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Preliminary Work for Low Temperature Phage Amplification

Claire Butkus & Alena Veigl

UES, Inc.

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711TH HUMAN PERFORMANCE WING,
AIRMAN SYSTEMS DIRECTORATE,
WRIGHT-PATTERSON AIR FORCE BASE, OH 45433
AIR FORCE MATERIEL COMMAND
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14. ABSTRACT

This project determined the growth curve for BL21 *Escherichia coli* and compared two propagation methods for T7 bacteriophage to determine which resulted in a higher titer solution of bacteriophage. A high titer working stock solution is desirable to ensure the lysate is stable for future use and development [19]. Both methods were adapted from Dr. Bonilla's Phage on Tap protocol [3]. The first method uses a set volume of 1 mL of bacteriophage to a 1 mL solution of *E. coli* culture. The second uses a volume determined by the multiplicity of infection (MOI), which is the ratio of *E. coli* colony forming units to the plaque forming units of T7 phage. It was determined that the propagation method using MOI appeared to result in a higher titer working stock solution; however, these results were not statistically different. The p-value obtained was close to the selected α-value, so it is believed that with a larger sample size the result should be statistical different. This project is part of a much larger effort. This project lays out the ground work for developing a genetically engineered bacteriophage capable of detecting and removing *E. coli* from water samples at room temperature (20-22°C).

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1.0 SUMMARY

This project determined the growth curve for BL21 *Escherichia coli* (E. coli) and compared two propagation methods for T7 bacteriophage to determine which resulted in a higher titer solution of bacteriophage. A high titer working stock solution is desirable to ensure the lysate is stable for future use and development [19]. Both methods were adapted from Dr. Bonilla's Phage on Tap protocol [3]. The first method uses a set volume of 1 milliliter (mL) of bacteriophage to a 1 mL solution of *E. coli* culture. The second uses a volume determined by the multiplicity of infection (MOI), which is the ratio of *E. coli* colony forming units (CFU) to the plaque-forming units (PFU) of T7 phage. It was found that the propagation method using MOI appeared to result in a higher titer working stock solution; however, these results were not statistically different. The p-value obtained was close to the selected α-value, so it is believed that with a larger sample size the result should be statistical different. This project is part of a much larger effort, and it lays the ground work for developing a genetically engineered bacteriophage capable of detecting and removing *E. coli* from water samples at room temperature (20-22 degrees Celsius (°C)).

2.0 Introduction/Background

Escherichia coli is a gram-negative, straight rod bacteria that plays an important role in human and animal overall gut health by suppressing the growth of harmful bacteria and synthesizing certain vitamins necessary for growth and survival such as vitamins K and B12 [2, 14, 24]. While this specific bacterium thrives in the intestinal tracks of humans and animals, it also has the ability to survive in the environment. *E. coli* can flourish in soil, manure, and contaminated waterways [5]. Not all strains of *E. coli* are harmful, but there are a handful of strains recognized as being pathogenic and responsible for several different health problems [4]. These harmful strains of *E. coli* can cause infections to wounds, the gastrointestinal tract, and the urinary, pulmonary, and nervous systems [4, 20]. These types of infections, most commonly gastrointestinal infections called Traveler's Diarrhea, are prevalent amongst travelers and deployed military personnel caused by contaminated drinking water. Standard treatment for *E. coli* infections are antibiotics, but antibiotic-resistant strains have emerged [20]. A novel approach for treating *E. coli* and other bacterial infections, specifically for wound care, has emerged using bacteriophages [20]. Bacteriophages infect and often kill the host bacteria [20]. However, new applications using a bacteriophage to detect and clean contaminated water have emerged [9, 10].

In this study, *E. coli* strain BL21 was used due to its harmlessness and rapid growth cycle [7]. Bacteria growth curves include a lag, log, stationary, and death phase. As each name indicates, the bacteria replicates slowly during the lag phase, but replicates exponentially during the log phase. Generally, after the log phase the bacteria has less space and nutrients causing its growth to slow before there is not enough space or nutrients and bacteria start dying. BL21 usually hits its log phase a few hours after the initial incubation period, always falling in between an optical density (OD) reading of 0.4-0.6 at 600 nanometers (OD_{600}). This phase is the ideal time for infecting the bacteria with a phage to result in high propagation.

Bacteriophages are a virus capable of infecting bacteria. Bacteriophages can be propagated in a lab by infecting host bacteria with the virus and isolating the replicated phage. A common bacteriophage capable of infecting most strains of *E. coli* is the T7 bacteriophage. The T7 phage uses its stubby tail to infect *E. coli* [26]. Once the host has been infected, the T7 phage highjacks the cell causing it to replicate the virus, eventually causing the bacteria to lyse, releasing all the virons [26]. Once lysed, T7 phage can have about 100 progeny per host cell making it an easy phage to propagate and produce a high titer working stock solution [22, 26].

Inspired by the work of Dr. Nugen at Cornell University, this study lays out the ground work needed to address the ability to rapidly test and decontaminate drinking water in austere environments without large lab equipment or temperature regulated storage capabilities. This project compares two phage propagation methods to determine which results in a higher working stock solution. The first method uses a set volume of one mL of bacteriophage to a one mL solution of *E. coli* culture. The second, uses a volume determined by MOI, the ratio of *E. coli* CFU to PFU of T7 phage [13]. As mentioned, *E. coli* should be infected with T7 phage when it is in its log phage (OD_{600} reading between 0.4-0.6). There is an exponential difference in the number of CFU between using a bacterial culture at an OD_{600} of 0.4 versus 0.6. However, any time in this range is acceptable for infecting the bacteria with a phage. This study evaluated the difference between these methods and whether one would result in more propagation and, therefore, a higher titer working stock solution of T7 phage.

3.0 Method

OD_{600} is a widely used technique to count cells in a liquid culture and the NanoDrop Microvolume Spectrophotometer was used to obtain OD_{600} measurements. Bacteria in a solution act like particles and cause light scattering. This scattering affects the light absorption of the solution and can be measured by the instrument. Using the Beer-Lambert Law, light absorbance directly correlates with solution concentration [11]. This holds true for low density cultures [11]. The intent of this measurement is to determine the growth phase (i.e., lag, log, stationary, or death phase) of the bacteria that correlates to the OD_{600} reading, as well as to determine the number of CFU present at that OD_{600} reading. This allows for the concentration of bacteria culture in future work to be standardized.

The MOI is the ratio of infectious virons to bacterial cells in a defined space [13]. The growth phase of the bacteria and the mechanisms for which the phage infect the bacteria all play a role in what the best MOI will be when looking at a bacterial infection [1]. If the MOI is too high, cytotoxicity could take place; if too low, 100 percent (%) infection is unachievable [1]. Establishing a good viron-to-bacteria infection ratio is key to developing a high titer working phage stock.

This work utilized OD_{600} and MOI to first establish the growth curve for BL21. Next, T7 phage were propagated using a set volume of phage, followed by propagation of T7 phage using MOI. All materials were purchased from Thermo Fisher Scientific unless otherwise stated. After the two propagation methods were completed, the results were analyzed using a Two-Sample t-Test.

3.1. Preparation

3.1.1. Luria Broth (LB) Preparation

One liter (L) of LB was prepared as two 500 mL bottles of broth in two separate one L autoclave bottles. This ensured that during the autoclaving process the LB broth did not boil over leading to losses and possible contaminations. Ten (10) grams (g) of LB powder and 500 mL of MilliQ water were measured and placed in a one L autoclave bottle. This was repeated, resulting in the two 500 mL solutions of LB broth. The ingredients were swirled to mix, though they did not dissolve completely. Orange caps (indicating suitable for autoclaving) were placed onto the bottles and left loose enough to allow for pressure equalization to occur while in the autoclave. A fresh piece of autoclave tape was placed over each cap. The bottles were placed in the middle of the autoclave, the autoclave was shut, and set to run on "LIQUID" cycle at 121 °C for 20 minutes. One hour after the autoclave depressurized and the bottles cooled enough to handle with heat resistant gloves, the broth was taken out and set on the benchtop to allow to cool to room temperature (RT). While the broth was cooling, 136 Falcon Tubes were labeled with the following: removal time, dilution factor, and date. After the broth cooled to RT, 9 mL of LB was pipetted into each of the labeled Falcon Tubes and set aside for later use [12, 18, 25].

3.1.2. LB Bottom Agar Preparation

One L of molten LB bottom agar was prepared as two 500 mL bottles of molten agar in two separate one L autoclave bottles. This ensured that during the autoclaving process the LB top agar did not boil over leading to losses and possible contaminations. 16 g of pre-mixed LB agar and 500 mL of MilliQ water were measure and placed in a one L autoclave bottle containing a clean stir bar. This was repeated resulting in the two 500 mL solution of LB bottom agar. Orange caps (indicating suitable for autoclaving) were placed onto the bottles and left loose enough to allow for pressure equalization to occur while in the autoclave. A fresh piece of autoclave tape was placed over each cap. The bottles were placed in the middle of the autoclave, the autoclave was shut, and set to run on "LIQUID" cycle at 121°C for 20 minutes. While agar sterilization was occurring, the benchtop was sprayed down with a 70 % ethanol solution, wiped down with a paper towel, and plates were set out in preparation for pouring. Once the molten agar was retrieved from the autoclave, it was placed on a stir plate and allowed to cool for 10-20 minutes or until the bottle reached a touchable temperature. About 10 mL of agar was hand poured into each plate, the lid replaced, and then tilted or gently swirled to allow for an even distribution of agar along the bottom of the plate. The plates were left to set overnight and the following day all 136 plates were labeled with the removal time, dilution factor, bacteria strain, and date. Each of the plates were then placed in a plastic bag or wrapped in parafilm. All were placed with the lid facing down in a 4°C refrigerator [21].

3.1.3. LB Top Agar Preparation

One L of molten LB top agar was prepared as two 500 mL bottles of molten agar in two separate one L autoclave bottles. This ensured that during the autoclaving process the LB top agar did not boil over leading to losses and possible contamination. Eight (8) g of pre-mixed LB agar was measured for each labeled one L autoclave bottle and added to 500 mL of MilliQ water. A clean stir rod was added to each bottle, capped, swirled, uncapped and recapped loosely to allow for pressure equalization during autoclaving, and taped with autoclave tape. The two bottles were placed into the autoclave and ran on the "LIQUID" cycle at 121°C for 20 minutes. Once cooled, the bottles were capped and set aside for future use [21].

3.1.4. SM Buffer Preparation

To make SM buffer, 2.9 g of sodium chloride (NaCl) and one g of hydrated magnesium sulfate ($MgSO_4 \cdot 7H_2O$) were measured twice and added to two separate one L autoclave bottles containing 400 mL of MilliQ water. Twenty-five (25) mL of Tris-Cl (1milli-Molar (mM), pH 7.5) was then added to each solution, followed by 75 mL of MilliQ water so each bottle reached 500 mL of solution in a one L bottle [6].

Once the solution was mixed, the cap was unscrewed to allow for pressure equalization, and a new piece of autoclave tape was placed over the top. The bottle was placed into the autoclave and run on the "LIQUID" cycle at 121°C for 20 minutes (unless a pre-mixed bottle noted otherwise). Once the autoclave had depressurized and cooled enough to handle contents, the bottles were retrieved and allowed to sit on the benchtop until they reached RT. Once at RT, the cap was tightened to seal the solution. Before using the buffer, it was passed through a sterile funnel with a 0.2 µm filter [3, 6].

3.1.5. *E. coli* Culture Preparation

Before preparing the *E. coli* cultures, the lab bench was cleaned using 70% ethanol. After the ethanol had dissipated, a Bunsen burner was then ignited to assist in maintaining the sterility of the workspace. Two LB agar plates were obtained from the refrigerator. Each plate was labeled on the bottom with the *E. coli* strain, date, and then allowed to sit for about 5 to 10 minutes to reach RT. After reaching RT, a glycerol stock of *E. coli* was obtained from the -80°C freezer. A sterile loop was used to touch and swirl through the glycerol stock briefly before plating. The loop, which had the bacteria on it, was gently spread over a section of the plate to create the first streak, see Figure 1. The old loop was then quickly disposed and a new loop was used to then spread the first section of bacteria to a new section on the plate (Streak 2). After Streak 2 was created, a third and final streak section was made with a new loop. Streaking the plate like this allows the bacteria to be more spread out to allow for the growth of single colonies and not chunks. These instructions were then repeated for the second plate [23].

Figure 1: Plate streaking [23]

Each plate was placed in an incubator set to 37°C overnight (20 hours). The next day before removing the overnight colonies, a liquid culture was prepared. To do so, two 15 mL Falcon Tubes with double click tops were obtained and labeled appropriately. Ten (10) mL of

liquid LB was pipetted into each and then set aside. After 20 hours of incubation, the plates were removed from the incubator and a single colony was selected as seen in Figure 2 [23].

Figure 2: Isolating a colony [23]

After choosing a single colony, which can be seen in Figure 2, a sterile loop was used to gently scrape the single colony from the LB agar plate. The loop was then placed into the liquid LB and swirled gently. The Falcon Tube was then capped, only being pressed down to the first click to allow for proper aeration. The tube was them placed in a shaking incubator set to 37°C and 180 revolutions per minute (rpm) for 18 hours [23].

4.0 *E. coli* Growth Curve

After 18 hours, the overnight liquid culture was removed from the shaking incubator. Before measuring the overnight culture on the nanodrop, it was cleaned using 70% ethanol. Two microliters (µL) of LB media was used as a blank. The nanodrop was cleaned again and two µL of overnight culture was measured at OD_{600} to ensure there was bacterial growth from the overnight solution. Once overnight growth was determined, 100 µL of the overnight liquid culture was pipetted into 100 mL of LB media in a 250 mL Delong Shaker Erlenmeyer flask. This was then fitted with a stainless steel cap, placed back into the shaking incubator at 37°C with shaking at 180 rpm and a timer began.

Following the breakdown of the time points outlines in Table 1, 1.5 mL of bacterial culture was withdrawn from the stock and dispensed into a new Falcon Tube. The stock culture was placed back into the incubator and the timer continued. From this sample two µL were used to measure the OD_{600} for that time point. Falcon Tubes were filled with 9 mL of LB and labeled accordingly for a serial dilution. Following Figure 3, one mL of the removed bacteria culture was pipetted into the first Falcon Tube and pipetted up and down 10 times to ensure mixing. One mL was pipetted from the first Falcon Tube and put into the next tube for the serial dilution. Again, it was pipetted up and down 10 times and the process was repeated for the number of dilutions outlined in Table 1. These dilutions were selected to ensure there was a countable plate (30-300 colony forming unit (CFUs)) for each time point.

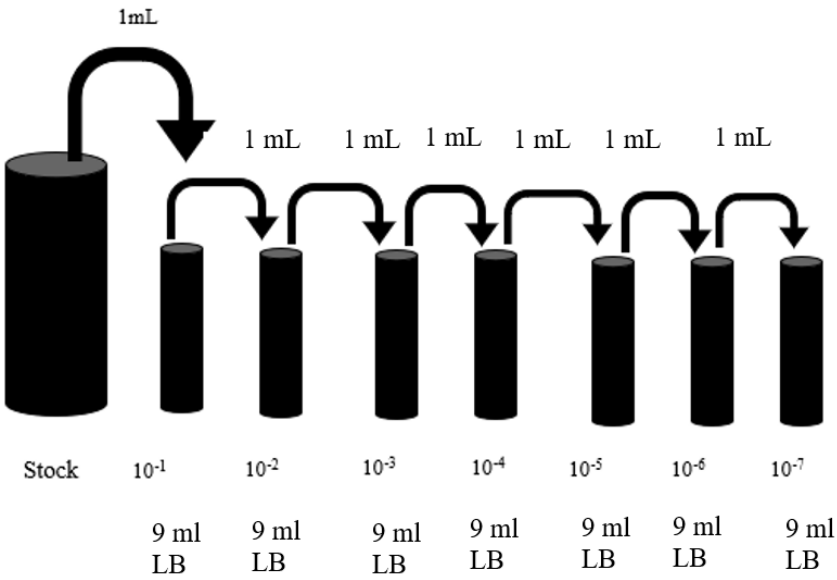

Figure 3: Illustration of serial dilution

Table 1: Break down of time, label, number of dilutions, number of plates, and amount of LB broth needed to complete growth curve

Break Down (hour)	Time of OD_{600} Reading (min)	Label	Plates								# Plates Needed	LB (mL)
0	0	T_0	T_0								1	9
	20	$T_{0.2}$	$T_{0.2}^{-1}$	$T_{0.2}^{-2}$	$T_{0.2}^{-3}$	$T_{0.2}^{-4}$	$T_{0.2}^{-5}$	$T_{0.2}^{-6}$			6	54
	20	$T_{0.4}$	$T_{0.4}^{-1}$	$T_{0.4}^{-2}$	$T_{0.4}^{-3}$	$T_{0.4}^{-4}$	$T_{0.4}^{-5}$	$T_{0.4}^{-6}$			6	54
1	20	$T_{1.0}$	$T_{1.0}^{-1}$	$T_{1.0}^{-2}$	$T_{1.0}^{-3}$	$T_{1.0}^{-4}$	$T_{1.0}^{-5}$	$T_{1.0}^{-6}$			6	54
	20	$T_{1.2}$	$T_{1.2}^{-1}$	$T_{1.2}^{-2}$	$T_{1.2}^{-3}$	$T_{1.2}^{-4}$	$T_{1.2}^{-5}$	$T_{1.2}^{-6}$	$T_{1.2}^{-7}$		7	63
	20	$T_{1.4}$	$T_{1.4}^{-1}$	$T_{1.4}^{-2}$	$T_{1.4}^{-3}$	$T_{1.4}^{-4}$	$T_{1.4}^{-5}$	$T_{1.4}^{-6}$	$T_{1.4}^{-7}$		7	63
2	20	$T_{2.0}$	$T_{2.0}^{-1}$	$T_{2.0}^{-2}$	$T_{2.0}^{-3}$	$T_{2.0}^{-4}$	$T_{2.0}^{-5}$	$T_{2.0}^{-6}$	$T_{2.0}^{-7}$		7	63
	10	$T_{2.2}$	$T_{2.2}^{-1}$	$T_{2.2}^{-2}$	$T_{2.2}^{-3}$	$T_{2.2}^{-4}$	$T_{2.2}^{-5}$	$T_{2.2}^{-6}$	$T_{2.2}^{-7}$	$T_{2.2}^{-8}$	8	72
	10	$T_{2.4}$	$T_{2.4}^{-1}$	$T_{2.4}^{-2}$	$T_{2.4}^{-3}$	$T_{2.4}^{-4}$	$T_{2.2}^{-5}$	$T_{2.2}^{-6}$	$T_{2.2}^{-7}$	$T_{2.2}^{-8}$	8	72
	10	$T_{2.5}$	$T_{2.5}^{-1}$	$T_{2.5}^{-2}$	$T_{2.5}^{-3}$	$T_{2.5}^{-4}$	$T_{2.5}^{-5}$	$T_{2.5}^{-6}$	$T_{2.5}^{-7}$	$T_{2.5}^{-8}$	8	72
3	10	$T_{3.0}$	$T_{3.0}^{-1}$	$T_{3.0}^{-2}$	$T_{3.0}^{-3}$	$T_{3.0}^{-4}$	$T_{3.0}^{-5}$	$T_{3.0}^{-6}$	$T_{3.0}^{-7}$	$T_{3.0}^{-8}$	8	72
	10	$T_{3.1}$	$T_{3.1}^{-1}$	$T_{3.1}^{-2}$	$T_{3.1}^{-3}$	$T_{3.1}^{-4}$	$T_{3.1}^{-5}$	$T_{3.1}^{-6}$	$T_{3.1}^{-7}$	$T_{3.1}^{-8}$	8	72
	10	$T_{3.2}$	$T_{3.2}^{-1}$	$T_{3.2}^{-2}$	$T_{3.2}^{-3}$	$T_{3.2}^{-4}$	$T_{3.2}^{-5}$	$T_{3.2}^{-6}$	$T_{3.2}^{-7}$	$T_{3.2}^{-8}$	8	72
	20	$T_{3.3}$	$T_{3.3}^{-1}$	$T_{3.3}^{-2}$	$T_{3.3}^{-3}$	$T_{3.3}^{-4}$	$T_{3.3}^{-5}$	$T_{3.3}^{-6}$	$T_{3.3}^{-7}$	$T_{3.3}^{-8}$	8	72
	20	$T_{3.4}$	$T_{3.4}^{-1}$	$T_{3.4}^{-2}$	$T_{3.4}^{-3}$	$T_{3.4}^{-4}$	$T_{3.4}^{-5}$	$T_{3.4}^{-6}$	$T_{3.4}^{-7}$	$T_{3.4}^{-8}$	8	72
4	20	$T_{4.0}$	$T_{4.0}^{-1}$	$T_{4.0}^{-2}$	$T_{4.0}^{-3}$	$T_{4.0}^{-4}$	$T_{4.0}^{-5}$	$T_{4.0}^{-6}$	$T_{4.0}^{-7}$	$T_{4.0}^{-8}$	8	72
	20	$T_{4.2}$	$T_{4.2}^{-1}$	$T_{4.2}^{-2}$	$T_{4.2}^{-3}$	$T_{4.2}^{-4}$	$T_{4.2}^{-5}$	$T_{4.2}^{-6}$	$T_{4.2}^{-7}$	$T_{4.2}^{-8}$	8	72
	20	$T_{4.4}$	$T_{4.4}^{-1}$	$T_{4.4}^{-2}$	$T_{4.4}^{-3}$	$T_{4.4}^{-4}$	$T_{4.4}^{-5}$	$T_{4.4}^{-6}$	$T_{4.4}^{-7}$	$T_{4.4}^{-8}$	8	72
5	20	$T_{5.0}$	$T_{5.0}^{-1}$	$T_{5.0}^{-2}$	$T_{5.0}^{-3}$	$T_{5.0}^{-4}$	$T_{5.0}^{-5}$	$T_{5.0}^{-6}$	$T_{5.0}^{-7}$	$T_{5.0}^{-8}$	8	72
Totals	300										136	1224

The titer $\left(\frac{\text{CFU}}{\text{mL}}\right)$ of the *E. coli* solution at a given time was calculated using Equation (1). To analyze the growth curve results, the titer was plotted against the OD_{600} measurements, for readings less than one, with its corresponding time point. A linear regression trend line was applied and the resulting equation used to calculate the titer for a given OD_{600} measurement [18, 25].

$$\frac{CFU}{mL} = \frac{\#\ of\ colonies\ per\ plate}{(volume\ of\ E.coli\ added\ to\ the\ plate)(dilution\ factor)} \quad (1)$$

4.1. Comparing Multiplicity of Infection and Set Volume Propagation

A phage stock solution was produced using two methods, 1) a set volume of phage and 2) a volume of phage determined by MOI. After the stock solutions were produced, the titer of each technique was determined and compared to suggest a procedure that yields a higher titer stock solution.

4.1.1. Phage Plaque Assay for Determining Titer

An overview of the propagation process can be seen below in Figure 4 was adapted from Dr. Natasha Bonilla's *Phage on Tap* protocol [3]. A liquid culture of BL21 *E. coli* was left to grow overnight at 37°C. Microcentrifuge tubes were filled with 900 µL of LB and labeled accordingly for a serial dilution. Following Figure 3 again, 100 µL of phage stock solution was pipetted into the first microcentrifuge tube and vortexed for 1 minute. Then 100µL was pipetted from the first microcentrifuge tube and put into the next tube for the serial dilution. Again, it was vortexed for one minute and the process was repeated seven times to obtain plates with countable plaques (30-300 CFUs).

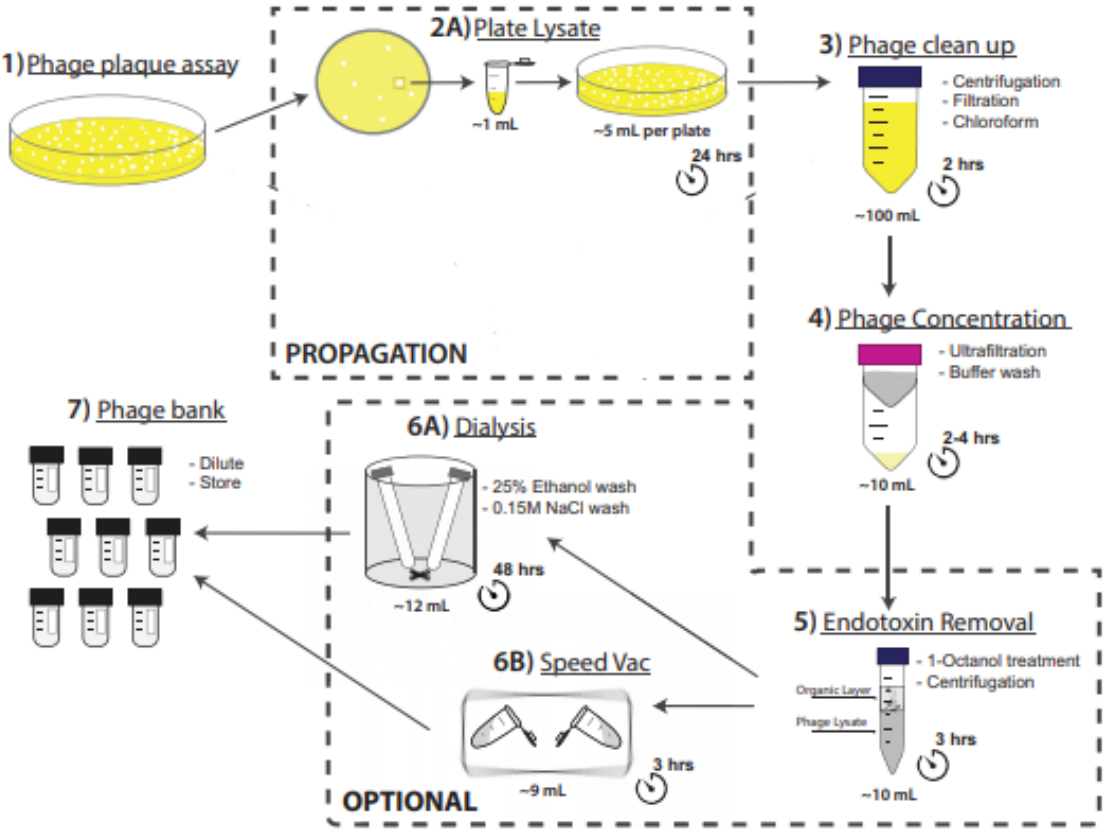

Figure 4: Overview of the phage propagation, cleaning, and concentrating protocol [3]

The LB top agar was melted and when ready, one mL of the overnight BL21 *E. coli* and one mL from one serial dilution was added to a Falcon tube. The solution was capped, gently shaken, and then three mL of the LB top agar was added to the Falcon tube. Again, the solution was shaken quickly and gently, then poured onto a labeled LB agar petri dish. This was completed for each phage dilution. The plate was allowed to solidify and then place inverted in an incubator overnight at 37°C. The following day, the dilution with countable plaques (30-300) was counted and the starting phage stock solution titer $\left(\frac{\text{PFU}}{\text{mL}}\right)$ was determined using Equation (2).

$$\frac{PFU}{mL} = \frac{\#\ of\ plaques\ per\ plate}{(volume\ of\ diluted\ phage\ added\ to\ the\ plate)(dilution\ factor)} \quad (2)$$

4.1.2. Phage Isolation and Propagation via Set Volume Plate Lysate

An overview of the propagation process can be seen in Figure 4, 2A) "Phage Lysate" as adapted from Dr. Natasha Bonilla's *Phage on Tap* protocol [3]. A single phage plaque from the plaque assay (step 2A) was collected using a sterile Pasteur pipette. The plaque was re-suspended into a microcentrifuge tube containing one mL of filtered SM buffer, vortexed for five minutes, and then centrifuged at 4,000 g for five minutes. To perform the plate lysate, BL21 *E. coli* was cultured in LB broth overnight at 37°C. Then 100 µL of the overnight liquid culture was added to 100 mL LB media and grown at 37°C with shaking at 210 rpm. After an hour and a half, the culture's OD_{600} was measured and allowed to grow until it reached an OD_{600} reading

between 0.4-0.6. The LB top agar was melted, and when ready, one mL of BL21 *E. coli* (at OD_{600} = 0.4-0.6) and one mL of phage was added to a Falcon tube. The solution was capped, gently shaken to allow for mixing, and then three mL of the LB top agar was added to the Falcon tube. Again, the solution was quickly and gently shaken, then poured onto a labeled LB agar petri dish. The plate was allowed to solidify and then placed inverted in an incubator overnight at 37°C [3].

The following day, five mL of SM buffer was added to the top layer of the plate and put on a plate rocker for 15 minutes. Using a pipette, the SM buffer and phage were collected from the plate and centrifuged at 4,000 g for five minutes. The supernatant was collected and the titer was checked using the above protocol [3].

4.1.3. Phage Isolation and Propagation via Multiplicity of Infection

An overview of the propagation process can be seen above in Figure 4, 2A) as adapted from Dr. Natasha Bonilla's *Phage on Tap* and Dr Nugen's *T7 Phages Stock Propagation Methods* protocol [3, 17]. A single phage plaque from the plaque assay (step 2A) was collected using a sterile Pasteur pipette. The plaque was re-suspended into a microcentrifuge tube containing one mL of filtered SM buffer, vortexed for five minutes, and centrifuged at 4,000 g for five minutes. To perform the plate lysate, BL21 *E. coli* was cultured in LB broth overnight at 37°C. Then 100 µL of the overnight liquid culture was added to 100 mL LB media and grown at 37°C with shaking at 210 rpm. After an hour and a half, the culture's OD_{600} was measured and allowed to grow until it reached an OD_{600} reading between 0.4-0.6. The LB top agar was melted and when ready one mL of BL21 *E. coli* (at OD_{600} = 0.4-0.6) and volume of phage at an MOI of 0.1 was added to a Falcon tube. The volume needed to achieve an MOI of 0.1 can be seen below in Equation (4). The solution was capped, gently shaken, and then three mL of the LB top agar was added to the Falcon tube. Again, the solution was quickly and gently shaken, then poured onto a labeled LB agar petri dish. The plate was allowed to solidify and then place inverted in an incubator overnight at 37°C [3, 17].

The following day, five mL of SM buffer was added to the top layer of the plate and put on a plate rocker for 15 minutes. Using a pipette, the SM buffer and phage were collected from the plate and centrifuged at 4,000 g for five minutes. The supernatant was collected and the titer was checked using the above protocol [3, 17].

$$MOI = \frac{\frac{PFU}{mL}}{\frac{CFU}{mL}} \tag{3}$$

$\frac{PFU}{mL}$: *from phage plaque assay for determination of titer*

$\frac{CFU}{mL}$: *from growth curve*

Thus, from Equation (3), to find the volume of phage required:

$$mL = \frac{(MOI \times CFU)}{\frac{PFU}{mL}} \tag{4}$$

5.0 Results & Discussion

5.1. Growth Curve

Having an established growth curve allows for a better understanding of the speed at which BL21 *E. coli* grows to help design future experiments aiming for a specific growth phase (see Appendix for raw data from growth curve). Additionally, an established growth curve allows for a known number of CFU to be used for future experiments at a given OD_{600}. The equation established by the trend line in Figure 5 allows for the CFU per mL to be calculated for a measured OD_{600}. This allows for a known ratio of phage PFU to bacterial CFU.

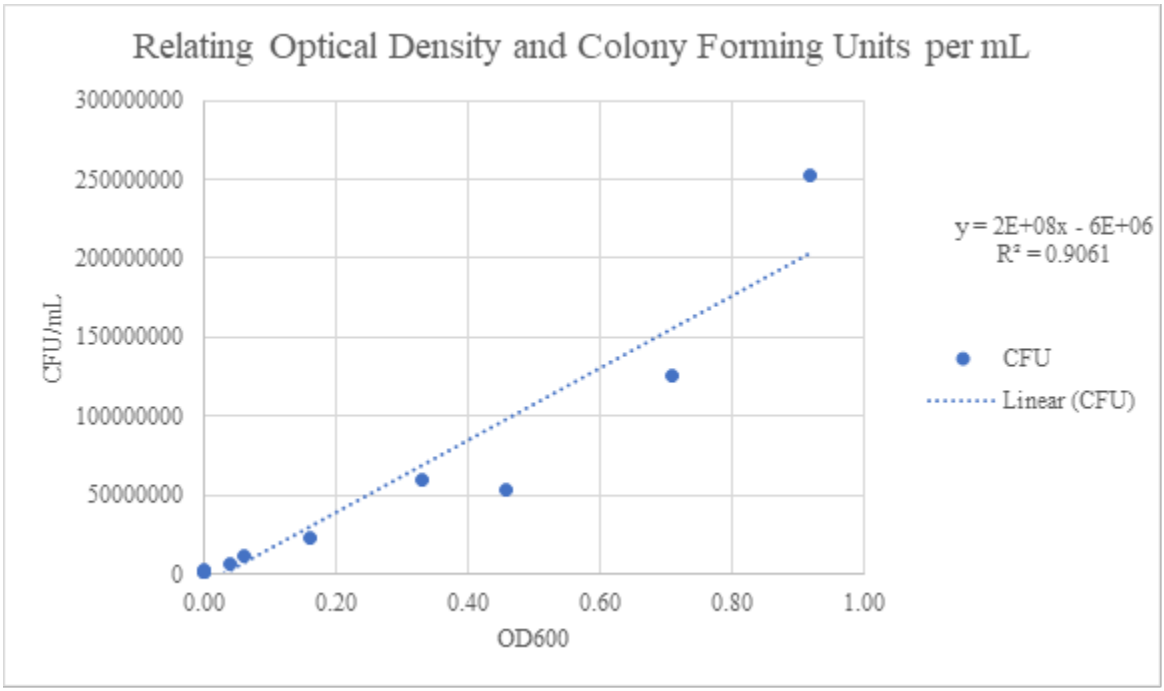

Figure 5: Plot of colony forming units versus optical density on a linear scale for OD_{600}

5.2. Propagation Comparison

The purpose of this work was to determine which propagation method resulted in a higher titer working stock solution. A high titer working stock solution is desirable to ensure the lysate is stable [19]. The Phage on Tap protocol was used in both methods because it is efficient and results in a homogenous, laboratory-scale, high titer, and endotoxin reduced stock solution; however, the volume of phage added to the bacterial culture was changed [3]. As seen in Figures 6 and 7 and in Table 2 it would appear that using an MOI of 0.1 opposed to a set volume of one mL of phage results in a consistently higher titer propagation of phage.

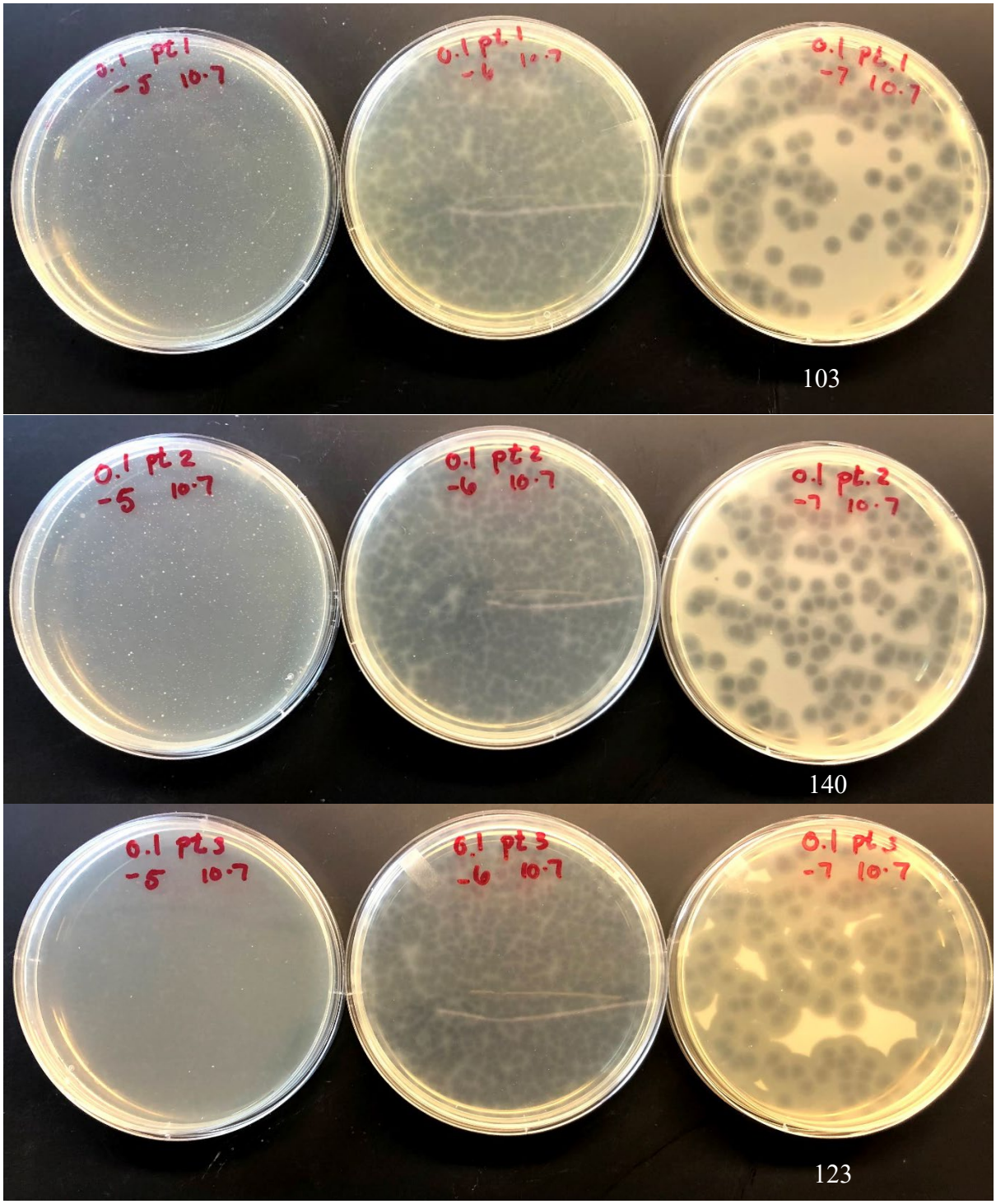

Figure 6: MOI of 0.1 final plates for phage propagation

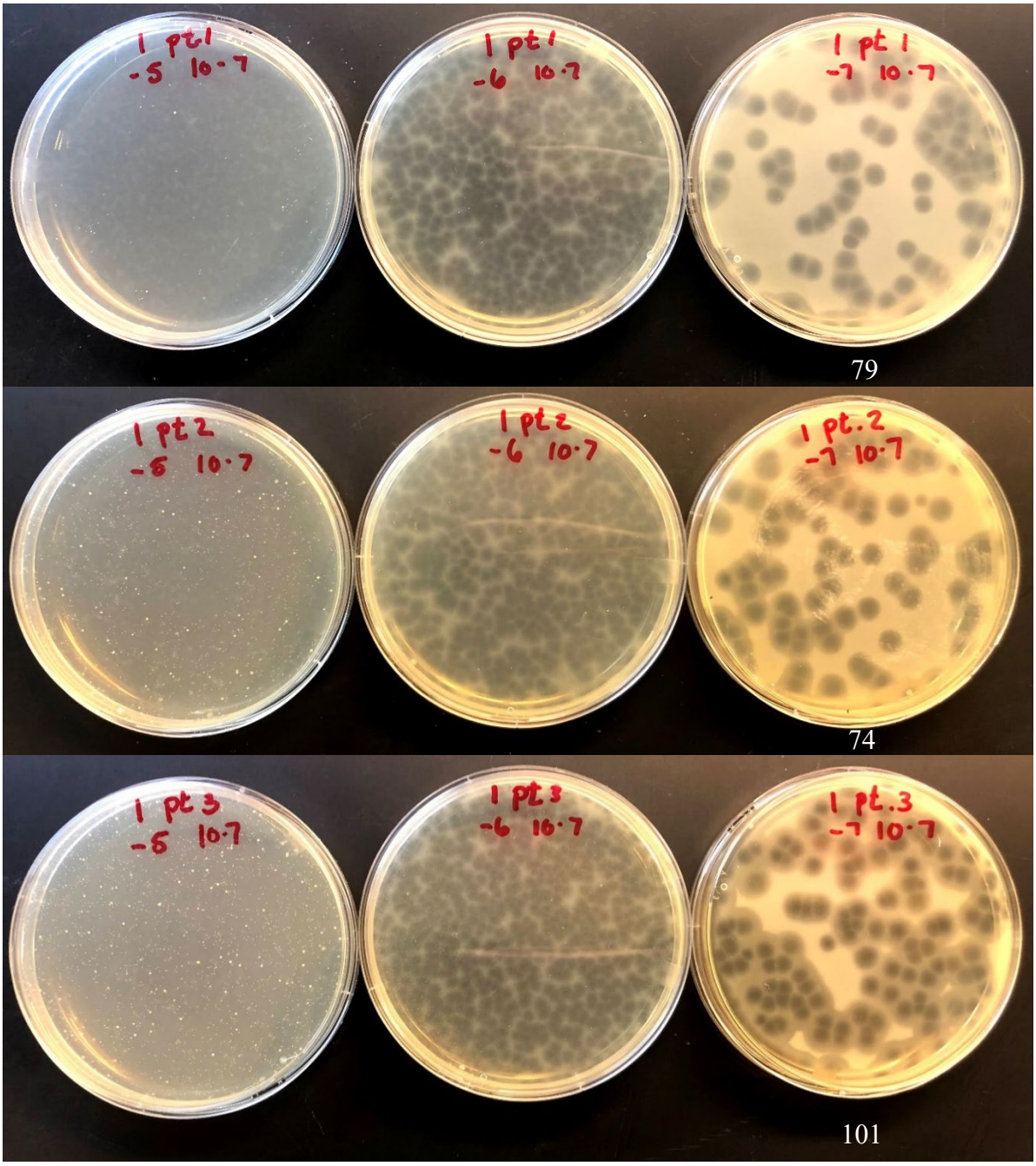

Figure 7: Set volume final plates for propagation

Table 2: Propagation Comparison

Propagation Method	MOI = 0.1			Set Volume		
Dilution Factor	0.0000001	0.0000001	0.0000001	0.0000001	0.0000001	0.0000001
PFU	103	140	123	79	74	101
PFU/mL	1,030,000,000	1,400,000,000	1,230,000,000	790,000,000	740,000,000	1,010,000,000
Average PFU/mL	1,220,000,000			846,666,666.7		
Standard Deviation	185,202,592			143,643,076		
95% Confidence Interval	209,572,611			162,544,456		

An independent two sample *t*-test with equal variance was used to determine if there was a significant difference between the two propagation methods. A two sample t-test assumes independent variables, normality, and equal variance [8]. The two propagation methods are independent of one another; however, statistical tests were used to ensure the data samples were normally distributed, did not contain outliers, and had equal variance. Initially, the data were tested for normal distribution and outliers to ensure the experiments were run correctly and to ensure the assumption of normality was correct for running the two sample *t*-test [16]. A Ryan-Joiner statistics test for normal distribution, similar to a Shapiro-Wilk test, was used due to the small sample size (n = 3) [15]. The Ryan-Joiner test for both propagation methods, seen below in Figures 8 and 9, resulted in *p*-values greater than α (α = 0.05), so the null hypothesis was not rejected and it was concluded that both data sets were normally distributed [16]. A Grubbs' test for outliers was used, seen below in Figure 10 and 11. Again, both propagation methods resulted in *p*-values greater than α (α = 0.05) so the null hypothesis was not rejected and it was concluded that both data sets did not contain outliers [16]. To determine which *t*-test to use, the data sets were tested for equal variance using an *F*-test, seen below in Figure 12. The *p*-value resulting from the *F*-test was greater than α (α = 0.05) so the null hypothesis was not rejected and it was concluded that the data sets had equal variance [16]. Finally, the independent two-sample *t*-test with equal variance was run. This resulted in a *p*-value (p = 0.051) equal to α (α = 0.05) meaning the two propagation methods were not significantly different from one another and the difference could be due to random variation [16]. However, when evaluating the data in Table 2 and Figures 6 and 7, it is clear to see that there is a difference between the two propagation methods; thus, it is possible that larger samples size would result in a statistically significant difference between the methods.

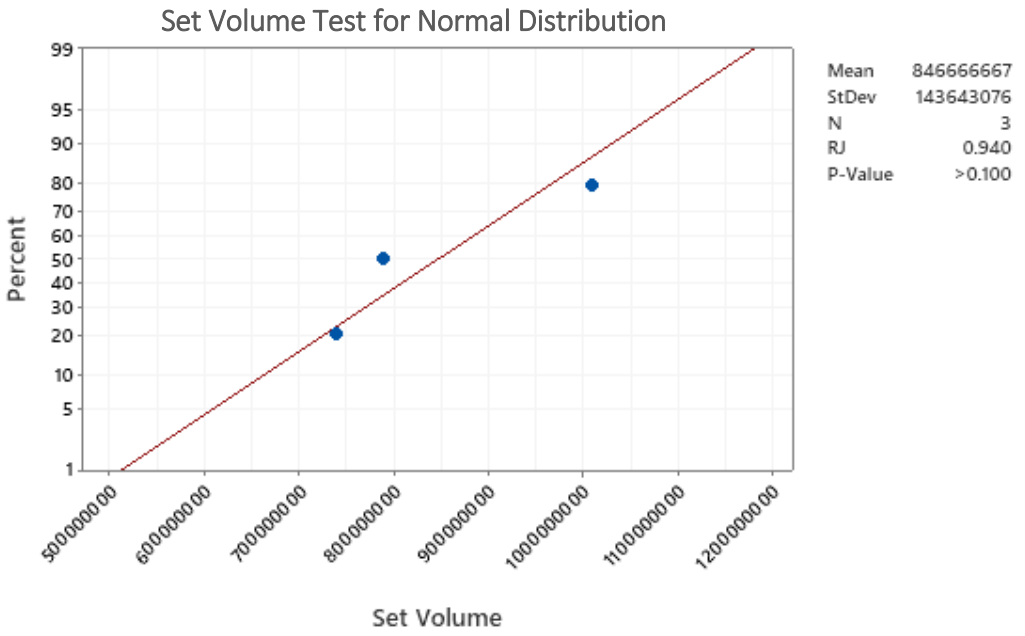

Figure 8: Set volume propagation method's Ryan –Joiner test for normal distribution

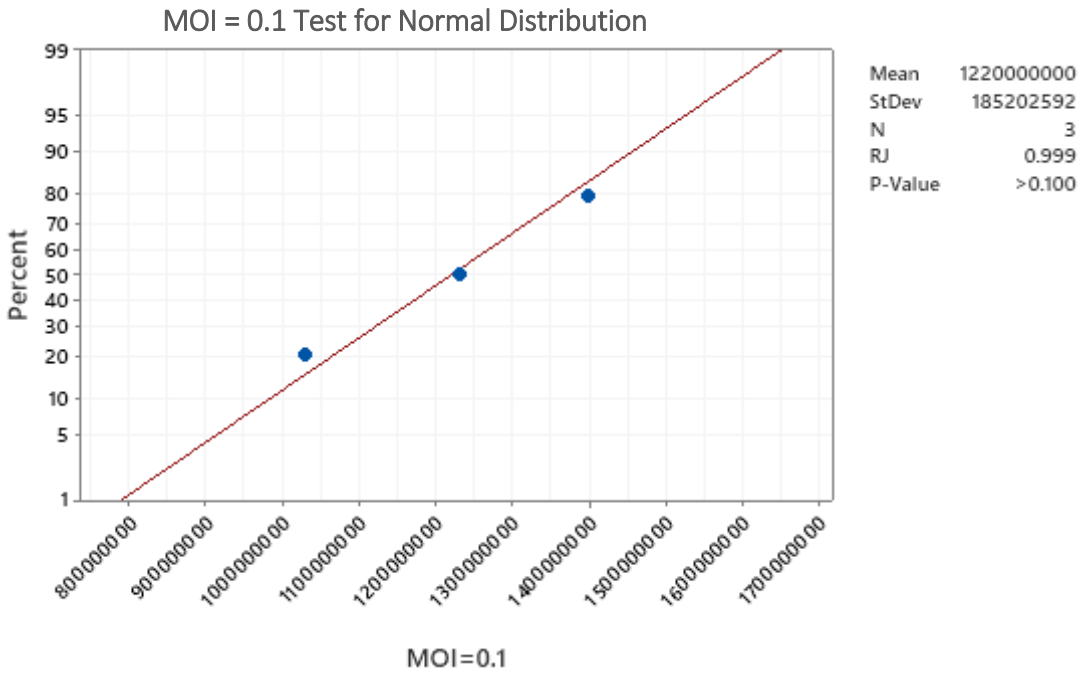

Figure 9: MOI = 0.1 propagation method's Ryan-Joiner test for normal distribution

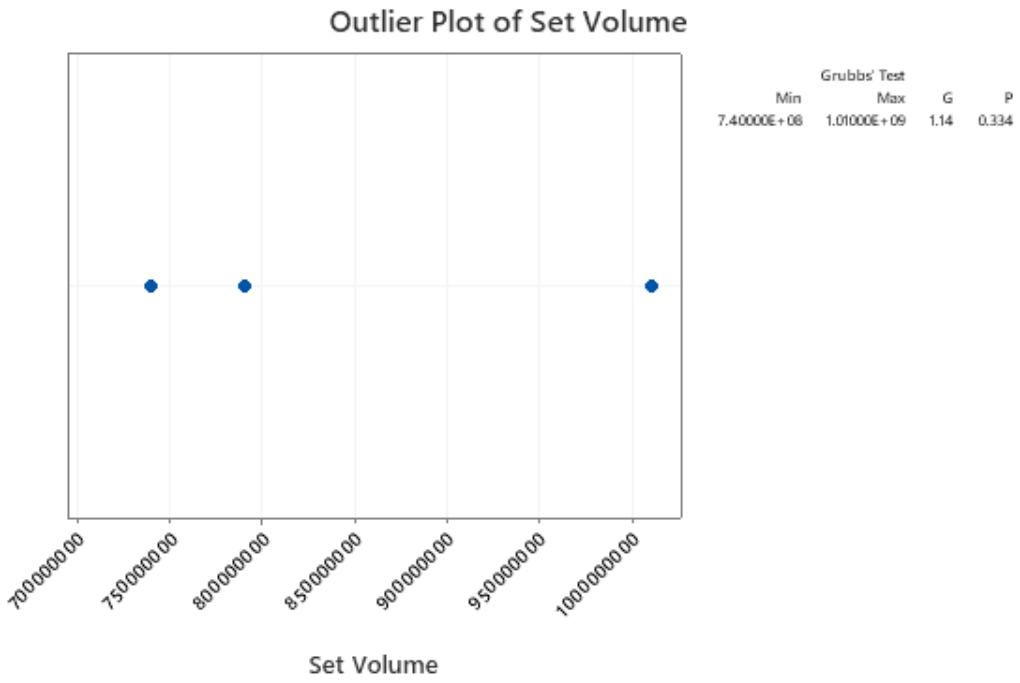

Figure 10: Set volume Grubbs' test for outliers

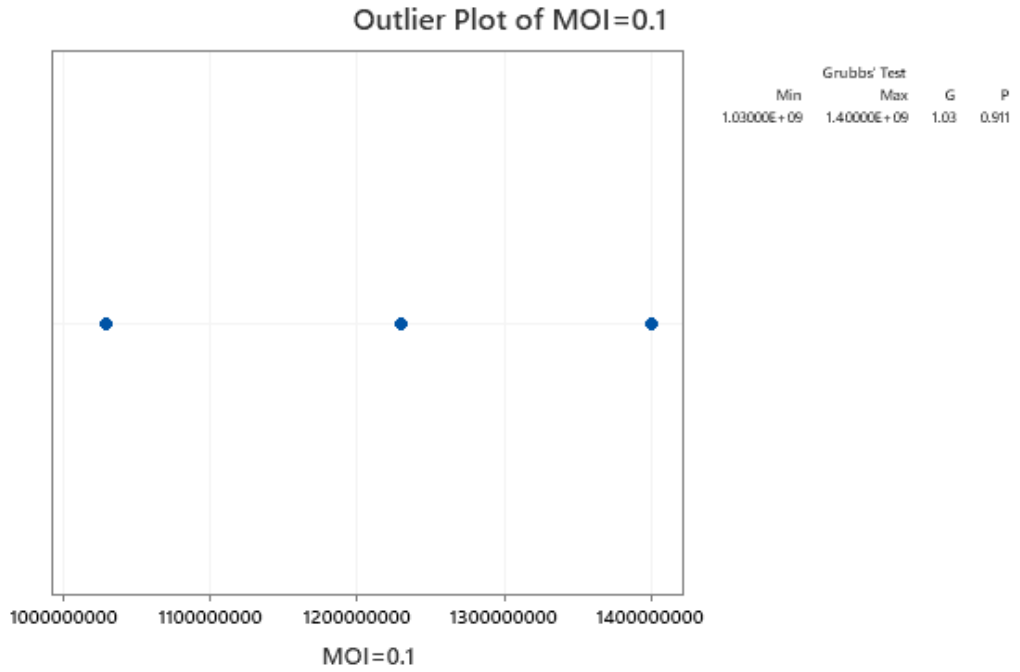

Figure 11: MOI = 0.1 Grubbs' test for outliers

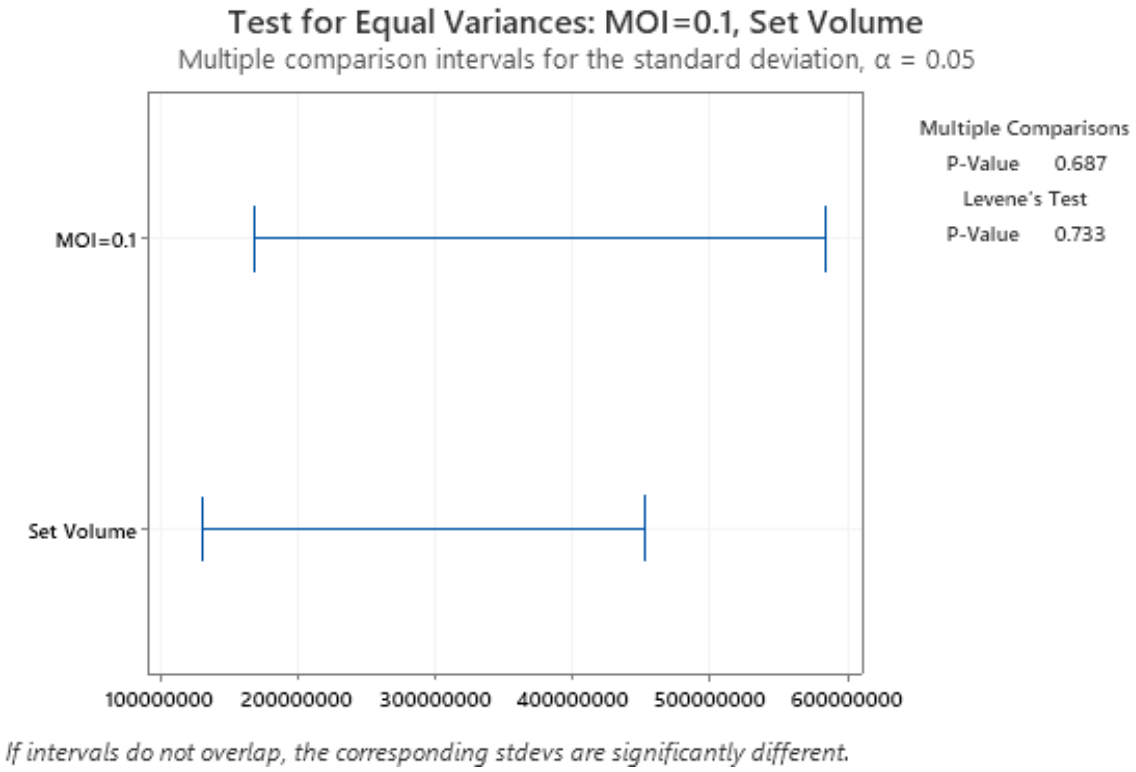

Figure 12: Test for equal variance

6.0 Conclusions

While it is likely that a larger sample size is needed, the MOI of 0.1 is indicated as being slightly better than the 1 mL that the Phage on Tap protocol designated using. Though these results do not show to be statistically significant, this is most likely due to the small sample size. Additional iterations of this exact comparison should be done at a later date to finalize the determination of significance. Also, it is recommended that a range of MOIs be tested to fully identify the most efficient ratio of T7 phage to BL21 *E. coli* for resulting in a high titer phage propagation.

The goal of this work was to determine the best practice of propagation in order to maximize the amount of working stock for later experiments. This was a small part of a much larger project – a project that will later be able to address the need to rapidly test and decontaminate drinking water in austere environments without lab or temperature-regulated storage capabilities.

7.0 References

[1] Applied Biological Materials. (2019, November 1). *Multipicity of Infection (MOI): What it is and how do I calculate it?* Retrieved from Youtube: https://www.youtube.com/watch?v=rfw_rcP5XOA

[2] Blount, Z. D. (2015, March 25). The unexhausted potential of E. coli. *eLife, 4*, e05826. doi:10.7554/eLife.05826

[3] Bonilla, N., Rojas, M. I., Cruz, G. N., Hung, S.-H., Rohwer, F., & Barr, J. J. (2016, July 26). Phage on tap–a quick and efficient protocol for the preparation of bacteriophage laboratory stocks. *PeerJ, 4*, e2261. doi:https://doi.org/10.7717/peerj.2261

[4] Centers for Disease Control and Prevention. (2021, March 11). *E. coli (Escherichia coli)*. Retrieved July 20, 2021, from Centers for Disease Control and Prevention: https://www.cdc.gov/ecoli/index.html

[5] Chekabab, S. M., Paquin-Veillette, J., Dozois, C. M., & Harel, J. (2013, April). The ecological habitat and transmission of Escherichia coli O157:H7. *FEMS Microbiology Letters, 341*(1), 1-12. doi:https://doi.org/10.1111/1574-6968.12078

[6] Cold Spring Harbor Protocol. (2006). *SM Buffer*. doi:doi:10.1101/pdb.rec8111

[7] *Escherichia coli Strains and NIH Guidelines*. (2019, June 21). Retrieved from UC San Diego: https://blink.ucsd.edu/safety/research-lab/biosafety/nih/e-coli.html

[8] Glen, S. (2021). *Independent Samples T Test (Unpaired Samples): Definition, Running*. Retrieved July 1, 2021, from StatistisHowTo: https://www.statisticshowto.com/probability-and-statistics/t-distribution/independent-samples-t-test/

[9] Hinkley, T. C., Garing, S., Singh, S., Le Ny, A.-l. M., Nicholas, K. P., Peters, J. E., . . . Nugen, S. R. (2018, July 11). Reporter bacteriophage T7NLC utilizes a novel NanoLuc::CBM fusion for the ultrasensitive detection of Escherichia coli in water. *Analyst, 143*, 4074-4082. doi:10.1039/c8an00781k

[10] Hinkley, T. C., Singh, S., Garing, S., Le Ny, A.-L. M., Nichols, K. P., Peters, J. E., . . . Nugen, S. R. (2018, September 21). A phage-based assay for the rapid, quantitative, and single CFU visualization of E. coli (ECOR #13) in drinking water. *Scientific Reports, 8*(14630). doi:https://doi.org/10.1038/s41598-018-33097-4

[11] Krumm, A. (2019, October 11). *Measure microbial growth using OD600*. Retrieved from BMG Labtech: https://www.bmglabtech.com/measure-microbial-growth-using-the-od600

[12] *LB Broth (Miller's LB Broth)*. (n.d.). (Interchim) Retrieved from Interchim: https://www.interchim.fr/ft/N/N1398A.pdf

[13] Lefers, M., & Holmgren Lab. (2004, July 26). *Multiplicity of Infection MOI*. Retrieved from North Western Glossary: https://groups.molbiosci.northwestern.edu/holmgren/Glossary/Definitions/Def-M/multiplicity_of_infection.html

[14] Lim, J. Y., Yoon, J. W., & Hoyde, C. J. (2010, Jan). A Brief Overview of Escherichia coli O157:H7 and Its Plasmid O157. *J Microbiol Biotechnol, 20*(1), 5-14. Retrieved July 20, 2021, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3645889/

[15] Mishra, P., Pandey, C. M., Singh, U., Gupta, A., Sahu, C., & Keshri, A. (2019). Decriptive statistics and normality tests for statistical data. *Ann Crd Anaesth, 22*(1), 67-72. doi:10.4103/aca.ACA_157_18

[16] NIST/SEMATECH. (2012). *e-Handbook of Statistical Methods.* doi:https://doi.org/10.18434/M32189

[17] Nugen, S. R. (2019). T7 Phages Stock Propagation Methods .
[18] Pepper, I., & Gerba, C. (n.d.). *Bacterial Growth Curve Analysis and its Envrionmental Applications*. (JoVE Science Education Library) Retrieved from https://www.jove.com/t/10100/bacterial-growth-curve-analysis-and-its-environmental-applications
[19] Phagehunting Program. (n.d.). *Titering*. Retrieved from https://phagesdb.org/media/workflow/protocols/pdfs/PDF-Tbox-4titering.pdf
[20] Pinto, A. M., Cerqueira, M. S., Banobre-Lopes, M., Pastrana, L. M., & Sillankorva, S. (2020, Febrary 20). Bacteriophages for chronic wound treatment: from traditional to novel delivery systems. *Viruses, 12*(2), 235. doi:10.3390/v12020235
[21] *Pouring LB Agar Plates*. (2016, September 14). (addgene) Retrieved from https://www.addgene.org/protocols/pouring-lb-agar-plates/
[22] Qimron, U., Tabor, S., & Richardson, C. C. (2011). New Details about Bacteriophage T7-Host Interactions. *American Society for Microbiogy Microbe Magazine*. Retrieved from https://web.archive.org/web/20110817192517/http://www.microbemagazine.org/index.php/03-2010-home/1428-new-details-about-bacteriophage-t7-host-interactions
[23] *Streaking and Isolating Bacteria on an LB Agar Plate*. (2017, February 28). (addgene) Retrieved from https://www.addgene.org/protocols/streak-plate/
[24] UniProt. (2021, March 7). *Proteomes - Escherichia coli O17:K52:H18 (strain UMN026 / ExPEC)*. Retrieved July 20, 2021, from UniProt: https://www.uniprot.org/proteomes/UP000007097
[25] Van Alst, A., LeVeque, R. M., Martin, N., & DiRita, V. J. (n.d.). *Growth Curves: Generating Growth Curves Using Colony Forming Units and Optical Density Measurements*. (JoVE Science Education Library) Retrieved from https://www.jove.com/t/10511/growth-curves-generating-growth-curves-using-colony-forming-units
[26] Youle, M. (2017). *Thinking Like a Phage* (1 ed.). San Diego, CA: Wholon. Retrieved July 20, 2021

Appendix A

A sample was taken from each time point, serially diluted, and plated to show a titer that corresponded to a given OD_{600} reading. BL21 grows relatively quickly, reaching the log phase in about two hours. Samples were taking every 20 minutes during the lag phase (OD_{600} 0-0.1) until the the log phase began (OD_{600} of around 0.4 – 0.6) in which samples were then collected every 10 minutes. To ensure the log phase had concluded, samples were collected at a time interval of 10 minutes until the OD_{600} reading reached two. At that time, the samples were again collected every 20 minutes to ensure that the solution was indeed reaching its stationary phase in the growth curve. Figures 1A-5A illustrate the plate cultures with their corresponding incubation times. Table 1A shows the corresponding OD_{600} reading, the number of CFUs counted, and the calculated titer. These plates are used to determine the number of colony forming units after a given incubation time and OD_{600} reading.

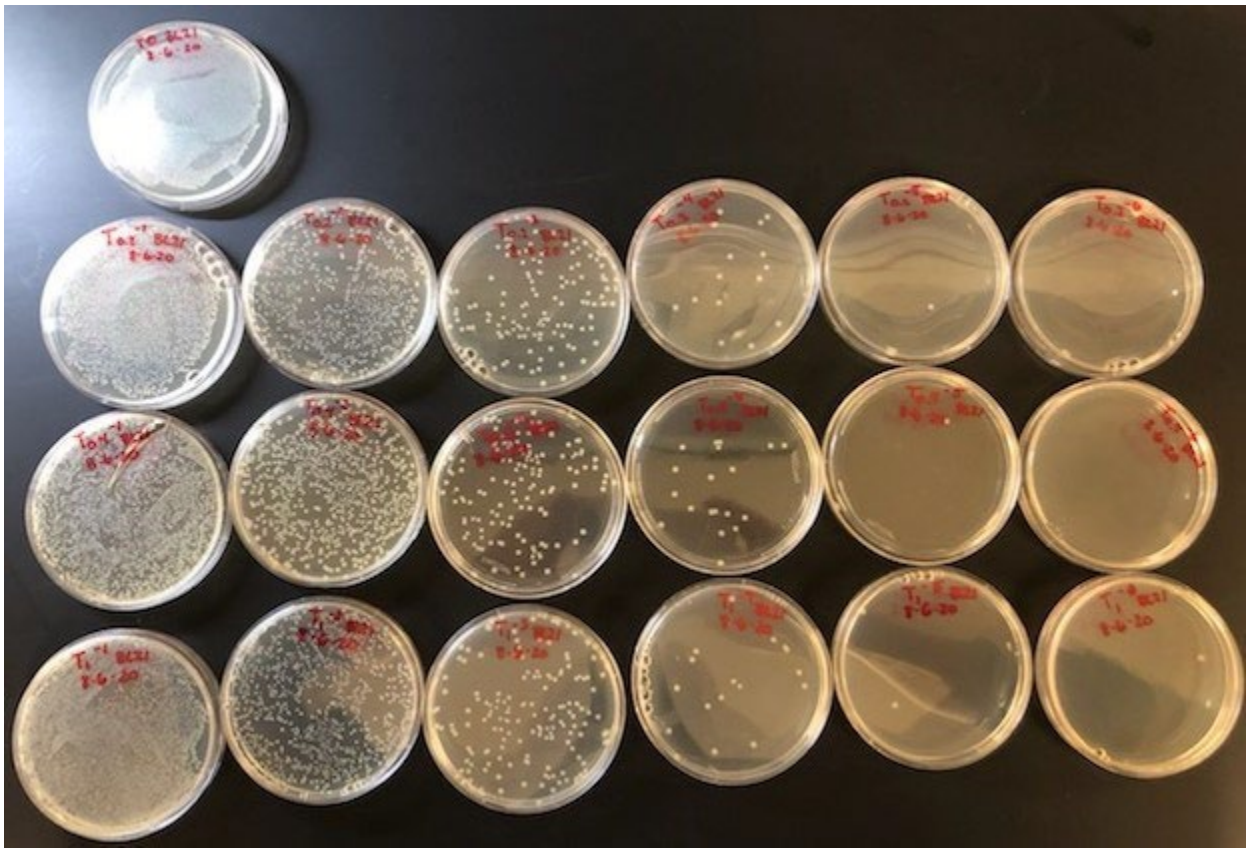

Figure 1A: Plates T_0-T_1 with dilution factors

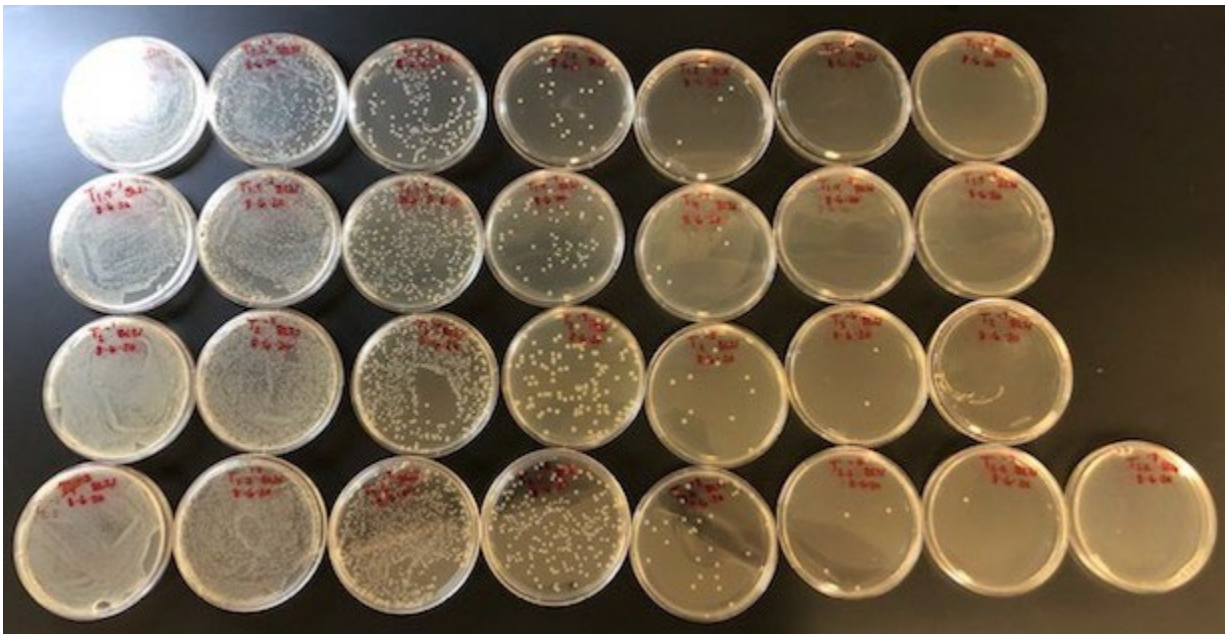

Figure 2A: Plates $T_{1.2}$-$T_{2.2}$ with dilution factors

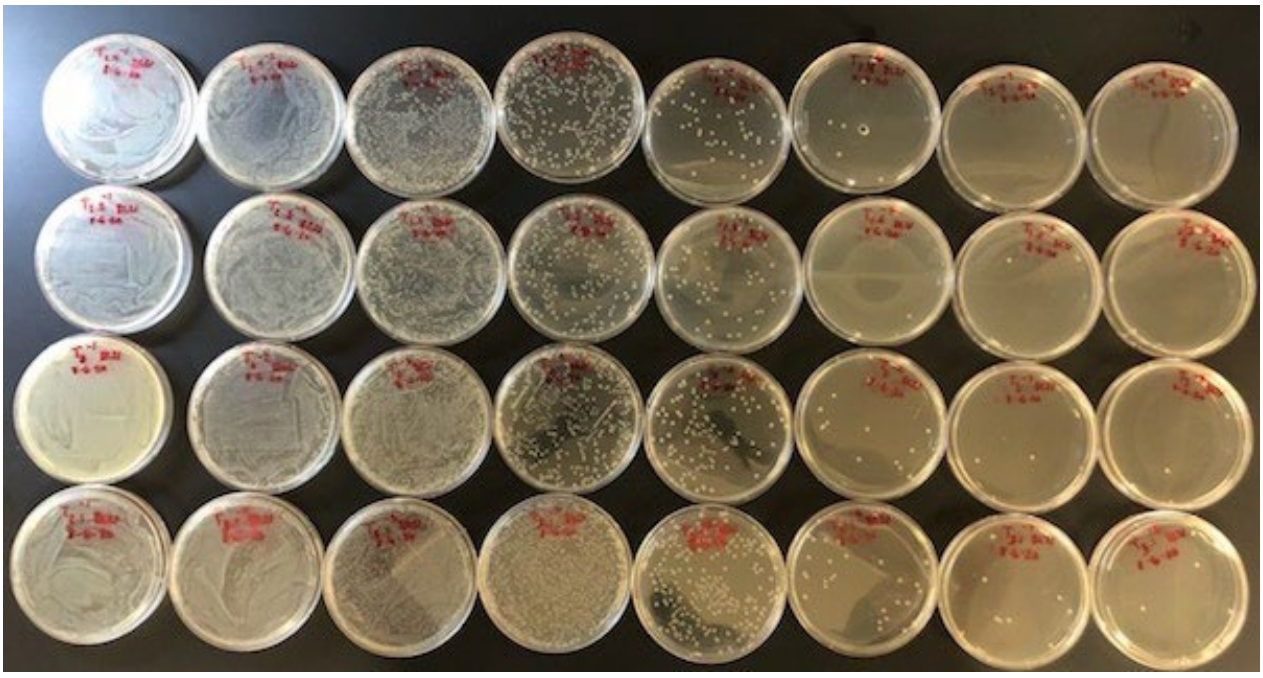

Figure 3A: Plates $T_{2.4}$-$T_{3.1}$ with dilution factors

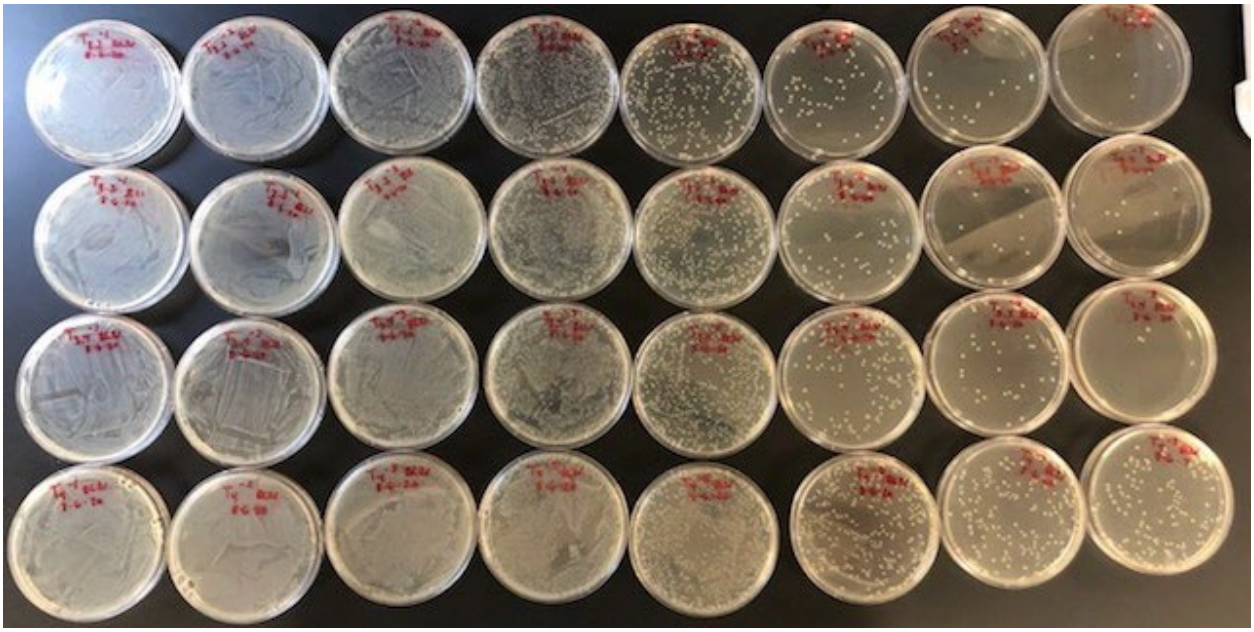

Figure 4A: Plates $T_{3.2}$-T_4 with dilution factors

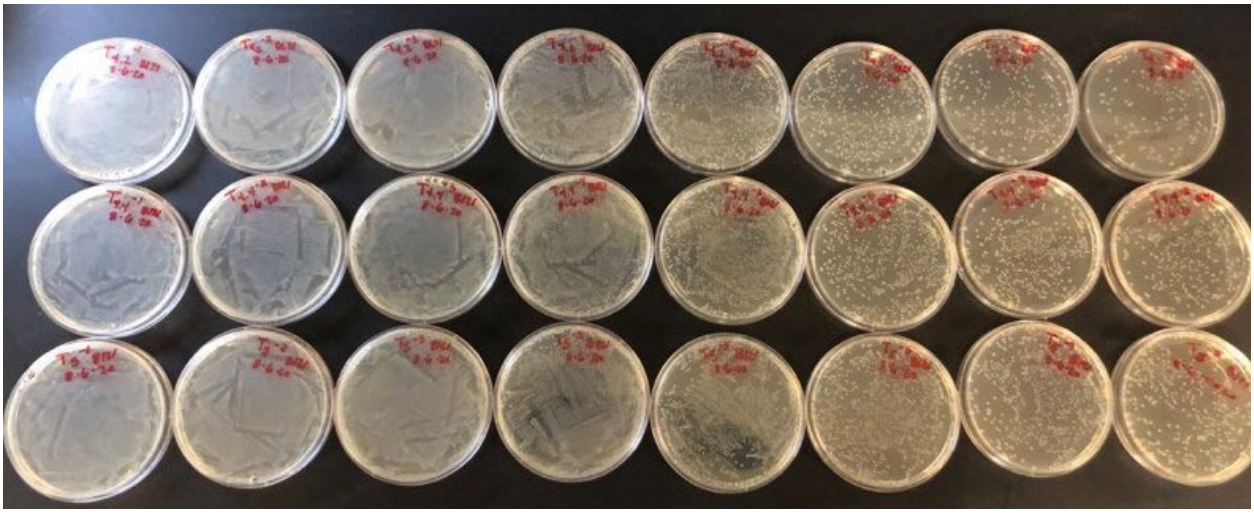

Figure 5A: Plates T_4-T_5 with dilution factors

Table 1A: Growth Curve by Time and Dilution Factor

Time Label	Time (minutes)	Dilution Factor	OD600	CFU	CFU/mL
T_0	0	1	0.01	>300	
$T_{0.2}$	**20**	10^{-3}	0.00	130	1300000
$T_{0.4}$	**40**	10^{-3}	0.00	133	1330000
$T_{1.0}$	**60**	10^{-3}	0.00	175	1750000
$T_{1.2}$	**80**	10^{-3}	0.00	267	2670000
$T_{1.4}$	**100**	10^{-4}	0.04	61	6100000
$T_{2.0}$	**120**	10^{-4}	0.06	118	11800000
$T_{2.2}$	**140**	10^{-4}	0.16	226	22600000
$T_{2.4}$	**160**	10^{-5}	0.33	60	60000000
$T_{2.5}$	**170**	10^{-5}	0.46	54	54000000
$T_{3.0}$	**180**	10^{-5}	0.71	126	126000000
$T_{3.1}$	**190**	10^{-5}	0.92	252	252000000
$T_{3.2}$	**200**	10^{-6}	1.24	60	600000000
$T_{3.3}$	**210**	10^{-6}	1.59	78	780000000
$T_{3.4}$	**220**	10^{-6}	1.93	148	1480000000
$T_{4.0}$	**240**	10^{-7}	2.88	176	17600000000
$T_{4.2}$	**260**	10^{-7}	3.72	249	24900000000
$T_{4.4}$	**280**	10^{-8}	4.06	296	296000000000
$T_{5.0}$	**300**	10^{-8}	4.18	>300	

LIST OF ABBREVIATIONS AND ACRONYMS

^{0}C	Degrees Celsius
%	Percent
CFU	Colony Forming Units
E. coli	*Escherichia coli*
g	Grams
L	Liter
LB	Luria Broth
MiliQ	Ultra-pure water of "type 1" with resistivity greater than 18.0
mL	Milliliter
MOI	Multiplicity Of Infection
OD_{600}	Optical Density at a wavelength of 600 nanometers
PFU	Plaque Forming Units
rpm	Revolutions Per Minute
RT	Room Temperature
µL	Microliter