High-Throughput Screening of a Metagenomic Library Using a Novel E. Coli Chip for Discovery of Antibiotic Resistance Genes

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HIGH-THROUGHPUT SCREENING OF A METAGENOMIC LIBRARY USING A NOVEL E. Coli CHIP FOR DISCOVERY OF ANTIBIOTIC RESISTANCE GENES

A Thesis
Presented to
The Faculty of the Department of Chemical and Materials Engineering
San José State University

In Partial Fulfillment
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Master of Science in Chemical Engineering

by
Richard Tiongco
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The Designated Thesis Committee Approves the Thesis Titled

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ABSTRACT

HIGH-THROUGHPUT SCREENING OF A METAGENOMIC LIBRARY USING A NOVEL E. COLI CHIP FOR DISCOVERY OF ANTIBIOTIC RESISTANCE GENES

by Richard Tiongco

The growing prevalence of antibiotic resistance is one of the greatest challenges facing humankind today. The Centers for Disease Control and Prevention (CDC) have estimated that about 2 million people in the US each year develop an infection caused by antibiotic-resistant bacteria, and about 23,000 people die. To combat this growing crisis, efforts geared toward the discovery of novel compounds, such as antimicrobial peptides (AMPs), have spurred. However, current approaches for identification of such compounds have proven to be costly, time-consuming and ineffective. This problem underscores the need for a new approach, one that is fundamentally different from traditional methods both in the content of the library being screened and in the screening methodology. As a first demonstration of this approach, we have developed an E. coli chip – a novel, robust, high-density nano-culture platform – and an associated high-throughput screening methodology to successfully screen for cells containing genes that confer resistance to ampicillin from a soil metagenomic library. This platform offers several advantages over the current industry standard, a 96-well microplate platform, including miniaturization, automation, reduced amount and cost of reagents and process time. It eliminates the need for more than one round of screening thus potentially speeding up the antibiotic discovery process. Further, a single E. coli chip can replace the work of approximately seven 96-well plates. These advantages make this technology ideal for further applications, including screening for AMPs.
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The growing prevalence of antibiotic resistance is one of the greatest challenges facing humankind today. The Centers for Disease Control and Prevention (CDC) have estimated that about 2 million people in the US each year develop an infection caused by antibiotic-resistant bacteria, and about 23,000 people die [1]. To combat this growing crisis, efforts geared toward finding compounds, such as antimicrobial peptides (AMPs), which have properties that not only kill or inhibit the growth or replication of bacteria but also combat its antibiotic resistance capabilities have spurred. These compounds can be identified through the high-throughput screening of a metagenomic library.

A metagenomic library is a highly diversified collection of genetic material extracted from an environmental sample, such as soil or sewage [2]. This library contains all kinds of genes including those which make a drug resistant to an antibiotic. The iterative high-throughput screening of a metagenomic library would lead to the identification of these antibiotic resistance genes. However, the traditional process of screening using 96-well plates takes multiple iterations and several months of intensive manual labor before genes can be successfully isolated due to low target sensitivity [3]. This issue underscores the need to miniaturize the assay to get better target sensitivity which can be resolved by utilizing a cellular microarray or an *E. coli* chip.

The objective of this research is to identify antibiotic resistance genes in metagenomic libraries using an indigenous high-throughput screening technology. To this end, we will develop an assay using an *E. coli* chip prepared with a robotic microarrayer, and analysis using a microarray scanner. We will determine the design considerations for
preparing a metagenomic library and *E. coli* chip, and benchmark the results from the novel *E. coli* chip assay against traditional 96-well plate assays.

1.1 Antibiotics and Antibiotic Resistance

Antibiotics are drugs that kill or inhibit the growth or replication of bacteria [4]. Since the discovery of the first antibiotic, penicillin, by Alexander Fleming in 1928, antibiotics have been used to successfully treat a wide range of bacterial infections [5]. In addition, antibiotics have also played a vital role in the development of a wide variety of medical advancements, including joint replacements, organ transplantation, cancer therapy and treatment of chronic diseases such as asthma, diabetes and rheumatoid arthritis [6, 7].

Antibiotic resistance is defined as the resistance of a bacteria to an antibiotic to which it was originally sensitive [5]. It is acquired when bacteria have developed defense mechanisms against that antibiotic [6]. Examples of these defense mechanisms include modifications of the antibiotic molecule, decreased antibiotic penetration and efflux, changes in target sites and global cell adaptations [6]. Over time, these bacteria will pass on these defense mechanisms to other bacteria, making them antibiotic-resistant as well [6]. Eventually, the antibiotic would no longer be effective and hence a new drug must be made. While the emergence of antibiotic resistance is mainly caused by interactions between bacteria and their environment, it is the overuse and misuse of these antibiotics that have greatly contributed to the widespread dissemination of bacterial strains which were able to resist the effect of these drugs [5, 6].

Table 1.1 shows a brief history of the discovery and introduction of various classes of antibiotics. It also shows the year in which resistance to that class of antibiotics was first
observed, the mechanism of those antibiotics for inhibiting bacterial formation and the activity of or the species targeted by those drugs.

Table 1.1. Timeline of the Discovery and Introduction of Antibiotics [8]

<table>
<thead>
<tr>
<th>Antibiotic class; example</th>
<th>Year of discovery</th>
<th>Year of introduction</th>
<th>Year resistance was observed</th>
<th>Mechanism of action</th>
<th>Activity or target species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfadrugs; prontosil</td>
<td>1932</td>
<td>1936</td>
<td>1942</td>
<td>Inhibition of dihydropter oate synthetase</td>
<td>Gram-positive bacteria</td>
</tr>
<tr>
<td>β-lactams; penicillin</td>
<td>1928</td>
<td>1938</td>
<td>1945</td>
<td>Inhibition of cell wall biosynthesis</td>
<td>Broad-spectrum activity</td>
</tr>
<tr>
<td>Aminoglycosides; streptomycin</td>
<td>1943</td>
<td>1946</td>
<td>1946</td>
<td>Binding of 30S ribosomal subunit</td>
<td>Broad-spectrum activity</td>
</tr>
<tr>
<td>Chloramphenicols; chloramphenicol</td>
<td>1946</td>
<td>1948</td>
<td>1950</td>
<td>Binding of 50S ribosomal subunit</td>
<td>Broad-spectrum activity</td>
</tr>
<tr>
<td>Macrolides; erythromycin</td>
<td>1948</td>
<td>1951</td>
<td>1955</td>
<td>Binding of 50S ribosomal subunit</td>
<td>Broad-spectrum activity</td>
</tr>
<tr>
<td>Tetracyclines; chlortetracycline</td>
<td>1944</td>
<td>1952</td>
<td>1950</td>
<td>Binding of 30S ribosomal subunit</td>
<td>Broad-spectrum activity</td>
</tr>
<tr>
<td>Rifamycins; rifampicin</td>
<td>1957</td>
<td>1958</td>
<td>1962</td>
<td>Binding of RNA polymerase β-subunit</td>
<td>Gram-positive bacteria</td>
</tr>
<tr>
<td>Glycopeptides; vancomycin</td>
<td>1953</td>
<td>1958</td>
<td>1960</td>
<td>Inhibition of cell wall biosynthesis</td>
<td>Gram-positive bacteria</td>
</tr>
<tr>
<td>Quinolones; ciprofloxacin</td>
<td>1961</td>
<td>1968</td>
<td>1968</td>
<td>Inhibition of DNA synthesis</td>
<td>Broad-spectrum activity</td>
</tr>
<tr>
<td>Streptogramins; streptogramin B</td>
<td>1963</td>
<td>1998</td>
<td>1964</td>
<td>Binding of 50S</td>
<td>Gram-positive bacteria</td>
</tr>
</tbody>
</table>
From Table 1.1, it can be shown that for 11 out of the 14 classes of antibiotics, bacterial resistance has been observed within 10 years from its discovery. In addition, for 12 out of the 14 classes, either resistance has been observed within 4 years from its year of introduction or there has already been observed resistance even before the drug has made into the market. Furthermore, the US CDC has reported extensively on the increasing rate of emergence of antibiotic resistance and has projected infections caused by antibiotic-resistant bacteria to result in 10 million deaths globally by 2050 [1]. This alarming threat underscores the need for compounds that not only possess antibacterial properties but also “antiresistance” properties as well.
1.2 Antimicrobial Peptides

Antimicrobial peptides (AMPs) are compounds which lower the rate of inducing bacterial resistance [9]. In addition, some AMPs could also function as antibiotics which inhibit bacterial formation through various mechanisms including cell membrane or cell wall disruption, as well as macromolecular synthesis inhibition and regulatory enzyme inhibition [6, 10]. Because of this dual nature of AMPs, they have emerged not only as a complement to traditional chemical antibiotics but also as an auspicious alternative for the treatment of bacterial infections through the years.

For example, resistance to tyrothricin, the first clinically used AMP, has yet to be reported despite having been used as a therapeutic for over 60 years [11-12]. This AMP, which is produced by the bacteria *Bacillus brevis*, is currently being used to treat various skin and oral and nasal cavity infections [12]. Furthermore, numerous pathogens such as *Staphylococcus aureus* (both the methicillin-susceptible and methicillin-resistant kind), *Staphylococcus haemolyticus*, *Streptococcus pyogenes*, *Enterococcus faecalis*, *Corynebacterium spec.*, *Candida albicans* and *Candida parapsilosis* have all shown to be highly susceptible to tyrothricin [12]. Another example is daptomycin, which has demonstrated superior and rapid activity against various Gram-positive pathogens including *Staphylococcus aureus* [13]. To this date, daptomycin is the only compound which has demonstrated activity against vancomycin-resistant enterococci (VRE) [13].

AMPs that possess little or no antibacterial properties have also been found to work synergistically with antibiotics. Synthetic peptides derived from natural peptides LL-37 and bactenecin coupled with antibiotics and have been found to be effective against pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) [14-15].
1.3 Drug Discovery and Development

The traditional or manual method of identifying a new drug from tens of thousands of potential candidates is often described as very slow, laborious, expensive and inefficient. Figure 1.1 shows a schematic of the drug discovery and development timeline.

![Drug discovery and development timeline](image)

Figure 1.1. Drug discovery and development timeline. Modified from [16].

At present, it takes about fifteen years total for a drug to be discovered from tens of thousands of compounds, pass pre-clinical and clinical trials and be approved by the Food and Drug Administration [16]. Further, it is estimated that only one drug reaches the market from about 10,000 compounds that were initially screened and that the cost of this complex process is around $1 billion [17]. The drug discovery process alone currently takes about three to five years to complete [17]. This is largely due to the laborious process of screening a traditional chemical library containing tens of thousands of compounds with varying amounts and concentrations. The need to accelerate the drug discovery process and make it more efficient and cost-effective has fueled the development of the high-throughput screening (HTS) method for drug discovery.
1.4 High-Throughput Screening

High-throughput screening (HTS) is a method used to accelerate the drug discovery process by allowing for the rapid analyses of thousands of chemical, biochemical, genetic or pharmacological substances [18]. It performs these functions using robotics, data processing and control software, liquid handling devices and sensitive detectors [19]. The selection of a high-throughput platform for screening potential drug candidates provides several benefits including rapid analyses, low cost, ease of automation, low working volume and amiability to miniaturization [18-21]. Because of these advantages, HTS became an invaluable tool in the arena of drug discovery. Over the past decade, it has flourished even more due to various technological advancements made including automation of liquid handling and dispensing for multi-well plates, creation of novel platforms such as microarrays and improvement of analytical tools for dealing with large quantities of data [22].

1.5 Metagenomic Libraries

Metagenomics, often referred to as environmental or community genomics, is the genomic analysis of microorganisms by direct extraction and cloning of DNA from a collection of microorganisms [23]. A metagenomic library is a highly diversified collection of genetic material, extracted from a variety of habitats including soil, sewage and the commensal microbiota of both healthy and diseased individuals from around the world [24-28]. This library is constructed by isolating DNA from a sample, cloning the DNA into a suitable vector, transforming the clones into a host bacterium [23]. The resulting transformants are then subjected to either a function-based or sequence-based
screening analysis [23]. The process for construction and screening of this type of library can be shown in Figure 1.2.

![Figure 1.2. Construction of a metagenomic library. Modified from [23].](image)

Screening a metagenomic library for drug discovery would entail being able to identify compounds which have properties that inhibit antibiotic resistance. A metagenomic library contains a plethora of genes including those which produce enzymes that promote drug metabolism, that is the breakdown of drugs. These genes are commonly known as antibiotic-resistance genes and are known to be possessed by living organisms. In humans, a variety of oxidative and conjugative enzymes involved in drug metabolism can be found in the lungs, kidneys and especially the liver [29-30]. The presence of these enzymes is the reason why compounds make it past the drug discovery stage but not during the subsequent pre-clinical and clinical trial stages. The traditional chemical libraries being screened during the drug discovery stage simply do not possess antibiotic-resistance genes. Iterative screening of a metagenomic library would lead to the identification of these antibiotic resistance genes and eventually the development of AMPs that would counteract its effects.
CHAPTER TWO
REVIEW OF RELATED LITERATURE

This present literature review examines the various components required for the high-throughput screening (HTS) of a metagenomic library using pathogen chips. These include metagenomic library preparation, microarray chip preparation and antimicrobial susceptibility testing. It also discusses studies that aimed to characterize the resistance of *E. coli* to various antibiotics and also efforts geared toward selecting for antibiotic resistance genes from metagenomic libraries using either an agar-plate or 96-well microplate format.

2.1 Metagenomic Library Preparation

A metagenomic library is prepared by inserting a vector containing DNA into a host bacterium. Over the past two centuries, numerous and comprehensive experiments have already been conducted on preparing, that is, culturing and purifying, bacterial cells. Only in more recent years have there been detailed studies on how to prepare a metagenomic library.

2.1.1 Cell Culture and Purification

Cell culture has been done in virtually the same way ever since the time of Pasteur in the 19th century. Frozen glycerol stocks of bacteria were subcultured onto agar plates filled with Luria Bertani (LB) agar, which may or may not contain some amount of drug, and incubated overnight. This culture was further subcultured into a new LB broth solution and again incubated overnight. The contents of this new overnight culture would then be purified by subjecting it to multiple centrifugation, washing and resuspension procedures. The resulting solution may be subjected to a cell count analysis to determine
the cell density or cell concentration. This solution would serve as the source of host bacteria for which the vector containing DNA would be inserted.

2.1.2 Metagenomic Library Construction

The construction of a metagenomic library involves three major steps: extracting DNA from an environmental sample, cloning the DNA into a suitable vector and transforming the clones into a host bacterium [23]. Hosokawa et al. describe a method of constructing a metagenomic library. In their study, 10 g of soil were taken from a Quercus serrata forest located in Mount Tsukuba in Ibaraki, Japan [2]. Soil DNA was then isolated and purified using ISOIL, a kit for extracting DNA from soil samples manufactured by Nippon Gene, headquartered in Tokyo, Japan [2]. The cloning of DNA was done using the CopyControl Fosmid Library production kit using the protocol developed by Gurgui and Piel [32]. The DNA fragments were then purified and joined together to form vectors [2]. These vectors were then inserted into Escherichia coli (E. coli) cells, which were then spread unto an agar plate containing LB agar augmented with 12.5 µg/mL of chloramphenicol. After incubating overnight, the vectors were then stored in tubes as library pools containing 1000 clones/mL, which were predetermined using a colony counting assay, of 50% (v/v) of aqueous glycerol solution at -80°C until screening [2].

2.2 Microarray Chip Preparation

A microarray chip is simply a chemically modified glass slide that serves as a platform for encapsulated bacterial cells during drug testing. These cells would be spotted on the glass slide either manually using a micropipette or through automation using a robotic spotter. The first major step for the preparation of a microarray chip is the
modification (i.e. sterilization and coating) of the slides to be used for the analysis. The next major step is the preparation of the inoculum (i.e. cells) and encapsulation material (i.e. collagen or alginate). Finally, the last major step is the preparation of the microarray chip itself, which entails the spotting of the inoculum and encapsulation material onto the modified glass slide. Among the advantages of utilizing a microarray chip over traditional 96-well microtiter plates are cost-effectiveness, efficiency and miniaturization [30].

2.2.1 Preparation of Functionalized Slides

The preparation of functionalized or chemically-modified glass slides involves the very intricate sterilization and coating of these glass slides. A procedure for developing this chip to screen for antimicrobial compounds, which encompasses both antibacterial and antifungal compounds, was formulated by Srinivisan et al. in 2013. A detailed schematic of the workflow used is shown in Figure 2.1. It is important to note that the workflow shown already includes the associated screening process for identifying drugs with antimicrobial activity.
Figure 2.1. Fabrication of a microarray chip and associated screening process for identifying antibiotics.

The first major step in developing the nano-biofilm microarray is modifying the surface of the glass slide. This was done by first treating the surface of the slide with ethanol and sulfuric acid. The treatment of the glass slide with sulfuric acid exposes the
silanol (Si-OH) groups, which results in surface activation [32]. The activated surface was then coated with 3-aminopropyltriethoxysilane (APTES) to yield an amine-functionalized siloxane group (-O-Si-O-) [32]. The slide was then subjected to spin coating with polystyrene co-maleic anhydride (PSMA) solution to provide a hydrophobic surface [32]. This is important for the subsequent spotting of Poly-L-Lysine (PLL) – Barium Chloride (BaCl₂) mixture, which acts as a “tie layer” for the bacterial spots to adhere to the glass slide surface, to ensure that the beads formed are spherical in shape.

2.2.2 Preparation of Inoculum and Encapsulation Material

The study done by Srinivasan et al. in 2012 utilized Candida albicans yeast cells encapsulated in a spherical matrix composed of collagen and yeast peptone dextrose (YPD) [31] for the creation of their own version of a microarray chip, which they called a Candida albicans biofilm chip or (CaBChip). In their study, a culture containing C. albicans was prepared and incubated overnight in an orbital shaker [31]. Then, 1 mL of C. albicans yeast cells were harvested and subjected to a series of centrifugation, washing and resuspension procedures [31]. The cell density of the resuspended cells was then determined using a hemocytometer and adjusted by dilution to obtain the desired concentration [31]. The yeast cells were then encapsulated in collagen and the resulting collagen-cell suspension was stored with ice to prevent potential gelation of the collagen before printing [31].

2.2.3 Preparation of a Microarray Chip

In the study done by Srinivasan (2013), the PSMA-coated slide was then treated with an aqueous solution of poly-L-lysine (PLL) mixed with barium chloride (BaCl₂) to provide a stable platform for both the alginate gel, containing the cells, and the PSMA to
attach on [32]. The treatment was performed using a robotic microarrayer, an automated device for spotting different materials, biomolecules or cells onto solid surfaces [18]. The same device was then used to print yeast cells encapsulated in an alginate matrix superimposing the PLL-BaCl₂ spots also known as the “tie layer” [32]. Contact angle goniometry may also be performed to confirm whether these surface modification processes resulted in a hydrophobic surface [32]. The alginate spots should be nearly hemispherical with a contact angle close to 90°C.

2.3 Antimicrobial Susceptibility Testing

After spotting the cells, the actual screening process may now be undertaken. This process, where the effect of a drug or combination of drugs on a bacterial sample will be tested at various concentrations, is often called antimicrobial susceptibility testing (AST).

2.3.1 Factors Crucial for a Successful Screening

Two factors which are deemed crucial for a successful screening are assay design and compound selection [33]. Assay design refers to determining the appropriate method for testing and measuring drug activity. The requirements of an effective screening assay include high target specificity and sensitivity, reproducibility and robustness [34]. There are two types of assay design methodologies, namely functional and biochemical. The goal of a functional assay is to simply determine if a drug is effective in inhibiting a bacterial sample. A biochemical assay would want to determine whether there is any enzyme activity going on in the sample. Also included in assay design is determining whether drug activity will be measured on either an absorbance basis or fluorescence basis. Compound selection, on the other hand, refers to the process of identifying compounds to be subjected to initial screening through either computational or
combinatorial chemistry methods [33]. For a functional-based analysis, it is important
that the compounds selected will render a high percentage of bacterial inhibition.

2.3.2 Antimicrobial Susceptibility Testing and Analysis

Srinivasan et al. (2017) describes a functional and fluorescence-based assay for
antimicrobial susceptibility testing. Following the schematic in Figure 2.1, the testing
process begins by printing drug dilutions that superimposes the cell spots [35]. The slides
were then incubated for 24 hours at 37°C for drug activity [35]. After incubation, the
microarray chip was then stained with FUN-1, a fluorescent cell viability dye, and was
incubated again at 37°C for 30 minutes. The chip was then washed and air-dried before
being read using a fluorescent scanner. Finally, a drug susceptibility profile was
constructed to identify potential drug candidates [35].

An antimicrobial/drug susceptibility profile, also known as a dose-response or
inhibitory concentration (or IC) profile, such as the one shown in Figure 2.2 (C), of a
bacterial species is a common method of depicting the effect of a drug on a sample
culture of that species. Specifically, it is a plot of normalized response against the
logarithm of the drug concentration. Normalized response is defined as the concentration
of bacteria remaining in the sample after a certain drug concentration is applied divided
by the concentration of the control sample (i.e. concentration of bacteria remaining when
no drug was applied). The formula, using fluorescence units, is shown on Equation 2.1.

\[
\text{Normalized Response} = \frac{F - F_o}{F_{\text{max}} - F_o} \times 100\%
\]

Equation 2.1. Normalized Response Formula in terms of Fluorescence [33]
where \( F, F_{\text{max}} \) and \( F_0 \) are the fluorescence intensities of drug-treated, control (no drug) and bleach-treated spots, respectively [35]. The intensities are given in terms of Resonance Fluorescence Units (RFU). The equation above can also be expressed in terms of absorbance units, as shown in Equation 2.2.

\[
\text{Normalized Response} = \frac{A - A_0}{A_{\text{max}} - A_0} \times 100\%
\]

Equation 2.2. Normalized Response Formula in terms of Absorbance

where \( A, A_{\text{max}} \) and \( A_0 \) are the fluorescence intensities of drug-treated, control (no drug) and bleach-treated spots, respectively.

A common quantity used to report drug effectiveness is known as the maximal inhibitory concentration (MIC). Two common types are MIC\(_{50}\) and MIC\(_{90}\), which are the amount of drug required to achieve 50% and 90% inhibition respectively. Table 2.1 shows the various MIC\(_{50}\) and MIC\(_{90}\) for common antibiotics. The lower the MIC of a drug, the more effective it is as you only require a lower concentration of that drug to kill a certain concentration of bacteria.
Table 2.1. MIC range, MIC₅₀ and MIC₉₀ for Common Antibiotics and the Percentage Resistance of 111 E. coli Strains to the Antibiotics [36]

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC range (mg/L)</th>
<th>MIC₅₀ (mg/L)</th>
<th>MIC₉₀ (mg/L)</th>
<th>Resistance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ampicillin</td>
<td>0.25-128</td>
<td>8</td>
<td>64</td>
<td>62.2</td>
</tr>
<tr>
<td>Ampicillin + sulbactam</td>
<td>0.25-32</td>
<td>1</td>
<td>4</td>
<td>1.8</td>
</tr>
<tr>
<td>piperacillin</td>
<td>0.5-64</td>
<td>2</td>
<td>8</td>
<td>3.6</td>
</tr>
<tr>
<td>piperacillin + tazobactam</td>
<td>0.25-4</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>cefazolin</td>
<td>1-32</td>
<td>1</td>
<td>4</td>
<td>7.2</td>
</tr>
<tr>
<td>cefuroxime</td>
<td>0.25-4</td>
<td>1</td>
<td>2</td>
<td>0.9</td>
</tr>
<tr>
<td>cefoxitin</td>
<td>0.25-2</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>cefoperazone</td>
<td>0.06-4</td>
<td>0.125</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>cefotaxime</td>
<td>0.03-0.5</td>
<td>0.06</td>
<td>0.125</td>
<td>0</td>
</tr>
<tr>
<td>ceftazidime</td>
<td>0.03-0.5</td>
<td>0.06</td>
<td>0.125</td>
<td>0</td>
</tr>
<tr>
<td>cefepime</td>
<td>0.03-0.5</td>
<td>0.06</td>
<td>0.125</td>
<td>0</td>
</tr>
<tr>
<td>meropenem</td>
<td>0.03-0.5</td>
<td>0.06</td>
<td>0.125</td>
<td>0</td>
</tr>
<tr>
<td>gentamycin</td>
<td>0.06-5</td>
<td>0.125</td>
<td>0.25</td>
<td>0</td>
</tr>
<tr>
<td>tobramycin</td>
<td>0.125-4</td>
<td>0.25</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>amikacin</td>
<td>0.25-8</td>
<td>0.5</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>ofloxacin</td>
<td>0.03-0.5</td>
<td>0.125</td>
<td>0.25</td>
<td>0</td>
</tr>
<tr>
<td>ciprofloxacin</td>
<td>0.001-0.25</td>
<td>0.06</td>
<td>0.125</td>
<td>0</td>
</tr>
<tr>
<td>colistin</td>
<td>0.125-1</td>
<td>0.25</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>tetracycline</td>
<td>1-64</td>
<td>16</td>
<td>32</td>
<td>81.1</td>
</tr>
<tr>
<td>chloramphenicol</td>
<td>0.25-128</td>
<td>2</td>
<td>4</td>
<td>5.4</td>
</tr>
<tr>
<td>cotrimoxazole</td>
<td>0.25-128</td>
<td>1</td>
<td>64</td>
<td>10.8</td>
</tr>
</tbody>
</table>

2.4 Characterization of Resistance in E. coli to Various Antibiotics

*Escherichia coli (E. coli)* is one of the most common causes of urinary tract infections and one of the leading causes of systemic infections including bacteremia, nosocomial pneumonia cholecystitis, infectious arthritis and neonatal meningitis [37-38]. Numerous studies have been done which have sought to observe the antimicrobial susceptibility of *E. coli* to various antibiotics. The study done by Cambrea found that *E. coli* had a low susceptibility (less than 40 % sensitivity) to ampicillin, amoxicillin, tetracycline and trimethoprim-sulfamethoxazol [39]. The results are shown in Table 2.2.
Another study done by Watkinson et al. has determined the antibiotic resistance of *E. coli* isolated from antibiotic-impregnated MI-R (tetracycline, ampicillin, cephalexin and sulfamethoxazole) plates [40]. The concentrations of the antibiotics added to the plates reflected their breakpoint concentrations, which are as follows: ampicillin, 32 µg/mL, tetracycline, 16 µg/mL, sulfamethoxazole, 350 µg/mL and ciprofloxacin, 4 µg/mL [41]. The same study has also reported the percentage of isolates from each plate that were resistant to multiple antibiotics. The method for determining resistance basically involves growing bacteria (50 colonies from each antibiotic-impregnated plate) on a control plate (without antibiotic treatment) and on an antibiotic plate (with antibiotic treatment). The
concentration of bacteria on both plates were counted after incubating for 24 hours at 37°C and the percent resistance was defined as follows:

\[
\% \text{ Resistance} = \frac{[E. \ coli] \text{ on antibiotic plate}}{[E. \ coli] \text{ on control plate}} \times 100\%
\]

Equation 2.3. Percentage Resistance Equation

The percentage resistance values from the study done by Watkinson et al. is shown in Table 2.3 [41].

Table 2.3. Antibiotic Resistance of *E. coli* Isolated from Antibiotic-Impregnated MI-R Plates [41]

<table>
<thead>
<tr>
<th>Antibiotic(s)</th>
<th>Ampicillin plate (n=50)</th>
<th>Tetracycline plate (n=50)</th>
<th>Sulfamethoxazole plate (n=50)</th>
<th>Ciprofloxacin plate (n=50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single antibiotics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ampicillin</td>
<td>100</td>
<td>78</td>
<td>73</td>
<td>70</td>
</tr>
<tr>
<td>cephalothin</td>
<td>60</td>
<td>42</td>
<td>43</td>
<td>28</td>
</tr>
<tr>
<td>ciprofloxacin</td>
<td>10</td>
<td>36</td>
<td>11</td>
<td>100</td>
</tr>
<tr>
<td>sulfafurazole</td>
<td>78</td>
<td>70</td>
<td>100</td>
<td>72</td>
</tr>
<tr>
<td>sulfamethoxazole-trimethoprim</td>
<td>62</td>
<td>64</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>Tetracycline</td>
<td>58</td>
<td>100</td>
<td>60</td>
<td>56</td>
</tr>
<tr>
<td>Multiple antibiotics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>two antibiotics</td>
<td>16</td>
<td>8</td>
<td>20</td>
<td>8</td>
</tr>
<tr>
<td>three antibiotics</td>
<td>6</td>
<td>16</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>four antibiotics</td>
<td>48</td>
<td>22</td>
<td>35</td>
<td>38</td>
</tr>
<tr>
<td>five antibiotics</td>
<td>20</td>
<td>26</td>
<td>28</td>
<td>26</td>
</tr>
<tr>
<td>six antibiotics</td>
<td>4</td>
<td>16</td>
<td>3</td>
<td>14</td>
</tr>
</tbody>
</table>

From the data in Table 2.3, it was concluded that resistance of *E. coli* to each of the antibiotics was observed and confirmed. All of the isolates tested showed some form of resistance against the other antibiotics tested [41]. In addition, it was reported that overall, the isolates demonstrated the highest occurrence of resistance to four antibiotics (36%) that were tested, followed by five (24%), three (13%), two (13%) and six (9%) [41].
2.5 Screening of a Metagenomic Library for Antibiotic Resistance Genes

Studies have been conducted aimed at retrieving antibiotic resistance genes from a natural environment using screening either through an agar plate or a 96-well microplate platform. In the agar plate platform, colonies of the metagenomic library grown on an agar plate containing the antibiotic for which the host bacteria (usually *E. coli*) alone would not survive (usually kanamycin) were streaked unto another agar plate containing the antibiotic of interest (i.e. that is the antibiotic used to test for resistance genes). The concentration of the antibiotic of interest is usually selected based on the MIC values. Studies done by Allen et al. [42] and D.F.K. dos Santos et al. [43, 44] have successfully employed this method to screen for genes containing resistance to carbenicillin while the one done by Miyazaki and Kitahara [45] was able to isolate spectinomycin resistance genes. The source of library, number of colonies, antibiotic concentration and number of resistance genes obtained are shown in Table 2.4.

For the 96-well plate platform, colonies of the metagenomic library grown on an agar plate containing the antibiotic for which the host bacteria alone would not survive were streaked and diluted with broth and arrayed across (usually 10) 96-well plates containing the antibiotic of interest. One such study is the one done by Mori et al. [46] that was able to successfully screen for genes which have conferred resistance to phleomycin. In their study, 3.2 Gb of DNA containing 96,000 unique clones were used. They were dispensed in 100 l of LB medium containing 12.5 g/mL chloramphenicol and 50 g/mL phleomycin and arrayed across ten 96-well plates, with each plate containing 100 clones per well. After an incubation period of 14 h at 37°C, there were three unique resistant clones that were identified. Their DNA was then amplified and subjected to gel
electrophoresis for validation. Studies done by Florez et al. [47] and Devirgillis et al. [48] have also been successful in isolating genes which conferred resistance to tetracycline and ampicillin respectively. The source of library, number of colonies, antibiotic concentration and number of resistance genes obtained are shown in Table 2.4.

Table 2.4. Metagenomic Library Screening for Antibiotic Resistance Genes

<table>
<thead>
<tr>
<th>Study</th>
<th>Source of metagenomic library</th>
<th>Number of clones tested</th>
<th>Clones per well (if using a 96-well plate)</th>
<th>Concentration of antibiotic used for screening (µg/mL)</th>
<th>Number of resistance genes obtained</th>
<th>Type of resistance genes obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mori et al., 2008 [46]</td>
<td>Activated Sludge</td>
<td>96,000</td>
<td>100</td>
<td>50</td>
<td>2</td>
<td>phleomycin</td>
</tr>
<tr>
<td>Florez et al., 2017 [47]</td>
<td>Cabrales cheese</td>
<td>850,000</td>
<td>100</td>
<td>10</td>
<td>4</td>
<td>tetracycline</td>
</tr>
<tr>
<td>Devirgillis et al., 2014 [48]</td>
<td>Food fermenting microbiota</td>
<td>20,000</td>
<td>10</td>
<td>25</td>
<td>6</td>
<td>ampicillin</td>
</tr>
<tr>
<td>Allen et al., 2015 [42]</td>
<td>Alaskan soil</td>
<td>500,000</td>
<td>n/a</td>
<td>50</td>
<td>1</td>
<td>carbenicillin</td>
</tr>
<tr>
<td>dos Santos et al., 2015 [43,44]</td>
<td>Brazilian Cerrado soil</td>
<td>150,000</td>
<td>n/a</td>
<td>50</td>
<td>1</td>
<td>carbenicillin</td>
</tr>
<tr>
<td>Miyazaki and Kitahara, 2018 [45]</td>
<td>Fermentation products, wood composts, activated sludge</td>
<td>2,000</td>
<td>n/a</td>
<td>40</td>
<td>4</td>
<td>spectinomycin</td>
</tr>
</tbody>
</table>

There were also studies done by Dantas et al. [24-28] which were able to successfully isolate novel antibiotic-resistant DNA from various environmental libraries, which include human, soil, sewage, animal and bacterial habitats. The source, number
and size of libraries as well as the number of resistance genes obtained are summarized in Table 2.5.

Table 2.5. Metagenomic Libraries and Resistance Genes in Dantas Lab [24-28]

<table>
<thead>
<tr>
<th>Habitat</th>
<th>Number of Libraries</th>
<th>Library Size (Gb)</th>
<th>Number of Resistance Genes per Gb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil</td>
<td>24</td>
<td>280</td>
<td>10.7</td>
</tr>
<tr>
<td>Human</td>
<td>83</td>
<td>334.9</td>
<td>7.3</td>
</tr>
<tr>
<td>Animal</td>
<td>14</td>
<td>21.4</td>
<td>7.1</td>
</tr>
<tr>
<td>Cultured Bacteria</td>
<td>12</td>
<td>24.4</td>
<td>23.9</td>
</tr>
<tr>
<td>Sewage</td>
<td>10</td>
<td>83.7</td>
<td>n.d.</td>
</tr>
<tr>
<td>Total</td>
<td>143</td>
<td>744.3</td>
<td>8.3</td>
</tr>
</tbody>
</table>

Despite the success of the screening methodology in terms of isolating for clones containing antibiotic resistance, the process still remains burdensome. For even with a small library containing $3 \times 10^5$ unique metagenomic inserts (i.e. unique clones), the screening procedure still required several months of intensive manual work thus underscoring the need of miniaturizing the assay.

2.6 Summary

Numerous studies have been done which have sought to determine the antimicrobial susceptibility of *E. coli* against several antibiotics. From these studies, it has been found that various strains of *E. coli* exhibited significant resistance against many antibiotics including ampicillin, tetracycline, amoxicillin etc. These dire results has spurred many efforts in isolating resistance genes from metagenomic libraries either through an agar-plate or 96-well plate platform. These studies have been able to select for indigenous genes which conferred resistance to various antibiotics including phleomycin, tetracycline, ampicillin, carbenicillin and spectinomycin. Though these studies have largely been successful, the general methodology however, showcased the difficulty in
using either platform to screen just even a relatively small library which underscores the need for assay miniaturization.
CHAPTER THREE
RESEARCH OBJECTIVE

The objective of this research is to identify antibiotic resistance genes in metagenomic libraries using an indigenous high-throughput screening technology. To this end, we will develop an assay using an *E. coli* chip prepared with a robotic microarrayer, and analysis using a microarray scanner. We will determine the design considerations for preparing a metagenomic library and *E. coli* chip, and benchmark the results from the novel *E. coli* chip assay against traditional 96-well plate assays. The following tasks will be performed to accomplish the objectives:

1. Screening metagenomic libraries using a 96-well plate assay to reproduce data from the Dantas lab
2. Preparation of *E. coli* chip and growth curves for *E. coli* nano-cultures
3. IC$_{50}$ of antibiotics against *E. coli* nano-cultures
4. Screening of metabolic libraries using the *E. coli* chip to generate antibiotic resistant hits
5. Identification of antibiotic resistant genes from the hits (in collaboration with Aura Ferreiro, Dantas Lab)

The metagenomic *E. coli* cells was prepared by Dr. Gautam Dantas, Washington University in St. Louis, Missouri. DNA was extracted from soil samples obtained from Kellogg Biological Station, Missouri and CLN, El Salvador respectively. For the 96-well plate platform, a Synergy$^\text{TM}$ Neo 2 Hybrid Multi-Mode Microplate Reader was utilized to carry out an absorbance-based assay. For the *E. coli* chip, a fluorescence-based assay
using a cell viability dye, will be conducted with the aid of an Axon Instruments’ GenePix Personal 4100A Microarray Scanner.
4.1 Process Flowchart

Figure 4.1 shows a schematic of the nano-culture antibiotic selection process workflow utilized in this study.

Figure 4.1. Schematic of nano-culture antibiotic selection process workflow.
4.2 Materials

Table 4.1 below shows the complete list of materials used in this research with the corresponding sources and catalog numbers.

Table 4.1. Sources and Catalog Numbers for Chemicals Used

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Source</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>Fisher Scientific</td>
<td>BP1421-2</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>Fisher Scientific</td>
<td>S641-212</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>Fisher Scientific</td>
<td>BP9727-500</td>
</tr>
<tr>
<td>Agar</td>
<td>Fisher Scientific</td>
<td>BP1423-500</td>
</tr>
<tr>
<td>Kanamycin sulfate</td>
<td>Fisher Scientific</td>
<td>11815024</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Fisher Scientific</td>
<td>BP1760-5</td>
</tr>
<tr>
<td>Deionized water</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Ethanol, Absolute (200 Proof)</td>
<td>Fisher Scientific</td>
<td>BP2818100</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Fisher Scientific</td>
<td>BP229-4</td>
</tr>
<tr>
<td>Sulfuric acid</td>
<td>Fisher Scientific</td>
<td>SA213</td>
</tr>
<tr>
<td>Air</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Toluene</td>
<td>Sigma-Aldrich</td>
<td>SIAL-244511</td>
</tr>
<tr>
<td>Polystyrene-co-maleic anhydride (PSMA)</td>
<td>Sigma-Aldrich</td>
<td>442399-250G-A</td>
</tr>
<tr>
<td>3-Aminopropyl Triethoxysilane (APTES)</td>
<td>Fisher Scientific</td>
<td>AC430941000</td>
</tr>
<tr>
<td>Phosphate-Buffered Saline 10x (PBS-10x)</td>
<td>Fisher Scientific</td>
<td>BP399-500</td>
</tr>
<tr>
<td>Calcium chloride dihydrate</td>
<td>Fisher Scientific</td>
<td>C70-500</td>
</tr>
<tr>
<td>Poly-L-Lysine (PLL)</td>
<td>Fisher Scientific</td>
<td>343810001</td>
</tr>
<tr>
<td>Barium chloride dihydrate</td>
<td>Fisher Scientific</td>
<td>B34-500</td>
</tr>
<tr>
<td>Alginic acid sodium salt</td>
<td>Alfa Aesar</td>
<td>B25266-250g</td>
</tr>
<tr>
<td>FUN®1 cell stain</td>
<td>Fisher Scientific</td>
<td>F7030</td>
</tr>
<tr>
<td>SYTO®9 nucleic acid stain</td>
<td>Fisher Scientific</td>
<td>S34854</td>
</tr>
</tbody>
</table>
4.3 Preparation of Culture and Reagents

4.3.1 Preparation of LB 2x Media with 50 µg/mL Kanamycin

LB 2x media was prepared by adding 2 g of tryptone, 2 g of sodium chloride and 1 g of yeast extract in 100 mL of DI water in a 250-mL beaker and allowing to mix in a hotplate for 5 minutes. The solution was then transferred into an agar bottle and autoclaved at 121°C for 20 minutes. When done, the solution was allowed to cool and 0.5 mL of 10 mg/mL kanamycin was added to it. The media was kept under a fume hood at room temperature.

4.3.2 Preparation of Overnight Culture

*E. coli* Mega X strain, stored as a glycerol stock kept at -80°C, was cultured on LB liquid media and grown in an orbital shaker at 30°C and 180 rpm for 12-18 h. Three kinds of Mega X strains were used throughout the experiment: wild-type, ampicillin-resistant and S18-3A metagenomic library.

4.3.3 Preparation of Functionalized Slides

Borosilicate glass slides (1 x 3 in.) were washed twice in a staining jar containing 99% (v/v in water) ethanol, air-dried and treated with concentrated sulfuric acid for 12-18 hours. The slides were sonicated for 30 minutes, washed with DI water for 30 minutes and treated with acetone for 5 minutes. Then, the slides were coated with 2.5 % (w/v in DI water) 3-aminopropyltriethoxysilane (APTES) followed by three washes with DI water for 15 minutes each. The slides were air-dried and baked in an oven at 120°C for 30 minutes. Finally, the slides were coated with 1.5 % (w/v in toluene) poly(styrene-co-maleic anhydride) (PSMA) using a spin coater (WS-400-6NPP-LITE, Laurell Tech. Ltd.) at 3000 rpm for 30 s and stored in a refrigerator at about 4°C.
4.3.4 Preparation of 3.0% (w/v in water) Alginate Stock

To prepare a 3.0% (w/v in water) alginate stock, 0.3 g of alginate was weighed and exposed to UV light for two hours to decontaminate. Sterilized DI water was then added to alginate, in a stepwise manner to prevent formation of alginate clumps in a 20-mL Erlenmeyer flask. The top of the flask was covered with parafilm and the contents were allowed to mix in a hotplate overnight.

4.3.5 Preparation of a 10 mM Calcium Chloride – PBS 10x Buffer Solution

To prepare a 10 mM CaCl$_2$ – PBS 10x buffer solution, 1.11 g of calcium chloride was added to 100 mL of phosphate buffered saline, PBS 10x and 900 mL of DI water in a 1000-mL Erlenmeyer flask. The top of the flask was sealed with parafilm and the contents were allowed to mix in a hotplate overnight.

4.3.6 Preparation of a 5 mg/mL Antibiotic Stock

A 5 mg/mL antibiotic (i.e. ampicillin, kanamycin) stock was prepared by first weighing 0.025 g of ampicillin then adding it to 5 mL of LB (2x) broth in a 15-mL falcon tube. The solution was then mixed using a vortex mixer for 30 seconds to allow the powder to dissolve. The solution was then transferred into a syringe and was filtered (using a 0.3 µm syringe filter) into 1.5-mL Eppendorf tubes and stored in a -20°C freezer until use.

4.3.7 Preparation of the Microarrayer for Printing

The printing of the tie-layer and alginate-encapsulated *E. coli* cell spots was performed using a robotic microarrayer (MicroSys, Digilab Inc.). All microarrayer functions such as priming, vacuum drying, aspirating, and printing were programmed using AxSys (Cartesian Technologies Inc.) To prepare the microarrayer, a conically
tapered 190 µm orifice ceramic tip was first loaded into a 10 mL scintillation vial and sonicated for 30 minutes. The microarrayer and its parts, including the source plate station, wash and vacuum station, vacuum slide deck were then cleaned with 70% (v/v in water) ethanol. The channels of the microarrayer were then primed twice with DI water and once with isopropyl alcohol (IPA). The same priming program was also performed upon completion of any experiment involving the microarrayer.

4.3.8 Preparation of 0.1% (v/v in water) Poly-L-Lysine (PLL) – 0.2 M Barium Chloride Mixture and Spotting of the Tie-Layer

Prior to printing the alginate-encapsulated cell spots, Poly-L-Lysine (PLL) – barium chloride (BaCl₂·2H₂O) spots were printed unto the PSMA-coated glass slides. These spots are known as the tie-layer. PLL is used for the alginate spots to attach to the slides while barium chloride is used for alginate gelation. From preliminary optimization experiments, it was determined that 0.1% (v/v in water) and 0.2 M are the minimum concentrations of PLL and barium chloride that would yield a tie layer that (A) adhered to the PSMA and (B) bound firmly to 0.1 µL of alginate-encapsulated cell spots such that the spots remained intact even after multiple washings with buffer solution. Figure 4.2 shows a schematic of the preparation of the E. coli chip.

To prepare the PLL-barium chloride mixture, 0.5 g of BaCl₂·2H₂O was weighed and added to 10 mL DI water in a 15-mL Falcon tube. 10 µL of PLL, stored at 4°C, was then added and the solution was vortex mixed for 1 minute to dissolve the pellets. The solution was then kept in a refrigerator at 4°C. When spotting (100 nL/spot) in the microarrayer, the solution was vortex mixed for 90 seconds prior to loading it in the well-plate. After printing, the spots were allowed to dry until formation of a white precipitate is visible.
The PLL-spotted slides were then placed in hybridization cassettes and then kept in a refrigerator at 4°C.

![Diagram of PLL-BaCl₂ bottom layer, PSMA, Glass slide, Alginate-cell spot](image)

*Figure 4.2. Schematic for the preparation of an E. coli chip.*

4.3.9 Preparation of Cells with Desired Cell Density

To prepare cells with a desired cell density, 100 µL of overnight culture and 100 µL of media were pipetted in triplicate into a 96-well plate and the absorbances were measured using a microplate reader (Synergy™ Neo 2 Hybrid, BioTek Instruments). From the absorbances, the cell concentrations were determined using Beer’s law and the rule of thumb that for *E. coli*, an OD₆₀₀ of 1.0 with a 1 cm path length is equivalent to 8x10⁸ cells/mL. Two 1 mL samples of overnight culture were then pipetted into 1.5 mL Eppendorf tubes and placed in a microcentrifuge at 8000 rpm for 15 minutes. The supernatant was discarded, and 0.2 mL of media (to increase the cell concentration five-fold) was added to the tubes and resuspended.
4.3.10 Preparation of 0.5 µM FUN1 Staining Solution

To prepare a 0.5 µM FUN1 staining solution, 2 µL of 10 mM FUN1 cell viability stain was added to 40 mL of CaCl$_2$-buffer solution in a 50-mL Falcon tube covered with aluminum foil and in the dark since the activity of FUN1 is reduced when exposed to light. The solution was then mixed using a vortex mixer.

4.4 Uniformity and Calibration Curves

Uniformity (or spot-consistency) curves, for both susceptible (wild-type) and ampicillin-resistant cells, were made in order to establish the premise that the fluorescence intensity of a spot is not affected by its spatial orientation in the slide. To do the experiment, 1 mL alginate-encapsulated *E. coli* samples (wild-type and ampicillin-resistant) were first prepared with a cell concentration of $9 \times 10^5$ cells/mL (or 90 cells/100 nL spot). When preparing samples, the media was added first, followed by the cells at the desired concentration and lastly 0.5 mL of 3.0% (w/v) alginate. The samples were then mixed using a vortex mixer for 90 seconds each. These samples were then loaded into the 96-well plate in the microarrayer and 100 nL spots were printed directly on top of the dried PLL-spots at a relative humidity of 100% to prevent drying of the alginate spots. The experiment was performed in duplicate with each slide consisting of 33 rows and 10 columns of spots. After printing, the spots were allowed to stick to the slides with the humidifier on for 10 minutes. The slides were then gently dunked into 40 mL of 0.5 µM FUN1 solution and placed in an incubator for 30 minutes. After incubation, the slides were then washed three times for two minutes each with CaCl$_2$-buffer solution and air-dried. The slides were scanned using a microarray scanner (GenePix Personal 4100A, Axon Instruments) at a gain of 400 and a wavelength of 532 nm to determine the
fluorescence intensities and the uniformity curves were then constructed using GraphPad Prism.

Calibration curves, for both susceptible (wild-type) and ampicillin-resistant cells, were constructed in order to determine the cell concentration corresponding to a particular fluorescence intensity. To do the experiment, alginate-encapsulated E. coli samples (wild-type and ampicillin-resistant) were first prepared with the following cell concentrations: 0 (blank), 1\times10^6, 5\times10^6, 1\times10^7 and 2.5\times10^7, 5\times10^7 cells/mL. When preparing 1-mL samples, the media was added first, followed by the cells at the desired concentration and lastly 0.5 mL of 3.0% (w/v) alginate. The samples were then mixed using a vortex mixer for 90 seconds each. These samples were then loaded into the microarrayer and 100 nL spots were printed directly on top of the dried PLL-spots at a relative humidity of 100% to prevent drying of the alginate spots. This experiment was performed in triplicate with each slide consisting of 6 blocks (with each block representing a particular cell concentration) and each block having 3 rows and 10 columns of spots each. After printing, the spots were allowed to stick to the slides with the humidifier on for 10 minutes. The slides were then gently dunked into 40 mL of 0.5 \mu M FUN1 solution and placed in an incubator for 30 minutes. After incubation, the slides were then washed three times for two minutes each with CaCl2-buffer solution and air-dried. The slides were scanned using a microarray scanner (GenePix Personal 4100A, Axon Instruments) at a gain of 400 and a wavelength of 532 nm to determine the fluorescence intensities and the calibration curves were then constructed using GraphPad Prism.
4.5 Growth Curves

Growth curves, for both susceptible (wild-type) and ampicillin-resistant cells, were constructed in order to observe the growth pattern of cells in a 24-hour period. To do the experiment, alginate-encapsulated *E. coli* samples (wild-type and ampicillin-resistant) were first prepared with the following cell concentrations: 0 and $9 \times 10^5$ cells/mL. When preparing 1-mL samples, the media was added first, followed by the cells at the adjusted concentration and lastly 0.5 mL of 3.0% (w/v) alginate. The samples were then mixed using a vortex mixer for 90 seconds each. The samples were then loaded into the microarrayer and 100 nL spots were printed directly on top of the dried PLL-spots at a relative humidity of 100% to prevent drying of the alginate spots. This was done in ten slides with two slides each representing a particular time period. After printing, the slides were immediately placed inside the incubator for growth except for the t=0 slides which will be left inside the microarrayer with the humidifier on for 10 minutes to give ample time for the spots to stick to the slides. The slides placed in the incubator will then be taken out at the following time periods: 6, 12, 18 and 24 hours. When the slides were taken out of the incubator, they were gently dunked into 30 mL of 0.5µM FUN1 solution and again placed in an incubator for 30 minutes. After incubation, the slides were then washed three times for two minutes each with CaCl$_2$-buffer solution and air-dried. The slides were scanned using a microarray scanner (GenePix Personal 4100A, Axon Instruments) at a gain of 400 and a wavelength of 532 nm to determine the fluorescence intensities and the growth curves were then constructed using GraphPad Prism.
4.6 Dose-Response (IC$_{50}$) Curves

Antibiotic susceptibility tests were conducted to generate dose-response curves, for both susceptible (wild-type) and ampicillin-resistant cells, with the purpose of identifying the concentration of the antibiotic suitable for metagenomic library screening. That is, it should be strong enough to kill wild-type (susceptible) cells but not the resistant cells. The antibiotic concentrations that would be tested are the following: 500, 100, 50, 10, 5, 1, 0.5, 0.1 and 0 µg/mL. To do the experiment, alginate-encapsulated *E. coli* samples (wild-type and ampicillin-resistant) were first prepared with the following cell concentrations: 0 and 9x10$^5$ cells/mL. When preparing 1-mL samples, the media was added first, followed by the cells at the desired concentration, then the antibiotic and lastly 0.5 mL of 3.0% (w/v in water) alginate. The samples were then mixed using a vortex mixer for 90 seconds each. The samples were then loaded into the microarrayer and 100-nL spots were printed directly on top of the dried tie-layer spots at a relative humidity of 100% to prevent drying of the alginate spots. This was done in triplicate with each slide containing 10 blocks with each block containing 2 rows and 10 columns of spots. Each block represents a specific antibiotic concentration to be tested including a no-drug (i.e. positive) control and a blank or no-cell (i.e. negative) control. After printing, the spots were immediately placed in the incubator. The slides were then gently dunked into 40 mL of 0.5 µM FUN1 solution and placed in an incubator for 30 minutes. After incubation, the slides were then washed three times for two minutes each with CaCl$_2$-buffer solution and air-dried. The slides were scanned using a microarray scanner (GenePix Personal 4100A, Axon Instruments) at a gain of 400 and a wavelength of 532 nm to determine the fluorescence intensities and the dose-response curves were then
constructed and the IC\textsubscript{50} values were calculated by fitting the Hill equation using GraphPad Prism.

4.7 Sensitivity to Resistant Phenotype

The activity of various resistant and susceptible \textit{E. coli} cell mixtures to 30 µg/mL ampicillin was determined in order to gain an understanding of how the fluorescence-based assay would be sensitive to the resistant phenotype. With each spot containing 90 cells, the various ratios of resistant to susceptible cells tested were the following: 0:90 (purely susceptible), 1:89, 2:88, 5:85, 10:80, 30:60 and 90:0 (purely resistant). To begin the experiment, 1-mL samples were initially prepared with a total cell concentration of 9\times 10^5 cells/mL. When preparing 1-mL samples, the media was added first, followed by the required number of susceptible and resistant cells respectively, then the antibiotic and lastly 0.5 mL of 3.0% (w/v in water) alginate. The samples were then mixed using a vortex mixer for 90 seconds each. The samples were then loaded into the microarrayer and 100 nL spots were printed directly on top of the dried PLL-spots at a relative humidity of 100% to prevent drying of the alginate spots. This was done in duplicate with each slide containing 8 blocks (representing the seven mixtures to be tested and one blank – no cell control) with each block containing 2 rows and 10 columns of spots. After printing, the spots were immediately placed in the incubator. The slides were then gently dunked into 40 mL of 0.5 µM FUN1 solution and placed in an incubator for 30 minutes. After incubation, the slides were then washed three times for two minutes each with CaCl\textsubscript{2}-buffer solution and air-dried. The slides were scanned using a microarray scanner (GenePix Personal 4100A, Axon Instruments) at a gain of 400 and a wavelength of 532
nm to determine the fluorescence intensities and the relative response profile was constructed using GraphPad Prism.

4.8 Metagenomic Library Screening

A metagenomic library (S18-3A) obtained from a soil environment, containing 1.7x10^9 cells/mL and 3x10^5 unique metagenomic inserts (UMIs) was prepared by Dr. Gautam Dantas, Washington University, St. Louis, MO. The library, saved as a 50% (v/v in water) glycerol stock, was kept stored in a freezer at -80ºC until use. Before the screening proper was conducted, a titer experiment was conducted to determine the actual concentration of the glycerol stock, which was determined to be (i.e. 1x10^9 cells/mL). To be 95% confident that all of the unique clones will be arrayed out, three times more cells (i.e. 9x10^5 cells) would be withdrawn from the stock. Arraying out this much cells into spots each having a concentration of 90 cells/spot, would require 10,000 spots and 16 slides. As a first demonstration of the capability of the microarray platform for screening, only 1890 spots would be printed, thus only requiring 3 slides.

A volume of 0.9 µL (9x10^5 cells) was withdrawn from the glycerol stock then added to 495.1 µL of LB 2x media in a 1.5 mL Eppendorf tube. 6 µL of 5 µg/mL ampicillin, equivalent to 30 µg/mL, followed by 0.5 mL of 3.0% (w/v) alginate, equivalent to 1.5% (w/v) alginate, was then added to the solution. 4 controls were prepared in parallel with the metagenomic library sample. These are the following: 1.) no-cell control (i.e. blank), 2.) ampicillin-susceptible cells with antibiotic, (3) ampicillin-resistant cells with antibiotic and (4) ampicillin-susceptible cells without antibiotic. The sample along with the controls were then mixed using a vortex mixer for 90 seconds each. The samples were then loaded into the microarrayer and 100 nL spots were printed directly on top of the
dried PLL-spots at a relative humidity of 100% to prevent drying of the alginate spots. This was done in 3 slides each containing 47 rows and 14 columns of spots, with the top 2 rows reserved for controls. This means each slide will contain 28 spots of controls and 630 spots of the metagenomic library.

After printing all of the spots in a slide, the slide was immediately taken out of the microarrayer, placed in a hybridization cassette, then placed in an incubator at 37°C for 24 hours. The slides were then gently dunked into 40 mL of 0.5 µM FUN1 solution and placed in an incubator for 30 minutes. After incubation, the slides were then washed three times for two minutes each with CaCl₂-buffer solution and air-dried. The slides were scanned using a microarray scanner (GenePix Personal 4100A, Axon Instruments) at a gain of 400 and a wavelength of 532 nm to determine the fluorescence intensities and the hit profile was then constructed using GraphPad Prism.

4.9 Safety Section

4.9.1 Culturing of E. coli Cells

It is highly imperative that any task involving bacteria be performed under a fume hood. This is to prevent contamination of the bacteria from airborne particulates in the outside environment. Before and after performing an experiment involving bacteria, the entire workspace must be sprayed with 70% (v/v in water) ethanol solution. The entire workspace includes the fume hood and all its contents including the fume hood handles, the micropipettes, the pipette tip box, etc. This is important to prevent any particulates which may be present in the fume hood or any equipment inside it from contaminating the bacteria samples. The recommended personal protective equipment (PPE) when working with bacteria are nitrile gloves, splash goggles and a lab coat. The chemicals that
are commonly used for growing bacteria including the antibiotics used are shown in the
SDS table in Table 4.2.

4.9.2 Preparation of Functionalized Slides

When preparing functionalized slides, the only major safety precaution to be observed
is when dunking the slides with a staining jar containing sulfuric acid. The recommended
PPE when working with sulfuric acid, nitrile gloves, chemical-resistant gloves, lab coat,
acid splash suit and a face shield. In addition, both the process of filling the staining jar
with sulfuric acid and the slide dunking process should be performed under a fume hood
to prevent inhalation of the fumes generated by the concentrated sulfuric acid. The
chemicals that are commonly used for preparing functionalized slides are shown in Table
4.2.

Table 4.2. Summary of SDS for Chemicals Used

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Health</th>
<th>Flammability</th>
<th>Reactivity</th>
<th>PPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>Splash goggles, Nitrile gloves, Lab coat, Dust respirator</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>Splash goggles, Nitrile gloves, Lab coat, Dust respirator</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>Splash goggles, Nitrile gloves, Lab coat, Dust respirator</td>
</tr>
<tr>
<td>Agar</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>Splash goggles, Nitrile gloves, Lab coat, Dust respirator</td>
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<tr>
<td>Kanamycin sulfate</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>Splash goggles, Nitrile gloves, Lab coat, Dust respirator</td>
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<tr>
<td>substance</td>
<td>concentration</td>
<td>toxicity</td>
<td>measures</td>
<td></td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>----------------</td>
<td>----------</td>
<td>--------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Ampicillin</td>
<td>2</td>
<td>1</td>
<td>Splash goggles, Nitrile gloves, Lab coat, Dust respirator</td>
<td></td>
</tr>
<tr>
<td>Deionized water</td>
<td>0</td>
<td>0</td>
<td>Safety goggles, Lab coat</td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>2</td>
<td>3</td>
<td>Splash goggles, Nitrile gloves, Lab coat, Vapor respirator</td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>2</td>
<td>1</td>
<td>Splash goggles, Nitrile gloves, Lab coat, Vapor respirator</td>
<td></td>
</tr>
<tr>
<td>Sulfuric acid</td>
<td>3</td>
<td>0</td>
<td>Nitrile gloves, Chemical-resistant gloves, Lab coat, Acid splash suit, Face shield</td>
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</tr>
<tr>
<td>Air</td>
<td>0</td>
<td>0</td>
<td>Vapor respirator</td>
<td></td>
</tr>
<tr>
<td>Toluene</td>
<td>2</td>
<td>3</td>
<td>Splash goggles, Nitrile gloves, Lab coat, Vapor respirator</td>
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</tr>
<tr>
<td>Polystyrene-co-maleic anhydride (PSMA)</td>
<td>1</td>
<td>1</td>
<td>Splash goggles, Nitrile gloves, Lab coat, Dust respirator</td>
<td></td>
</tr>
<tr>
<td>3-Aminopropyl Triethoxysilane (APTES)</td>
<td>3</td>
<td>1</td>
<td>Splash goggles, Nitrile gloves, Lab coat, Dust respirator</td>
<td></td>
</tr>
<tr>
<td>Phosphate-Buffered Saline (PBS)</td>
<td>1</td>
<td>0</td>
<td>Splash goggles, Nitrile gloves, Lab coat</td>
<td></td>
</tr>
<tr>
<td>Calcium chloride dihydrate</td>
<td>2</td>
<td>0</td>
<td>Safety goggles with side shields, Chemical-</td>
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</tr>
<tr>
<td></td>
<td>Required Protective Gear</td>
<td>Remarks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------------</td>
<td>--------------------------</td>
<td>----------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poly-L-Lysine (PLL)</td>
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<td>Safety goggles, Chemical-resistant gloves, Lab coat, Dust respirator</td>
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<td></td>
</tr>
<tr>
<td>Barium chloride dihydrate</td>
<td>3 1 0</td>
<td>Safety goggles with side shields, Chemical-resistant gloves, Lab coat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alginic acid sodium salt</td>
<td>0 0 0</td>
<td>Safety goggles, Nitrile gloves, Lab coat, Dust respirator</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FUN®1 cell stain</td>
<td>1 2 0</td>
<td>Safety goggles, Butyl rubber gloves, Lab coat, Vapor respirator</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SYTO®9 nucleic acid stain</td>
<td>1 2 0</td>
<td>Safety goggles, Chemical-resistant gloves, Lab coat, Vapor respirator</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER FIVE
RESULTS AND DISCUSSION

5.1 Evaluation of Uniformity and Calibration of Fluorescence Intensities of *E. coli* Nanocultures

Uniformity curves, shown in Figure 5.1 (A), were constructed to confirm that the fluorescence intensity of a spot with a set concentration is independent of its spatial orientation in the chip. In order to develop a correlation between cell concentration and fluorescence intensity, calibration curves were constructed as shown in Figure 5.1 (B).

![Uniformity curves](image1)

![Calibration curves](image2)

Figure 5.1. (A) Uniformity (spot-consistency) curves for susceptible (wild-type) and ampicillin-resistant *E. coli* cells along with corresponding microarray scanner images (B) Calibration curves for susceptible and ampicillin-resistant *E. coli* cells along with corresponding microarray scanner images.
For both susceptible and resistant curves in Figure 5.1 (A), it can be shown in the figure that the total fluorescence values are identical (coefficient of variation for susceptible and resistant cells are 9.38% and 9.85% respectively). An unpaired t-test shows that there is no statistically significant difference between the susceptible and resistant cell fluorescence values (P value = 0.2121). This verifies the premise that the cell-type is independent of fluorescence reading. The figure also shows the corresponding microarray scanner images where a consistent level of brightness is clearly observed among the spots.

From Figure 5.1 (B), we observed that there is a linear relationship between fluorescence and cell count for both types of cells as evidenced by their $R^2$ values (0.9959 and 0.9956 for susceptible and resistant cells). It was also observed that the two curves nearly superimpose each other, thus validating the premise that the cell-type is independent of fluorescence reading. The figure also shows the corresponding microarray scanner images where an increase in brightness is clearly observed among the spots.
5.2 Estimation of *E. coli* Growth on the Chip

Figure 5.2 depicts the growth kinetics for both susceptible and resistant *E. coli* cells for a 24-hour period.

![Figure 5.2. Growth curves for susceptible (wild-type) and resistant *E. coli* cells along with corresponding microarray scanner images.](image)

From Figure 5.2, it was observed that the two curves nearly superimpose each other, thus validating the premise that the cell type is independent of fluorescence reading. As shown in the figure, the cells on the chip grow rapidly and maximum growth is reached at 24 h for both susceptible and resistant cells. The corresponding scanner images further validate the results as evidenced by the increase in brightness of the spots up to 24 h. Based on the calibration curve, the number of cells per spot obtained after 24 h is estimated to be 1500 to 1900. This corresponds to about 5 doublings. The growth pattern is also consistent with that obtained in a standard 96-well plate format (See Figure A.1, Appendix A), where maximum growth was observed at 24 h.
5.3 Dose-Response of *E. coli* Nanocultures to Ampicillin

Figure 5.3 depicts the dose-response profiles for both susceptible and ampicillin-resistant *E. coli* cells against various concentrations of the antibiotic for a 24-hour growth period.

![Dose-Response Profiles](image)

Figure 5.3. Dose-response profiles for susceptible and resistant *E. coli* cells against ampicillin evaluated after a 24 h incubation period along with corresponding microarray scanner images.

The dose-response curves shown in Figure 5.3 are similar to the ones obtained from a 96-well plate format (See Figure A.2, Appendix A) but are not as steep. The IC$_{50}$ values obtained for the susceptible and resistant cells were $1.57 \pm 0.03 \mu g/mL$ and $24.64 \pm 4.78 \mu g/mL$ respectively. These values were four times lower than those obtained in a standard 96-well plate format at $6.28 \pm 1.24 \mu g/mL$ and $98.39 \pm 8.22 \mu g/mL$, respectively (See Table 5.1). The lower IC$_{50}$ values could be explained by the reduced culture size resulting in fewer cells being able to grow in the spots. It could also be explained by the fact that the 96-well plate platform was subjected to agitation in an orbital shaker at 180 rpm and 37°C, thus promoting lateral gene transfer while the *E. coli* chip platform was not. From the data,
it is postulated that an ampicillin concentration of 30 µg/mL would be enough to kill a majority of the susceptible cells but only about half of the resistant cells. This would be the antibiotic concentration that would be utilized for metagenomic library screening.

Table 5.1. Comparison of IC₅₀ Values for the *E. coli* Chip and 96-well Plate Platforms

<table>
<thead>
<tr>
<th>Cell-Type</th>
<th>IC₅₀ Values (µg/mL)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>E. coli</em> (microarray) chip</td>
<td>96-well plate</td>
</tr>
<tr>
<td>Susceptible</td>
<td>1.57 ± 0.03</td>
<td>6.28 ± 1.24</td>
</tr>
<tr>
<td>Resistant</td>
<td>24.64 ± 4.78</td>
<td>98.39 ± 8.22</td>
</tr>
</tbody>
</table>

5.4 Sensitivity of Fluorescence Intensities of Nanocultures to Resistant Phenotype

The relative response profile, showing the activities of various resistant and susceptible *E. coli* cell mixtures to 30 µg/mL ampicillin, is shown in Figure 5.4. The various ratios of resistant to susceptible cells tested were the following: 0:90 (purely susceptible), 1:89, 2:88, 5:85, 10:80, 30:60 and 90:0 (purely resistant). Each spot contains 90 cells.

![Figure 5.4](image)

Figure 5.4. Relative response of susceptible and resistant *E. coli* cell mixtures to 30 µg/mL ampicillin evaluated after a 24 h incubation period along with corresponding microarray scanner image. The total number of cells in each spot is 90 cells.
In Figure 5.4, it can be seen that as the number of resistant cells per spot increases, the net fluorescence relative to that of the purely susceptible one increases as well. In addition, a 40% increase in net fluorescence was observed for the 1:89 resistant to susceptible cell mixture compared to the purely susceptible one. This also suggests that the presence of one resistant cell in one 100-nL spot in the initial sample is enough to cause a significant increase in net fluorescence after a 24-hr incubation period. Also, it is important to note that the values for the purely susceptible and purely resistant cells were consistent with those obtained from the dose-response experiment, further validating the results. The relative response profiles may be compared to those obtained using a 96-well plate format (See Figure A.3, Appendix A). The results are comparable; however, the absorbance-based well-plate assay shows more sensitivity to the resistant phenotype than the fluorescence-based microarray chip assay.
5.5 Screening of a Metagenomic Library for Ampicillin Resistance

The hit profile from the screening of the S18-3A metagenomic library arrayed out in three slides is shown in Figure 5.5. The hit cut-off was designated to be 7000, which is about 1.4 times the upper limit of the net fluorescence of the susceptible control w/ ampicillin based on the result of the previous experiment where a 40% increase in net fluorescence was observed for a 100-nL spot with one resistant cell as opposed to that with none.

Figure 5.5. Hit profile for the screening of a S18-3A metagenomic library. The red line represents the cut-off point for hits. Black dots above the red line represent the hits (i.e. spots containing E. coli cells with apparent resistance to ampicillin). Red squares represent ampicillin-resistant E. coli cells with antibiotic. Blue circles represent wild-type E. coli cells with antibiotic. Green triangles represent wild-type E. coli cells w/o antibiotic. The corresponding scanner images are shown below the hit profile.

Based on the chosen hit cut-off, it was determined that after a 24 hour incubation period, there were 37 out of 1890 spots (~1.96%) that are considered as apparent hits. This hit ratio was significantly higher compared to that obtained from the first round of screening in a standard 96-well plate format at 3 out of 880 wells (~0.34%, See Figure A.4, Appendix A). This may seem to indicate that there may be a high number of false positives which have resulted from the screen. It is important to note however, that the values for the
three controls used in the experiment (susceptible cells with ampicillin, resistant cells with ampicillin and susceptible cells without ampicillin) were consistent with those obtained from prior experiments (growth and dose-response), thus validating the results.
CHAPTER SIX
CONCLUSION AND FUTURE WORK

An *E. coli* chip, a novel high-density cellular nanoculture platform, was developed and used for cell growth, antibiotic susceptibility testing and screening of a metagenomic library. This new technology offers several advantages over the current industry standard, a 96-well microplate platform, including miniaturization, automation, reduced amount and cost of reagents and process time. For the purpose of screening a metagenomic library, it also eliminates the need for more than one round of screening, potentially speeding up the antibiotic discovery process. Furthermore, a single *E. coli* chip can replace the work of approximately seven 96-well plates.

In order for the *E. coli* chip to be able to screen a larger library size, smaller-sized droplets (<100 nL) and more slides should be used. In addition, performing post-screening experimental processes such as alginate separation, DNA extraction, PCR and gel electrophoresis are highly recommended for the purpose of identifying the presence of antibiotic resistance genes in the hits. Finally, further validation of the presence of ampicillin-resistant DNA is also recommended. This can be done through sequencing of the amplicons obtained from the PCR or transformation of competent *E. coli* cells using these amplicons and performing the dose-response experiment and comparing the IC\(_{50}\) value generated to the one obtained in this study.
REFERENCES


APPENDIX A

Figure A.1. Growth curves for susceptible and resistant *E. coli* cells grown on a shake flask whose absorbances were measured using a microplate reader. Initial concentration of cells was $10^5$ cells/mL.

Figure A.2. Dose-response curves for susceptible and resistant *E. coli* cells grown on a 96-well plate whose absorbances were measured using a microplate reader. Initial concentration of cells was $9 \times 10^5$ cells/mL.
Figure A.3. Dose-response curves for various mixtures: (A) 2:98, (B) 5:95, (C) 10:90 of ampicillin-resistant to susceptible *E. coli* cells grown on a 96-well plate whose absorbances were measured using a microplate reader. Initial concentration of cells was $9 \times 10^5$ cells/mL.

Figure A.4. Hit profile for the (A) first and (B) second rounds of screening of a S18-3A metagenomic library grown on a 96-well plate. The red line represents the cut-off point for hits. Black dots above the red line represent the hits (i.e. spots containing *E. coli* cells with apparent resistance to ampicillin). Orange triangles represent ampicillin-resistant *E. coli* cells with antibiotic. Blue triangles represent wild-type *E. coli* cells with antibiotic. Initial concentration of cells was $9 \times 10^5$ cells/mL.