurements for comparison.

### Bacterial Plating:

- At 40-minute intervals, bacterial plates will be made from the growing culture. Plates should be made at the following time points: T<sub>20</sub>, T<sub>60</sub>, T<sub>100</sub>, T<sub>140</sub>, T<sub>180</sub>, T<sub>240</sub>.
- The prepared culture tubes of sterile TSB will be needed here. After taking absorbance measurements, pipette 100 µL of the culture in the sample cuvette to the first culture tube containing 9.9 mL of sterile TSB.
- Vortex the first tube to ensure a cell suspension is created.
- Transfer 1 mL of the solution in the first tube to the second, and vortex again. Transfer 1 mL of the second tube to the third tube, vortex, and then transfer 1 mL of the third to the fourth tube. This creates the serial dilutions, up to a factor of 10<sup>-5</sup>.
- From each tube, pipette 100 µL on to two TSA plates. There will be a total of eight plates for each time point. Use the bacterial spreader to evenly distribute the culture on the plate. Before spreading, dip the spreader in the ethanol and then in the flame to sterilize. Allow the spreader to cool before touching the plate to prevent killing the cells. A schematic of the dilution process is below.





- Label the plates using the following convention: tXXX, dilution factor, plate# (eg. T<sub>100</sub>, 10<sup>-3</sup>, #1). The date should also be included.  
*Tip: Only write on the plate not the lid to save the information incase the lids get separated.*
- Place the plates in the incubator at 37°C overnight.

Day 3:

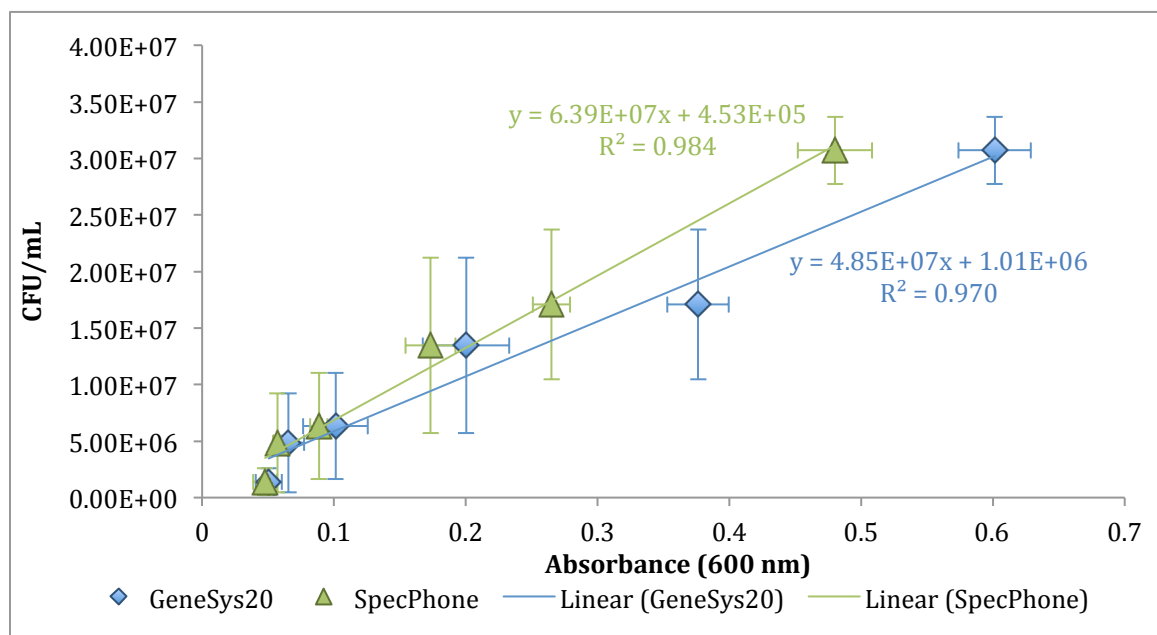
### Plate Counting & Data Analysis:

- Following incubation, count the number of colonies on each plate. Only those plates that have between 30-300 colonies should be counted, as this is the best window for statistical accuracy.  
*Tip: If a plate has more than 300 colonies, label it as 'Too Many To Count.'*
- Count the colony number for both plates from each dilution factor for the six time points. Average the two colony counts for each dilution factor.
- Calculate the number of colony forming units per mL (CFU/mL) using the following formula:

$$\frac{CFU}{mL} = \frac{\text{Average Colony Number}}{(mL \text{ Plated}) \times (\text{Dilution Factor})}$$

*Tip: The mL Plated for all is 100  $\mu$ L or 0.1 mL.*

- Images taken with the SpecPhone can be imported to a computer containing ImageJ (Rasband, 2016) and a photo browser. Follow the instructions for image analysis provided in the *Image Processing and Calibration* protocol (Smith, 2015). A table is included in the **Appendix** to organize the data and calculations.
- Create the standard curve by plotting the absorbance value for each of the six time points on the X-axis, and the calculated CFU/mL value on the Y-axis. An example standard curve appears below:



6. If an unknown K12 *E. coli* culture is given, the number of CFU/mL can be calculated using the equation of the line of best fit for the standard curve, substituting the known absorbance value in for X and solving for Y (CFU/mL).

*Important: The data obtained from these trials can only be used to quantify unknown cultures of K12 E. coli. If another bacterial species is to be analyzed, a standard growth curve for that species must be constructed as each bacterium has its own growth characteristics.*

## **Safety Guidelines:**

Given that this protocol deals with bacteria, the safety guidelines are somewhat stricter than when completing the other SpecPhone protocols. When choosing a bacterial strain to work with, we recommend K-12 *E. coli*, as this is a standard laboratory strain that has been determined to be non-pathogenic for humans and other mammals (U.S. Environmental Protection Agency, 1997).

Working with nonpathogenic organisms falls under the Biosafety Level 1 (BSL-1) guidelines from the CDC. Extensive guidelines can be found on the CDC website (U.S. Centers for Disease Control and Prevention, 2009). Briefly, all personnel should wash their hands before entering and leaving the lab, and wear gloves throughout the entire procedure. Gloves should not be worn outside of the lab, and should be immediately replaced if any damage occurs. If possible, wearing lab coats is preferable to protect personal clothing. No food or drink should be kept or consumed in the area where the experiment is being conducted. At the end of the lab period, disinfect all surfaces and any equipment that came in contact with the bacterial cultures. All students completing the protocol should be instructed to report any mistakes, spills, injuries etc. to the laboratory coordinator who will decide the appropriate action. Any individual institution practices that extend beyond these should be followed as well.

## **Protocol Expansions:**

There are a number of modifications that can be made to this protocol to further increase the educational potential. One such modification would be the addition of an antibiotic to one culture, and to then compare the growth rate of this culture to one that did not contain the drug. If a more nuanced comparison is desired, differing classes of antibiotic could be compared. For example, one culture could be supplemented with a bactericidal antibiotic, which will kill the cells and another could be supplemented with a bacteriostatic antibiotic, which does not kill the bacteria but rather prevents further division. Two different antibiotics of the same class could also be compared to determine if one is more effective than another.

Another potential expansion would be to supplement one culture with a glucose solution and compare its growth to that of a non-supplemented culture. Glucose is the preferred carbon source for *E. coli*, and it is able to enter directly into central metabolism. This addition is expected to increase the growth rate of the supplemented culture. Finally, the growth rates of differing species of bacteria could also be compared. A potentially interesting comparison would be between a Gram-negative species and a Gram-positive

species. However, this would involve different strains of bacteria that carry differing safety recommendations, which should be carefully considered before any additional trials are performed or any protocol modifications are made.

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**Appendix:**

Use the following tables to organize the plate counting and absorbance data. Remember, only count those plates that have 30-300 colonies. Use the provided formula to calculate the CFU/mL.

<b>T<sub>20</sub></b>				
<b>Absorbance Value:</b>				
Dilution Factor:	$10^{-2}$	$10^{-3}$	$10^{-4}$	$10^{-5}$
<i>Plate 1</i>				
<i>Plate 2</i>				
<b>Average</b>				
CFU/mL				

<b>T<sub>60</sub></b>				
<b>Absorbance Value:</b>				
Dilution Factor:	$10^{-2}$	$10^{-3}$	$10^{-4}$	$10^{-5}$
<i>Plate 1</i>				
<i>Plate 2</i>				
<b>Average</b>				
CFU/mL				

<b>T<sub>100</sub></b>				
<b>Absorbance Value:</b>				
Dilution Factor:	$10^{-2}$	$10^{-3}$	$10^{-4}$	$10^{-5}$
<i>Plate 1</i>				
<i>Plate 2</i>				
<b>Average</b>				
CFU/mL				

<b>T<sub>140</sub></b>				
<b>Absorbance Value:</b>				
Dilution Factor:	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>
<i>Plate 1</i>				
<i>Plate 2</i>				
<b>Average</b>				
CFU/mL				

<b>T<sub>180</sub></b>				
<b>Absorbance Value:</b>				
Dilution Factor:	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>
<i>Plate 1</i>				
<i>Plate 2</i>				
<b>Average</b>				
CFU/mL				

<b>T<sub>240</sub></b>				
<b>Absorbance Value:</b>				
Dilution Factor:	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>
<i>Plate 1</i>				
<i>Plate 2</i>				
<b>Average</b>				
CFU/mL				

## Bacterial Growth Curves - Cuvette Cultures

### Materials:

- Laboratory strain (K-12) of *Escherichia coli*
- Trypticase Soy Broth (TSB) (~20mL)
- Culture tube, sterile (1)
- p5000 & p1000 Pipettes and pipette tips
- Bunsen burner or other flame
- Ethanol
- Incubator, capable of reaching 37°C and 265 rpm
- Bacteria spreader
- Plastic cuvettes (2), 4.5 mL 1 cm. x 1 cm.
- Lens Cloth (KimWipes)
- SpecPhone apparatus with necessary additions
- Light Source (desk lamp)
- iPhone or another camera-containing mobile device
- Laboratory notebook
- Commercial spectrometer (if available)



### Introduction:

This protocol provides steps for creating a bacterial culture and measuring growth within a plastic cuvette. A stock culture of *E. coli* is created and then used to inoculate sterile media to grow a new culture. At regular intervals along the process, the culture is placed in the SpecPhone and an absorbance measurement is taken. When all the data has been collected, the absorbance measurements across the timespan can be used to solve for the number of bacteria using the equation of the trend line of a standard curve. The advantage of this protocol is a reduced amount of time and resources needed for completion.

As the culture grows, it becomes increasingly turbid. This increasing turbidity causes more light to be scattered by the culture, resulting in a decrease in the intensity of the light recorded by the SpecPhone. Light scatter is directly dependent on the density of bacterial cells present in the culture. The amount of light allowed to pass through the culture is the percent transmission (%T), which can be converted to an absorbance value using the equation:  $A = -\log(\%T)$ . The convention is to measure bacterial cultures at 600 nm.

A special note should be made that this protocol requires two days for completion. One day is needed to create the stock bacterial culture and allow it to incubate as well as preparations. A second day will be spent taking the actual measurements.

## Procedure:

### Day 1:

#### **Creation of Stock Bacterial Culture:**

1. Prepare a culture tube full of sterile TSB, and bring it to a temperature of 37°C.
2. If the *E. coli* are initially on a bacterial plate, select a single colony from the plate with a loop and inoculate the TSB. Flame the loop before touching the plate to ensure sterility. If the *E. coli* are initially in broth, pipette 100  $\mu$ L of the culture into 9.9 mL of warm TSB.
3. Incubate this culture for 24 hours, shaking at a speed of 265 rpm, and at a temperature of 37°C.

### Day 2:

#### **Preparation for Experiment:**

1. This protocol involves growing a bacterial culture in a cuvette. Fill a plastic cuvette with 3 mL of warm TSB, which will be later inoculated to create the culture. This will be the sample cuvette.
2. Prepare the SpecPhone apparatus. Ensure the surroundings are as free of outside light as possible, and that the light source being used is bright enough. Place the mobile device in the SpecPhone and launch the camera app.
3. None of the camera pixels should be saturated by the light source, and the spectrum should have even intensity across the colors. Once adequate and appropriate lighting is found, tape the device in place or make marks on the surface to record the proper positioning. Take great care to keep the apparatus stationary throughout the trials to ensure accuracy across all measurements.
4. Fill a second cuvette with 3 mL of TSB, this will serve as the blank.

#### **Taking Absorbance Measurements:**

1. Inoculate the sample cuvette of warm TSB with 100  $\mu$ L of the previously created stock bacterial culture, transferring using the p1000 pipette.
2. Vortex to ensure mixing and immediately begin taking spectrum images with the SpecPhone.
3. Place the blank cuvette in the SpecPhone and capture a few spectrum images. Replace the blank with the culture cuvette, and capture several more spectrum images. Record the exact time of measurement in the lab notebook, as well as denote which pictures in the camera roll correspond to blanks or culture samples. This is the  $T_0$  measurement.

*Tip: It may be useful to photograph some object at the beginning and end of the series of photos to denote the sequence in the phone's camera roll.*

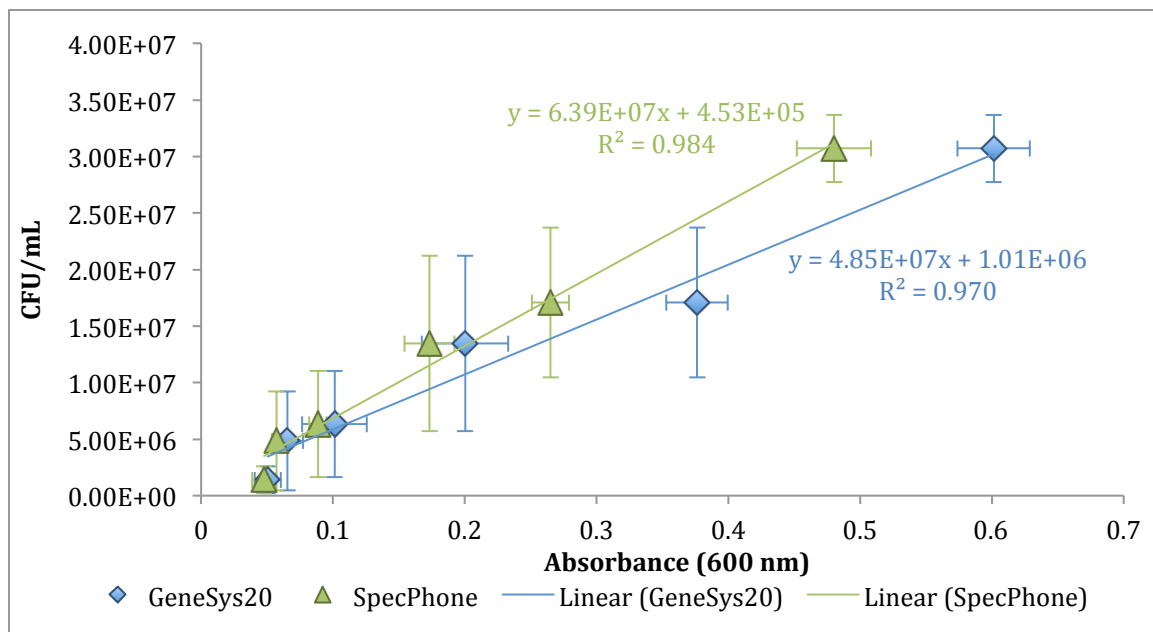
4. Depending on the availability of laboratory equipment, there are two options for completing this step:
  - a. If a shaking incubator is available: Secure the sample cuvette with tape in the incubator at 37°C and a speed of 200 rpm.

- b. If a stationary incubator is the only option: Place the sample cuvette in the incubator, uncovered. At 5-minute intervals, remove the culture cuvette and vigorously shake by hand, placing a lid on the cuvette to prevent spilling. Replace the cuvette in the incubator until it is time for absorbance measurement.
- At 10-minute intervals, remove the culture cuvette from the incubator and take a series of absorbance spectrum images. A series of captures of the blank cuvette should be done before the culture cuvette is placed in the device. Keep detailed records in the laboratory notebook, or using the table in the **Appendix**.
  - This process will continue over a period of 2.5 hours, with absorbance measurements being taken at the following time points: T<sub>0</sub>, T<sub>10</sub>, T<sub>20</sub>, T<sub>30</sub>, T<sub>40</sub>, T<sub>50</sub>, T<sub>60</sub>, T<sub>70</sub>, T<sub>80</sub>, T<sub>90</sub>, T<sub>100</sub>, T<sub>110</sub>, T<sub>120</sub>, T<sub>130</sub>, T<sub>140</sub>, T<sub>150</sub>.

### Image Analysis:

- Images can be imported to a computer containing ImageJ (Rasband, 2016) and a photo browser. Follow the instructions for image analysis provided in the *Image Processing and Calibration* protocol (Smith, 2015).
- By comparing the ratio of intensities for the blank cuvette and the culture cuvette, absorbance values are calculated for the culture at 10-minute intervals. A table is available in the **Appendix** to organize the data.
- From these absorbance measurements, the number of bacteria present in the culture at that time point can be determined by using the equation of a line of best fit for a standard curve. A sample standard curve appears below.

*Construction of a standard curve requires dilutions of the culture at regular time points and bacterial plating. A separate protocol (Creating a Standard Bacterial Growth Curve) is available which outlines the procedure for creation of the standard curve.*





## **Safety Guidelines:**

Given that this protocol deals with bacteria, the safety guidelines are somewhat stricter than when completing the other SpecPhone protocols. When choosing a bacterial strain to work with, we recommend K-12 *E. coli*, as this is a standard laboratory strain that has been determined to be non-pathogenic for humans and other mammals (U.S. Environmental Protection Agency, 1997).

Working with nonpathogenic organisms falls under the Biosafety Level 1 (BSL-1) guidelines from the CDC. Extensive guidelines can be found on the CDC website (U.S. Centers for Disease Control and Prevention, 2009). Briefly, all personnel should wash their hands before entering and leaving the lab, and wear gloves throughout the entire procedure. Gloves should not be worn outside of the lab, and should be immediately replaced if any damage occurs. If possible, wearing lab coats is preferable to protect personal clothing. No food or drink should be kept or consumed in the area where the experiment is being conducted. At the end of the lab period, disinfect all surfaces and any equipment that came in contact with the bacterial cultures. All students completing the protocol should be instructed to report any mistakes, spills, injuries etc. to the laboratory coordinator who will decide the appropriate action. Any individual institution practices that extend beyond these should be followed as well.

## **Protocol Expansions:**

There are a number of modifications that can be made to this protocol to further increase the educational potential. One such modification would be the addition of an antibiotic to one culture, and to then compare the growth rate of this culture to one that did not contain the drug. If a more nuanced comparison is desired, differing classes of antibiotic could be compared. For example, one culture could be supplemented with a bactericidal antibiotic, which will kill the cells and another could be supplemented with a bacteriostatic antibiotic, which does not kill the bacteria but rather prevents further division. Two different antibiotics of the same class could also be compared to determine if one is more effective than another.

Another potential expansion would be to supplement one culture with a glucose solution and compare its growth to that of a non-supplemented culture. Glucose is the preferred carbon source for *E. coli*, and it is able to enter directly into central metabolism. This addition is expected to increase the growth rate of the supplemented culture. Finally, the growth rates of differing species of bacteria could also be compared. A potentially interesting comparison would be between a Gram-negative species and a Gram-positive species. However, this would involve different strains of bacteria that carry differing safety recommendations, which should be carefully considered before any additional trials are performed or any protocol modifications are made.

**References:**

Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <https://imagej.nih.gov/ij/>, 1997-2016.

Smith, A. W. (2015, August 28). Processing SpecPhone Images and Wavelength Calibration. Retrieved February 16, 2017, from SpecPhone Protocols: <https://smithlab.uakron.edu/files/2015/09/Protocol-1-Image-Processing-and-Calibration.pdf>

U.S. Centers for Disease Control and Prevention. (2009). *Biosafety in Microbiological and Biomedical Laboratories* (5th ed.). (C. Chosewood, & D. Wilson, Eds.) Atlanta, Georgia.

U.S. Environmental Protection Agency. (1997, February ). *Attachment I- Final Risk Assessment of Escherichia coli K-12 Derivatives*. Retrieved February 24, 2017, from EPA United States Environmental Protection Agency: <https://www.epa.gov/sites/production/files/2015-09/documents/fra004.pdf>

**Appendix:**

Use the following tables to organize the spectrum data and to create a growth curve. The actual time of measurement will be the time on the clock when the absorbance measurement was taken using the SpecPhone.

Time Point	Actual Time of Measurement	SpecPhone Absorbance Value
T <sub>0</sub>		
T <sub>10</sub>		
T <sub>20</sub>		
T <sub>30</sub>		
T <sub>40</sub>		
T <sub>50</sub>		
T <sub>60</sub>		
T <sub>70</sub>		
T <sub>80</sub>		
T <sub>90</sub>		
T <sub>100</sub>		
T <sub>110</sub>		
T <sub>120</sub>		
T <sub>130</sub>		
T <sub>140</sub>		
T <sub>150</sub>		